Molecular determinants and prevalence of the different body composition phenotypes in a Maltese cohort

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I hereby declare that all material in this research project is my original work.
References to other works are documented.

This project is dedicated to my late father, Carmel, a stalwart ophthalmologist and my role model for hard work, personal sacrifices, and self-discipline, who encouraged me to set high goals and the perseverance, resilience and confidence to achieve them.

'Education is the most powerful weapon which you can use to change the world'

Nelson Mandela

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Abstract

Background

Obesity is accompanied by metabolic abnormalities which increases risk for type 2 diabetes (T2 DM), cardiovascular diseases (CVD) and some cancers. However, some individuals with obesity may present a favourable metabolic profile (metabolically healthy obese (MHO)) while, paradoxically, a subset of normal weight individuals exhibit an adverse cardiometabolic phenotype (the metabolically unhealthy normal weight (MUHNW)). Thus, the presence or absence of metabolic health (MH) creates different body composition phenotypes with metabolically healthy normal weight (MHNW) at one end of the spectrum, metabolically unhealthy obese (MUHO) at the other end, and MUHNW and MHO somewhere in between. To date there is still no standard definition of what constitutes MH, leading to heterogeneity both in prevalence as well as in long term outcomes between studies. The commonly used definitions are those based on the metabolic syndrome (Met S) criteria, the presence of insulin resistance (IR), or a combination of the two. Furthermore, impaired mitochondrial function is implicated in the pathogenesis of several chronic metabolic conditions including IR, Met S, T2DM, and obesity. Quantification of mitochondrial DNA copy number (mtDNA CN) is increasingly used as a biomarker of mitochondrial function and has been observed to correlate with visceral adiposity, body mass index (BMI), hyperlipidaemia, CVD, and mortality. However, the association between mtDNA CN and the different metabolic subtypes of obesity has not been clearly evaluated so far.

Aims

From an epidemiological perspective, this research sets out to investigate, for the first time, the prevalence, sex distribution and characteristics of the different body composition phenotypes within a Maltese Caucasian population. This study also aimed to compare the prevalence when using different definitions to identify MH and to explore which one of them most strongly associates with IR in males and females. Another purpose of this study was to explore the discriminatory power and respective cut-points of various readily available anthropometric and biochemical parameters in predicting IR. The molecular analysis entailed the assessment of the relationship between peripheral blood leukocyte mtDNA CN, Met S and the different body composition phenotypes using various definitions of MH.

Methodology

A cross-sectional study consisting of 521 individuals (63.3% females) aged 41±5 years was conducted. Body composition phenotypes were created based on the combined consideration of each participants' BMI category and MH, defined as the presence of ≤1 components of the NCEP ATPIII criteria. Four body composition phenotypes were generated: metabolically heathy normal weight (MHNW), metabolically unhealthy normal weight (MUHNW), metabolically healthy overweight or obese (MHOW/O), and metabolically unhealthy overweight/obese (MUHOW/O), and subsequently participants with overweight and obesity were considered as separate categories. Relative leukocyte mtDNA CN was determined by qPCR and corrected for leukocyte and platelet count.

Results

Overall, 70% of the studied population were living with overweight or obesity and 32.8% of participants exhibited the metabolically unhealthy phenotype. The population prevalence for each of the body composition phenotypes was as follows: MHNW 27.8%, MUHNW 2.1%, MHOW 28.6%, MUHOW 8.1%, MHO 10.7%, MUHO 22.6%. Generally, the MHOW/O phenotype presented a worse anthropometric and cardiometabolic profile than MHNW, and, in turn, the MUHNW displayed a worse cardiometabolic profile than MHOW/O. Males exhibited the metabolically unhealthy phenotype more frequently than females (41.3% vs 27.8% respectively), were more likely to be insulin resistant (i.e., having a HOMA-IR ≥2.5) (22.9% vs 15.3% respectively), and overall presented a worse anthropometric and metabolic profile compared to females even when classified as being metabolically healthy. Furthermore, significant differences in sex distribution were noted for each body composition phenotype. The lifestyle determinants for the MHOW/O phenotype were regular physical activity and alcohol consumption, non-smoking status and age <40 years. No significant associations were observed for the MUHNW phenotype. When using different definitions to define metabolic health, the prevalence of MHO ranged from 2.1 to 19.0% and that of MUHNW from 0.6 to 13.5%. In females, adopting the presence of ≤2 Met S components of the NCEP ATPIII definition had the highest odds for predicting IR (OR 19.7, 95%CI 16.6-22.3), whereas the Aguilar-Salinas et al. definition had the strongest association in males (OR 18.7, 95%CI 12.3-21.9). With respect to anthropometric and biochemical parameters, the lipid accumulation product (LAP), visceral adiposity index (VAI) and waist circumference (WC) had the best discriminatory power to detect IR in both males and females, however, the cut-off for WC was observed to be lower than those currently used in both

sexes. A lower mtDNA CN was observed in individuals with Met S ($p<0.05$), however no difference in copy number was detected between MHOW/O and MUHOW/O. Moreover, compared to MHNW, a significantly lower mtDNA CN was observed in both metabolically healthy and unhealthy overweight/obese phenotypes ($p<0.001$).

Conclusion

A high prevalence of the metabolically unhealthy phenotype was observed in this relatively young population which may result in increased CVD burden in the future unless timely assessment and management of modifiable risk factors are implemented. This study also demonstrates that the MHO phenotype is not completely benign, and that its risk may lie somewhere between that of MHNW and MUHNW. Furthermore, the prevalence of the various body composition phenotypes is definition dependent highlighting the need for having standard criteria. Since normal weight males were more inclined to be metabolically unhealthy than normal weight females, BMI cut-offs may need to be lowered in males. Additionally, cut-offs for WC may also need to be lowered in both sexes at least in this population. Furthermore, this study expands on the spectrum of associations between reduced leukocyte mtDNA CN, obesity, and Met S in different populations. Moreover, the presence of obesity irrespective of whether it is healthy or unhealthy, is associated with a reduced mtDNA CN (and therefore a degree of mitochondrial dysfunction), implying that the distinction between these two phenotypes may not be directly explained by pathophysiological changes at the level of the mitochondrion.

Ethics Declaration

This study was conducted in accordance with the 1964 Helsinki declaration and its later amendments and comparable ethical standards. All participants gave their written informed consent stating willingness to participate in this study as well as to undergo physical examination and biochemical testing including molecular analysis. Ethical and data protection approvals were granted from the University of Malta Research Ethics Committee (Ref No: 06/2016) of the Faculty of Medicine and Surgery and the Information and Data Protection Commissioner respectively. To ascertain participant anonymity, all data collected for the purposes of this study were stored using a cross indexing system where the primary identifiers were securely filed by the study supervisor.

A copy of the ethical approval and the consent form is attached in **Appendix 1A and B** of this thesis.

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	List of Abbreviations
AACE	American Association of the College of Endocrinologists
ABSI	A Body Shape Index
AC	Arm Circumference
ACE	American College of Endocrinology
ADP	Adenosine Diphosphate
AIP	Atherogenic Index of Plasma
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AMA	American Medical Association
AMI	Acute Myocardial Infarction
AMPK	Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AUC	Area Under Curve
AVI	Abdominal Volume Index
BAI	Body Adiposity Index
BMI	Body Mass Index
BP	Blood Pressure
BRI	Body Roundness Index
CI	Conicity Index
cIMT	Carotid Intima Media Thickness
CM	Cardiometabolic
CNS	Central Nervous System

COSI	Childhood Obesity Surveillance Initiative
CVD	Cardiovascular Disease
DEXA	Dual Energy X-ray Absorptiometry
DNA	Deoxyribonucleic Acid
DVT	Deep Vein Thrombosis
EDTA	Ethylenediaminetetraacetic Acid
eGFR	estimated Glomerular Filtration Rate
EGIR	European Group for the study of Insulin Resistance
EHIS	European Health Interview Survey
ELISA	Enzyme Linked Immunosorbent Assay
ETC	Electron Transport Chain
FBC	Full Blood Count
FFA	Free Fatty Acid
FPG	Fasting Plasma Glucose
FPLD	Familial Partial Lipodystrophy
FT3	Free Triiodothyronine
FT4	Free Thyroxine
G6PDH	Gluocse-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDM	Gestational Diabetes Mellitus
GERD	Gastro Esophageal Reflux Disease
GGT	Gamma Glutamyl Transpeptidase
GH	Growth Hormone
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like Polypeptide-1

GWAS	Genome Wide Association Studies
HAART	Highly Active Anti-Retroviral Treatment
HBA1c	Glycated Haemoglobin
HBB	nuclear gene coding Haemoglobin subunit ß
HC	Hip Circumference
HCC	Hepatocellular Carcinoma
HDL-C	High Density Lipoprotein Cholesterol
HIV	Human Immune deficiency Virus
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
ICH	Intracerebral Haemorrhage
IDF	International Diabetes Federation
IGF-1	Insulin-like Growth Factor -1
IL	Interleukin
IMCL	Intramyocellular lipid
IQR	Inter Quartile Range
IR	Insulin resistance
ISI	Insulin Sensitivity Index
LAP	Lipid Accumulation Product
LDL-C	Low Density Lipoprotein Cholesterol
LVH	Left Ventricular Hypertrophy
Met S	Metabolic Syndrome
MH	Metabolic Health
MHNW	Metabolically Healthy Normal Weight
MHO	Metabolically Healthy Obese
MHOW	Metabolically Healthy Overweight

MHOW/O	Metabolically Healthy Overweight/Obese
MMP	Matrix Metallopeptidase
MSD	Mediterranean Style Diet
MT-CYB	Mitochondrial gene encoding Cytochrome B
mtDNA CN	Mitochondrial DNA Copy Number
MUHNW	Metabolically Unhealthy Normal Weight
MUHO	Metabolically Unhealthy Obese
MUHOW	Metabolically Unhealthy Overweight
MUHOW/O	Metabolically Unhealthy Overweight/Obese
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steato Hepatitis
NC	Neck Circumference
NCD	Non-Communicable diseases
NCEP ATPIII	National Cholesterol Education Program Adult Treatment Panel III
NHANES	National Health and Nutritional Examination Survey
NHLBI	National Heart Lung and Blood Institute
NLR	Neutrophil Lymphocyte Ratio
OA	Osteoarthritis
OSA	Obstructive Sleep Apnoea
PCA	Principal Component Analysis
PCOS	Polycystic ovarian Syndrome
PCR	Polymerase Chain Reaction
PE	Pulmonary Embolus
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
RAAS	Renin Angiotensin Aldosterone System

ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
SAT	Subcutaneous Adipose Tissue
T2DM	Type 2 Diabetes Mellitus
TBF	Total Body Fat
TC	Thigh Circumference
TChol	Total Cholesterol
TG	Triglycerides
TL	Telomere Length
TNF-α	Tumour Necrosis Factor alpha
TSH	Thyroxine Stimulation Hormone
TyG	Triglyceride glucose Index
UA	Uric Acid
UCP	Uncoupling protein
VAI	Visceral Adiposity Index
VAT	Visceral Adipose Tissue
WC	Waist circumference
WHO	World Health Organisation
WHR	Waist to hip ratio
WHtR	Waist to height ratio
WI	Waist index
WTR	Waist to thigh ratio

Conference Proceedings and Publications in Peer Reviewed Journals

I. Poster Presentation at the Obesity Update 2020:

Prevalence and determinants of metabolic health and different body composition phenotypes in a Maltese cohort
Obesity Abstracts (2020) **2** P5 | DOI: 10.1530/obabs.02.P5
Rachel Agius, Nikolai Pace & Stephen Fava

II. Poster presentation at the Obesity Update 2021:

Gender Differences in Cardiometabolic Abnormalities across Different BMI Categories
Rachel Agius, Nikolai Pace & Stephen Fava
https://www.obesity-abstracts.org/ob/0003/ob0003p3
Published: 2021-06-16

III. Oral presentation at the Mata Medical School Conference, November 2019:

Prevalence and determinants of Metabolic Health and different Body Composition Phenotypes in a Maltese Cohort
Rachel Agius (presenter), Nikolai Paul Pace, Stephen Fava

IV. Publication

Sex differences in cardiometabolic abnormalities in a middle-aged Maltese population
Canadian Journal of Public Health
2022-06 | Journal article
DOI: 10.17269/s41997-021-00592-7
Rachel Agius; Nikolai Paul Pace; Stephen Fava

V. Publication

Characterisation of body size phenotypes in a middle-aged Maltese population
Journal of Nutritional Science**,** 2021
DOI: 10.1017/jns.2021.74
Rachel Agius; Nikolai Paul Pace; Stephen Fava

VI. Publication

Prevalence rates of metabolic health and body size phenotypes by different criteria and association with insulin resistance in a Maltese Caucasian population
BMC Endocrine Disorders
BMC Endocr Disord. 2022;22(1):160. Published 2022 Jun 15.
doi:10.1186/s12902-022-01071-x
Rachel Agius; Marie Claire Fava, Nikolai Paul Pace, Stephen Fava

VI. Publication

Reduced leukocyte mitochondrial copy number in metabolic syndrome and metabolically healthy obesity.
Frontiers in Endocrinology
Front Endocrinol (Lausanne). 2022;13:886957. Published 2022 Jul 25.
doi:10.3389/fendo.2022.886957
Rachel Agius, Nikolai Paul Pace and Stephen Fava

VII. Publication

Phenotyping obesity : A focus on Metabolically healthy Obesity and Metabolically unhealthy normal weight.
Diabetes Metab Res Rev. 2023;e3725.
https://doi.org/10.1002/dmrr.3725
Rachel Agius, Nikolai Paul Pace, Stephen Fava

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Chapter 1 – Introduction

1-1 Introduction

The prevalence of obesity has increased to pandemic proportions globally over the past 30 years making it an urgent public health concern. Obesity is now recognised as being a chronic, complex and relapsing disease entity within its own right and not just a risk factor for other non-communicable diseases (including metabolic, mental, mechanical and malignant diseases). Furthermore, an increased understanding of the role of the adipocyte in the pathogenesis of obesity and its associated morbidities has led to the perception that obesity is a heterogenous condition with multiple different phenotypes. Therefore this literature review aims to discuss different aspects of obesity in terms of its epidemiology, definition, complications as well as a description of the different obesity phenotypes.

The literature review is based on data obtained from cross-sectional, prospective/longitudinal studies and consensus obtained from systemic review and meta-analyses using PubMed/Medline and Google Scholar databases from inception up to February 2023. The search terms (medical subject headings [MeSH]) used for Section 1-2 'Epidemiology and Prevalence of Overweight and obesity' were: 'obesity', 'overweight', and 'epidemiology'. For Section 1-3 'Definition and indices of measurement of obesity' the MeSH terms used were: 'obesity', diagnostic criteria' and 'obesity assessment'. For Section 1-4 'Complications of obesity' the MeSH terms were: 'obesity', 'metabolic disorders', insulin resistance', 'adipose tissue', 'adiposopathy', obesity-related adipose tissue disease'. For section 1-5 'Metabolically healthy obesity (MHO) and metabolically unhealthy normal weight (MUHNW)' the MeSH terms used were: 'metabolically healthy obesity', 'unhealthy obesity', 'metabolically unhealthy

normal weight', in combination with 'cardiometabolic disease', 'type 2 diabetes', 'mortality', 'transition', 'interventions'. For Section 1-6 the following MeSH terms were used: 'mitochondrial DNA copy number', 'mtDNA', 'mitochondrial dysfunction', in combination with 'metabolic diseases', 'insulin resistance', 'obesity', 'lipotoxicity', 'type 2 diabetes'. Additionally, a manual search of the bibliographies for each of the retrieved articles was also carried out. Only articles written in the English language and published in peer-reviewed journals were considered.

1-2 Epidemiology and Prevalence of Overweight and Obesity

Recent data by the World health Organization (WHO) has shown that globally overweight and obesity prevalence has nearly tripled over the past forty years. In 2016 it was estimated that around 1.9 billion adults aged 18 years and older were overweight of whom 650 million were obese, translating to around 39% and 13% of the global population respectively. Within the paediatric population the problem of excess body weight is also prevalent with recent statistics stating that more than 240 million children and adolescents between the ages of 5 and 19 were either overweight or obese. Moreover, the problem of excess body weight is also afflicting low-and middle-income countries where previously the major contributors towards morbidity and mortality were from under nutrition and communicable diseases (WHO, 2015; WHO, 2016a).

In view of such revelations WHO has since issued a statement stating that we are now facing the 'double burden of disease' (WHO, 2016b). This is characterized by the coexistence of undernutrition along with overweight/obesity (also known as diet related non-communicable diseases [NCDs]) within individuals, households and populations especially in rural areas and countries with low-income status (Min et al., 2018). Thus, whilst such territories are still combatting infectious diseases, they are also experiencing a rapid surge in non-communicable disease and lifestyle-related chronic diseases such as T2DM and CVD. As an example of such countries, both Ethiopia and Nepal have seen an increase in prevalence of overweight with a concomitant reduction in prevalence of underweight during the 1990s and 2000s (Bygbjerg, 2012). In addition to urbanization and industrialization and the associated changes in food systems, other mechanisms have been postulated as potential drivers for this double burden of disease. Biological

processes including the theories centring around the developmental origins of adult disease (such as the Baker hypothesis, developmental programming and the thrifty phenotype) have helped explain coexistence of such conditions especially at individual level. Evidence has shown that undernutrition during early fetal life or rapid weight gain during the first years of life is associated with increased predisposition to overweight/obesity and cardiometabolic disease later in life (Uauy et al., 2011).

Locally, within the Maltese archipelago recent data from the European Health Interview Survey (EHIS) has revealed that obesity concerns more than one in four adults totalling to a prevalence rate of around 28.7% (Eurostat 2019). This signifies that the Maltese islands have the highest burden of obesity among the EU member states. Moreover, a cross-sectional study done in 2016 by Maltese authors has shown that nearly two-thirds of the Maltese population is overweight or obese with males having a significantly higher prevalence for both conditions and with a higher predilection in the working age group (35 to 44 years) (Cuschieri et al., 2016a). Similarly, a recent study done by the European Childhood Obesity Surveillance Initiative (COSI) also revealed that Malta had the highest rate of severe childhood obesity (about 1 in 3 children) in Europe with boys being more significantly affected then girls (Spinelli et al., 2019).

In tandem with this worldwide surge in prevalence rates of obesity and overweight, it is worth also mentioning here the parallel rise observed in the prevalence rate of type 2 diabetes (T2DM), making it one of the most common metabolic diseases within the European Union (Tamayo et al., 2014). Obesity is closely associated with an increased risk of developing T2DM mainly through insulin resistance (IR) and thus it comes as no surprise that the exponential increase in obesity prevalence has been mirrored by that

of T2DM. In fact, it has been estimated that 80-90% of individuals with T2DM are overweight or obese and that increments in BMI above that of 25 kg/m^2 leads to exponential increases in the risk of developing this metabolic condition (Chan et al., 1994; Colditz et al., 1995). Such is the strong association between the two that Astrup and Finer proposed the term 'diabesity' be adopted to reflect both aetiological and clinical presentation (Astrup and Finer, 2000). They argue that obesity and diabetes share common aetiological lifestyle factors (such as excess calorie intake, sedentary lifestyle, increased dietary intake of saturated fat) and that in genetically susceptible individuals may lead to development of one or both of these diseases. Moreover, a number of intervention trials have also shown that weight loss by diet, exercise, pharmacological agents or bariatric surgery prevented the onset of T2DM in individuals living with obesity and at high risk of developing this condition (Heymsfield et al., 2000; Pan et al., 1995; Sjöström et al., 1999).

The situation with regards to prevalence rate of T2DM in the Maltese islands is very reminiscent to the trends seen in most European Countries (Savona-Ventura, 2001). Malta is a European island strategically situated in the middle of the Mediterranean Sea with a total population standing at approximately 520,000 of predominantly Caucasian decent and a westernized type of lifestyle and diet (Cuschieri and Mamo, 2014; National Statistics Office [NSO], 2022). In the 2016 cross-sectional prevalence study by Cuschieri et al., the authors found that one in eight adults aged between 25 and 64 years suffered from T2DM and more alarmingly they also note that approximately 10,000 of these individuals were unaware of the diagnosis. Furthermore, they carried out projected prevalence rates for T2DM and obesity for the year 2050 using the projected EUROSTAT

2050 Maltese population. The authors report that while the overall total Maltese population appears to decrease by 2050, the diabetes and obese population, however, will increase by around 28 and 15% respectively. They also looked at the cost burden of these two diseases and found that the diabetes and obesity economic health burdens are expected to increase exponentially the total health care expenditure in Malta by the year 2050 reflecting the surge in prevalence rates of both diseases (Cuschieri et al., 2016a; Cuschieri et al., 2016b).

Thus, in view of such revelations the term 'globesity' was coined in 2016 in order to reflect this global increase in prevalence rate of obesity that is affecting both children and adults. Moreover, if not addressed properly in terms of establishing effective preventive strategies and screening programs and immediate action taken, the worldwide prevalence will continue to increase with the consequence that an increased number of individuals will be afflicted from an array of serious health comorbidities associated with excess weight (Costa-Font and Mas, 2016).

1-3 Definition and indices of measurement of Obesity

Obesity is now recognised as being a chronic, relapsing, multifactorial, neurobehavioral disease entity within its own right by several regulatory bodies (including the American Medical Association (AMA), the American Association of Clinical Endocrinologists (AACE), the World Obesity Federation (WOF) and WHO) since it is observed to fit the criteria common to all definitions that constitute a disease namely that it a) leads to an impairment of the normal functioning of some aspect of the body (such as dysregulation of appetite and energy balance, endocrine dysfunction (such as insulin resistance, and infertility), altered physiological function (including adipose tissue inflammation and dysregulated adipokine signalling) as well as physical impairments resulting from an increase in body fat mass (such as osteoarthritis, immobility and lymphedema) b) it has characteristic signs and symptoms primarily resulting from the physical accumulation of fat mass (such as joint pain. Immobility and sleep apnoea) and c) leads to harm or morbidity as a result of the physical increase in fat mass and/or the physiological and metabolic derangements associated with obesity (such as type 2 diabetes, cardiovascular disease, cancer and death) (Mechanic et al., 2012; Sbraccia and Dicker 2023; World Obesity Federation, 2017; Pollack, 2013).

Over the past few decades, an accumulation of biomedical knowledge has led to a better understanding of the pathophysiology of obesity. A wealth of data now demonstrates that molecular, genetic, and endocrine process in combination with lifestyle, socio-economic and behavioural practices all contribute to the creation of an obese phenotype. The fundamental defect in obesity is that of an imbalance between energy intake (in the form of calories consumed) an energy expenditure (calories expended)

Blüher, 2019). While there are clear behavioural, socio-economic and lifestyle determinants of obesity (some which are under voluntary control such as diet preferences and physical inactivity / sedentary lifestyle while others are outside of individual control such as availability of healthy foods, sociocultural attitudes and customs and exposure to environmental endocrine disruptors) it is recognised that there are also biological and genetic factors controlling appetite, food craving as well as storage and mobilization of energy. With respect to genetics, early observations from twin and adoption studies showed that obesity might be a result of an inherited dysregulation of energy homeostasis and that the heritability of BMI was as high as 40-70% (Stunkard et al., 1990; Borjeson, 1976). Compounding these findings are the discoveries of monogenic forms of obesity following the detections of mutations in genes encoding several hypothalamic proteins such as melanocortin 4 receptor, pro-opiomelanocortin, leptin receptor and gut hormones (leptin) involved in the hypothalamic regulation of appetite and satiety. While these mutations are associated with early onset and severe obesity, they are rare. Furthermore, results from genome wide association (GWAS) studies observed 97 loci associated with BMI all of which affect genes expressed within the central nervous system. However, these single nucleotide polymorphisms could only explain 2% of the BMI variability (Locke et al., 2015). Therefore, while genetic alterations clearly cannot account for the current obesity pandemic, they underpin the importance of biological factors in the pathogenesis of obesity (Farooqi and O' Rahilly 2005; Bluher 2020). In fact, an improved understanding of the neurohormonal control of energy homeostasis led to the discovery of key areas within the hypothalamus (notably the arcuate nucleus) which are involved in the

integration of hormonal, metabolic and mechanical signals from peripheral tissues such as adipose tissue and the gastrointestinal tract, and which are ultimately responsible for caloric intake and energy expenditure. While this homeostatic system ensures maintenance of adequate caloric intake for survival, it can be overridden by hedonic (reward) pathways whereby the emotional sphere and the sight, taste, and smell of certain palatable foods lead to increased caloric intake regardless of energy needs. While these regulatory mechanisms work well in situations of weight reduction, they tend to be more permissive towards weight gain especially in the current obesogenic environment where food scarcity is rare (Heymsfield and Wadden, 2017; Murray et al., 2014; Farooqi, 2014). Therefore, these data reinforce that obesity is an altered pathophysiological state resulting from genetic/epigenetic, biological, hormonal and environmental interactions.

Thus, in view of the above observations several organisations and regulatory bodies felt the pressing need of a 'new diagnostic and more medically meaningful definition of obesity' (Bray et al., 2017; Heymsfield and Wadden, 2017; Jastreboff et al., 2019; Schwartz et al., 2017). Thus in 2017 a position statement was issued by the American Association of Clinical Endocrinologists (AACE) and American College of Endocrinology (ACE) proposing the use of the term 'adiposity based chronic disease or ABCD' to better characterize the pathophysiological basis and chronicity of the disease as well as to avoid the stigma related to the use of the term 'obesity' (Mechanick et al., 2017). Traditionally overweight and obesity are assessed by measuring the body mass index (BMI) which is the person's weight (in kilograms) divided by the square of their height (in meters). The observation that weight varies across individuals as height squared has been first

reported by Adolphe Quetelet in 1842 and further studies consolidated this seminal observation thus creating a shape index which is independent of height (Gadde et al., 2018; Mechanick et al., 2017; Upadhyay et al., 2018). Therefore, a BMI of greater than or equal to 25 kg/m^2 denotes overweight and a BMI of 30 kg/m^2 or more denotes obesity (WHO, 2016b).

The use of this parameter by most leading institutions (including WHO and the National Institutes of Health) for classifying weight status stems from several epidemiological observations which demonstrate a relationship between rising BMI values and cardiometabolic risk and mortality (Cornier et al., 2011; Klein et al., 2007). Direct associations between adiposity (as reflected by a high BMI) and diseases such as T2DM, hypertension, dyslipidaemia and coronary heart disease have been well documented. Moreover, each 5 kg/m^2 rise in BMI above the normal value of 25 kg/m^2 is associated with increased cause-specific mortality including vascular mortality by 40%, and renal, diabetic and hepatic mortality by 60% to 120% (Upadhyay et al., 2018). However, the relationship between BMI and all-cause mortality has been somewhat controversial. One landmark study which evaluated data from the National Institutes of Health-AARP Diet and Health Study, showed that in both males and females (who were never smokers), irrespective of age, rising BMI and weight gain in early adulthood was positively associated with mortality (Adams et al., 2017; Livingston, 2012; Malnick and Knobler, 2006). However, other studies which have also delved into the correlation between BMI and mortality rate have shown inconsistent results, with several epidemiological studies reporting a positive, J-shaped, U-shaped, non-existent or even an inverse relationship (Malnick and Knobler, 2006; McGee, 2005). Specifically, in the

overweight category some studies have actually shown little increase in risk or even a small protective effect from being overweight (Flegal et al., 2013; McGee, 2005). One potential explanation for this is the fact that the BMI does not truly identify excess adiposity since it is unable to differentiate fat from lean mass and may therefore misclassify individuals who have increased muscle mass (which increases insulin sensitivity and may therefore be protective from an increase in fat mass). Attesting to this is a meta-analysis of 32 studies which included nearly 32,000 individuals , where it was observed that the BMI had a sensitivity and specificity of 50% and 90% at identifying excess adiposity, implying that half of individuals with excess adiposity were not correctly identified as obese (Okorodudu et al., 2010). Furthermore, the BMI does not account for ethnic, sex and age-related difference in adiposity and more importantly gives no insight into adipose differentiation (ectopic/visceral fat vs subcutaneous fat) as explained in more detail later on in this section

In fact, it has been thought that obesity itself does not increase the risk of death, but rather it acts via intermediate risk factors (Livingston, 2012; Wilson et al., 1998). This has been shown in a sub-analysis of the Framingham study population where multivariate analysis showed that certain cardiometabolic parameters such as blood pressure and lipid levels proved to be much more powerful at predicting CVD then obesity on its own (Hubert et al., 1983). Such observations have shed light on the fact that a mild degree of excess adiposity might actually be beneficial for overall survival. In fact studies in individuals living with overweight or class I obesity i.e., having a BMI of 30-34.9 kg/m^2 (especially those involving older people or people with CVD), have been shown to have more favourable prognoses when compared with normal weight

individuals (Strandberg et al., 2009). This has been termed the 'obesity paradox' or reverse epidemiology, that is the theoretical advantage in terms of morality that overweight and mild obesity confers in some pathological states such as heart failure. Whilst obesity is well known to cause both functional and structural changes on the heart including increased stroke volume, ventricular hypertrophy, abnormalities in systolic and diastolic function and eventual heart failure, obese patients seem to have more favourable clinical outcomes in terms of survival rates. Several possible mechanisms have been postulated for such observations including a more preserved systolic function, attenuated natriuretic peptide response (leading to earlier expression of heart failure symptoms), increased nutritional and metabolic reserve and that leanness could be the surrogate marker of other underlying disease states. Moreover, other traditional cardiovascular risk parameters usually associated with obesity such as hypertension and hyperlipidaemia have been shown to be protective for heart failure development, hence the term 'reverse epidemiology'. In addition to this, it is also worth mentioning that obesity has also been shown to protect against a number of other disease processes namely patients with end stage renal disease and as well as dialysis and cancer patients. However, more studies are needed to further elucidate whether this relationship is causal or merely an association (Clark et al., 2014; Dulloo et al., 2010; Horwich et al., 2001; Lavie et al., 2005; Ryan, 2005; Strandberg et al., 2009).

In a systematic review and meta-analysis, Flegal *et al.,* concluded that individuals with a BMI of >30kg/m^2 and especially at values higher then 35kg/m^2 were associated with a significantly increased risk of all-cause mortality after adjustments for age, sex and smoking. Interestingly overweight again was associated with significant lower all-cause

mortality implying that use of predefined standard BMI categories would facilitate comparisons between different studies (Flegal et al., 2013).

While keeping in mind these confounding issues, the BMI continues to be universally accepted as the 'gold-standard' and most practical population-level measure of human adiposity due to its simplicity and ease in measurement (Klein et al., 2007). There are several important reasons for the limitations of using BMI alone to assess adiposity in the clinical setting. One important reason for this is that obesity is a very heterogeneous condition (Cornier et al., 2011). For instance, the BMI does not take into account body composition (muscle mass versus fat mass) and furthermore it does not differentiate between total body fat mass and regional adiposity such as visceral adipose tissue, subcutaneous tissue or ectopic fat deposition (for example in skeletal muscle, liver and other organs) (Goossens, 2017; Peiris, 1989). It has been well-documented in several studies that body fat distribution, particularly abdominal adiposity, is an independent predictor of metabolic aberrations as well as of cardiovascular morbidity and mortality (Camhi et al., 2011, p. 20; Lapidus et al., 1984). Also, the use of universal BMI cut-off points to classify individuals as normal weight, overweight and obese does not reflect adiposity in different demographic groups. This is particularly seen in South Asian individuals, who for the same BMI display a greater amount of body fat than Caucasians (Peiris, 1989). Thereafter several studies went on to show that for a given BMI the amount of body fat is significantly influenced by age, sex and race (Camhi et al., 2011). Moreover, it also overlooks people who by BMI criteria are considered normal weight but who may also harbour unhealthy visceral fat, the so-called metabolically unhealthy normal weight (MHNW) individuals (Ruderman et al., 1981). At the other extreme the

BMI also does not identify those who are obese but nonetheless show trivial or no metabolic complications at all – the metabolically healthy obese (MHO) individuals (Sims, 2001; Stefan et al., 2008b). One study has shown the futility of BMI at accurately predicting body composition at values below 30kg/m^2. Here, a significant number of individuals who were not obese by BMI criteria were found to have obese levels of body fat by bioelectrical impedance and thus would have had their obesity status misclassified (Kuk et al., 2006). Thus, measurement of BMI alone may not truly identify all cases of obesity mainly because it fails to assess body composition or distribution of body fat (Cornier et al., 2011; Frankenfield et al., 2001).

As stated earlier, variation in body fat distribution (upper body [abdominal region] vs lower body [gluteofemoral region] deposition have significant implications on the development of obesity-related comorbidities (Peiris, 1989). Population studies have shown that abdominal obesity significantly predicts obesity-related comorbidities and mortality (independently of BMI), whereas peripherally distributed fat deposits are associated with a protective lipid and glucose profile and decreased cardiovascular and metabolic disease prevalence (Grundy et al., 2013). However, precise measurement of regional fat distribution (by bioelectric impedance, dual energy x-absorptiometry, MRI scanning or CT imaging) can be a laborious and expensive task which would not be practical for everyday clinical use. In view of this, other tools have been generated for better assessment of distribution of adiposity (Cornier et al., 2011; Neeland et al., 2019). One such commonly used and inexpensive yet effective parameter is the waist circumference (WC). It is easily measured with a tape measure placed at the midpoint between the iliac crest and the lowest rib (Cornier et al., 2011; Han, 2001). WC is often

used as a surrogate marker of centripetal obesity since it has been shown to correlate well with abdominal fat mass (especially visceral adiposity) on abdominal imaging and thus better at identifying individuals at increased cardiometabolic risk (Cornier et al., 2011; Janssen et al., 2002; Pouliot et al., 1994). In fact, for a given BMI individuals with higher WC values are considered to be at greater relative health risk than those with lower WC values implying that WC adds to an individual's risk of disease to that predicted by BMI alone (Janssen et al., 2002; Schneider et al., 2007).

Furthermore, other anthropometric indices have been put forward to assess abdominal adipose accumulation and body fat distribution and thereafter examined for their ability to diagnose and predict the metabolic syndrome (Met S) and cardiovascular risk (Bener et al., 2013; Schneider et al., 2007). These include circumferential measurements of the neck, hip, thigh and arm; subcutaneous skinfold thicknesses; and various indices of central adiposity and body fat distribution including: waist-hip ratio (WHR), waist to height ratio (WHtR), waist to thigh ratio (WTR), waist index (WI), visceral adiposity index (VAI), conicity index (CI), abdominal volume index (AVI), body adiposity index (BAI), a body shape index (ABSI) and body roundness index (BRI) (Amato et al., 2014; Ben-Noun and Laor, 2003; Cornier et al., 2011; Goh et al., 2014; Seidell et al., 1990; Valdez, 1991; Wang et al., 2017).

The hip circumference (HC) is measured at the level of the widest circumference over the buttock and is mainly used to calculate the waist to hip ratio (WHR) (Han and Lean, 2001). Thigh circumference is measured at 1cm below the gluteal fold (Cornier et al., 2011). It has been shown to be inversely associated with mortality in males and larger thigh circumferences are associated with lower risk of T2DM in both sexes (Mason et

al., 2008; Snijder et al., 2003). Neck circumference (NC) is another anthropometric parameter that has been found to be related to CVD risk. Several studies have found it to correlate well with WC, WHR and BMI as well as to components of the Met S in both sexes. It also provides risk assessment for obstructive sleep apnoea (OSA) as well as severity of OSA independent of obesity (Kawaguchi et al., 2011; Preis et al., 2010).

Overall, both circumferential and ratio measurements have all been shown to a certain extent to be positively correlated to adverse cardiovascular risk factor parameters (such as diastolic blood pressure, serum total cholesterol, low HDL-cholesterol, serum triglycerides (TG) and insulin levels) in different ethnic groups; however, circumferential measurements particularly of the breast, waist and thigh had the strongest correlation with cardiometabolic biochemistry (Barzi et al., 2010; Borruel et al., 2014; Meisinger et al., 2006). Moreover, in the study by Taylor *et al*, BMI, WHR, WHtR and WC were all found to have similar magnitudes for association with CVD risk factors (Taylor et al., 2010). However, in the study by Lim *et al.,* the authors investigated the relationship of general adiposity indices (as expressed by the BMI) and central adiposity indices (including WC, WHR and WHtR) in an Asian cohort with dysglycaemia, and concluded that for a given BMI, indices of central obesity were associated with increased all-cause and CVD-related mortality suggesting that BMI alone does not accurately represent mortality risk in this population (Lim et al., 2015). Interestingly, in one analysis of the National Health and Nutrition Examinations Survey (NHANES), the WTR ratio was found to have the greatest discriminating power and the strongest association with presence of T2DM compared with other indices (such as WHR, WHtR and WC) (Li et al., 2010). These findings were echoed in the meta-analysis by Lee et al., which confirmed that

measures of central obesity, in particular the WHtR are better discriminators for certain cardiovascular risk factors (including hypertension, diabetes and dyslipidaemia) and that BMI was the poorest discriminator (Lee et al., 2008). On the other hand, a Spanish study assessing such indices within a young adult cohort noted that the BMI and WC have the strongest correlations with ultrasonographic measurements of visceral adiposity (Borruel et al., 2014). Moreover, in a study by Kvist *et al*., it was noted that the reported correlations of waist-hip ratios to visceral adipose tissue volume (as assessed by CT imaging) were imperfect (Kvist et al., 1988). This seems to imply that although these anthropometric parameters all have different discriminatory abilities in determining cardiovascular morbidity and mortality, the predictive power to which each of them exerts their effect seems to depend (at least in part) on ethnicity, age and sex of the population studied (Delvarianzadeh et al., 2017; Kato et al., 2008; Molarius and Seidell, 1998; Wang et al., 2017).

Thus, while ratios, especially those involving the use of WC measurement (such as WHR and WHtR) reflect distribution of body fat and despite having been shown to be similar (if not superior) to other anthropometric parameters at predicting coronary heart disease incidence overall, it still remains debatable whether they should be incorporated routinely in the clinical assessment of adiposity. . More recently the National Institute of Health and Care Excellence (NICE) has demonstrated evidence for the use of the WHtR (alongside the BMI) as a practical measure for central adiposity particularly in individuals with a BMI under 35 kg/m^2. This stems from that fact that the WHtR offers a truer estimate of central (visceral) obesity by virtue of having the waist circumference in its calculation and furthermore has the advantage of being accurate in people with high

muscle mass or in older individuals. Accordingly, the committee purports that a WHtR of less than 0.5 (i.e., having a waist circumference which is less than half the individual's right) irrespective of sex or ethnicity, to be associated with no increased health risk (specifically with regards to T2 DM, hypertension and CVD). However it also recommends that institution of interventions should be individualised taking into account other factors such as the person's individual needs and preferences and other factors such as ethnicity, the presence of weight related comorbidities, family history and their socioeconomic status regardless of WHtR (NICE, 2023).

1-4 Complications of Obesity

The explosive increase in the number of people with overweight and obesity has also been paralleled by the increase in number of several medical conditions. It is now well known that obesity brings with it a host of comorbidities which affect many different physiological processes within the body (Upadhyay et al., 2018). The underlying mechanisms for most of these conditions stem from the fact that excess adiposity causes harm by two main processes, either via excessive fat-mass mechanisms (as occurs in conditions such as obstructive sleep apnoea and osteoarthritis) or due to adipose tissue dysfunction – also called adiposopathy or 'sick fat' which in turn leads to abnormal endocrine and immune responses that may directly promote CVD or indirectly through the onset of metabolic disease (for exampleT2DM, hypertension and dyslipidaemia). Thus, it can be said that obesity targets nearly all organ systems. It has been also implicated in the pathogenesis of certain cancers; it also affects patients psychologically (including increased risk of depression and anxiety as well as social stigmatization and discrimination) (Bays, 2011; Fruh, 2017; Malnick and Knobler, 2006; Yumuk et al., 2015). A summary of the major comorbidities associated with obesity are described in **Table 1.1**. While precise underlying mechanisms have not been demonstrated for all of these comorbidities, the fact that weight loss causes considerable amelioration in these conditions clearly shows that obesity plays a crucial role in their development (Bays, 2011; Vague, 1996).

Interestingly, a review of the literature shows that knowledge of the pathological potential of adipose tissue and the relationship between fat distribution and metabolic ill health has been acknowledged since the 1940s. Seminal work from Vague and

colleagues showed that there are ‘obesities which prevail on the upper half of the body’ and which are linked to hypertension and hyperglycemia and associated with adipose tissue hypertrophy and ‘obesities which are predominant on the lower half of the body’ and are not associated with hypertension or disturbances in glycoregulation. They also went on to explain that sexual differences in fat topography also exist, (which could be explained by the different influences of sex hormones) with android obesity in males exhibiting the former features and gynaecoid obesity in females relating to the latter (Bays, 2011; Vague, 1996).

Subsequently, in the decades that followed there was increased interest in adipose tissue embryology, anatomy and functionality and how these three components are interrelated and could potentially contribute to the onset of metabolic disease. It was noted that embryologically, adipocytes share a common genetic lineage with cardiomyocytes such that they both originate from a common pluripotent mesenchymal stem cell and that fat cell turnover is a dynamic process which is dependent on the balance between adipogenesis and apoptosis. While this might have potential therapeutic implications in future, (adipose tissue being a relatively accessible source of mesenchymal cells with propensity to differentiate into heart and blood vessel cells) and could well represent a possible treatment modality in CVD regenerative medicine, it also has other clinical implications (Bays, 2011). While previously adipogenesis was thought to be a static process which ceases in early life giving a ‘fixed adipocyte number’ to individuals, it is now known that fat-cell turnover is a dynamic process and adipogenesis occurs throughout adult life whereby the progenitor mesenchymal stem cell undergoes

lineage commitment, pre-adipocyte proliferation and differentiation into fully functional mature adipocytes (Roche, 1981).

Normally, when caloric intake exceeds energy expenditure, the excess energy is initially stored through adipocyte hypertrophy. This is then followed with an adipogenic period through the process of recruitment, proliferation and differentiation of preadipocytes leading to the creation of new and mature fat cells. However, during periods of persistent positive calorie balance, the energy supply might exceed the storage capacity of adipocytes and this process of adipogenesis may be disrupted propelling existing adipocytes to undergo excessive hypertrophy and an overall increase in fat cell size with resultant abnormal metabolic and immune responses that ultimately lead to metabolic consequences. This has notoriously been observed in studies involving obese patients with T2DM whereby decreased expression of adipogenic genes was noted with associated decreases in proliferation and differentiation of adipocytes (Bays et al., 2008; Dubois et al., 2006; Francisqueti et al., 2017; Yumuk et al., 2015).

The whole process of adipogenesis and the predilection toward either adipocyte hypertrophy or hyperplasia is undoubtedly dependent upon genetic and environmental predisposition together with the actions of multiple adipocyte or non-adipocyte regulatory factors and hormones which either promote or impair adipogenesis, with some of them also exerting different effects within the adipogenic process (for example impair proliferation and promote differentiation) (Bays et al., 2008; Smas and Sul, 1995). Of these, the most notorious adipogenic inhibitors include several interleukins (IL): IL-1, IL-6, IL-8 and IL-11, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α, hormones such as androgens, glucagon-like peptide-1 and

glucocorticoids as well as several growth factors (including fibroblast growth factor, transforming growth factor-ß, platelet-derived growth factor and tumour growth factor-ß). On the other hand, adipose and non-adipose tissue factors which facilitate adipogenesis include adiponectin, insulin-like growth factor-I (IGF), angiotensin converting enzyme, angiotensinogen, hormone sensitive lipase, autotaxin and free fatty acids, as well as hormones such as oestrogens, insulin, prolactin and thyroid hormone (Bays et al., 2008). Thus, any disruption at any point within the adipogenic process (recruitment, proliferation or differentiation) will lead to unhealthy / dysfunctional adipose tissue which is ultimately responsible for the downstream abnormal endocrine and immune responses which contribute to the onset of metabolic disease.

From a clinical point of view, whether fat is stored either by means of adipose tissue hyperplasia (healthy, smaller, functional adipose tissue) or hypertrophy (sick, bloated, unhealthy adipose tissue) in response to positive caloric balance can be translated (and may in part explain) to the paradoxical finding of metabolically healthy but obese individuals and conversely the metabolically unhealthy normal weight individuals. This is also implying that 'how' the fat is stored (hypertrophied adipocytes with production of 'sick fat') may be more important in terms of metabolic disease risk than simply the amount of fat stored (fat mass disease) (Bays, 2011; Bays et al., 2008; Haller et al., 1979).

As has already been alluded to previously, another important aspect in the pathogenesis of metabolic diseases depends on where the fat is being stored. It has been shown that the pathogenic potential of the fat depot depends also on its location. From an anatomical standpoint, individuals who store their fat preferentially in the visceral adipose tissue (VAT) (specifically intraperitoneal, extraperitoneal or intrapelvic) as

opposed to the subcutaneous adipose tissue (SAT) are more prone to develop metabolic disease (such as hypertension, dyslipidaemia and T2DM) than their counterparts with less visceral adiposity (the so-called the metabolically healthy obese). Similarly, at the other end of the spectrum are those patients who although have normal weight are metabolically obese (and thus exhibit metabolic disease) due to increased deposition of fat in the VAT (the metabolically unhealthy normal weight individuals). The reasons for these observations have been postulated by the inherent differences in intrinsic activities of VAT and SAT. In fact VAT is genetically predetermined to be metabolically more active then SAT and differs in the production of bioactive adipokines/cytokines and certain enzymes, hormones and immune molecules, expression and activity of various adipocyte receptors, and in enzymatic processes involving fat metabolism all of which eventually lead to abnormal downstream endocrine and immune processes which contribute to the onset of metabolic disease (Bays, 2014; Bays and Ballantyne, 2006; Tchkonia et al., 2013). A comparison of the different characteristics and function between VAT and SAT is summarized in **table 1.2.**

Furthermore, fat depots in areas other than the VAT or SAT such as in peri-organ or intra- organ regions (including pericardial, perimuscular, perivascular, orbital and paraosseal fat depots) have also been deemed to have pathogenic potential by virtue of abnormalities in metabolic and inflammatory processes and may well have an intrinsic activity somewhere in between that of peripheral SAT and VAT (Schäffler *et al.*, 2006; Bays *et al.*, 2008; Bays, 2011). Pericardial and perivascular adiposity has been shown to exert pathogenic effects on the myocardium in two manners, either via the secretion of vasoactive and pro-inflammatory factors or directly via an 'outside to inside' approach

(Baker et al., 2006; Bays, 2011). Fat deposition within the liver (intrahepatic) and muscle has been shown to contribute towards metabolic disease by way of adiposopathic fat accumulation which leads to abnormal release of inflammatory factors, increased lipolysis and circulating free fatty acids (FFA) causing lipotoxicity which ultimately leads to insulin insensitivity and IR. Similarly, fat accumulation within the pancreas also contributes to metabolic disease presumably due to beta cell dysfunction leading to decreased release of insulin and insulinopenia.

Thus, it can also be said that how adipose tissue interacts or 'cross talks' with other body organs also contributes to metabolic disease onset such that if an organ is inflexible towards storage of excess TG, then the resultant increase in FFA influx leads to a lipotoxic organ which promotes the onset of metabolic abnormalities (Bays et al., 2008; DeFronzo, 2010).

Another interesting observation is the sex differences on fat distribution. It is well-documented that when adjusted for age, males are overall at higher CVD risk than females. This is in accordance with the knowledge that males are more inclined to store fat in the visceral region - the so called 'android' (or upper body) adipose tissue distribution and conversely, females more often store fat in the peripheral subcutaneous region – the so called 'gynoid' (or lower body) adipose tissue distribution. The underlying reason for such differences in fat distribution is probably explained by the different effects of sex hormones on adipose tissue distribution. Androgens are thought to have an increased predilection of storing fat in the VAT and oestrogens in the peripheral SAT (Bays et al., 2008; Kirschner et al., 1990; Kitabchi and Buffington, 1994).

Table 1.1: Morbidities associated with Obesity

Organ/event	Comorbidity
Respiratory:	OSA, obesity hypoventilation syndrome, increased infections, asthma, hypoventilation, increased risk of pulmonary emboli
Cardiovascular:	Coronary artery disease, hypertension, obesity-associated cardiomyopathy, cor pulmonale, atherosclerosis, congestive heart failure, LVH, pulmonary hypertension
Gastrointestinal:	GERD, NAFLD, NASH, gall bladder disease, pancreatitis
Metabolic:	T2DM, metabolic syndrome, IR, dyslipidaemia
Reproduction:	Women: PCOS, anovulation, menstrual disorders, early puberty, hyperandrogenism and infertility Men: Hypogonadotropic hypogonadism, decreased libido and sexual dysfunction
Renal:	Functional hyperfiltration, albuminuria, glomerulomegaly and glomerulosclerosis, nephrolithiasis, poor renal graft survival, worse course of CKD regardless of primary renal disease
Obstetric and Perinatal:	Pregnancy-related hypertension, macrosomia, low birth weight, preterm births, increased caesarean delivery rates, GDM, pelvic dystocia
Neurological:	Stroke, ICH, dementia, meralgia paresthetica
Musculoskeletal:	OA, chronic lumbago, plantar fasciitis, coxa vara, immobility, gout, flat feet, falls
Genitourinary:	Stress incontinence
Skin and Integument:	Acanthosis nigricans, intertrigo, cellulitis, carbuncles, venous varicosities, venous stasis ulcers, venous and/or lymphatic oedema, psoriasis, hirsutism, stasis pigmentation of legs, panniculitis
Surgical:	Increased surgical risk and risk of post-operative complications (ex. pneumonia, DVT/ PE), wound infection and dehiscence
Malignancy:	Postmenopausal endometrial and breast cancer, ovarian, prostate, colon and rectum, gall bladder, oesophageal and pancreatic cancer
Psychological:	Depression/anxiety, personality disorder, social stigmatisation, body image disturbance

OSA; obstructive sleep apnoea, LVH; left ventricular hypertrophy, GERD; gastro oesophageal reflux disease, NAFLD; non-alcoholic fatty liver disease, NASH; non-alcoholic steatohepatitis, T2DM; type 2 diabetes mellitus, IR; insulin resistance, PCOS; polycystic ovary syndrome, GDM; gestational diabetes mellitus, ICH; idiopathic intracranial hypertension, OA; osteoarthritis, DVT; deep vein thrombosis, PE; pulmonary embolism

(Source: Kushner, Lawrence and Kumar, 2013; Fruh, 2017)

In addition to the type and location of fat storage another determinant of adipose tissue pathogenicity is its relationship with the extracellular matrix (ECM) and vascularity. During periods of excess cellular hypertrophy, fat accumulation may outgrow its vascular supply leading to a lack of blood flow resulting in adipose and peri-adipose (ECM) tissue hypoxia. This also leads to metabolic disease in view of organ dysfunction and ECM instability /remodelling dysfunction causing impaired fat storage leading to the increased release of free fatty acids and further lipotoxicity (Henegar et al., 2008).

The common pathway by which adipose tissue hypertrophy and VAT accumulation lead to adiposopathy and metabolic disease has been thought to rise from the abnormal handling of lipid metabolism. During periods of positive energy balance impaired storage of excess fat in the form of TG leads to increased adipocyte hydrolysis resulting in the net release of high levels of circulating FFA. Chronic and sustained increases in circulating FFA lead to 'lipotoxicity' which is deemed to be the contributing factor in the array of metabolic diseases seen in clinical practice. This 'lipotoxic' effect on peripheral tissues such as the liver (leading to hepatosteatosis), pancreas and muscle leads to IR and abnormal glucose metabolism shifting the concept of T2DM from a 'glucocentric' view to a 'lipocentric' view (Jensen, 2006; Bays *et al.*, 2008). FFAs, have also been implicated in the pathogenesis of hypertension, either due to their effect on IR or due to abnormalities in endothelial function. FFAs also lead to the typical dyslipidaemia found in the Met S (hypertriglyceridemia, low high-density lipoprotein-cholesterol and abnormalities in lipoprotein particle size) (Fagot-Campagna, 1998; Tchkonia et al., 2013; Yu and Ginsberg, 2005).

Finally, while VAT in conjunction with visceral fat hypertrophy has been implicated as the major contributor towards metabolic disease with SAT being 'protective', it can be noted that even excessive SAT may eventually become pathogenic through two different mechanisms. Firstly, while SAT through the process of adipogenesis is capable of storing excess fat in the form of small, functional adipocytes, this process may become overwhelmed leading to the formation of enlarged dysfunctional adipocytes which also contribute to development of metabolic disease. Secondly, although VAT is the major contributor of portal free fatty acids and thus metabolic disease, FFAs also originate from the SAT.

SAT represents approximately 80% of total body fat (in contrast to the 20% from VAT) and it accounts for the majority of the postabsorptive systemic free fatty acids. During periods of ever-increasing positive calorie balance there may be impaired storage of post-absorptive FFAs leading to increased delivery of FFAs to the portal system which then contributes to the lipotoxic effects on the liver. In addition, when SAT fat storage is impaired (due to dysfunctional subcutaneous fat) the resultant increase in net FFAs into the circulation can also adversely affect via lipotoxicity in other non-hepatic organs such as muscle and pancreas Thus it can be said that during periods of chronic positive calorie balance both visceral and subcutaneous adipose tissue have the potential to be pathogenic (Bays and Ballantyne, 2006; Jensen, 2006).

In summary, it is not simply the amount or the actions of adipose tissue that lead to adverse metabolic consequences but is rather the degree of dysfunction of body organs (liver, pancreas, muscle) as a result of potential pathogenic adipose tissue in the genetically and environmentally susceptible individual that will give rise to metabolic disease.

Table 1.2: Comparison of the characteristics and functions of SAT and VAT

Characteristic / Function	Adipose Tissue	
	Subcutaneous	Visceral
General Characteristics		
Fat amount relative to TBF	↑	↓
Metabolic activity	↓	↑
Adipogenesis	↑	↓
Apoptosis	↓	↑
Lipolysis/Lipogenesis		
Lipolysis inhibition by insulin	↑	↓
Lipolysis inhibition by prostaglandins	↑	↓
Lipolysis inhibition by adenosine	↑	↓
Portal vein drainage into liver	↓	↑
Stimulation of catecholamine mediated lipolysis	↓	↑
Storage of postprandial FFA	↑	↑
Increased FFA release into portal vein	↓	↑
Increased hepatic triglyceride and glucose production	↓	↑
Adipocyte receptors		
Lipolytic B-adrenergic receptors	↓	↑
Antilipolytic A 2-adrenergic receptors	↑	↓
Glucocorticoid receptors	↓	↑
PPAR -γ	↓	↑
Estogen receptors	↑	↓
Androgen receptors	↓	↓
Adipocyte factors		
Adiponectin	↓	↑
Leptin	↑	↓
IL-6	↓	↑
Plasminogen activator inhibitor-1	↓	↑
Cholesteryl ester transfer protein	↑	↓
UCP-1	↑	↓
UCP-2	↑	↓
IGF-1	↔	↔
IGF-1 binding protein	↔	↔
Angiotensinogen	↓	↑
Acylation stimulating protein	↓	↑
Monobutyrin	↔	↔
Retinol binding protein	↔	↔
Lipoprotein lipase	↔	↔
Hormone sensitive lipase	↑	↓
TNF-α	↓	↑

SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue, TBF: total body fat, UCP: uncoupling protein, TNF-α: tumour necrosis factor, IGF-1: insulin like growth factor -1, IL-6: interleukin-6, PPAR-γ: peroxisome proliferators-activated receptor-γ, FFA: free fatty acid
(Source: Bays *et al.*, 2008)

Excess adiposity is now also known to be a major determinant in the increasing incidence and prevalence of certain cancers and is also thought to surpass that of smoking as a preventable cause of cancer (Avgerinos et al., 2019; Ligibel et al., 2014). Overweight and obesity have been linked to an increase in cancer risk in at least 13 anatomical sites within the body, including cancers of the gastrointestinal tract (colon, rectum, gastric cardia, gall bladder, oesophageal, pancreas and liver), uterine, ovarian, and postmenopausal breast cancer in females, prostate in males and some haematopoetic cancers such as multiple myeloma and certain lymphomas (Avgerinos et al., 2019; Marseglia et al., 2014; Upadhyay et al., 2018). Data from the WHO International Agency for Research on Cancer estimated that excess body weight and inactivity accounted for at least a quarter of all cancers of the breast, endometrium, colon and oesophagus (Bianchini et al., 2002; Malnick and Knobler, 2006). Epidemiological studies have also shown that incremental increases in BMI values are associated with a parallel increase in cancer risk so much so that a BMI > 40kg/m^2 is associated with a 70% increase in lifetime risk of malignancy (Upadhyay et al., 2018). The same can be said for cancer mortality whereby a study by Calle *et al* found that mortality rate was increased by 52% in males with obesity and even more in females (62%) when compared to normal weight individuals (Calle et al., 2003). These data have all been confirmed by a number of longitudinal studies as well as meta-analyses and systematic reviews (Ahlgren et al., 2006; Avgerinos et al., 2019; Calle et al., 2003; Colditz et al., 1995). The meta-analysis by Renehan *et al.,* also showed significant sex differences particularly in relation to colon cancer which has an increased prevalence in males presumably due to the fact then males are more prone to visceral (central) adiposity then females, which is in turn

associated with IR and hyperinsulinaemia and that endogenous oestrogens in females may have a protective effect (Renehan et al., 2008b).

Interestingly, some studies found the association between cancer risk and obesity to be greater in non-smokers, implying that there might be an interaction between smoking and BMI (Renehan et al., 2008b). Furthermore, in a paradoxical manner, emerging epidemiological data are showing that obesity may actually be a protective factor for the incidence of certain cancer types. This was particularly observed in cancers of the lung (non-small cell lung cancer), head and neck cancers and premenopausal breast cancer in females. Again, this phenomenon has been termed the 'obesity paradox' similar to that encountered in cardiovascular and renal studies. However, there are a lot of putative explanations for such findings, the most plausible being inadequate adjustment for confounding bias especially for tobacco smoking. Smoking is known to be associated with lung cancer and reduced weight and thus is likely to account for this observed inverse relationship. Also, cancer cachexia and resultant weight loss may confound BMI at diagnosis of cancer. Another mechanism may be that of competing mortality risks (Avgerinos et al., 2019; Renehan et al., 2008a).

It is also important to note that while adult obesity is usually associated with cancer risk, it is not the sole driver for the increased risk. Research has shown that excess body weight during childhood and adolescence has been associated with increased risk of many cancers linked to adult weight such as pancreatic cancer (independent of diabetes), colon cancer in females and multiple myeloma (Avgerinos et al., 2019). Furthermore, they were associated with a younger age of onset and a lower survival rate overall. However, one key exception has been observed with respect to breast cancer

risk. It was noted that childhood adiposity was inversely related to the risk of pre- and post-menopausal breast cancer even after adjusting for adult weight gain. The reasons for this relationship are still unclear, however these observations should not allow for the adverse effects of childhood obesity to be underestimated especially in an era where the incidence and prevalence of childhood obesity is on the rise but should rather help in identifying pathways linking childhood exposures to breast cancer risk (Ahlgren et al., 2006; Renehan et al., 2008a).

While the exact role of obesity in cancer aetiopathogenesis, prognosis and survival is not fully understood for all different types of cancers, several putative factors have been cited as potential underlying pathophysiological mechanisms (Colditz and Peterson, 2018). These pathways generally involve similar mechanisms with those linked to the onset of metabolic and CVD . As described previously, adipose tissue is an active endocrine and metabolic organ and during periods of chronic positive energy balance releases FFAs and other pro-inflammatory factors and hormones which promote IR and also cause alteration in metabolism of sex steroids (oestrogens, androgens and progesterone) which seem to be at the forefront for the onset of many of the malignancies associated with obesity (Bianchini et al., 2002; Renehan et al., 2008a). Thus it can be summarized that the major pathways linking obesity to cancer comprise 1) underlying genetic factors, 2) alteration in adipokines pathophysiology and secretion, 3) chronic low grade inflammation and oxidative stress leading to increased levels of reactive oxygen species and oxidative DNA damage, 4) abnormalities in insulin/IGF-1 signalling, 5) factors derived from ectopic distribution of fat, 6) abnormalities in sex hormone biosynthesis and pathways and possibly also the role of certain dietary

nutrients, an alteration in gut microbiota, disrupted circadian rhythms, and the mechanical factors associated with excess adiposity (Avgerinos et al., 2019; Ligibel et al., 2014).

Genetic factors determine the variation in BMI and ectopic fat deposition (ex. intrahepatic and intramyocellular) which may cause alterations in deoxyribonucleic acid (DNA) repair and gene function through multiple channels involving metabolic, inflammatory or immunologic pathways leading to epigenetic changes which permit malignant transformation (Marseglia et al., 2014).

The three most studied candidate systems with respect to cancer risk are perhaps the insulin-cancer hypothesis, the role of sex steroids and alterations in adipocytokine pathophysiology and shall be discussed here.

Over a decade ago two scholars noted that the risk factors for westernized cancers were similar to those predisposing IR with the major underlying driving force being hyperinsulinaemia. Chronic elevation of serum levels of insulin (which has a growth-promoting effect) has been linked to increased levels and activity of insulin-like growth factor-1 (IGF-1) (a peptide hormone with a similar structure to insulin and regulates cellular proliferation) predominantly in the liver (which is the main source of circulating IGF-1) with concomitant reductions in insulin-like growth factor binding proteins (IGFBP-1, IGFBP-6) (which bind IGF-1 and dampen its action). Such elevations in free IGF-1 and insulin levels are known to cause changes in the cellular environment promoting cell cycle progression, inhibition of apoptosis and tumorigenesis (Avgerinos et al., 2019; Giovannucci, 2003; Renehan et al., 2006). Translating this into clinical practice, T2DM (a

metabolic disorder characterized by IR and hyperinsulinaemia in its early stages) has been consistently shown to increase the risk of pancreatic, kidney, colon and endometrial cancer in females independently of obesity and in individuals with T2DM there was a greater cancer mortality rate when compared to non-diabetic controls (Gallagher et al., 1996; Larsson et al., 2005). Furthermore, measurements of surrogate markers of IR (for example by HOMA [homeostasis model assessment] and C-peptide respectively as a measure of hyperinsulinaemia in lieu of serum insulin (which can be more daunting to measure on its own) have also been strongly linked to colorectal cancer in several epidemiological studies (Avgerinos et al., 2019; Calle et al., 2003; Kaaks et al., 2002). Moreover, one case-control study by Yang *et al* in 2004 fuelled a lot of controversies with regards to cancer screening in individuals with T2DM when the results showed that insulin therapy was associated with increased incidence of colorectal adenoma risk. However, it is also important to note that these associations did not reflect causality (Gallagher and LeRoith, 2013; Yang et al., 2004). Conversely patients who are treated with the insulin sensitizing drug metformin have a lower risk of cancer incidence and cancer-related mortality. These observations are further consolidated in studies involving caloric restriction and weight loss whereby concomitant reductions in circulating insulin and IGF-1 levels have consistently shown suppressed cancer incidence rates (Hursting et al., 2010; Vucenik and Stains, 2012).

The molecular mechanisms by which insulin and IGF-1 are presumed to promote carcinogenesis is due to activation of two major pathways implicated in tumourigensis. These are the phosphatidylinositol 3-kinase (PI3K) - AKT-mammalian target of rapamycin (mTOR) pathway and the Ras-Raf-MEK-Mitogen activated Protein Kinase (MAPK)

pathway which are involved in regulating cell growth, differentiation, and proliferation. It is thus thought that insulin or IGF-1 activates the insulin receptor or IGF-1R (both of which have tyrosine kinase activity), which in turn off sets the PI3K pathway culminating in mTOR activation leading to mitogenic and antiapoptoic effects (Hursting et al., 2010; Renehan et al., 2008a; Yang et al., 2004). It is important to note that the MAPK pathway is usually largely unaffected by IR and therefore becomes hyperactive in hyperinsulinaemic states thereby driving cell growth and proliferation.

Another pathway by which obesity can cause increased risk of certain malignancies is through alterations in the metabolism of sex steroids. Peripheral adipose tissue is also known to influence synthesis and bioavailability of sex hormones (notably oestrogen) through at least three mechanisms (Calle et al., 2003). Firstly, adipose tissue is known to be responsible for a process called steroid aromatization. Essentially this involves converting androgens and androgenic precursors to oestradiol by means of the aromatase enzyme. In obese states, the excess adipose tissue leads to increased levels of aromatase activity which in turn leads to higher conversion rates of androgens to oestrogens which could be the driving force for the increased incidence of postmenopausal breast cancer and uterine cancer seen in females with obesity (Avgerinos et al., 2019; Crosbie et al., 2010; Renehan et al., 2008a). Second, excess adiposity is associated with high levels of insulin and IGF-1 which cause a reduction in hepatic synthesis of sex hormone binding globulin (SHBG) which is the major carrier protein for testosterone and oestrogen in the plasma. This consequently leads to a higher amount of unbound (free) sex-steroids available for bioactivity. In both sexes this translates into higher levels of active oestrogen and in females it also leads to increased

levels of bioavailable testosterone. The opposite is seen however in males, whereby decreases in SHBG lead to decreased levels of bioavailable testosterone, the reason for this is probably strong oestradiol-mediated reduction in gonadotropic stimulation of testosterone production from the testicles (Kokkoris and Pi-Sunyer, 2003; Pugeat et al., 1991). Lastly, hyperinsulinaemia has been postulated as being one of the underlying causes of the polycystic ovary syndrome. Here, high insulin levels promote the formation of ovarian and adrenal androgens (such as dehydroepiandrosterone or its sulphate [DHEA/ DHEAs] and androstenedione) which causes clinical features of hyperandrogenism, anovulation and progesterone deficiency (Colditz and Peterson, 2018; Dunaif, 1997).

There are a number of experimental studies from in vitro and animal models which show that oestrogens have a mitogenic effect on mammary tissues (Renehan et al., 2008a; Travis and Key, 2003). Notably in human epidemiological studies there is sufficient association to explain the correlation between anthropometric indices of excess weight (such as BMI) and breast cancer to the circulation levels of sex steroids (Calle et al., 2003; Key et al., 2003). Both oestrogen and the oestrogen receptor have been implicated as mutagens in the initiation of breast cancer either directly or indirectly through free-radical mediated DNA damage and genetic instability as well as cell mutations. Oestrogen-related pathways (supporting the 'unopposed oestrogen hypothesis') have now been established as the most important driving forces for breast cancer in postmenopausal females. The Endogenous Hormones and Breast Cancer Collaborative Group (EHBCCG), which is a pooled analysis of nine prospective studies, showed that risk of postmenopausal breast cancer increased with increasing circulating levels of sex

steroids further consolidating the hypothesis that the association of BMI to breast cancer risk is driven by the increased circulating levels of oestrogen with rising BMI values (Renehan et al., 2008a;2008b).

With respect to androgens, there have been some conflicting data regarding breast cancer risk. Generally, obese females have been associated with raised blood levels of circulating testosterone and some studies have shown an association with increased risk of breast cancer in females regardless of menopausal status fuelling other potential mechanisms by which obesity is associated to breast cancer. However, in animal studies testosterone exerts both inhibitory and stimulatory effects on mammary epithelia leading to inconclusive results as to the effect of testosterone on mammary tissue (Liao and Dickson, 2002; Renehan et al., 2008a).

In a similar fashion to breast cancer risk, the underlying factors driving endometrial cancer are probably also related to pathways associated with a greater life-time exposure to oestrogens thus also supporting the 'unopposed oestrogen hypothesis' for endometrial cancer (Calle et al., 2003). Similar to postmenopausal breast cancer risk, higher oestrogen levels in obese females have been linked to a 2.6-fold increase in risk of postmenopausal endometrial cancer (Avgerinos et al., 2019; Shaw et al., 2016). During the follicular phase of a normal menstrual cycle, there is increased production of unopposed oestrogen from the ovaries leading to endometrial epithelial tissue and stromal fibroblasts to proliferate. This process of proliferation continues till ovulation where oestrogen levels reach a peak. Thereafter, in the luteal phase oestrogen levels and actions start to decline due to the concomitant increase in levels of progesterone which mitigate the proliferative actions of oestrogen. Thus, increased and unopposed

oestrogen levels in obese postmenopausal females have been linked to increased cellular proliferation and inhibition of apoptosis of uterine cells. Moreover, the proliferative actions of oestrogen are probably also mediated by increased production of uterine IGF-1 which then acts on the endometrium in a paracrine fashion, with progesterone on the other hand opposing the effects of oestrogen by increasing production of IGFBP-1 which leads to reduced IGF-1 levels (Giudice et al., 2013; Kaaks et al., 2002; Renehan et al., 2008a).

Another postulated mechanism linking obesity to endometrial neoplasia is through hyper-secretion of ovarian androgens such as androstenedione and testosterone. Clinically this is seen in pre- and post-menopausal females with polycystic ovary syndrome (PCOS) and obesity possibly due to a number of overlapping pathways involving oestrogen and androgen hyper-secretion, raised IGF-1 levels and chronic hyperinsulinemia (Calle and Kaaks, 2004). The polycystic ovary syndrome is characterized by a triad of anatomic, clinical, and metabolic manifestations. The anatomic aberrations include the presence of polycystic ovaries usually defined as having more than 12 follicles per ovary (even though they need not be present to establish a diagnosis of PCOS). Clinically, females may present with oligo- or amenorrhea and cutaneous manifestations of hyperandrogenaemia such as hirsutism, acne, and male pattern hair-loss. Biochemical aberrations include raised androgen levels (including serum free testosterone, free androgen index and DHEA levels), IR and sustained elevations in serum oestrogen levels. Moreover, a substantial proportion of females with PCOS are overweight or obese with increased predilection towards visceral adiposity and exhibit acanthosis nigricans (the cutaneous manifestation of IR), have

impaired glucose tolerance and may also display features of the Met S thus making them at increased risk of CVD (Chittenden et al., 2009; Rosenfield and Ehrmann, 2016). This condition has also been associated with increased risk of some gynaecological cancers. One of the causes for this increased risk is due to the presence of anovulatory cycles which lead to chronic exposure of unopposed oestrogen (the unopposed oestrogen hypothesis) which is known to induce endometrial hyperplasia and cancer formation. Further to this, females with PCOS also carry other known risk factors for endometrial cancer including obesity, T2DM and nulliparity which together with chronic elevations in serum oestrogen levels and disturbances in concentrations of other steroid hormones, could increase the risk of uterine cancer and also of other hormone sensitive tumours especially of the breast and ovary. One meta-analysis recently reported a threefold increased risk for endometrial cancer in females with PCOS than in those without the condition, whereas results from a single study showed that females with PCOS were twice as likely to develop ovarian cancer but with regards to breast cancer there did not seem to be any increase in risk in females with PCOS (Chittenden et al., 2009).

White adipose tissue (WAT) which is the major component of adipose tissue is known to be a highly active metabolic and endocrine organ with the production of in excess of 50 different types of adipokines (polypeptide hormones), some of which allow for the adipocyte to communicate with satiety centres in the hypothalamus and thus have an important role in the feedback regulation of appetite and energy expenditure and in the development of IR and atherogenic processes (Fischer-Posovszky et al., 2007; Renehan et al., 2008a). Among the most studied are leptin, adiponectin, resistin, retinol-binding

protein 4 (RBP4) and visfatin (MacDougald and Burant, 2007). Of these, leptin and adiponectin are the most abundantly produced adipokines and their blood levels reflect the amount and distribution of adipose tissue within the body. They are also the most studied adipokines with respect to cancer development. In obesity, hypoxia of the adipose tissue results in a chronic inflammatory state which triggers alterations of normal leptin and adiponectin levels and together with other changes such as infiltration of macrophages and mitochondrial dysfunction may be associated with promotion of certain cancers such as colorectal cancer in obese individuals (Avgerinos et al., 2019; Spyrou et al., 2018).

Leptin is a 167 amino acid product of the *ob* gene. Leptin interacts with both orexigenic and anorexigenic pathways in the central nervous system (CNS) to modulate food intake and energy expenditure. Systemic leptin concentrations are proportional to the degree of body fat stores and its principal role is thought to be that of defending minimum body weight such that low leptin levels are associated with increased energy intake and storage; however, in obese states when there is a surplus of fat mass further rises in serum leptin have a limited ability to suppress food intake suggesting there might be a degree of leptin resistance or relative leptin deficiency. Leptin is also a highly pleiotropic hormone and has been implicated in the onset of various cancers. It has mitogenic properties on various cell types such as haematopoietic progenitor cells and vascular endothelial cells and may also exert anti-apoptoic effects. It is also a potent pro-inflammatory agent and has been associated with upregulation of aromatase enzyme activity favouring increased production of oestrogen and increased breast cancer risk. There are several forms of leptin receptors of which one (the long form [LRb]) activates

the PI3 kinase and MAPK signalling pathways which are involved in cellular proliferation and differentiation. In breast cancer there is increased leptin receptor expression especially in oestrogen receptor (ER) positive breast cancer cells which probably implies that the effect is primarily mediated through ER actions (Rose et al., 2004; Vona-Davis and Rose, 2007).

Adiponectin is a 247 amino acid peptide and is secreted mainly from visceral adipose tissue and is the most abundant protein hormone within the adipocyte. Unlike leptin it is produced from mature adipocytes and has anti-proliferative, pro-apoptotic and anti-angiogenic properties as well as having insulin-sensitizing and antiatherogenic actions (Fasshauer and Paschke, 2003; Goldfine and Kahn, 2003). Contrary to leptin, serum levels of adiponectin are negatively correlated with BMI and tissue hypoxia; it has been shown that serum levels of this hormone correlate strongly with insulin sensitivity such that in obese mice and human individuals, levels of adiponectin are markedly decreased. In fact, in experiments involving adiponectin-deficient mice, it was noted that they are both insulin-resistant and prone to development of diabetes but when administered a recombinant form of adiponectin there is marked improvement in insulin sensitivity with concomitant improvements in plasma FFA and triglyceride content and also exhibited modest weight loss thus consolidating its fundamental role in glucose homeostasis and IR (Goldfine and Kahn, 2003; Maeda et al., 2002; Vasseur et al., 2003). In contrast to leptin, several epidemiological studies show an inverse association between circulating levels of this hormone and risk of cancer especially with respect to uterine and breast cancer risk in females (Petridou et al., 2003). While the exact mechanism of adiponectin in tumour inhibition is not fully understood, one

potential pathway is thought to be through inactivation of MAPK pathways and several murine studies confirm its inhibitory effect on primary tumour growth. Thus, in summary adiponectin is a key regulator of glucose and lipid metabolism and its levels are regulated by the degree of obesity and IR and may have an important component in the link between obesity and tumour development (Zhang et al., 2015).

Other potential mechanisms by which obesity induces tumorigenesis worth mentioning include disruption of circadian rhythms such as reduced quantity and quality of sleep results in altered glucose regulation and energy balance increasing the risk for obesity and malignancy due to metabolic and obesity-related factors (Nedeltcheva and Scheer, 2014). Furthermore, obesity itself is also associated with disturbed sleep patterns, which in turn has also been linked to increased risk for certain cancers thus showing a bidirectional relationship between obesity and sleep disorders (Cao et al., 2019; Kakizaki et al., 2008a; Kakizaki et al., 2008b).

Recently much interest has been shown in the role the gut microbiome plays in onset of obesity and cancer. The human gut microbiome consists of four phyla: Bacteriodetes and Probacteria which are Gram negative and Acenetobacteria and Firmicutes which are Gram positive (Avgerinos et al., 2019; Nam et al., 2011). Their prevalence within the gut is determined by BMI, diet and other environmental factors. They also exert an important role in the intestinal metabolism and absorption of ingested nutrients and thus participate in the pathogenesis of certain metabolic diseases such as T2DM and CVD as well as obesity and cancer (Carvalho and Saad, 2013). It has been thought that alteration in the gut microbiome can give rise to obese states and this is reflected in diet

induced obese mice such that when their gut microflora was transferred to normal weight mice these acquired the phenotype of the former mice (Turnbaugh et al., 2006).

The proposed mechanisms linking gut microbiota to obesity and cancer can be either due to promotion of inflammation or production of cancer-promoting substances. A process called endotoxinemia (which implies leakage of bacterial derived substances into the blood stream) is currently considered one of the pivotal mechanisms for the initiation of inflammation (Cani and Jordan, 2018). An example of how gut microbacteria induces inflammation and tumourigensis is derived from studies of patients with hepatocellular carcinoma (HCC) (White et al., 2012). Altered intestinal micro flora and gut barrier dysfunction are the main triggers for onset of non-alcoholic steato-hepatits (NASH) which in turn lead to downstream activation of pro-inflammatory pathways which up-regulate myogenic factors thought to be central to the onset of liver cancer pathophysiology. The second mechanism by which gut microbiota causes cancer promotion is from the generation of toxic metabolites. Obese states with a high fat diet are known to have changes in intestinal microbiome leading to altered bile acid metabolism and increased production of a toxic substance called deoxycholic acid (DCA). DCA causes DNA damage through formation of reactive oxygen species creating a cancer promoting environment favouring the onset of HCC as well as colorectal cancer (Ma et al., 2022; Payne et al., 2007).

Thus, accumulating epidemiological data continues to show that excess body weight is a key factor in the increased risk and prognosis of several common adult cancers. In an era where the global burden of obesity is starting from a young age, the impact of weight on cancer risk is undoubtedly underestimated in current literature in view of the fact

that data comes from cohorts of adult-onset obesity. However, increasing evidence also shows that weight loss through either caloric restriction or increased physical activity improves a number of chronic non-communicable disease including CVD and diabetes. Similarly, preventive measures based on lifestyle modification (such as incorporating hypocaloric or ketogenic diets) have also shown to be important for cancer prevention (Avgerinos et al., 2019).

Lastly bariatric surgery, which is proving to be the most significant modality to cause long-term weight loss and resolution of certain comorbidities, has also been shown to reduce the incidence of many cancers especially that of the breast and endometrium in females (Adams et al., 2017).

In conclusion, there is now strong evidence linking obesity-driven chronic inflammation, IR, disrupted adipokines function and altered gut microbiome with cancer and that reversing such processes with lifestyle intervention or bariatric surgery could be of public health relevance with respect to cancer risk.

1-5 Metabolically Healthy Obesity (MHO) and Metabolically Unhealthy Normal Weight (MUHNW)

As discussed previously, obesity is conducive to the development of several metabolic abnormalities including hyperinsulinemia and IR, dysglycaemia, hypertension, and dyslipidaemia, which in turn put an individual at higher risk of T2 DM, CVD and mortality. However, it has also become increasingly recognised that obesity is a heterogenous disease due to interindividual variability in body composition (fat mass vs fat-free mass), adipose tissue distribution (central/visceral vs peripheral/subcutaneous) and adipocyte function, metabolic profile and the degree of cardiometabolic risk (Neeland et al., 2018). Attesting to this are a subset of individuals with obesity who, despite having excessive amounts of body fat, appear to be resilient to the development of these metabolic abnormalities and are thus termed metabolically healthy obese (MHO) (Blüher, 2020; Karelis, 2008; Phillips, 2013a; Sims, 2001; Stefan et al., 2013). On the other hand, since the early 1980s, Ruderman *et al*, and others thereafter, described the occurrence of individuals who, despite a normal BMI, exhibited increased visceral adiposity, were hyperinsulinemic and insulin resistant and displayed an abnormal metabolic profile similar to that found in individuals living with obesity, thereby rendering them at a higher risk for all-cause mortality and cardiovascular events (Conus et al., 2007; Ding et al., 2016; Dvorak et al., 1999; Klitgaard et al., 2020; Ruderman et al., 1998; St-Onge et al., 2004). These individuals are described as metabolically unhealthy normal weight (MUHNW) or metabolically obese normal weight (MONW) individuals and thus support the presence of a 'lipodystrophy-like' phenotype in the general population (Stefan et al., 2017). Therefore, for a given BMI, individuals exhibit a variability in metabolic risk since

the pathogenic potential of excess adipose tissue does not rely on increased fat mass alone but also on its distribution and function. This has led to the concept of different body composition phenotypes which take into account both the individuals' body size as well as their metabolic profile such that metabolically healthy normal weight (MHNW) individuals are at one end of the spectrum and metabolically unhealthy obese (MUHO) individuals at the other end, with MUHNW and MHO lying somewhere in between (Blundell et al., 2014). An increased understanding of the aetiopathogenesis of these different obesity phenotypes has provided insight into the presence of risk factors which are independent of adiposity-induced abnormalities (as occurs in MHO) and also risk factors which are essentially independent of overall obesity (as occurs in MUHNW).

1-5.1 Prevalence and definition of MHO and MUHNW

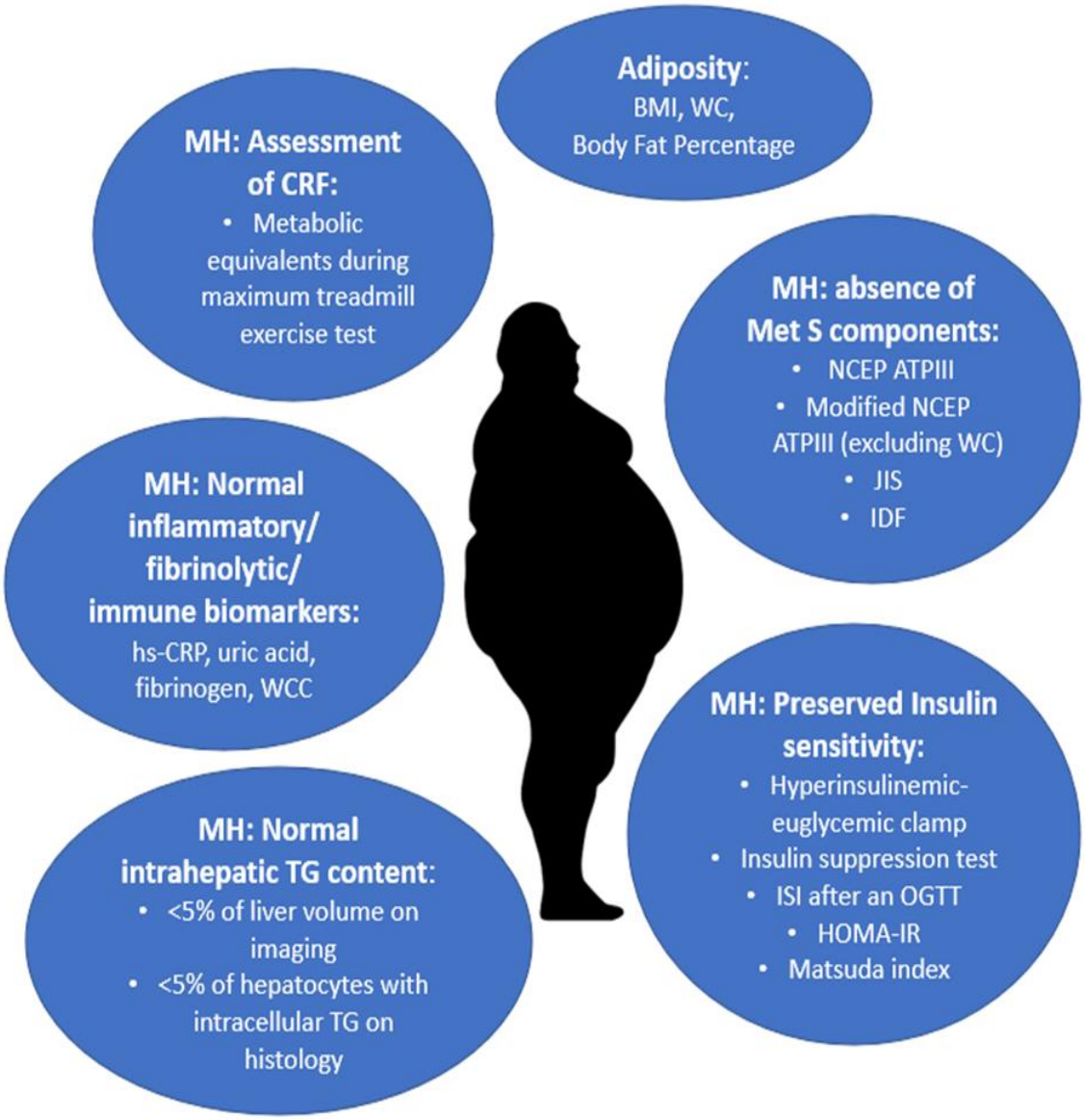
Although both MUHNW and MHO have been extensively studied and documented worldwide and in different ethnic populations for over four decades, there is still a considerable amount of conflicting and incongruent data surrounding these two phenotypes particularly regarding their prevalence, aetiopathogenesis and long-term health implications which led some authors to question the very existence of these phenotypes (Lopez-Miranda and Perez-Martinez, 2013; Muñoz-Garach et al., 2016; Smith et al., 2019). The major reason for such world-wide discrepancies stems from the fact that to date there is yet no universally standardized definition of what constitutes metabolic health (Brandão et al., 2020; Tsatsoulis and Paschou, 2020). In fact, different studies use different criteria for assessing obesity and metabolic health (both in terms of choice of risk factor parameters and their respective cut-off values). For example, most studies use a BMI of <25 kg/m^2 to denote normal weight and a BMI of ≥25 kg/m^2

as the threshold to denote overweight/obesity. However, other studies use WC (with sex-specific and ethnic-specific cut-offs) or body fat percentage (BF%) as assessed either by Dual Energy Absorptiometry (DXA), bioelectric impedance, skin fold thickness or by hydrostatic weighing as well as visceral fat area to assess adiposity levels. This consequently leads to marked differences in both obesity and in MHO/MUHNW prevalences within and between different populations (Choi et al., 2012; De Lorenzo et al., 2007; Hyun et al., 2008; Ortega et al., 2013; Romero-Corral et al., 2010; Shea et al., 2012, 2011). For example, the study by Ortega *et al.,* found that the overall prevalence of obesity within the same population was much lower when using standard BMI criteria compared to BF% (using cut-offs of ≥25% for males and ≥30% for females) subsequently leading to a marked difference in the prevalence of MHO (30.8% and 46.3% respectively) (Ortega et al., 2013).

With respect to metabolic health, the most frequently used definitions in the literature are based either on Met S criteria (mainly as defined by WHO (World Health Organization, 1999), the European Group for the study of IR [EGIR] (Balkau et al., 2002), the National Cholesterol Education Program, [NCEP] Adult Treatment Panel III [ATPIII] (NCEP, 2001), the American Association of Clinical Endocrinologists [AACE] (Einhorn et al., 2003), the International Diabetes Federation [IDF] (Alberti et al., 2006) or by the Joint Interim Statement [JIS] (Alberti et al., 2009)) **(appendix 2)**, by measuring IR or a combination of both (Durward et al., 2012; Karelis et al., 2004a; Kuk and Ardern, 2009; Meigs et al., 2006; Wildman et al., 2008). A review of the literature also confirms different approaches to measuring IR in published studies including; a) the hyperinsulinemic-euglycemic clamp, b) insulin sensitivity index (ISI) following an oral

glucose tolerance test, c) the insulin suppression test, and d) the derived indices: the Matsuda index and the homeostatic model assessment of IR [HOMA-IR]); with different studies also citing different (and sometimes arbitrary) cut-off values for each of these methods (Appleton et al., 2013; Brochu et al., 2001; Calori et al., 2011; Klöting et al., 2010a; Tracey McLaughlin et al., 2007; Messier et al., 2010; Sesti et al., 2011; Stefan et al., 2008b; Wang et al., 2021a; Wildman et al., 2008, 2008). Additionally, other authors also took into consideration inflammatory, fibrinolytic, and immune function abnormalities (such as circulating levels of hs-CRP, white blood cell count, fibrinogen and uric acid levels) to differentiate the healthy from the unhealthy phenotype while others also included assessment of cardiorespiratory fitness (Hamer and Stamatakis, 2012; Iacobellis et al., 2005; Karelis and Rabasa-Lhoret, 2008; McAuley et al., 2010; Wildman et al., 2008) **(Figure 1.1).**

Generally, MHO individuals exhibit a better metabolic and biochemical profile compared to their unhealthy counterparts, including a favourable lipid profile (higher HDL-C, lower total cholesterol, low density lipoprotein-cholesterol [LDL-C], TG and apolipoprotein B levels as well as a lower TG:HDL-C ratio), lower levels of glucose and insulin and lower systolic and diastolic blood pressure values thus allowing several authors to use these parameters when classifying MHO individuals (Iacobini et al., 2019; Karelis et al., 2004b; Phillips, 2013a). Additionally, studies also report improved renal function, lower levels of hepatic enzymes and a favourable adipokine profile (characterised by a high adiponectin and low leptin levels) in MHO individuals compared to MUHO (Messier et al., 2010; Sesti et al., 2011).



BMI: body mass index, WC: waist circumference, MH: metabolic health, Met S: metabolic syndrome, NCEP ATPIII: national cholesterol education program adult treatment panel III; JIS: joint interim statement, IDF: international diabetes federation, ISI: insulin sensitivity index, OGTT: oral glucose tolerance test, HOMA-IR: homeostasis model assessment of insulin resistance, TG: triglycerides, hs-CRP: high sensitivity - C-reactive protein. WCC: white cell count, CRF: cardiorespiratory fitness

Figure 1.1 Different criteria used to define adiposity and metabolic health in the literature

Hormonal differences within the entero-insular axis following a glucose load (reduced glucose-dependent insulinotropic polypeptide (GIP) and glucagon plasma levels and higher levels of glucagon-like peptide-1 (GLP-1)) have also been reported in MHO individuals (Calanna et al., 2013). Such findings may in part explain the lower propensity for T2DM observed in MHO individuals. Yet, other investigators also observed that adherence to a Western dietary pattern is predictive of the MUHO phenotype and IR (Mirzababaei et al., 2019). However, these parameters are not used in current definitions of metabolic health.

There are wide variations in the reported prevalence of body size phenotypes. Overall, the global prevalence estimates quoted in the literature stand at 35% for MHO among individuals living with obesity and approximately 30% for MUHNW among normal weight individuals (Lin et al., 2017; Wang et al., 2015). As explained above, these statistics are partly definition-dependent and may merely reflect the *a priori* criteria used to define metabolic health. For example in the US National Health and Nutrition Examination Surveys (NHANES) study (which included 5440 US civilians over the age of 20), the authors found that 31.7% and 51.3% of obese and overweight individuals respectively to be metabolically healthy when metabolic health was defined by the presence of ≤1 cardiometabolic abnormalities from the following: elevated BP, elevated serum TG levels, high fasting plasma glucose, elevated hs-CRP and presence of IR as defined by HOMA-IR. Furthermore 23.5% of normal weight individuals carried an unhealthy metabolic phenotype while utilising the same criteria to classify metabolic health (Wildman et al., 2008). As expected, when the same authors used more stringent criteria (i.e. the presence of no cardiometabolic abnormalities) to define metabolic

health, only 16.6% of obese individuals could be classified as metabolically healthy. Moreover, when using sex-specific WC cut-offs to denote obesity, a higher percentage of non-abdominally obese individuals were metabolically unhealthy (28.3%), while a lower percentage of individuals with abdominal obesity could be defined as metabolically healthy (36.4%).

Such discrepancies were also corroborated in a study coming out of China, which reported a 3-fold range in the prevalence of MHO when different definitions for metabolic health were applied to the same population (Liu et al., 2019). Furthermore, the systematic review by Rey-Lopez *et al.,* which evaluated 27 prospective studies worldwide (including studies form Europe, Oceania, North America and Asia) found that the prevalence rate of MHO ranged from 6% to 75% and identified 30 different definitions of metabolic health (Rey-López et al., 2014). These findings thus keep highlighting that prevalence estimates for each body composition phenotype are definition dependent. Typically, the MHO phenotype is more prevalent in females, younger aged individuals and in non-Hispanic whites while the prevalence of MUHNW is higher in males and increases with age (Lin et al., 2017; Rey-López et al., 2014; Wang et al., 2015; Wildman et al., 2008).

These studies also underscore that biological, genetic, ethnic, and secular lifestyle changes could all also be accountable for differences in the prevalence of body composition phenotypes reported in the literature. Accordingly, many studies concur that MHO status is inversely related to age. Increasing age is associated with decreased muscle mass, increased percentage body fat and a predilection to visceral fat deposition (Gallagher et al., 1996; Kuk et al., 2009). Females are also consistently found to have a

higher MHO prevalence. This is in agreement with previous studies (as explained earlier in this chapter) which observed females to have favourable body fat distribution patterns (lower visceral adipose tissue and higher subcutaneous gluteofemoral fat deposition) compared to males by virtue of the different predilection of the sex hormones on adipose tissue deposition, which renders them at lower cardiometabolic risk (Bays et al., 2008; Vague, 1996). However, the finding of a higher prevalence of MHO in Asian individuals as per the systematic review by Rey-Lopez *et al.* is surprising since Asians have an increased tendency to accumulate fat in visceral and ectopic areas which is conducive to higher metabolic risk compared to other ethnicities (Lee, 2009). Furthermore, factors such as psychosocial stress, population differences in dietary habits, other behavioural variables (including smoking, alcohol consumption and physical activity), and genetic differences affecting body fat distribution undoubtedly also contribute to the variability in prevalence rates of the MHO phenotypes. In 2018, Carl Lavie *et al.* attempted to standardize the concept and definition of MHO by proposing a 'harmonized definition'. They suggested that individuals be classified as MHO if they are obese by BMI criteria and meet zero out of four Met-S criteria (elevated TG, reduced HDL-C, elevated BP and elevated fasting glucose but excluding WC). There was an emphasis on the fact that an individual must exhibit *none* of the latter parameters as they argued that 'a person with high blood pressure or T2 diabetes cannot be considered 'healthy' (Lavie et al., 2018). More recently, Zembic et al. derived an empirical definition for metabolic health based on anthropometric and metabolic factors known to be associated with increased cardiovascular and total mortality risk utilizing the NHANES III and UK Biobank dataset. Using this definition, MHO individuals

were not at increased risk of cardiovascular or all-cause mortality when compared to healthy normal weight individuals , while those classified as metabolically unhealthy, irrespective of their BMI, displayed increased risks (Zembic et al., 2021).

Likewise, an extensive variation (5-45%) in the global prevalence of MUNW has been reported, and this, too, is also dependent on the population and criteria used (Ding et al., 2016; Dvorak et al., 1999; Lee, 2009; Wildman et al., 2008; Zheng et al., 2020). For example, in the Spanish study by Goday *et al*., a prevalence of 2% was reported among normal weight persons when a modified NCEP-ATPIII definition for metabolic health was applied. This however rose to 46.4% when more stringent criteria were used (Goday et al., 2016). Moreover, the study by Gujral *et al.* noted that the prevalence of MUHNW was significantly higher in all racial/ethnic minority groups compared with whites (Gujral et al., 2017). However, the meta-analysis of Wang *et al.* observed a higher prevalence of MUHNW among European people (Wang et al., 2015). Again, this is in contradiction to what has been previously reported in the literature, namely that this phenotype is more prevalent in Asians especially those of Indian, Korean and Chinese descent, due to their tendency to accrue more fat intra-abdominally even though they exhibit a normal BMI as well as having a reduced muscle mass thus placing them at higher cardiometabolic risk (Ding et al., 2016; G and AS, 2021; Mathew et al., 2016). The authors however do acknowledge that most of the Asian studies included in the meta-analysis were conducted in Korea and may thus not be a true reflection of the entire Asian population. Overall, most studies report the presence of MUNHW to be higher in males, older age individuals, and in those with lower physical activity levels and larger WC. In the study by Conus *et al*., the authors observed that in participants with a BMI

ranging between 25 to 27 kg/m^2 and a family history of T2 DM and/or hypertension, the presence of abdominal obesity, high serum cholesterol and triglyceride levels and hypertension would point towards the presence of the MUHNW phenotype (Conus *et al.,* 2007).

More recently, Lee *et al.* proposed a novel and simple diagnostic criterion to identify individuals with the MUHNW phenotype. They suggested the use of the triglyceride glucose index (TyG) which is the product of fasting triglyceride and glucose levels to help in discriminating normal weight individuals are higher risk of metabolic diseases (Lee et al., 2015). They observed that it correlates well with levels of IR and thus argue that since individuals with the MUHNW phenotype have higher degrees of IR, then, using this simple parameter might be an easier way to identify such individuals. Other researchers suggested the use of serum ferritin concentration levels to identify MUHNW individuals . This was based on the fact that ferritin is an acute phase protein (similar to CRP) and thus levels increase with an inflammatory environment as occurs in MUHNW and which in turn leads to increased IR and cardiometabolic risk (Ren et al., 2022). Others also suggested the assessment of hepatic fat content as it was observed to be strongly correlated with incident T2DM than was being overweight or obese (Sung et al., 2012a; Urata et al., 2020). However, these studies made use of ultrasonography to quantify hepatic fat which may be impractical and expensive as a screening tool in clinical practice, readily available calculated indices such as the Fibrosis-4 Index (FIB4) and the NAFLD fibrosis score (NFS) which predict metabolic abnormalities, cancer and overall mortality are more practical can thus be used as biomarkers for hepatic fat (Önnerhag et al., 2019; Taylor et al., 2022)

Of note, de Lorenzo and colleagues also described the occurrence of another obese phenotype: the normal weight obese individual (NWO) (De Lorenzo et al., 2006). People belonging to this class are also typically lean with a normal BMI, however thy have a higher total body fat content (>30%) and a lower amount of lean muscle mass. Furthermore, these individuals are characterised by higher fasting glucose and lipid levels, IR, higher oxidative stress and a proinflammatory state (such as increased serum levels of TNF-α, IL-8 and IL-6) when compared to healthy normal weight individuals, which also renders them at higher cardiometabolic risk even after adjusting for central obesity (De Lorenzo et al., 2007; Kapoor et al., 2020; Mohammadian Khonsari et al., 2022).

As alluded to above, most studies used either Met S components and/or IR to define metabolic health. While IR is a core feature of the Met S and considered to be the unifying pathogenic mechanism responsible for its occurrence (including its individual components), one would expect that individuals stratified as metabolically healthy by Met S criteria to also be insulin sensitive. Interestingly, several studies found minimal overlap in prevalence rates when using Met S criteria or HOMA-IR to define metabolic health in the same population. For example, Kuk *et al*. found a higher prevalence of MHO when the NCEP-ATPIII criteria for Met S was adopted than when using HOMA-IR (38.4% vs 30.2% respectively). Furthermore, only 6.0% of obese individuals did not exhibit Met S parameters or IR simultaneously (Kuk and Arden, 2009). , Such discrepancies cast a doubt on the strength of the association between IR and presence of Met S or its components and may imply that other factors might be contributing to the development of this syndrome (Cheal et al., 2004; Tracey McLaughlin et al., 2007). In a similar fashion,

Eckel *et al.* observed that only 30.5% of normal weight individuals who developed diabetes had the presence of the Met S as defined by NCEP-ATPIII at baseline (Eckel et al., 2015). This observation seems to suggest that the traditional definition of the Met S may be insensitive to correctly identify those normal weight but at-risk individuals and thus it might be more pertinent to look at individual components of the Met S to better characterise such individuals. Moreover, within this study those participants with incident diabetes had mean levels of WC, TG and HDL-C which were much lower than the cut-offs used for the Met S, again implying that the traditional Met S definition might be insufficient to identify individuals with 'true' MUHNW and that the cut-offs for these parameters might need to be revised downwards.

1-5.2 Characteristics and determinants of metabolic health

Biological mechanisms

Several factors are implicated in the metabolic heterogeneity observed across different BMI categories. One of the most recognized contributing factors is dysfunction of adipose tissue (adiposopathy), however other factors including environmental, behavioural and genetic factors all have been implicated through the modulation of fat mass and fat cell biology/function (Bays, 2014; Goossens, 2017; Stefan, 2020a). It is increasingly recognised that preservation of metabolic health and insulin sensitivity is associated with a favourable adipocyte morphology and functionality pattern as opposed to adiposopathy in metabolically unhealthy individuals. The main adipocyte features which contribute towards a healthy metabolic state during periods of caloric overload include preferential expansion of insulin-sensitive subcutaneous adipose tissue

(particularly of gluteofemoral areas) through hyperplasia, lower visceral and ectopic fat deposition in tissues such as the liver and skeletal muscle, preserved adipocyte remodelling (including efficient extracellular matrix remodelling, increased angiogenesis and adipogenesis) and a favourable inflammatory status (including lower levels of proinflammatory adipocytokine secretion (CRP, TNF-α, IL-6, PAI-1, leptin, chemerin, visfatin and resistin) and immune cell infiltration (M1 macrophages and T_H cells, and a higher level of adiponectin (Cho et al., 2022; Iacobini et al., 2019; Tsatsoulis and Paschou, 2020). On the other hand, individuals with adiposopathy are characterised by a reduced capacity of subcutaneous adipose tissue expansion, increased visceral adiposity through hypertrophy, impaired adipose tissue remodelling, altered lipid metabolism and lipotoxicity, fat overspill into ectopic sites such as liver and skeletal muscle, as well as elevated adipose tissue and systemic inflammation (meta-inflammation) together with a proinflammatory adipocytokine profile and a high immune cell infiltration (Ahmed et al., 2021; Badoud et al., 2015; Phillips, 2017; Teixeira et al., 2015) **(Figure 1.2).** Thus, these observations may be summed up via three hypotheses: 1) the adipose tissue expandability hypothesis 2) the lipotoxicity hypothesis and 3) the inflammation and adipokine hypothesis.

Over the last few decades research has shown that different body fat depots exert different effects on health outcomes (Stefan, 2020a). While the subcutaneous adipose tissue (SAT) compartment is the main fat storage depot, adipose tissue at other anatomic regions has critical roles in the development of cardiometabolic disorders (Mongraw-Chaffin et al., 2017; Neeland et al., 2019; Piché et al., 2018; Smith et al., 2001). These regions include visceral adipose tissue (VAT) and adipose tissue in ectopic

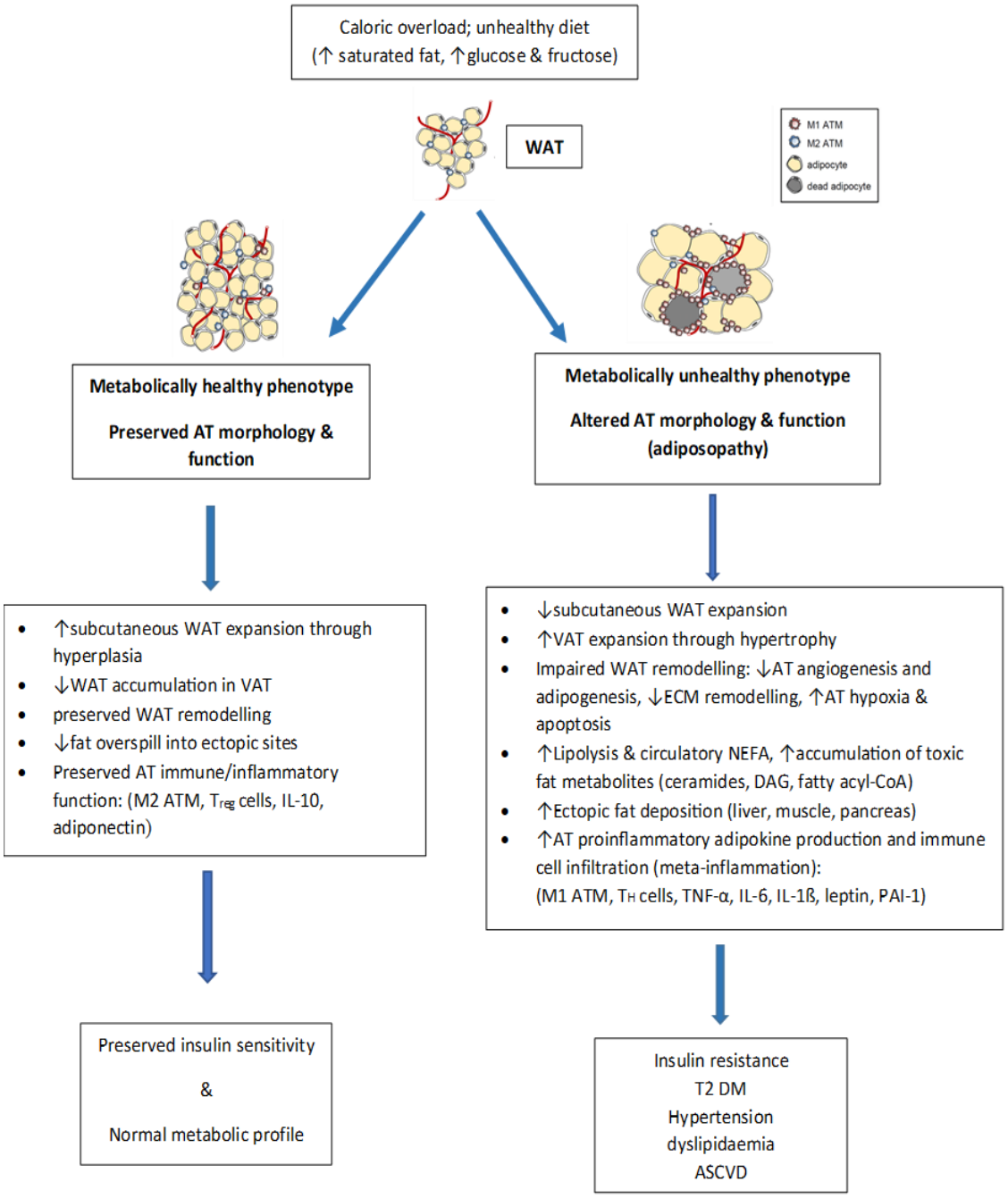
sites such as the liver, heart, pancreas, and skeletal muscle (Shulman, 2014). Advanced imaging techniques have enabled improved characterisation of these depots, which were previously considered as part of total body fat (Neeland et al., 2019; Thomas et al., 2012). These depots differ in endocrine function and immune function and may in part be responsible for the varying metabolic abnormalities observed in different obesity phenotypes (Bays et al., 2008; Teixeira et al., 2015; Thomas et al., 2012). As the primary TG reservoir, subcutaneous adipocytes expand through hyperplasia in response to caloric excess, leading to an increase in the proportion of small adipocytes with normal functionality and preserved insulin sensitivity (hyperplastic obesity). Such findings are characteristic of individuals with MHO (Blüher, 2010; Klöting et al., 2010a; O'Connell et al., 2010). However, SAT has a ginite expansion capacity resulting in an overspill into visceral/ectopic areas leading to the onset of systemic inflammation, IR and hyperglycaemia. (Mathieu et al., 2009; Tan and Vidal-Puig, 2008; Tchernof and Després, 2013).

Thus, the *adipose tissue expandability hypothesis* implies that during periods of sustained excess calorie intake, a limited degree of SAT plasticity predisposes towards the accumulation of fat in visceral and ectopic sites thereby leading to lipotoxicity and the subsequent development of metabolic disturbances typically observed in obese states. (Blüher, 2013; Carobbio et al., 2017). Several lines of evidence support this hypothesis. In lean individuals, a lower proportion of subcutaneous gluteofemoral fat mass is the strongest predictor of the MUHNW. Furthermore, increased leg fat mass correlates with higher insulin sensitivity and a favourable metabolic profile (Stefan et al., 2017). The Women's Health Initiative Study demonstrated that in females with a

normal BMI, a reduced gluteofemoral fat mass is associated with a higher incidence of cardiometabolic diseases independent of truncal fat mass, further supporting the notion that adipose tissue expansion within peripheral gluteofemoral areas allows for the thelahty storage of excess fat. (Chen et al., 2019; Klitgaard et al., 2020). Similarly, despite an identical BMI and high amounts of body fat, MHO individuals have a lower WC than MUHO individuals due to reduced VAT and hepatic fat. In response to weight gain, MHO individuals preferentially expand the SAT compartment whereas MUHO individuals are more likely to gain fat in the visceral regions. Such differences in location of fat accumulation are thought to be responsible for the preserved metabolic function observed in MHO (Brochu et al., 2001; Karelis et al., 2004b; Klöting et al., 2010). Thus, although the exact mechanisms by which visceral fat contributes to the dysmetabolic state are not fully elucidated, the overall evidence points towards VAT as being a dysfunctional depot characterised by abnormal adipogenesis (hypertrophied adipocytes rather than hyperplasia), increased lipolytic activity and fatty acid accumulation into ectopic sites as well as a dysregulated inflammatory /immune milieu which ultimately leads to IR and end organ damage (such as T2DM and ASCVD) (Blüher, 2013; Crewe et al., 2017; Pluta et al., 2022).

The predilection towards storage of fat in subcutaneous vs visceral regions depends on multiple factors. Increasing age, male sex, senescence, smoking, physical inactivity and high fat or fructose diets favour an increase in VAT (Brandão et al., 2020; Iacobini et al., 2019; Ortega et al., 2013; Teixeira et al., 2015). Studies which looked at ectopic fat accumulation in different body composition phenotypes found differences in levels of fat accumulation particularly in liver and skeletal muscle of MHO individuals compared

to MUHO counterparts which may partly explain the varying cardiometabolic risk found among these two groups (Pimentel et al., 2015; Stefan et al., 2008b). Other studies went on to show that levels of hepatic fat were strong predictors of both the MUHO phenotype and of IR (Ogorodnikova et al., 2013; Stefan et al., 2008b).



ASCVSD, Atherosclerotic cardiovascular disease; AT, adipose tissue; ATM, adipose tissue macrophage; DAG, diacylglyceride; ECM, extracellular matrix; IL, interleukins; NEFA, non esterified fatty acids; PAI-1, plasminogen activator inhibitor -1; T2 DM, type 2 diabetes; $T_H$cells, T helper cells; T_{reg} cells, regulatory T cells; TNF- α, tumor necrosis factor α; WAT, white adipose tissue

Figure 1.2: Schematic diagram illustrating the potential mechanisms by which adipose tissue mediates the development of the metabolically healthy and unhealthy phenotypes

The relationship between expansion of VAT/ectopic fat depots and IR can be partially explained by the *lipotoxicity hypothesis*. Increased VAT is prone to lipolysis leading to the excess release of non-esterified fatty acids (NEFA) into the circulation which subsequently accumulate in ectopic sites (Boden, 1997; Manu et al., 2012; Nielsen et al., 2004). Within skeletal muscle, an accumulation of biologically active lipids (such as ceremides, diacylglycerols and fatty acyl CoA) negatively affects insulin signalling pathways (via an impairment in GLUT 4 translocation) which induces muscle IR resulting in a decrease in glucose uptake and glycogen synthesis (Stefan et al., 2017; Zaid et al., 2008). On the other hand, increased NEFA delivery to pancreatic β-cells leads to impaired insulin secretion which further exacerbates lipolysis and therefore propagates the influx of NEFA within the circulation (Ahmed et al., 2021; Succurro et al., 2008; Teixeira et al., 2015). Delivery of NEFA to the liver increases with increasing amounts of VAT and contributes to the development of hepatic IR which manifests by an increase in VLDL synthesis, increased triglyceride concentration in the blood, increased glycogenolysis and gluconeogenesis and the development of non-alcoholic fatty liver disease (Karpe et al., 2011; Klein, 2004; Klöting et al., 2010; Stefan et al., 2008a; Teixeira et al., 2015). Furthermore, fat accumulation in the liver is associated with an increased production of the hepatokine fetuin-A, a proinflammatory cytokine which impairs insulin signalling through the activation of Toll-like receptors resulting in IR and onset of T2DM (Stefan and Häring, 2013a). A lower level of fetuin A level was observed in individuals with MHO compared with MUHO and this was found to be associated with improved insulin sensitivity and glucose homeostasis thereby making it a potential player in the link between liver fat and IR (Stefan et al., 2008a; Stefan and Häring,

2013b). Thus these factors may all potentially explain the complex relationship observed between abdominal adiposity, IR and metabolic dysfunction. Moreover, NEFA are pro-inflammatory, leading to reactive oxygen species (ROS) generation, endothelial cell damage and the formation of a proatherogenic vascular milieu that exacerbates cardiovascular risk (Wang et al., 2009).

The *inflammation and adipokine hypothesis* centres around the pro-inflammatory and immune responses induced by dysmorphic adipose tissue including the recruitment of M1 macrophages and the secretion of multiple inflammatory adipocytokines (such as TNF-α, IL-6, plasminogen activator inhibitor-1 (PAI-1), resistin, CRP RBP4, leptin and angiotensin II) leading to chronic low grade systemic inflammation (meta-inflammation), endothelial dysfunction and the onset of IR and the metabolic abnormalities associated with obesity (Ahmed et al., 2021; Blüher, 2013; Esser et al., 2014; Fontana et al., 2007).. Several studies have demonstrated that the adipose tissue of individuals with obesity is associated with a switch from anti-inflammatory alternatively activated M2 macrophages to proinflammatory classically activated M1 macrophages which accumulate in crown-like structures (CLS) around necrotic adipocytes leading to systemic IR and higher levels of TG, LDL-C and lower levels of HDL-C (Apovian et al., 2008; Cancello et al., 2006; Farb et al., 2011; Heilbronn and Campbell, 2008; Lumeng et al., 2007). Studies which looked at levels in the MHO phenotype observed this group to have lower macrophage infiltration in both VAT and SAT compared to individuals with MUHO or T2DM which may be responsible for the improved cardiometabolic profile in MHO (Blüher, 2010; van Beek et al., 2014). Additionally, data from animal and human studies also demonstrated the presence of several types of T cells (including T helper

[T_H]cells, cytotoxic T [T_c] cells and regulatory T [T_{reg}] Cells) in adipose tissue which could also be contributing to the immune and inflammatory processes observed in obese states thereby providing additional evidence which consolidates the link between dysfunctional adipose tissue, insulin-resistant states and a dysregulated metabolic milieu (Duffaut et al., 2009; Feuerer et al., 2009; Kintscher et al., 2008; Winer et al., 2009).

Likewise MUHNW individuals , also present with an abnormal body composition and fat distribution patterns including a higher VAT, liver and muscle fat content and a lower skeletal muscle mass, increased oxidative stress together with a proinflammatory and thrombotic state as well as IR, hyperinsulinemia and an adverse metabolic milieu (Conus et al., 2007; Dvorak et al., 1999; Karelis et al., 2004b; Katsuki et al., 2003; Klitgaard et al., 2020; Pluta et al., 2022; Ruderman et al., 1998). Specifically, in MUHNW, a greater proportion of body fat has been documented, with the risk of metabolic abnormality correlating with body fat percentage in both males and females and furthermore, in young lean individuals , the percentage body fat was observed to be the single strongest predictor for low insulin sensitivity (Karelis et al., 2004b; Shea et al., 2012). Conflicting findings have also been reported, with some investigators failing to identify differences in percent body fat between healthy and unhealthy normal weight individuals implying that metabolic dysfunction in MUHNW may occur independent of total body adiposity (Ding et al., 2018; Hyun et al., 2008). Building up on these findings was the elegant study by Stefan and coworkers. (Stefan et al., 2017). Interestingly the authors reported different risk phenotypes among the metabolically unhealthy lean and overweight/obese individuals . Specifically, within lean individuals, insulin secretion

failure and low percentage subcutaneous leg fat mass were the major drivers for metabolic ill-health whereas visceral adiposity and steatohepatitis to be the underlying drivers for the metabolically unhealthy phenotype in overweight and obese groups. Notwithstanding this, several studies also report greater ectopic fat deposition including higher levels of VAT depots and a greater intrahepatic triglyceride content among MUHNW individuals than among controls which were matched for age and BMI (Ding et al., 2018; Dvorak et al., 1999; Furukawa et al., 2017; Katsuki et al., 2003; Takeno et al., 2016). Remarkably though, no differences in intramuscular fat content were observed (Ding et al., 2018). An important caveat which may in part explain these discrepancies lies in the fact that in most studies MUHNW individuals had a significantly greater BMI than 'matched' healthy controls despite both categories falling within a 'normal' BMI range. This bias is significant, since the incidence of Met S and T2DM increases even within the normal weight BMI range making comparisons between these two phenotypes difficult to interpret making it a challenging exercise to ascertain whether the observed differences in metabolic profiles are driven primarily by the MUHNW phenotype or due to differences in body weight. Nevertheless, in the NHANES III cohort, normal weight individuals with central obesity (as determined by a raised WHR) had the worst long-term survival outcome compared to all other body composition phenotypes (Sahakyan et al., 2015). Moreover, a similar degree of subclinical inflammation is observed between MUHNW and MUHO individuals. (Di Renzo et al., 2010; Piya et al., 2013). Such findings thus reinforce that within the general population, some lean individuals exhibit features akin to the lipodystrophy syndromes (paucity of subcutaneous fat, hepatic steatosis, and severe IR) which drive

cardiometabolic risk. Furthermore, the combination of lower expansion of peripheral subcutaneous fat with an increased accumulation of fat in central (abdominal) areas may be the landmark trait which characterises metabolic dysfunction in normal weight individuals.

Genetic and early life factors

There is on-going and extensive research to identify the underlying genetic mechanisms which could contribute towards the development of different obesity phenotypes. Obesity is a primarily polygenic disease regulated by complex gene-environment interactions and transgenerational epigenetic mechanisms. Although multiple pathways have been implicated in the development of obesity, the different fat distribution patterns and in the development of comorbidities, the exact underlying genetic mechanisms are still largely undetermined (T. McLaughlin et al., 2007; Pérez-Echarri et al., 2007; Schleinitz et al., 2014; Speakman et al., 2011; Yasuda et al., 2008).

Both genome-wide association studies (GWAS) and gene-expression studies have demonstrated that body fat distribution is influenced by a number of genetic loci independent of the BMI. Specifically, GWAS recently identified 14 genetic variants which are associated with the regulation of body fat distribution and a lower risk of developing metabolic abnormalities and cardiometabolic diseases despite a BMI in the obese range (Iacobini et al., 2019). GWAS has also enabled the construction of polygenic risk scores characterizing different obesity phenotypes (Scott et al., 2014; Yaghootkar et al., 2016). Typically, GWAS-identified variants exert small effect sizes on BMI when considered in isolation and which explains a minimal proportion of the phenotypic

variance in BMI (Yengo et al., 2018). However, when considering polygenic risk scores which aggregate the effect of multiple variants as a single polygenic predictor, a more robust stratification of the risk of severe obesity and cardiometabolic disease in adults is observed (Khera et al., 2019). For example, the WHR has been associated with a significant heritability of up to 60%, and a locus near IRS1 is associated with lower subcutaneous fat compared to visceral fat and an adverse metabolic profile in males (Iacobini et al., 2019; Kilpeläinen et al., 2011). Additionally, recent GWAS also identified polymorphisms in developmental genes which are strongly related to body fat distribution (including TBX15, RSP03, HOXC13) implying that fat distribution may be determined from a very early stage in life (Gesta et al., 2006; Rask-Andersen et al., 2019). Principally, the MHO phenotype is associated with genetic variants relating to lower risk of development of metabolic abnormalities such as dyslipidaemia and hypertension despite having a higher BMI. This reinforces the notion of a distinct genetic component driving different adipose distribution patterns (Scott et al., 2014; Yaghootkar et al., 2016) (151,152). A recent meta-analysis of GWAS found that heritability of fat distribution was generally stronger in females than in males, and that approximately one-third of all signals were sexually dimorphic (Pulit et al., 2019). Functionally, adiposity-related signals identified by GWAS are enriched for genes involved in adipocyte differentiation, adipogenesis, and transcriptional regulation of insulin signalling and lipolysis (Iacobini et al., 2019). Furthermore, distinct adipocyte gene expression patterns were observed which distinguish the MHO from MUHO phenotype. Importantly, differentially-expressed transcripts are linked to CVD, inflammatory

pathways and branched chain amino acid catabolism (Das et al., 2015; Yaghootkar et al., 2016).

Genetic factors also impact on the various ways by which adipose fat depots store energy. The *adipose tissue expandability hypothesis* outlined earlier relates to the transition of normal adipose tissue to one which leads to metabolic derangement characterised by adipocyte hypertrophy. Strong support for this hypothesis comes from pharmacological studies of peroxisome proliferator-activated receptor- γ (PPARγ) activation in both murine and human studies (Mathew et al., 2016; Stefan et al., 2013). Knock-out studies with PPARγ lipodystrophy murine models identified altered adipokine secretion patterns following saturation of adipose tissue. Treatment with thiazolidinediones (PPARγ agonists) results in an increase in adiponectin concentrations, expansion of subcutaneous adipose tissue, a decrease in liver fat content, and an increase in insulin sensitivity. Thus, thiazolidinediones might be a promising treatment approach in insulin-resistant individuals with non-alcoholic fatty liver disease or an increased risk of CVD (Lincoff et al., 2007; Tan and Vidal-Puig, 2008). Moreover, the genetics of obesity extends beyond adipocyte biology to loci involved in satiety and appetite regulation. Recently, 27 variants previously associated with obesity have also been implicated in the regulation of food intake, energy expenditure as well as food reward pathways (Phillips, 2017).

Additional molecular mechanisms are implicated in MHO. The systemic proinflammatory state and oxidative stress accompanying obesity and the Met S have been associated with telomere attrition. Moreover, dynamic changes in adiposity lead to changes in telomere length (TL) such that weight loss leads to an increase in telomere

length whereas weight gain accelerates telomere attrition (García-Calzón et al., 2014). Limited data exists on telomere length in MHO vs MUHO, although MHO has been associated with higher TL (Lejawa et al., 2021).

Mitochondrial bioenergetics and body composition are also interrelated. A reduction in mtDNA copy number is generally associated with poor health through detrimental effects on ATP production, changes in mitochondrial gene-expression or via an altered oxidative stress response. A reduced skeletal muscle mtDNA content in T2DM patients has been described (Antonetti et al., 1995a; Lee et al., 1998; Silva et al., 2000; Xu et al., 2012). Additionally, a reduction in leukocyte mtDNA copy number is associated with obesity, IR and the Met S (Meng et al., 2016; Skuratovskaia et al., 2018; Zheng et al., 2015a). Other studies further support mtDNA as a potential biomarker of metabolic disease and obesity, with mtDNA copy number independently associated with visceral fat accumulation in healthy young adults (Lee et al., 2014a; Skuratovskaia et al., 2019b). Bordoni *et al*. report the association between lower buccal mtDNA copy number and unfavourable body composition profile (Bordoni et al., 2019). Further investigations are warranted to evaluate further the role of mtDNA as a Met S risk biomarker in obesity.

Much of the molecular evidence supporting MUHNW comes from studies which address a lipodystrophy-like phenotype in the general population. Seminal work by Yaghootkar *et al.* identified common alleles at loci (such as *IRS1*, *GRB14* and *PPARG*, *ARL15*) which demonstrate a genetic basis for the metabolically abnormal normal weight individual. A 'polygenic lipodystrophy' score composed of 11 common genetic variants shows associations with adverse metabolic traits, including an increased risk of hypertension,

T2DM, and CAD despite a lower BMI (Yaghootkar et al., 2016, 2014). Recently Lotta et al. also reported several loci which are associated with metabolic risk and coronary heart disease in individuals with lower percentage body fat, BMI, and lower gynoid and leg fat mass thus providing additional evidence of the genetic contribution towards a 'lipodystrophy-like' phenotype in the general population (Lotta et al., 2017).

Accumulating evidence also reinforces the shared contribution of early-life environmental and genetic factors. An adverse intrauterine milieu of either under- and over-nutrition has been linked to the 'thrifty phenotype' that predisposes to adult obesity and Met-S. In the early 90s, Hales and Barker postulated that poor foetal and early post-natal nutrition 'imposes mechanisms of nutritional thrift on the growing individual' leading to impaired development of the endocrine pancreas and increased susceptibility to T2DM. Furthermore, Barker also suggested that infants whose 'birth weights were at the lower end of the normal range, who were thin or short at birth, or who were small in relation to placental size have increased rates of coronary heart disease' (Barker, 1990; Hales, 1997). These factors act through complex epigenetic modifications (Heijmans et al., 2008) . More recent work suggests that there may be a U-shaped relationship between birth weight and risk of cardiometabolic abnormalities, such that extremes of birth weight are both associated with obesity and Met-S in adolescence and adulthood (Agius et al., 2013; Tam et al., 2015). However, conflicting findings have been reported. Some studies showed that higher birth weight and early postnatal weight gain was associated with higher insulin sensitivity and lower hepatic IR reminiscent of the MHO phenotype, although the mechanisms around this relationship are unascertained (Bouhours-Nouet et al., 2008). More studies are required in this area

to further identify modifiable risk factors for the MUHO phenotype. Another hypothesis is the "thrifty genotype" hypothesis. Here, evolutionary selection of genes originally thought to be beneficial for energy storage during times of starvation could partly explain the current obesity and T2DM epidemics in the Westernised world, where caloric excess and sedentary behaviour predominate (Neel, 1962).

Lifestyle and behavioural determinants

A growing body of research also indicates that certain environmental/lifestyle factors also contribute towards metabolic health and obesity. Most commonly studies focussed on the role of diet, fitness, age, tobacco smoking and alcohol intake as being a few of the potential modifiable risk factors which may in part explain the heterogeneity of metabolic abnormalities among individuals with obesity (Camhi et al., 2015b, 2013; Iacobini et al., 2019; Lopez-Garcia et al., 2013; Matheson et al., 2012; Phillips et al., 2013). Changes in dietary intake and physical activity have both contributed towards the obesity epidemic and may partly explain the metabolic heterogeneity in lean and obese individuals. The overconsumption of calorie dense food, in tandem with global increase in urbanisation and sedentary behaviours results in a state of positive energy imbalance leading to increased accumulation of adipose tissue and progression to overt obesity (Camhi et al., 2015b, 2013; Cuschieri and Mamo, 2016).

Matheson *et al.,* show that adoption of four healthy lifestyle habits (modest alcohol intake, non-smoking, 30 minutes of exercise daily and eating five portions of fruit /vegetables daily) results in identical mortality risk in obese and lean individuals (Matheson et al., 2012). However, the precise contribution of each of these lifestyle

factors on MHO is still unclear. Most studies did not report any differences in total energy intake, dietary macronutrient composition and quality between MHO and MUHO individuals . However, better compliance with the food pyramid recommendations increased the likelihood of the MHO phenotype (Phillips, 2017). Moreover, in a recent analysis of the data from the NHANES survey, Manu et al., noted that dietary energy intake and composition as well as alcohol consumption was similar between MHO and MHNW in males, however, MHO females consumed less fibre (Manu et al., 2012). Additionally, another study which investigated Mediterranean Diet Scores (MDS) found lower consumption of red meat and dairy products among MHO individuals (Park et al., 2016). The beneficial effects of physical activity on incidence of T2DM, CVD, obesity, cancer and all-cause mortality as well as the adverse effects of a sedentary lifestyle on metabolic outcomes are well documented (Lee et al., 2012; Phillips, 2013a; Sattelmair et al., 2011). Physical activity and cardiorespiratory fitness characterises and maintains MH in obese individuals, as supported by evidence from meta-analysis (Ortega et al., 2013; Prince et al., 2014). Furthermore, physical activity is associated with increased fatty acid oxidation and higher fat utilization, leading to lower fat accumulation in the liver and visceral compartments (Pujia et al., 2016). Smoking and alcohol consumption are widely studied modifiable lifestyle factors that impact on MH. Wildman et al. and others show that modest alcohol intake was associated with the MHO. This association is however disputed as conflicting findings showing no differences in alcohol and smoking habits between MHO and MUHO individuals have been published (Martínez-Larrad et al., 2014). The effects of modest alcohol intake on both glucose and HDL-C levels are also well documented, however the adverse effects of excessive alcohol

consumption include raised TG levels and increased abdominal obesity. The link between alcohol consumption and MH may thus be bimodal (Kroenke et al., 2003).

Certain sleep habits such as sleep quality and quantity have also been implicated in the onset of obesity, IR and the Met-S with most studies reporting short sleep duration to be associated with a number of metabolic abnormalities (Choi et al., 2008; Gangwisch et al., 2005; Koren and Taveras, 2018; Spaeth et al., 2013). Interestingly, even a single night of sleep deprivation was observed to decrease insulin sensitivity by 19-25% in hepatic and peripheral tissues as well as increase NEFA levels in healthy normal weight adults (Donga et al., 2010). The pathophysiological mechanisms linking sleep deprivation with obesity and metabolic dysfunction include changes in appetite regulatory hormones; lower levels of leptin [an anorexigenic hormone] and higher levels of ghrelin [an orexigenic hormone], loss of diurnal variation in cortisol secretion as well as increased catecholamine production (Spiegel et al., 1999; Taheri et al., 2004). Furthermore, sleep restriction also results in loss of the beneficial effects of growth hormone on muscle mass and fat distribution (Stich et al., 2022). Sleep debt is also associated with an increased predilection towards the consumption of high-fat or carbohydrate rich foods as well as reduced physical activity (Spiegel et al., 2005; St-Onge et al., 2014). With respect to the relationship between sleep habits and the different body composition phenotypes, one study which analysed data from the Korean National Health and Nutrition Examination Survey V (KNHANES V) observed that individuals living with obesity had significantly shorter sleep durations compared to normal weight individuals (irrespective of metabolic health), yet the MUHO phenotype had the shortest sleep duration compared to the others (Ryu et al., 2015). On the other hand, data

derived from the U.S. NHANES cohort, did not reveal any relationship between overall sleep quality and quantity in the MHO phenotype, however the authors did report that certain sleep characteristics such as trouble falling asleep, waking up during the night, feeling unrested during the day and feeling overly sleepy during the day to be associated with lower odds of having the MHO phenotype (Kanagasabai et al., 2017).

While it is acknowledged that limited data regarding the characteristics and determinants of MHO are currently available, the behavioural and lifestyle characteristics of the MUHNW phenotype have been even less well studied. Eckel et al. observed that the traditional risk factors for T2DM commonly observed in individuals with obesity (including male sex, smoking, increasing age, physical inactivity, an unfavourable fat distribution and an adverse cardiometabolic profile) are also contributors to T2DM in lean individuals (Eckel et al., 2015). Furthermore, while most studies also did not observe a significant difference with respect to total energy and macronutrient intake between healthy and unhealthy normal weight participants , others observed that MUHNW females were more likely to have a higher intake of saturated fats and a lower polyunsaturated /saturated fatty acids ratio accompanied by lower dietary fibre intake than healthy normal weight females (Dvorak et al., 1999; Hyun et al., 2008; Klitgaard et al., 2020). With respect to physical activity, studies show that MUHNW individuals have significantly lower cardiorespiratory fitness (as assessed by VO_2max) compared to their healthy peers and, moreover, individuals within the lowest tertile of handgrip strength were reported to have a worse cardiometabolic risk profile than those in the upper tertile, suggesting that inadequate engagement in physical activity to be an important component of the MUHNW phenotype (Dvorak et al., 1999;

Kim et al., 2013a; Takeno et al., 2016). Most studies conducted in different ethnic populations also found smoking status, alcohol consumption and lower education levels to be potential contributors to the unhealthy phenotype (Hajian-Tilaki and Heidari, 2018; Lee, 2009). These findings were also supported in the meta-analysis by Wang *et al*. (Wang et al., 2015).

Role of gut & skin microbiota

Over the past two decades findings from observational epidemiological studies as well as cellular and high throughput human omics-based studies (including metagenomics and metabolomics data) show that gastrointestinal tract (GIT) by way of its bacterial flora may contribute to the metabolic health of humans especially with regards to energy homeostasis, body adiposity, inflammation, glucose regulation, insulin sensitivity and hormone secretion. Specifically, analyses from cause-and-effect studies demonstrate that gut microbiota and their associated microbial compounds to be causative to the pathogenesis of common metabolic diseases and that interventions aimed at targeting the gut microbiome lead to improvements in metabolic health (Dabke et al., 2019; Fan et al., 2021).

The GIT is home to a diverse number of microorganisms which form the gut microbiome, some of which include the Gram negative Bacteroidetes and Probacteria and the Gram positive Acenetobactieria and Firmicutes (Nam et al., 2011). Typically, these microbes act in a symbiotic fashion with the human host by maintaining a healthy gut immune system, aiding breakdown of complex non-absorbable plant-derived polysaccharides and also for maintaining energy homeostasis. It has been shown that alteration in the

gut microbiome (as occurs by way of diet such as a high-fat intake, genetics or environmental factors such as exposure to antibiotics in early life) is associated with increased risk of obese states. In fact, human and animal studies confirm a 50% reduction of *Bacteroidetes* species and consequently a higher proportion of *Firmicutes* in obese vs lean states. Conversely, weight loss with a low-calorie diet leads to an abundance of *Bacteriodetes* (Bäckhed et al., 2004; Ley et al., 2006). Furthermore, the obese microbiome is transmissible such that when caecal bacteria is harvested from genetically obese mice to germ-free (GF) lean mice, they exhibited a greater increase in adiposity than when colonised with a 'lean' microbiome (Turnbaugh et al., 2006). The proposed mechanisms linking gut microbiota to obesity is through a process of metabolic endotoxinemia. Studies show the obese microbiome is associated with an increased absorption of calories from the gut even in the absence of excess food intake (Ley et al., 2005). It has been postulated that high-fat diets lead to the observed microbial imbalances which consequently cause an increase in the intestinal permeability and translocation of certain bacterial products (endotoxin lipopolysaccharide [LPS]) to the circulation. This incites hypertrophy of the mesenteric adipocytes causing increased gene expression of proinflammatory and oxidative cascades and the generation of pro-inflammatory cytokines which indirectly are thought to contribute to the downstream development of metabolic abnormalities (Brandão et al., 2020; Erridge et al., 2007). This relationship has been replicated in both human and animal studies with positive correlations between intestinal permeability markers and certain anthropometric indices as well as degree of liver and visceral fat and levels of insulin and HOMA indices (Cani et al., 2007; Lam et al., 2012). Interestingly

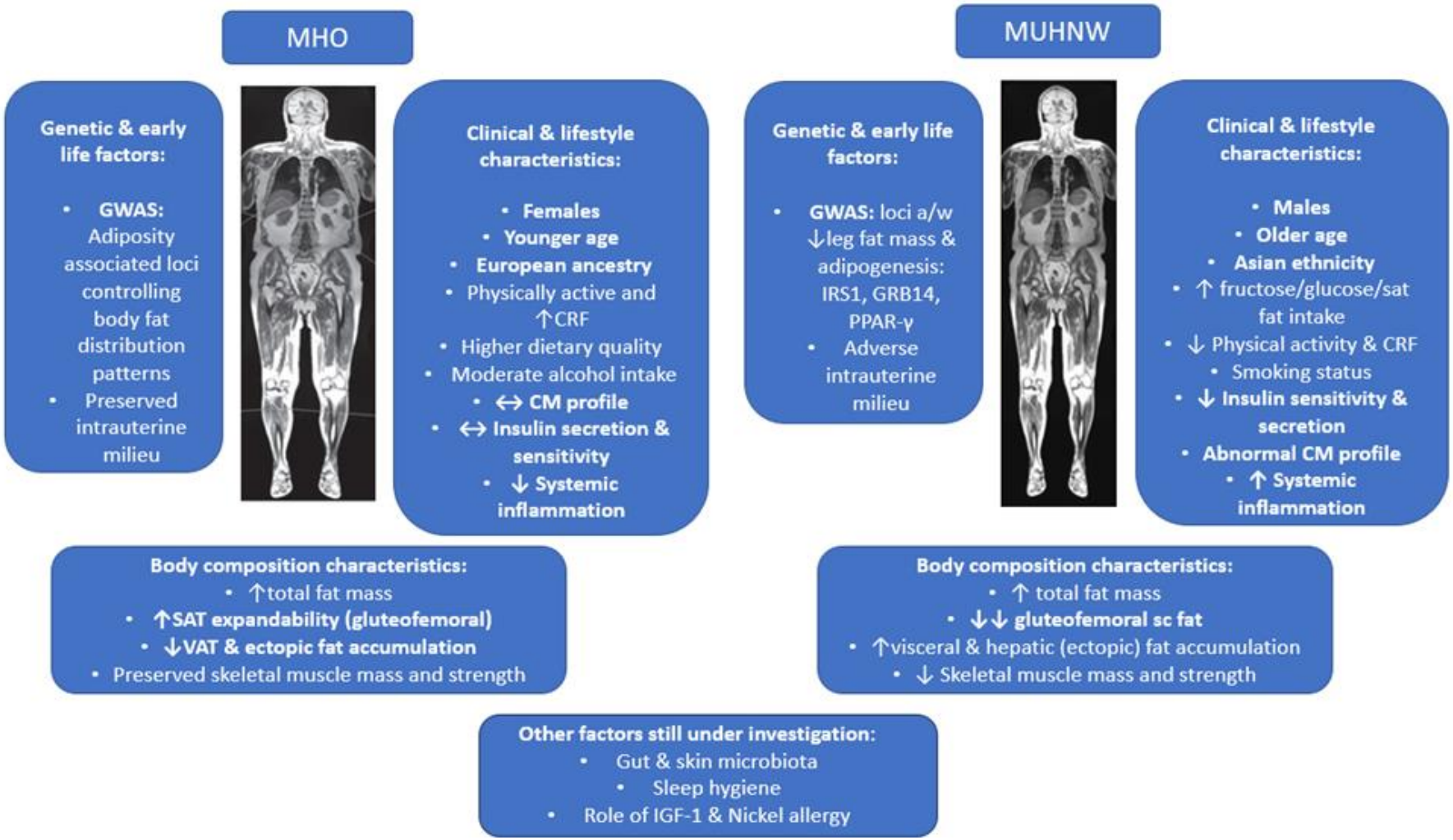
the presence of *Akkermansia Muciniphilia* has been reported as being a beneficial bacterium. It reduces gut barrier disruption and thus has an inverse relationship with obesity and cardiometabolic diseases and growing evidence shows that it might protect against low-grade inflammation (Derrien et al., 2011).

Recently, differences in the microbial diversity and gut microbiota composition were also reported between MHO and MUHO phenotypes (Kim et al., 2020). Furthermore, metagenomics analysis in a large European population with severe obesity found altered bacterial biotin status to be associated with an inflammatory phenotype and an altered metabolic profile (Fan et al., 2021). Depletion of gut microbiota by antibiotics in mice confirms the microbial contribution to host biotin levels. Additionally, oral biotin improved glycaemia in high-fat diet-fed mice (Belda et al., 2022). Dietary fibre-induced changes in the gut microbiota can also significantly increase short-chain fatty acids production and decrease metabolically detrimental compounds including indole and hydrogen sulphide thereby improving the metabolic profile (Zhao et al., 2018; Ojo et al., 2020). However, it is acknowledged that more work in this field is required both in terms of basic as well as translational research with the ultimate aim being that of preventing and /or treating common human metabolic disorders.

Emerging endocrine disruptors and additional factors

Recent studies have also shown that individuals with obesity, particularly those with increased visceral adiposity, tend to have lower serum IGF-1 levels and a blunted growth hormone response on dynamic testing. Furthermore, a diminished IGF-1 level in the serum was observed to be associated with a worse metabolic profile (Miller et al.,

2005). IGF-1 is a peptide hormone produced primarily by the liver and has a molecular structure similar to that of insulin. It primarily mediates the effects of growth hormone (GH) through its mitogenic and anabolic actions and is currently being investigated for its potential role as a predictor of metabolic health. Interestingly, a recent study found that MHO individuals have significantly higher values for IGF-1 compared to MUHO subject. Furthermore, incorporating a surrogate marker of IGF-1, the IGF-1 z Standard of Deviation Score (zSDS), as a variable into a machine learning model increased accuracy for predicting the MHO and MUHO phenotypes, suggesting that it could be a novel biomarker for identifying those clinical phenotypes at highest risk of adverse cardiometabolic outcomes (Masi et al., 2022). Of late, several pre-clinical studies have also implicated the heavy metal Nickel (Ni) in its role as an endocrine disruptor, to be associated with dysregulation of both energy and glucose metabolism as well as disruption of the GH-IGF-1 axis in human individuals . In agreement with this, a recent study found the presence of Nickel allergy to be a more frequent occurrence among Italian individuals living with obesity. Furthermore, individuals with morbid obesity and nickel allergy exhibited a worse overall metabolic and body composition profile and an impaired growth hormone response on dynamic testing (Watanabe et al., 2018). Such findings prompted the authors to speculate the possible role of Ni exposure in the pathogenesis of obesity and hormonal dysregulation. Future research in this field is anticipated with the aim being that of elucidating the precise mechanisms underlying such a relationship in order to better clarify the role of Ni in metabolic health **(Figure 1.3).**

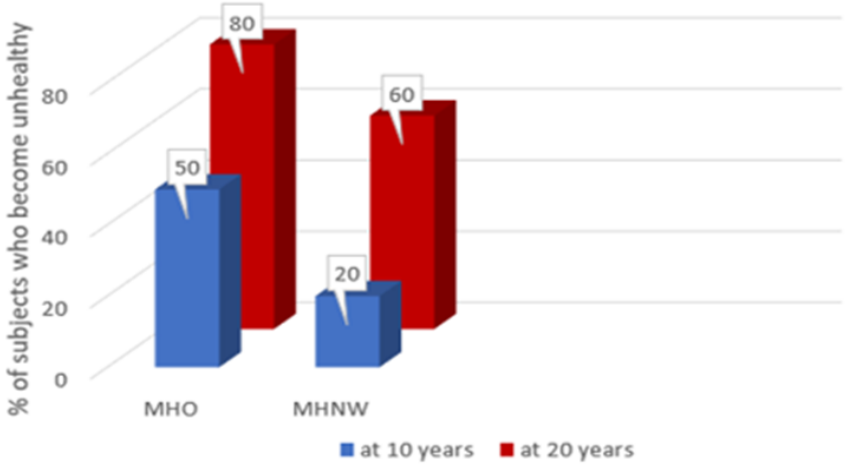


MHO: metabolically healthy obesity, MUHNW: metabolically unhealthy normal weight. CRF: cardiorespiratory fitness, CM: cardiometabolic, GWAS: genome wide association studies, SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue, IGF-1: insulin like growth factor-1,

Figure 1.3: Characteristics and determinants of MHO and MUHNW phenotypes

1.5.3 Natural course and long-term outcomes of MHO and MUHNW

Another area which has been intensively investigated and widely critiqued in the literature relates to the long-term trajectories of MHO and MUHNW, particularly their natural course and clinical implications on cardiometabolic diseases and mortality. A number of prospective studies have shown that the MHO phenotype is not a permanent state and, given enough time, tends to degenerate to an unhealthy metabolic status (Soriguer et al., 2013). In fact, up to 50% of individuals with MHO were observed to convert to the MUHO phenotype within 10 years of follow up whereas only 6% of females remained metabolically healthy after 30 years of follow-up. Whilst such studies were conducted mostly on Caucasians, similar relationships were found in other ethnic communities including Asian cohorts (Eckel et al., 2018; Hamer et al., 2015; Hwang et al., 2015; Kouvari et al., 2019) **(Figure 1.4).**



Bell et al., 2015, *J Am Coll Cardiol*; Eckel et al., 2015, *Lancet Diabetes Endocrinol;* Soriguer et al., 2013, *J Clin Endocrinol Metab*

Figure 1.4: Transition to a metabolically unhealthy state in MHO and MUHNW

Interestingly, preservation of MH is also transient among normal weight individuals. While some studies report that a good proportion (up to ~80%) of MHNW individuals remain metabolically healthy after 8 years, the Nurses' Health Study observed that only approximately 30% of MHNW individuals remained metabolically healthy after 20 years of follow up and even less (around 15%) remained healthy after 30 years of follow up (Eckel et al., 2018) **(Figure 1.4).**

Whilst most studies focused on a single transition, namely that of metabolic deterioration, it is possible for some individuals to experience other transitions in both metabolic and weight statuses throughout their life course as well as maintenance of one's initial status. Recently, Zhang et al., used a multistate Markov model to explore all possible transitions among six different metabolism-weight phenotypes. They found that over a follow-up period of nearly 1 year, less than half of participants maintained their MHO status while MUHO and MHNW were relatively stable states. Interestingly, the MHO phenotype had the shortest mean sojourn time (1.16 years) and a 50% chance of deteriorating to a metabolically unhealthy state after 6 years pointing towards MHO being an unstable and dynamic trait across the life span (Zhang et al., 2022).The major determinants of metabolic deterioration across studies are increasing anthropometric measures (such as BMI, WHR, and WC), baseline lipid concentrations (TG and HDL-C), and IR as defined by HOMA-IR, however others also found the presence of non-alcoholic fatty liver disease (NAFLD), visceral adiposity and the presence of a proinflammatory profile to be significant factors in the conversion of MHO to MUHO (Eshtiaghi et al., 2015; Hwang et al., 2015; Kouvari et al., 2022; Schröder et al., 2014). Furthermore, the Multi-Ethnic Study of Atherosclerosis (MESA) showed that both obesity duration

(assessed as the cumulative number of visits with obesity) and severity (using BMI cut-points) were strongly and consistently associated with progression of MHO to incident Met S (Mongraw-Chaffin et al., 2016). Interestingly, the Bogalusa Heart Study (which followed-up children aged between 5-7 years for approximately 24 years) showed that while only 13% of MHO children maintained a healthy obese phenotype in adulthood, they were 2.7-9.3 times more likely to be metabolically healthy obese adults than children in any other BMI/metabolic categories, suggesting that the MHO phenotype begins in childhood and persists into adulthood (Li et al., 2012).

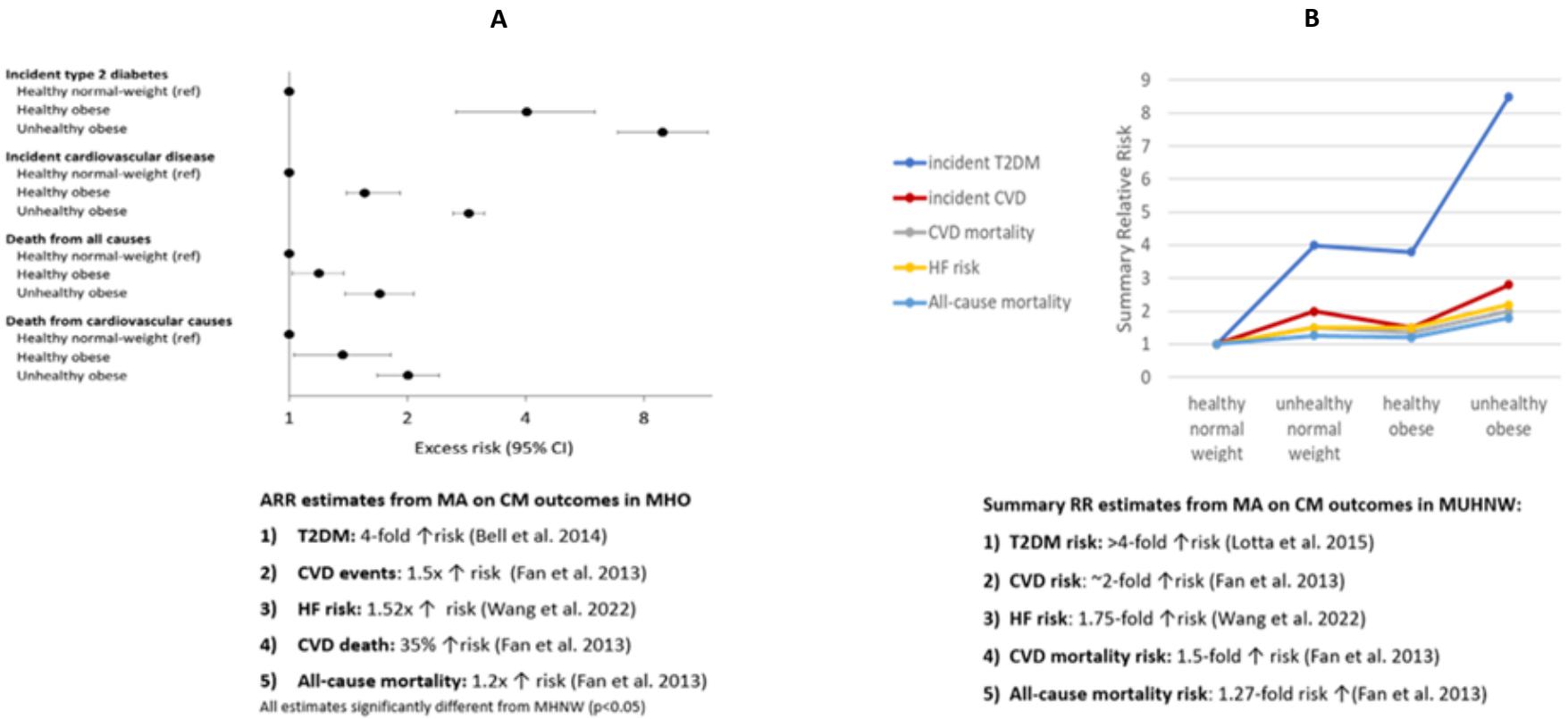
On the other hand, younger age, the female sex, lower baseline BMI and body weight and incorporation of a healthy lifestyle (a composite of diet, leisure time physical activity and smoking) were associated with sustained MH in individuals living with obesity (Moussa et al., 2019). Lately, Elias-Lopez et al., reported several novel factors which could be responsible for the risk of progressing to a unhealthy metabolic phenotype particularly in individuals with a BMI ≥25kg/m^2. These mainly consisted of sociodemographic and lifestyle variables including history of childhood obesity, number of pregnancies, socioeconomic status, a high carbohydrate diet, physical inactivity, low intake of vegetables and consumption of sweetened beverages. Contrariwise, higher HOMA-S (sensitivity) and HDL-C levels and weight loss ≥5% were associated with increased probability of reverting back to a healthy metabolic state (Elías-López et al., 2021). Moreover, evidence also shows that fluctuations in body weight including both weight gain and weight loss are risk factors for mortality, cardiovascular events and T2DM which may be partly explained by the adiposopathy that follows from weight cycling (Lei et al., 2022).

Thus, since the MHO phenotype tends to convert to the unhealthy metabolic state over time, one would expect it to result in increased cardiometabolic disease risk. In fact, meta-analyses of longitudinal studies across different ethnicities have shown that the MHO phenotype has an intermediate cardiometabolic disease risk between that of MHNW and MUHO (Bell et al., 2014; Eckel et al., 2016; Jung et al., 2015; Kramer et al., 2013) **(Figure 1.5).** However, there are other studies which report no excess risk when compared to MHNW, while others suggest that the accumulation of cardiometabolic risk factors rather than BMI, confers the greatest risk (Appleton et al., 2013; Dhana et al., 2016; Guo and Garvey, 2016; Hamer and Stamatakis, 2012; Kip et al., 2004; Song et al., 2007; Wildman et al., 2011b). For example Al-khalidi et al., reported that MHO was not associated with an increased risk of all-cause or cardiometabolic mortality when a harmonised definition of MH was used (Al-khalidi et al., 2018). More recently Zembic et al., also report no increased risk in total and CV disease mortality risk among individuals with MHO within two large data sets (NHANES III and UK Biobank) (Zembic et al., 2021).

Several factors have been implicated which might explain these observed differences. For example, the Zembic et al. definition used the WHR rather than the WC which incorporates the hip circumference i.e. lower-body fat and therefore has a stronger predictive power in terms of mortality risk; furthermore, other studies allowed up to 2 metabolic risk factors to be present while the Zembic et al. definition entailed that MHO individuals fulfil all 3 criteria in order to be considered healthy. Allowing the presence of 1 or 2 metabolic risk factors in the definition of metabolic health arbitrarily implies that the risk factors are metabolically equivalent with respect to cardioembolic disease risk;

however one study which investigated 1.8 million participants from 97 prospective cohort studies found that for each 5kg/m^2 increase in BMI, the excess risk for coronary heart disease and stroke was mostly mediated by blood pressure (accounting for 31% and 65% respectively) whilst serum cholesterol and glucose only accounted for 10%, and 15%, of excess risk respectively (Global Burden of Metabolic Risk Factors for Chronic Diseases Collaboration [BMI Mediated Effects], 2013). On the other hand, in The Health Improvement Network (THIN) study which consisted of 3.5 million participants, the authors observed that irrespective of BMI status, increasing number of metabolic abnormalities (from 0 to 3) led to a dose dependent increase in risk for CVD. Notably even when MHO was defined as having no metabolic abnormalities, the risk for several cardiovascular events (including stroke, heart failure and coronary heart disease) was still elevated compared to healthy normal weight individuals (Caleyachetty et al., 2017). Furthermore, while MHO individuals are observed to have a favourable metabolic profile, some studies noted subtle differences in cardiometabolic risk factors parameters (including larger WC, higher blood pressure, TG and insulin levels and lower HDL-C) relative to MHNW (Bell and Hamer, 2016; Marini et al., 2007; Mongraw-Chaffin et al., 2016). These differences also extend to subclinical CVD risk. A handful of cross-sectional studies show that MHO (even when defined as having no metabolic abnormalities including preserved insulin sensitivity) was associated with increased severity of carotid atherosclerosis and angiographic coronary artery disease (as assessed by increased carotid intima media thickness [cIMT]) and coronary artery calcification), compared to MHNW individuals , both of which are surrogate markers of increased subclinical CVD burden, (Chang et al., 2014; Kwon et al., 2013; Sinn et al., 2020).

However, in the study by Lin et al., increased risk of subclinical atherosclerosis only occurred in MHO individuals who transitioned to the unhealthy obese phenotype during a follow-up period of 4.4 years suggesting that preservation of metabolic health may be a therapeutic target for the prevention of CVD even in the absence of overt weight loss (Lin et al., 2020). Moreover, several studies found that number of metabolic abnormalities permissible at baseline and hence the criteria used to categorise the healthy obese individuals to also influence the relationship with cardiovascular outcomes (Caleyachetty et al., 2017; Guo and Garvey, 2016; Mongraw-Chaffin et al., 2016).



ARR: adjusted relative risk, MA: metanalyses, CM: cardiometabolic, MHO: metabolically healthy obese, MUHNW: metabolically unhealthy normal weight, T2DM: type 2 diabetes, CVD: cardiovascular disease, HF: heart failure

Figure 1.5: Long term outcomes of A) MHO and B) MUHNW phenotypes

A) The MHO phenotype has an intermediate cardiometabolic risk between that of healthy normal weight and metabolically unhealthy obese phenotypes

B) The MUHNW phenotype has a risk profile which is intermediate to that of metabolically healthy obesity and metabolically unhealthy obesity

Duration of follow-up has also been implicated as an important determinant in the relationship of MHO with long term cardiometabolic outcomes. Studies which followed-up MHO for more than 10 years found a higher risk compared to MHNW individuals , whilst those with a shorter duration (<10 years) found a similar risk, implying that a certain time lag may be necessary before the full effect of metabolic status is manifest (Appleton et al., 2013; Eckel et al., 2018; Hinnouho et al., 2015; Kip et al., 2004). These observations were emphasized in the meta-analysis by Fan et al., whereby the risk for cardiovascular events was reported to be approximately 1.2 and 1.5 times higher in participants living with healthy overweight or obesity respectively, compared to healthy normal weight individuals. Moreover this risk was higher in those studies which had a follow-up period of >15 years (pooled relative risk of 1.47 and 2.00 respectively) (Fan et al., 2013). Subsequent meta-analyses also came to a similar conclusion however, the one by Zheng et al., also observed that when the MHO phenotype was described as having zero cardiometabolic risk factors, it did not present excess CV risk. Additionally, MHO individuals were also not associated with increased risk of all-cause mortality (HR 1.07, 95% CI 0.92 to 1.25). The authors postulated that MHO individuals are associated with higher levels of cardiorespiratory fitness and the potential 'protective' effect of overweight and class I obesity (BMI 30-35 kg/m^2) on all-cause mortality which could in part explain these findings (Eckel et al., 2016; Kramer et al., 2013; Zheng et al., 2016).

Interestingly, maintenance of metabolic health or transition to an unhealthy phenotype was also associated with different effects on long-term risk (Appleton et al., 2013; Eckel et al., 2018; Lee et al., 2022; Mongraw-Chaffin et al., 2018). The Nurses' Health Study showed that females with obesity who progressed from a healthy to unhealthy

metabolic state over 20 years where at higher CVD risk than those who maintained the MHO phenotype, yet those who maintained their MHO status over the same time period were still at higher risk for CVD when compared to stable healthy normal weight females (Eckel et al., 2018). Conversely data from the ATTICA study observed that while approximately half of their MHO participants maintained a healthy metabolic status (defined as absence of all NCEP ATPIII criteria), excess CVD risk was only observed in the subset of participants who converted to an unhealthy metabolic status (Kouvari et al., 2022). Furthermore, several studies agree that the MHO phenotype is not protective from heart failure risk. In the HUNT study (Nord-Trondelag Health Study) the authors observed that while individuals with severe or long-lasting MHO were not at increased risk for acute myocardial infarction compared to healthy normal weight individuals, those with unhealthy or healthy subtypes of obesity had similar increased risk for incident heart failure (Mørkedal et al., 2014). Other reasons for the observed incongruencies in results could also in part be explained by the different populations being studied including differences in ethnicity, background prevalence of obesity and other sociodemographic factors known to impact on cardiometabolic disease risk (such as education level, dietary quality and economic status).

There is some evidence that protection from the onset of CVD in MHO individuals might be due to the presence of preserved cardiorespiratory fitness (CRF). Accordingly, Ortega et al. observed that individuals who were metabolically healthy but had a high body fat per cent had a better level of CRF (as assessed by a maximal treadmill exercise test) which led to a 30-50% lower risk for cardiovascular outcomes compared to metabolically unhealthy but obese individuals. Furthermore no difference in risk was observed

between MHO individuals and metabolically healthy normal-fat participants (Ortega et al., 2013). Additionally, a recent meta-analysis by the same authors confirmed that MHO individuals engaged in more physical activity, had higher levels of CRF and spent less time in sedentary behaviour compared to unhealthy individuals with obesity. Notably, risk for all-cause and cardiovascular mortality and morbidity became insignificant after adjusting for physical activity (Ortega et al., 2018).

Studies which evaluated the risk of T2DM in MHO individuals found an approximate 4-fold increase in risk compared to MHNW participants . Furthermore, this risk was observed to be higher in Asian populations and was independent of the presence of metabolic abnormalities, such that a BMI ≥25 kg/m^2 conferred a significantly higher risk for T2DM even when no metabolic risk factors were present (Bell et al., 2014; Jung et al., 2015; Twig et al., 2014). Moreover, individuals who progressed to MUHO were at higher risk for developing T2DM compared to those who retained the MHO status, yet this risk was lower when compared to those with stable MUHO (Song et al., 2022). Contradicting this is a study by Wang et al. which reported that individuals with stable and persistent MHO were not at increased risk for incident T2DM compared to stable MHNW individuals after 6 years of follow-up, albeit with very wide 95% confidence intervals in the odds ratio (0.20-1.40) (Wang et al., 2018). Others also noted that the risk for T2DM in MHO varied according to the degree of subclinical systemic inflammation (meta-inflammation), insulin resistance and beta-cell function as well as the presence of liver and visceral abdominal fat, further demonstrating that MHO itself is a very metabolically heterogeneous condition (Ampuero et al., 2020; Hjelmgren et al.,

2020; Hwang et al., 2015; Jung et al., 2016; Rydén et al., 2019; Sung et al., 2012b; Wu et al., 2022).

Few studies are available which assess long-term cardiometabolic trajectories in MUHNW. By definition, these individuals exhibit an unfavourable anthropometric, cardiometabolic and inflammatory parameters as well as adverse adiposity profiles (including BMI and parameters of body fat distribution) and are thus described as being 'fatter' than metabolically healthy normal weight individuals (Dvorak et al., 1999; Eckel et al., 2015; Wildman et al., 2011a; Xia et al., 2017). As has been alluded to earlier, studies have shown that the presence of metabolic abnormalities lead to a dose-dependent increase in cardiometabolic and mortality risk compared to MHNW (Caleyachetty et al., 2017; Guo and Garvey, 2016; Wang et al., 2018; Wang et al., 2021b). One meta-analysis showed that metabolically unhealthy individuals carried a significantly higher risk of incident T2DM compared with metabolically healthy participants across all BMI categories particularly in East Asian ethnic groups (Lotta et al., 2015).

Additionally it was observed that the MUHNW phenotype carries the same, if not greater, risk for adverse long-term outcomes as that of MUHO, yet others observed it to present a worse cardiometabolic prognosis when compared to MHO (Choi et al., 2013; Guo and Garvey, 2016; Kramer et al., 2013) **(Figure 1.5).** Furthermore, Wang et al. observed that within a cohort of rural Chinese adults, compared to stable MHNW individuals , stable MUHNW conferred a higher risk for diabetes after 6 years of follow-up than did transition from MHO to MUHO (HR 5.78, 95% CI 3.15-10.62 vs 4.52, 95% CI 2.42-8.47 both $p<0.001$, respectively) (Wang et al., 2021b).

With respect to incident CVD risk, Fauchier et al. showed that in normal weight participants , even the presence of just one metabolic abnormality elevated the risk for a number of CV events (including MACE-HR [a composite of myocardial infarction, heart failure, ischaemic stroke or cardiovascular death], cardiovascular death, myocardial infarction, ischemic stroke, new-onset heart failure and new onset atrial fibrillation) compared to normal weight individuals with no metabolic abnormalities. Furthermore, this risk was higher than that observed in participants with obesity but with no metabolic abnormalities (Fauchier et al., 2021). Similar findings were also observed in a Chinese study whereby after adjusting for a number of potential confounding factors, non-obese individuals with more than 2 metabolic risk factors were at a higher risk for incident CVD compared to MHO when using metabolically healthy normal weight participants as the referent group (HR= 2.31, 95% CI 1.70-3.14 vs 1.76, 95% CI 1.23-2.51 respectively) (Wang et al., 2021a).

The worse cardiovascular outcomes observed in both MUHNW and MUHO individuals is partly attributable to a higher burden of liver fat and increased subclinical cardiac systolic and diastolic dysfunction. This suggests that BMI may not be the sole driver for myocardial dysfunction but rather the overall metabolic profile (Dobson et al., 2016). Additionally, the metabolically unhealthy normal weight phenotype was associated with higher prevalence and severity of angiographic coronary artery disease even after controlling for potential confounding factors (Kwon et al., 2013). Studies have also shown the MUHNW phenotype to be associated with increased risk of heart failure. In fact, Voulgari et al. observed that lean participants with Met S were at approximately 2.5 fold increased risk for incident heart failure while individuals with overweight and

obesity and without Met S had the lowest risk compared to normal weight individuals without Met S (HR: 2.33, CI: 1.25-4.36; 1.12, CI: 0.35 to 0.33 and 0.41, CI: 0.10-1.31respectively) (Voulgari et al., 2011). All things considered, these associations reinforce that both MHO and MUHNW are not benign conditions, rather they are dynamic states that represent a spectrum of adverse cardiometabolic disease risk with the risk of MHO being intermediate to that of MHNW and MUHNW.

1-5.4 Management of MHO and MUHNW

At present most scientific organisations advocate a weight loss of around 5%–10% from baseline body weight (using a combination of lifestyle interventions, pharmacotherapy or bariatric procedures) for all individuals living with obesity since it is associated with improvements in several cardiometabolic risk factors (Garvey et al., 2016; Jensen et al., 2014). With respect to lifestyle modifications, one meta-analysis found that in individuals with MHO, caloric restriction led to reductions in BMI, systolic and diastolic blood pressure, and triglycerides, but not in glucose, insulin resistance, or CRP (Stelmach-Mardas et al., 2016). Specifically, adherence to a Mediterranean-Style diet (characterised by an abundant consumption of monounsaturated fat in the form of olive oil and nuts), has been consistently associated with a lower incidence of CVD, T2DM, all-cause mortality and cancer mortality, even in the absence of significant weight loss (Di Daniele et al., 2017). Moreover, in the study by Park et al., MHO individuals who complied with the Mediterranean diet had a lower all-cause mortality risk over a median follow-up of 18.5 years. Interestingly however, such findings were not observed among MUHO individuals, suggesting that this phenotype may require alternative therapeutic strategies to reduce mortality risk (Park et al., 2016). Recently, a randomised controlled

study by Lean et al. showed that the use of a low-calorie diet (by way of a low energy formula replacement diet) within a primary care setting was associated with remission of T2DM in participants with overweight or obesity (Lean et al., 2018). However, it is acknowledged that achieving and maintaining weight loss can be challenging for many patients and therefore another plausible treatment strategy would be one which shifts the attention towards the improvement or maintenance of cardiometabolic health rather than solely focussing on weight loss (Hall and Kahan 2018). Given the aforementioned findings, incorporating a Mediterranean style diet (which consists of qualitative rather than quantitative changes to the macronutrient component) can be a more acceptable treatment option for patients and may also encourage adherence to lifestyle changes (Gaesser et al., 2011).

Over the past decade the US Food and Drug administration (FDA) has approved a handful of pharmaceutical agents as part of the treatment armamentarium for the chronic management of obesity in conjunction with lifestyle modification. Recently, semaglutide (a glucagon like peptide -1 receptor agonist [GLP-1 RA]) was observed to induce a mean weight loss of approximately 15% as well as improvements in a number of cardiometabolic endpoints in the phase 3 Semaglutide Treatment Effect in People with Obesity (STEP) clinical programme (Wilding et al., 2021). Furthermore, studies have shown that this agent also exerts positive effects on the cardiovascular system through weight-independent mechanisms, including improved vascular endothelial function, ischaemic conditioning and reduction in systemic inflammation (Dai et a., 2013; Zhao et al., 2006; Zhao et al., 2021). Furthermore, it was also observed to have a positive impact on body composition through significant improvements in VAT, fat mass index (FMI),

epicardial fat and hepatic steatosis, thus making it an appealing drug in the management of different obesity phenotypes (Volpe et al., 2022; Volpe et al., 2022a; Wilding et al., 2021a).

Up till now, bariatric surgery is still deemed to be the most effective strategy for weight loss and weight loss maintenance (Buchwald et al., 2004). It has also been associated with improvements in cardiometabolic risk factors, improvement or remission of T2 DM, reduction in CVD events, total mortality, and cancer incidence (Sjostrom, 2014; Sjostrom 2008). Both sleeve gastrectomy and gastric bypass are associated with significant reductions in visceral fat depot sizes and in adipose tissue inflammation which may account for the favourable effects observed on glucose metabolism in insulin sensitive tissues (Cancello et al., 2005). Genua et al., noted that the MHO phenotype was associated with a higher percentage of total weight loss when compared with MUHO and this was independent of the baseline BMI and type of surgery performed (Genua et al., 2021). On the other hand, Goday et al. assessed the metabolic benefits of bariatric surgery in participants who were metabolically healthy but morbidly obese (Goday et al., 2014). They observed significant improvements in blood pressure, lipid profiles, plasma glucose and HOMA-IR values 1 year after bariatric surgery, despite them having lost a comparable amount of weight to their unhealthy counterparts. Overall, these studies show that there is a role for bariatric surgery even in people living with obesity but who are metabolically healthy since they still tend to gain from a metabolic standpoint.

The management of MUHNW may prove to be more challenging in clinical practice (Rubin, 2018). Such individuals have a higher amount of visceral and liver fat compared to healthy normal weight individuals and an abnormal cardiometabolic and inflammatory profile (rendering them at heightened cardiometabolic disease risk) despite exhibiting normal body weight and/or BMI and thus may present to clinical practice at more advance stages of disease. Management of this obesity phenotype would involve early identification and treatment of the different metabolic abnormalities by way of diet, exercise and pharmacotherapy for hypertension, dyslipidaemia and dysglycaemia in order to off-set both the onset of obesity and the progression to overt cardiometabolic diseases. Whilst some studies have shown that weight loss through caloric restriction to be associated with improvements in metabolic dysfunction and body composition in people with MUHNW, others have demonstrated that it is the quality rather than the quantity of calories consumed to be the key factor in reducing cardiovascular disease risk and mortality in this cohort of patients (Park et al., 2016a). Analogous to individuals with acquired or inherited lipodystrophies, management of patients with MUHNW would involve some form of treatment which would promote adipocyte differentiation and insulin sensitivity to restore MH. Such individuals also have low percentage leg fat mass, implying impaired subcutaneous adipose tissue expansion. Accordingly, Stefan et al. suggested the use of PPAR-γ agonists such as pioglitazone since these drugs have insulin-sensitising effects as well as improved adipocyte differentiation (Stefan et al., 2017; Stefan et al., 2020a).

To date current obesity management guidelines do not distinguish between management of different subclasses of obesity, in particular there is no mention of the

assessment of body fat distribution or visceral adiposity in the work-up towards risk stratification and targeted treatment strategies for individuals with excess adiposity.

Furthermore, sole use of BMI as the definition of obesity would miss identification and management of those normal weight individuals with abnormal metabolic profiles who are also at increased risk of morbidity and mortality. Therefore, this underscores the pressing need to characterize body composition (using either imaging-based modalities such as bioimpedance analyses or non-imaging based anthropometric markers of central adiposity such as the WC, WHR or WHR) in addition to measurement of BMI in order to allow for better risk stratification of individuals as well as the tailoring of personalised and cost-effect therapeutic options rather than the traditional 'one size fits all' approach.

1.6 Potential role of mitochondrial DNA in obesity and metabolic health

1-6.1 Introduction: Overview of the role of mitochondria in health and disease

Mitochondria are ubiquitous subcellular organelles with their own circular genome and are the primary metabolic platform within eukaryotic systems. They play a key role in cellular energy production and in maintaining metabolic homeostasis in a number of mammalian tissues such as skeletal and cardiac muscle, liver and adipose tissue by generating adenosine triphosphate (ATP) through the process of oxidative phosphorylation (Johannsen and Ravussin, 2009). In essence, mitochondria modulate energy metabolism by utilizing substrates generated from the catabolism of nutrients (including lipids, proteins and carbohydrates) in order to produce ATP (and other by-products such as heat and water) via a process involving the transfer of electrons through complexes of the electron transport chain (ETC). Beyond nutrient metabolism, they also play an important role in other cellular processes including signal transduction and insulin metabolic signalling, cell proliferation, differentiation and apoptosis and are also thought to be associated with several biosynthetic pathways (including the synthesis of macromolecules such as nucleotides, heme and steroid hormones) thus making them indispensable for maintaining the overall health of an organism (Guha and Avadhani, 2013; Vakifahmetoglu-Norberg et al., 2017; Wallace, 2018). Furthermore, mitochondria within eukaryotic cells are able to undergo adaptive responses under conditions of environmental stress such as cell growth and death by regulating their number or morphology or by remodelling their organisation and distribution (Lee et al., 2019; McBride et al., 2006; Wang and Youle, 2009). Whilst it is known that the dynamics of mammalian nuclear and mitochondrial genome differ from each other, there are still

gaps in the knowledge of mitochondrial genome regulation. The nuclear genome contains only two copies of DNA per cell, while the mitochondrial genetic system (which is exclusively maternally inherited) is polyploid with up to several thousand copies of DNA strands per cell depending on the type of cell and its energy demands such that those having high ATP requirements (as occurs in cardiac and skeletal myocytes) would contain higher mtDNA copies than would low energy requirement cells such as the spleen and liver (Castellani et al., 2020; Chabi et al., 2003).

Primary (inherent) disorders of the mitochondria involving qualitative changes to its genome such as mutations, insertions and deletions result in rare metabolic and neurodegenerative diseases some of which have diabetes as an accompanying feature, and which are associated with severe morbidity or early death. For example, a point mutation in the gene coding for $tRNA^{Leu(A\ to\ G)}$ at position 3243 is commonly associated with maternally inherited diabetes and deafness. Such individuals are more likely to have a mother affected with diabetes, generally present with diabetes at a younger age and typically require insulin treatment. However these clearly cannot account for the majority of metabolic diseases and therefore can only explain a small proportion of people with T2DM (Ballinger et al., 1992; Johannsen and Ravussin, 2009; Reardon et al., 1992; Suzuki et al., 1994). It has thus become increasingly apparent that qualitative and quantitative changes of a milder nature within the mitochondrial genome contribute towards the patho-aetiology of more common chronic diseases. Mitochondria are highly susceptible to oxidative stress leading to inefficient cellular energy production and increased formation of reactive oxygen species (ROS), resulting in both qualitative and quantitative changes which ultimately lead to mitochondrial damage and dysfunction

(Larsson and Clayton, 1995; Rösen et al., 2001). Under normal circumstances the process of oxidative phosphorylation can generate between 0.2 to 2% of ROS, however conditions which may cause defects in the transfer of electrons through the ETC can lead to the accumulation of electrons within these complexes and enhanced ROS production which may exceed the antioxidant capacity of the mitochondria leading to cell damage or death (Harper et al., 2004; Wallace, 1999). In fact, there is considerable data which shows that conditions associated with increased oxidative stress (as occurs with a variety of chronic disorders such as cardiovascular and neurological diseases, cognitive decline, chronological age and cancer) to be associated with alterations of mitochondrial biogenesis and function (Johannsen and Ravussin, 2009; Nicolson, 2014; Ren et al., 2010; Runge et al., 2007; Short et al., 2005). Specifically, a growing body of evidence has implicated the important role of mitochondrial bioenergetics in metabolic disorders typically associated with insulin resistant states such as the Met S, obesity, T2DM and metabolic cardiomyopathy (a condition characterized predominantly by diastolic dysfunction) citing an array of abnormalities in mitochondrial metabolism ranging from reduced expression of genes associated with mitochondrial biogenesis, lower protein subunits of respiratory chain complexes, reduced oxidative enzyme activity, as well as decreased mitochondrial size/number and density (Cheng and Ristow, 2013; Kim et al., 2008; Ørtenblad et al., 2005; Petersen et al., 2005; Ren et al., 2010). This thus essentially underscores the pivotal role of mitochondria across different cellular pathways and throughout major organ systems such that any perturbation of their function will incite downstream deficits in vital functions such as skeletal muscle contraction, hepatocyte metabolism, insulin production and metabolic signalling, neuronal health and cardiac

function (Pinti et al., 2019). This literature review will specifically focus on mitochondrial (dys)function and how it relates to IR, metabolic diseases, and obesity.

Under normal physiological conditions, insulin acts to maintain glucose homeostasis during both the fed (pre-absorptive) and fasted (post-absorptive) states either through glucose uptake or via hepatic glucose production via glycogenolysis or from gluconeogenic precursors (such as glycerol and amino acids) respectively. Circumstances which are notably associated with increased lipid supply (for example increased lipid availability due to a high-energy diet or increased lipolysis) cause defects in insulin metabolic signalling compromising both glucose-mediated insulin secretion from the pancreas and in insulin-stimulated glucose disposal within various insulin-responsive tissues (such as skeletal muscle, liver and adipose tissue) as well as abnormalities in hepatic glucose production (Kim et al., 2008; Lowell et al., 2012; Ren et al., 2010; Saltiel and Kahn, 2001). Furthermore, findings from clinical and experimental human and rodent studies were able to demonstrate that elevated circulating level of FFAs as occurs when there is an imbalance between energy intake and expenditure such as during high-fat feeding, leads to the intracellular accumulation of toxic metabolites including long chain acyl-CoA, diacylglycerol and acylcarnitine and overspill of fat in non-adipose tissues such as the liver and muscle as ectopic fat (Boden et al., 1991; Han et al., 1997; Kelley et al., 2022; Szendroedi and Roden, 2008). These perturbations in lipid metabolism generate a cascade of maladaptive processes within the mitochondria leading to inefficient oxidative phosphorylation resulting in an imbalance between ATP/ADP generation and an increase in ROS production shifting the cellular environment towards a more oxidized state as well as causing defects at the protein and

transcriptional levels including those associated with mitochondrial biogenesis (Anderson et al., 2009; Boden, 2006; Kelley et al., 2002b). Collectively, these processes contribute towards global mitochondrial dysfunction by way of a reduced mitochondrial density, size/number and a decrease in mitochondrial metabolic efficiency. This, in turn, stimulates inflammatory pathways which interfere with downstream insulin metabolic signalling giving rise to IR and the onset of several metabolic diseases such as T2DM and fatty liver disease (Kim et al., 2008; Runge et al., 2007). In fact, initial studies from the 1990s had already demonstrated oxidant-mediated repression of mitochondrial transcription and a lower mitochondrial DNA content in mice models with acquired diabetes (Kristal et al., 1997). Following these studies were a series of others carried out on isolated mitochondria from muscle biopsies of human individuals with a personal history of obesity and T2DM as well as in first-degree relatives of individuals with T2DM which also implicated abnormalities of mitochondrial metabolism in the pathogenesis of T2DM (Befroy et al., 2007; Morino et al., 2005; Petersen et al., 2005; Ritov et al., 2005). Taken together, the theory that seemed to be evolving from these observations was that mitochondrial oxidative capacity is reduced in individuals with T2DM and furthermore, the accumulation of intracellular toxic lipid metabolites subsequently leads to perturbations of insulin signalling in insulin-sensitive tissues. Interestingly however, some studies challenged this theory. For example, Phelix and co-workers observed a reduction in insulin sensitivity in participants with obesity and T2DM however sensitivity was not related to mitochondrial dysfunction in skeletal muscle or to the intramyocellular lipid content and furthermore intracellular lipid levels did not differ between individuals with diabetes, their first degree relatives or normoglycemic

healthy volunteers (Phielix et al., 2008). Additionally, another study reported a similar level of mitochondrial activity (as assessed by several methods including citrate synthase activity and maximal mitochondrial ATP production rate) within Indian participants irrespective of their diabetes status but this was higher when compared with non-diabetic Northern European Americans suggesting a dissociation between mitochondrial dysfunction and IR. Still, these observations highlight the importance of considering race/ethnicity when investigating mitochondrial dysfunction within the context of IR and also shed light on the fact that at least within certain ethnic groups, IR may develop independently of mitochondrial function and that other pathways may be responsible for the development of IR (Nair et al., 2008).

Alterations in the oxidative capacity of the mitochondria have also been linked with body weight regulation. As alluded to previously, energy homeostasis is highly preserved within the mitochondrial milieu by maintaining a balance between nutrient metabolism and ATP generation. Under normal circumstances and depending on the energy requirements of the body, the process of oxidative phosphorylation allows for inefficient coupling of nutrient substrate oxidation with ATP formation. This incomplete coupling gives rise to a 'proton leak' which causes energy in substrates to be lost as heat. This process occurs through the action of three uncoupling proteins (UCP) and is collectively responsible for up to 20-25% of the body's basal metabolic rate as well as body weight regulation, adaptive thermogenesis, and also safeguards against ROS generation and oxidative damage (Harper et al., 2004; Vidal-Puig et al., 2000). Thus, mitochondrial coupling efficiency determines the proportion of calories from substrate metabolism that are eventually used to generate energy as ATP or heat (and may in part explain the

variation in body weight observed in individuals with similar nutrient intake) such that individuals with high coupling efficiency will predominantly generate energy as ATP with little being lost as heat thereby causing excess energy to be stored as fat, while those with low coupling efficiency will have a greater proportion of energy being dissipated as heat and ATP required for normal cellular function must either come from additional substrate metabolism or from adipose stores leading to weight loss (Harper et al., 2004; Johannsen and Ravussin, 2009).

Furthermore, in addition to aberrations of nutrient metabolic signalling and oxidative stress it is worth mentioning genetic and environmental factors (including diet, lack of exercise, aging and stress), inappropriate activation of the renin-angiotensin-aldosterone system (RAAS), as well as infective processes and use of certain medications to be other putative factors which may predispose at least in part to mitochondrial dysfunction and to the onset of IR and its associated complications and which may thus contribute towards a common pathophysiologic aetiology for many chronic diseases (Castellani et al., 2020; Kim et al., 2008; Ritz and Berrut, 2005). Additionally, pharmacological interventions targeted at improving IR and glucose metabolism such as the use of thiazolidinediones and metformin have been also associated with enhanced mitochondrial function and mitochondrial biogenesis corroborating further the notion that alterations in mitochondrial integrity plays a central role in insulin metabolic signalling as well as in the downstream onset of an array of metabolic and cardiovascular diseases (Airaksinen et al., 2005; Cleasby et al., 2004; Kim et al., 2008) **(Appendix 3).**

Nevertheless, reliable measures of mitochondrial dysfunction in clinical practice remain a limiting factor. Recently, quantification of mtDNA copy number (mtDNA CN) which is

a surrogate index of cellular mitochondrial DNA content, has been increasingly employed in clinical and population studies as a biomarker of mitochondrial function and which may thus reflect the degree of mtDNA damage (Afshan N Malik and Czajka, 2013). This stems from findings which demonstrated a direct correlation between levels of mtDNA CN and mitochondrial oxidative stress and energy reserve (Guha and Avadhani, 2013). Thereafter, a series of studies followed which were designed to explore the relationship between quantitative mtDNA status and the presence of common chronic diseases. Some of the earliest research was conducted with the aim of finding a relationship between mtDNA CN, insulin sensitivity andT2DM; however this was accompanied with conflicting results (Lee et al., 1998; Singh et al., 2007). Later came a number of other studies which showed that reduced levels of mtDNA CN in several tissues (including skeletal myocytes, leukocytes, hepatocytes and adipocytes) to be associated with BMI, visceral adiposity and hyperlipidaemia, while others were able to demonstrate that changes in mtDNA CN precede the onset of a number of age-related conditions such as atherosclerotic CVD, chronic kidney disease, neurodegenerative disorders, cognitive decline, and cancer (Dai et al., 2012; Koller et al., 2020; Lee et al., 2010; Lee et al., 2014a; Mengel-From et al., 2014). This lends support to the notion that mtDNA regulation is causative to the development of a variety of chronic diseases (Castellani et al., 2020). However, while the precise mechanisms linking mitochondrial dysfunction to chronic diseases remain yet to be elucidated, the purported theories revolve around alterations in cell signalling pathways and changes in inflammatory dynamics and immune function (Castellani et al., 2020). Abnormalities in mitochondrial oxidative function as occurs in the context of a reduction in mtDNA CN has been linked

to chronic inflammation by way of recruitment of pro-inflammatory M1 macrophages rather than anti-inflammatory M2 macrophages. Complementing the macrophage hypothesis is the immune hypothesis since many of the diseases associated altered mtDNA CN (such as atherosclerotic CVD) are also associated with immune dysfunction on top of a chronic pro-inflammatory state. Furthermore, dysfunctional mitochondria (as occurs in conditions associated with a reduction in mtDNA CN) are associated with a disruption of the mitochondrial membrane potential leading to changes in nuclear gene expression through retrograde signalling. This allows the nucleus to undergo adaptive responses by way of an altered nuclear gene expression profile leading to changes in cell physiology and morphology thereby increasing the risk for chronic diseases (Castellani et al., 2020; Guha and Avadhani, 2013).

Overall, while considerable evidence is available indicating the central role of mitochondrial metabolism in the onset of a number of chronic diseases there are still inconsistencies in findings thus making it challenging to draw any definite conclusions at this point. Furthermore, most of the data comes from cross-sectional studies thus limiting the inference of direction of the relationship. Thus, more prospective clinical trials are required in order to fully determine whether mitochondrial dysfunction is indeed the perpetrator rather than the consequence of metabolic disorders.

1-6.2 Mitochondrial DNA copy number (mtDNA CN) and its association with insulin resistance, metabolic disorders and obesity

The recent observations that mitochondrial dysfunction may be at the centre for the development of several chronic metabolic disorders and that mtDNA CN is a surrogate of mitochondrial function prompted investigators to explore further the nature of the relationship of this parameter with IR, T2DM, and obesity as well as to try and shed light into the causal mechanisms linking the conditions together. Some of the earliest studies revolved around quantification of mtDNA CN in both healthy and diabetic individuals and how this related with insulin metabolic signalling as well as metabolic variables associated with IR, fuel metabolism and T2DM (Antonetti et al., 1995b; Lee et al., 1998; Lim et al., 2001; Morino et al., 2006; Soo et al., 2001, 1999). One such study was that by Antonetti and colleagues which showed a 50% reduction in mtDNA CN within skeletal muscle of individuals with T2DM (Antonetti et al., 1995b). This was subsequently confirmed in a study by Lee and co-workers which observed a quantitative decrease of up to 35% in mtDNA content within the peripheral blood of individuals with noninsulin dependent diabetes mellitus compared to normal individuals. More importantly this reduction preceded the development of diabetes and significant inverse associations were observed between mtDNA content and parameters linked with IR including WHR, fasting glucose level and blood pressure ($p<0.05$). Interestingly, however, while the same authors found a correlation between parameters of fuel metabolism (positively with changes in fat oxidation rate and negatively with changes in carbohydrate oxidation rate) and mtDNA content under euglycemic clamp conditions, they did not find any

association between mtDNA content and indices of IR within healthy lean individuals (Soo et al., 1999). These findings however, were not supported in two subsequent studies by the same authors on healthy young volunteers which confirmed a negative correlation between several surrogate indices of IR and insulin secretion such as WHR, HOMA-IR score, ratio of fasting glucose to insulin concentration and fasting insulin levels (Lim et al., 2001). This led the researchers to suggest that mtDNA content may be related with both clinical and metabolic parameters of IR. Moreover, since lower mtDNA levels (and hence an increased oxidative state) precede the onset of diabetes, then, quantitative reductions in mtDNA content is casual to rather than a consequence of, diabetes, and other mechanisms may be responsible for the increase in oxidative stress found in diabetes (Lee et al., 1998). To assess this relationship further, the same authors went on to investigate whether the amount of mtDNA content in metabolically more important tissues other than peripheral blood could have an impact on glucose and insulin metabolism. They and others were able to confirm that mtDNA depletion in human hepatic cells leads to an attenuation in glucose uptake and utilization via a reduction in the expression of all nuclear-encoded glucose transporters and in enzymes associated with glucose metabolism (including hexokinase, GAPDH and G6PDH) while mtDNA depletion in rodent pancreatic beta cells is associated with insulin secretory defects leading to glucose intolerance and the development of diabetes (Park et al., 2001; Soejima et al., 1996).

Subsequently, several other researchers went on to explore the direct relationship between mtDNA CN and T2DM and how this varies within different peripheral tissues,

yielding conflicting associations. A number of studies corroborated the findings of a reduced mtDNA CN within skeletal muscle, peripheral blood and adipose tissue from participants with T2DM (DeBarmore et al., 2020; Memon et al., 2021; Morino et al., 2005; Song et al., 2001; Xu et al., 2012). Furthermore a lower mtDNA content was also observed in the offspring of individuals with T2DM who exhibited normal or impaired glucose tolerance and was the main predictor of insulin sensitivity in this cohort, suggesting a heritable trait controlling mtDNA content (Song et al., 2001). Additionally the authors also found that a decrease in mtDNA CN in peripheral blood predates the onset of T2DM, thus lending further support for a potential transgenerational role of mitochondrial dysfunction in the pathogenesis of IR and T2DM (Song et al., 2001).

By the turn of the century, many studies had consistently shown that measures of intramuscular triglyceride stores (also known as intramyocellular lipid) to be strongly associated with IR in skeletal muscle of healthy individuals as well as in insulin-resistant offspring of parents with T2DM. Furthermore, studies on individuals with defects in adipocyte metabolism as occurs in individuals with lipodystrophy showed a preferential accumulation of fatty acids within the liver and these patients typically present with severe IR. However, the mechanisms responsible were still poorly understood (Kelley et al., 2002a; Lowell et al., 2012; Petersen et al., 2002). Building up on these findings as well as trying to elucidate the underlying mechanisms responsible for the onset of IR in young lean individuals of relatives with T2DM was the elegant study by Petersen and colleagues. Here, the authors measured intramyocellular lipid and intrahepatic

triglyceride content by using proton magnetic resonance spectroscopy and thereafter subjected the participants to either euglycemic clamp studies to assess tissue responsiveness to insulin as well as indirect calorimetric testing to assess basal and stimulated rates of whole body energy expenditure, fat and glucose oxidation or a ^{31}P magnetic resonance spectroscopy study to assess rates of muscle mitochondrial phosphorylation (Petersen et al., 2004). A significantly lower rate of insulin-stimulated glucose uptake was observed in the insulin-resistant individuals vs insulin-sensitive controls, and this was accompanied by an approximately 30% reduction in mitochondrial oxidative function and an associated 2-fold increase in intramyocellular lipid (IMCL) content. Taken together the authors speculated that dysregulation of fatty acid metabolism in these individuals may be the mediator linking mitochondrial dysfunction with severe muscle IR. Following this hypothesis were several other studies supporting the concept that abnormalities of mitochondrial metabolism cause an accumulation of fatty acids in important tissues such as muscle and liver and which in turn are responsible for the onset of IR and T2DM (Petersen et al., 2005). This theory lends further support in establishing the importance of normal mitochondrial function for the maintenance of blood glucose homeostasis.

Central to their role in glucose sensing in the liver and skeletal muscle, the mitochondria also play a role in pancreatic beta cell function mainly by maintaining normal responsiveness of beta cells to glucose and ensuring the release of appropriate amounts of insulin. Mitochondrial oxidative function is central to glucose-stimulated insulin secretion. Under normal circumstances ATP generated from glucose oxidative metabolism leads to a chain of events within the pancreatic beta cells which are involved

in the secretion of insulin (namely closure of cellular ATP/ADP-regulated potassium channel, plasma membrane depolarisation and the opening of voltage-gated calcium channels leading to an influx of calcium and the secretion of insulin) (Maechler and Wollheim, 2001). Thus, any abnormalities in mitochondrial function which impacts on the ATP/ADP ratio or in conditions associated with mitochondrial DNA depletion will lead to reduced beta cell mass resulting in impaired insulin secretion and development of frank diabetes (Soejima et al., 1996).

On the other hand, some studies found no relationship between mtDNA content and prevalent or incident T2DM, while others observed positive associations between mtDNA content and glucose metabolism (Lindinger et al., 2010; Malik and Czajka, 2013; Reiling et al., 2010; Weng et al., 2009). For example the study by Weng and colleagues demonstrated an increase leucocyte mtDNA CN as well as markers of oxidative stress with a progressive deterioration in glucose metabolism (from normal glucose tolerance vs impaired fasting glucose vs fank diabetes) even after adjusting for typical confounding factors such as age, sex and BMI (Weng et al., 2009). Furthermore, a positive correlation was observed between mtDNA content and glucose dysregulation even after correcting for potential confounding variables with hyperglycaemia emerging as the only predictor of mtDNA copy number in cases of glucose dysregulation. Hsieh and colleagues however, reported tissue specific differences in mtDNA content in patients with T2DM with a higher leukocyte and lower muscle mtDNA content (Hsieh et al., 2011).

One plausible explanation for the directionally inconsistent associations observed could be attributed to the different tissue-specific effects of oxidative stress on rates of

mitochondrial turnover and copy number such that cells with a longer lifespan are associated with higher mtDNA levels (Liu et al., 2003).

Interestingly another study reported a 35% reduction in *in-vivo* ADP-stimulated mitochondrial respiration in patients with T2DM after normalising for mitochondrial content, implying that mitochondrial dysfunction is mediated by inherent mitochondrial defects at the level of oxidative phosphorylation and electron transport chain rather than due to variations in mtDNA CN. Furthermore, a recent study comprising of approximately 11 thousand participants from the Atherosclerosis Risk in Communities (ARIC) study found that lower mtDNA CN measured from buffy coat to be associated with prevalent diabetes but not with incident diabetes. This sheds some light on the direction of causality by suggesting that mitochondrial dysfunction (at least in peripheral blood leukocytes) may not be the primary cause of T2DM, but rather, the presence of diabetes is likely to result in lower levels of mtDNA CN (DeBarmore et al., 2020).

Several studies have also investigated the relationship between mtDNA CN and the Met S. These report an overall lower mtDNA CN in individuals with the Met S and a higher number of Met S components correlates with a lower mtDNA CN within the general population (Huang et al., 2011; Kim et al., 2012). Furthermore, as already alluded to above, mitochondrial dysfunction including abnormalities in biogenesis and energetics also plays an important role in the pathophysiology of cardiometabolic diseases and obesity (Bournat and Brown, 2010; Nisoli et al., 2007). Metabolic perturbations within adipocytes as occurs with excessive caloric intake causes the mitochondria to respond by an alteration in number and morphology of the mitochondrion as well as by

modifying its metabolic and enzymatic capacity and/or its mitochondrial DNA content (Ritov et al., 2005).

Over the last decade a handful of epidemiological studies investigated the relationship between mtDNA CN within various tissues and how it relates with anthropometric measures of adiposity assessment and weight change. However, in a similar fashion to that of T2DM, there are inconsistencies in the literature regarding the relationship between mtDNA CN and obesity. Most researchers found a negative correlation between mtDNA CN and BMI while others did not find any relationship and with others even finding a positive association between mtDNA copies and BMI in participants living with obesity and T2DM depending on the tissue studied (Kaaman et al., 2007; Lee et al., 2014a; Lindinger et al., 2010; Xu et al., 2012). One particular study by Skuratovskaia and colleagues found that mean mtDNA CN was significantly lower in peripheral blood mononuclear cells (irrespective of BMI status) compared to other tissues (including liver, greater omentum, mesenterium and SAT); furthermore BMI correlated positively with mtDNA abundance in SAT but negatively with peripheral blood leukocytes and hepatocytes (Skuratovskaia et al., 2018). The authors attributed the findings within SAT to be associated with increased mitochondrial biogenesis as a compensatory feedback mechanism to counterbalance oxidative defects resulting from dysfunctional mitochondria in the face of weight gain. In a follow-up study on a cohort of participants living with obesity and T2DM, the same group of authors were able to demonstrate a dynamic relationship between mtDNA levels and BMI. Essentially, levels of mtDNA levels in peripheral blood increased to a statistically similar level to that of healthy normal weight patients one year after bariatric surgery (Skuratovskaia et al., 2019a). The

study by Lindinger and co-workers revealed a positive association between mtDNA content and BMI such that a higher BMI was associated with a 56% increase in mtDNA count in omental tissue of individuals with obesity (BMI>30 kg/m^2) vs those without obesity (BMI <30 kg/m^2) (Lindinger et al., 2010). Furthermore, there was no association of mtDNA count with age, sex, seasonal change or with markers of energy metabolism such as basal metabolic and fat oxidation rates. The reasons implicated for the observed variations in mtDNA content between different tissues could be due to the specialised function and cell turnover of the tissue under examination. For example, skeletal muscle cells are associated with high mitochondrial activity and turn over in order to generate enough energy required for contraction whilst the adipocyte's main function is that of energy storage and is thus associated with a lower overall cell turnover. In another study, Meng and co-workers were able to demonstrate significant inverse relationships between a number of anthropometric variables and leucocyte mtDNA CN (including weight, WC, BMI and WHR) and a bidirectional and inverse relationship between peripheral blood leukocyte mtDNA CN and weight gain (Meng et al., 2016).

Interestingly however, while the relationship between mtDNA CN and various cardio-metabolic risk factors and outcomes has been intensively investigated, few studies explored its relationship between healthy and unhealthy subtypes of obesity. Healthy obesity is typically described in individuals with a BMI >30 kg/m^2 and the presence of few or no Met S parameters or with preserved insulin sensitivity, whilst people with unhealthy obesity are those individuals who harbour the cardiometabolic risk factors associated with the Met S or who present with IR. Likewise a subset of normal weight individuals may also present with an unhealthy metabolic profile and are termed as

being metabolically unhealthy normal weight (Karelis et al., 2004b; Stefan et al., 2017, 2008b).

A study by Kim and colleagues showed that participants with healthy overweight/obesity as expected, exhibited a better metabolic profile than normal weight individuals with the Met S, and that the latter where at higher odds of having increased oxidative stress (Kim et al., 2013b). This finding is in agreement with previous studies which show an association between oxidative stress and presence of the Met S (Ren et al., 2010; Runge et al., 2007). On the other hand, a recent study which involved the recruitment of two large cohorts of European females found no consistent evidence for associations between mtDNA CN and a wide range of cardiometabolic parameters even after controlling for a range of confounding variables (including laboratory covariates and sociodemographic confounders) as well as cellular heterogeneity. Furthermore on using a random-effects model and meta-analysing all participants from both cohorts a weak association between higher mtDNA CN and lower blood pressure was observed, which however disappeared after multiple testing correction (Guyatt et al., 2018). These findings overall are not in support of previously published literature which lead authors to speculate that mtDNA CN is not an important predictor of cardio-metabolic risk at least in females of European descent.

Thus, in view of the inconsistencies in the literature which currently surround the association between mtDNA CN, obesity and T2DM, no definitive conclusions can be inferred at this point in time. Future larger and population-based prospective studies in individuals of different ethnicities are required to fully clarify the role of mitochondrial

bioenergetics in the pathogenesis of obesity and metabolic disease in particular whether variation in mtDNA CN is a cause or consequence of disease development.

1-6.3 Putative cellular and molecular mechanisms linking mitochondrial dysfunction to insulin resistance and obesity

The last two decades has led to an increased understanding of the cellular mechanisms responsible for the relationship between adipose tissue, its microenvironment and the onset of IR and metabolic disease.

It is now well established that mitochondria play a central role in the regulation of whole body energy homeostasis (Johannsen and Ravussin, 2009). Analogous to that of other organs, adipose tissue mitochondria provide cellular energy by generating ATP via the process of oxidative phosphorylation to support a variety of metabolic pathways such as triglyceride synthesis, gluconeogenesis and fatty acid re-esterification, thus the presence of an intact number/size as well as normally functional mitochondria is crucial (Kim et al., 2015; Lee et al., 2019). Specifically, studies have also shown that adipose tissue mitochondria are intimately associated with several other adipocyte-specific functions including adipogenesis and adipocyte differentiation, apoptosis and autophagy, substrate catabolism, insulin sensitivity, and adaptive thermogenesis (Boudina and Graham, 2014; Lee et al., 2019). Furthermore, they are the locus of convergence for multiple signalling pathways particularly those relating to insulin metabolic signalling and maintenance of glucose homeostasis and also enable crosstalk between various insulin-sensitive tissues and adipocytes (Boudina and Graham, 2014;

Keuper et al., 2014). However, mitochondria are highly vulnerable organelles and under various pathological conditions, changes in their oxidative capacity, biogenesis, density and dynamics within adipocytes is associated with the development of obesity, IR and other metabolic diseases (Chen et al., 2010; Rong et al., 2007; Vamecq et al., 2012; Wilson-Fritch et al., 2004).

In obese states, excessive lipogenesis occurs in the face of an increased lipid supply coming from a high energy diet which consequently increases the total lipid pool (Choo et al., 2006; Sun et al., 2011). This generates oxidative stress which triggers adipocyte mitochondria to undergo maladaptive processes inciting a cascade of events which lead to a reduction in mitochondrial oxidative capacity, reduced fatty acid beta-oxidation and lipid clearance and a decrease in overall energy expenditure (Lee et al., 2019; Sergi et al., 2019). Thus mitochondrial dysfunction consequently leads to the intracellular accumulation of toxic fatty acid metabolites such as diacylglycerol and ceramides and ectopic lipid overspill causing defects of insulin metabolic signalling and increased susceptibility to the development of IR in non-adipose tissues such as skeletal and cardiac myocytes, liver and pancreatic beta cells (Bournat and Brown, 2010; Kim et al., 2008; Saltiel and Kahn, 2001; Vamecq et al., 2012; Wang et al., 2010).

Furthermore, other mitochondrial aberrations including ultrastructural abnormalities as well as reductions in mitochondrial mass/size caused by abnormalities relating to mitochondrial biogenesis within insulin-resistant tissues are also associated with mitochondrial dysfunction by way of an impairment in mitochondrial oxidative capacity (Boudina and Graham, 2014; Kim et al., 2008; Nisoli et al., 2007; Ritov et al., 2005).. These observations were deduced from *in vivo* studies on genetic mice models of obesity

where approximately half of the gene transcripts encoding mitochondrial proteins were downregulated in adipocytes with the onset of obesity (Wilson-Fritch et al., 2004). Following this study were several others who also evaluated mitochondrial mass, structure, function, and biogenesis in adipose tissue of genetic mice models of obesity and diabetes as well as in high-fat diet fed mice. Accordingly,these studies observed abnormalities in mitochondrial morphology, function, and abundance and by using microarray technology, a comprehensive transcriptional profiling showed a lower expression of genes regulating several mitochondrial metabolic functional pathways including ATP production and energy uncoupling, as well as downregulation of genes associated with mitochondrial structural proteins, mitochondrial biogenesis and replication as well as lower mitochondrial DNA levels in both mice models of diabetes/obesity compared to wild-type mice (Choo et al., 2006; Rong et al., 2007).

Mitochondrial biogenesis involves the integration of multiple transcriptional pathways which regulate both nuclear and mitochondrial gene expression. The molecular mechanisms underlying defects of mitochondrial biogenesis have been in part attributed to reduced gene expression of mitochondrial regulatory protein peroxisome proliferator-activated receptor (PPAR)-γ co-activator 1α (PGC 1α), dubbed the master regulator of mitochondrial metabolism and biogenesis (Bogacka et al., 2005; Lee et al., 2019; Uldry et al., 2006). PGC-1α is a transcription coactivator and interacts with other transcription factors to regulate the expression of genes involved in mitochondrial biogenesis, metabolic substrate metabolism and adaptive thermogenesis. For example, PGC-1α is associated with activation of downstream transcriptional regulatory circuits such as nuclear respiratory factor -1 and -2 (NRF-1 & NRF-2) which in turn regulate

several mitochondrial genes involved in oxidative phosphorylation as well as mitochondrial transcription factor A (TFAM) which plays a key role in mitochondrial replication and transcription (Wu et al., 1999). Additionally, it also regulates genes involved in the cellular uptake of fatty acids and subsequent fatty acid beta oxidation by acting as a coactivator of PPAR α and δ (Sergi et al., 2019). In fact, studies show that individuals with IR and obesity display fewer and smaller skeletal muscle mitochondria possibly due to decreased PGC-1α expression. Indeed, DNA microarray studies show that expression of PGC-1α responsive genes are downregulated in skeletal muscle of individuals with obesity and a family or personal history of T2DM compared to healthy controls (Kim et al., 2008; Mootha et al., 2003; Patti et al., 2003; Ren et al., 2010). Conversely, overexpression of PGC- 1α in human myocytes enhances insulin sensitivity, increases mitochondrial density, and protects against lipotoxicity. These studies thus support the concept that PGC-1α may be at the interphase between the development of mitochondrial dysfunction, ectopic lipotoxic lipid accumulation and the onset of IR. However, one study did observe similar mRNA expressions of PGC-1α and other transcription factors including nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (TFAM) despite significant reductions of mitochondrial function in insulin-resistant offspring of parents with T2DM compared with control groups. This may suggest that reduced mitochondrial biogenesis cannot be fully explained by abnormalities of mitochondrial function underscoring the importance that both abnormalities of mitochondrial function and biogenesis are intricately linked with energy metabolism and insulin metabolic signalling (Morino et al., 2005). Nevertheless, animal and human therapeutic intervention trials found that use of PPAR-γ agonists

(glitazones) was associated with increased mitochondrial biogenesis and the resetting of mitochondrial mass, which led to improvements in many metabolic processes including regulation of lipid mobilisation, gluconeogenesis in the liver, glycerol production and glucose uptake in pancreatic, hepatic, skeletal and adipose tissues thus establishing themselves as potent insulin-sensitizers (Bogacka et al., 2005; Kim et al., 2008; Matsui et al., 2004; Rong et al., 2007; Wilson-Fritch et al., 2004). Glitazones modulate adipocyte dynamics by activating PGC-1α in subcutaneous adipose tissue leading to adipocyte differentiation and remodelling. While they are associated with an increase in overall fat mass they are associated with a favourable redistribution of body fat, increased fatty acid oxidation and maintenance of systemic lipid homeostasis as well as enhanced UCP-1 expression and WAT browning (Carey et al., 2002; Hock and Kralli, 2009; Tonelli et al., 2004). Taken together, these drugs would be an attractive option in the pharmacological armamentarium for both the prevention and treatment of metabolic diseases by way of their favourable effects on adipose mitochondria, but this has to be balanced against their known side-effects.

Obesity, in particular visceral fat accumulation, is also characterized by a low-grade inflammatory state with increased levels of various pro-inflammatory cytokines such as TNF-α, the presence of which has been associated with smaller and condensed mitochondria, aberrant ATP synthesis and mitochondrial dysfunction (Chen et al., 2010). Furthermore, the presence of TNF-α is associated with decreased expression of genes of protein complexes involved in oxidative phosphorylation and fatty acid oxidation (Dahlman et al., 2006). Other studies have also implicated dysregulation of mitochondrial dynamics through an imbalance in fusion and fission processes as well as

altered mitophagy and mitochondrial turnover in adipocytes in the presence of metabolic diseases (Kovsan et al., 2011; Lee et al., 2019; Liesa and Shirihai, 2013). However, further studies in these areas are needed to fully understand both the physiological and pathophysiological roles of mitochondrial dynamics in the context of adipocyte metabolism. In summary, disruptions in the oxidative capacity, density, biogenesis, and dynamics of adipose tissue mitochondria increases the susceptibility to the development of obesity, IR and metabolic diseases.

From a cellular point of view, abnormalities of mitochondrial function, quantity or biogenesis within adipocytes is associated with defects of insulin metabolic signalling, reduced insulin-mediated glucose uptake and hyperglycaemia (Sutherland et al., 2008; Wang et al., 2013). Insulin is a major anabolic hormone, and its primary function is that of glucose uptake in metabolically active tissues such as myocytes and adipocytes i to maintain whole body metabolic homeostasis. Insulin resistance on the other hand is the blunted response of classical insulin target tissues to insulin leading to a dysregulation of nutrient metabolism and homeostasis. Briefly, canonical insulin signalling is initiated by the binding of insulin to its cognate receptor. The insulin receptor is a cell surface receptor having tyrosine kinase activity and is characterised by the presence of two extracellular ligand-binding domains (α subunits) and two intracellular tyrosine kinase β domains (Lee et al., 2014b). Thus, upon binding to the extracellular α subunits, insulin induces a conformational change in the β-subunit and the activation of a signal transduction cascade involving PDK1 and mTORC2 which results in tyrosine phosphorylation (pY) of insulin receptor substrate-1 protein (IRS). This in turn activates proteins containing SRC homology 2 (SH2) domains such as phosphatidylinositol 3-

kinase (PI3K) whose main action is to convert phosphatidylinositol 4,5-bisohosphte (PIP_2) to phosphatidylinositol 3,4,5-tripohsophate (PIP_3). PIP_3 subsequently leads to the downstream activation of other kinases (such as phosphoinositide-dependent protein kinase 1 [PDK1] and protein kinase B [Akt]) which ultimately culminates in the pleiotropic metabolic actions of insulin (Sergi et al., 2019; Zick, 2005).

In circumstances of high levels of glucose or free fatty acid availability, as occurs during periods of hyper-nutrition , adipose tissue mitochondrial dysfunction leads to ectopic fat storage in insulin-sensitive tissues (lipotoxicity) and an attenuation of insulin-mediated glucose uptake. Thus, the presence of lipotoxicity is thought to be the underlying mechanism bridging the gap between increased nutrient availability, impaired metabolic substrate oxidation and the development of IR. Furthermore, reduced mitochondrial oxidative capacity and the subsequent impairment of fuel oxidation (particularly fatty acid oxidization) appears to be the primary defect which triggers a cascade of events culminating with an increase in levels of lipotoxic metabolites such as fatty acyl CoA, ceramides and diacylglycerol in classical insulin target tissues and the subsequent impairment of insulin metabolic signalling, (Samuel et al., 2010). In effect, intramyocellular accumulation of lipids has been found to be a better predictor of muscle IR than the degree of adiposity (Krssak et al., 1999).

At the molecular level, the build-up of these lipotoxic molecules within skeletal muscle subsequently leads to abnormal insulin signalling either directly through the activation of protein kinase C (PKC) and serine phosphorylation of IRS-1 or through the activation of various proinflammatory stress pathways (Morino et al., 2005; Zick, 2005). Increased phosphorylation of IRS-1 on serine residues (pS) hampers insulin-mediated IRS-1

tyrosine phosphorylation which in turn inhibits PI3K activity leading to suppression of insulin mediated glucose transport, lending further support to the notion that intracellular accumulation of lipotoxic species such as diacylglycerol is more pathophysiologically relevant than accumulation of TG *per se* in the onset of IR (Dresner et al., 1999; Sergi et al., 2019). Furthermore, mitochondrial dysfunction in the face of free fatty acid accumulation also stimulates several pro-inflammatory stress pathways such as nuclear factor kappa light chain enhancer of activated B cells (NF-κβ), c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK) which in turn incite an immune and inflammatory response via an increase in pro-inflammatory cytokines (such as IL-1B, IL-6, TNF-1a and monocyte chemoattractant protein-1 (MCP-1)) (Lee et al., 2019; Liebert, 2005; Lowell et al., 2012).

Another putative mechanism linking mitochondrial dysfunction to IR is mediated by the presence of reactive oxygen species (ROS). ROS generation within adipocytes is thought to be a mandatory by-product of mitochondrial oxidative metabolism with their production being kept in check by the intracellular antioxidant system. However, the presence of an excess nutrient supply and catabolism overwhelms the electron transport chain leading to a high proton gradient and if not matched to an increase in ATP synthesis culminates with greater ROS production. Thus, mitochondrial ROS generation induces oxidative damage to mitochondrial cellular structures such as DNA, lipids and proteins, leading to mitochondrial dysfunction (Anderson et al., 2009; Schieber and Chandel, 2014). Furthermore, ROS generation *per se* can also directly induce IR via alterations in insulin signal transduction pathways through the stimulation of various serine kinases. In keeping with these observations are studies which showed

improved mitochondrial function and insulin signalling following a decrease in ROS generation by the use of antioxidants or increased expression of UCP2/3 (Morino et al., 2005; Nishikawa and Araki, 2007). Thus, mitochondrial dysfunction induced by excess lipid accumulation impairs insulin signalling both directly and indirectly through the generation of excess ROS. (**Appendix 4**).

However, despite the mounting and well-documented evidence described so far demonstrating the relationship between mitochondrial dysfunction and IR, some authors failed to validate this association and thus the question of whether mitochondrial dysfunction is causal to or a consequence of IR still remains a matter of debate. For example, Trenell and co-workers failed to show abnormalities of mitochondrial function including resting and maximal ATP turnover in human individuals with obesity and T2DM compared to individuals without diabetes; however increased physical activity led to an improvement in lipid oxidation in individuals with T2DM independent of mitochondrial activity (Trenell et al., 2008). Another study found that while high-fat feeding induced IR and increased oxidative stress in healthy individuals without obesity , no change was observed in any of the markers of mitochondrial content including protein levels of PGC-1α, and subunits of complex I, II and V of the electron transport chain within skeletal myocytes. This supports the possibility that while IR may be explained at least in part by the increase in oxidative stress, IR may arise independently of mitochondrial dysfunction (Samocha-Bonet et al., 2012a). Similarly, there are animal studies which also failed to confirm a direct cause-effect relationship between defective mitochondrial oxidative function and IR. One particular study showed that a high fat diet was associated with a gradual increase in skeletal muscle

mitochondria via upregulation of PGC-1α while others observed an increase in fatty acid oxidative capacity and an increase in mitochondrial proteins involved in oxidative metabolism (Garcia-Roves et al., 2007; Turner et al., 2007; Williams et al., 2014). These findings might be explained by the fact that increased mitochondrial function may be a compensatory mechanism in response to nutrient oversupply and acts as a protective mechanism against the development of IR. Collectively these observations seem to allude to the fact that mitochondrial dysfunction may not be a prerequisite for the onset of IR.

The downstream molecular consequences of a variation in mtDNA content which are thought to explain the mechanisms linking mitochondrial dysfunction to the onset of chronic diseases involves several theories centring round immune (macrophage) dysfunction, inflammation and altered cell signalling (Castellani et al., 2020). There is robust evidence causally linking changes in mtDNA CN to adipose tissue inflammation and oxidative stress. Mitochondrial-mediated changes in respiratory capacity can impact on macrophage polarization and results in chronic subclinical inflammation which is associated with both the onset of the Met S and atherosclerotic disease. Pro-inflammatory M1 macrophages generate ATP primarily through glycolysis, while anti-inflammatory M2 macrophages rely on oxidative phosphorylation via the mitochondrial electron transport chain (Viola et al., 2019). Reduced mtDNA CN results in insufficient OX-PHOS proteins and a block in the reprogramming of M1 macrophages to the M2 subtype (Castellani et al., 2020). Consequently, in obese states, an increased number of M1-activated adipose tissue macrophages (ATMs) infiltrate adipocytes secreting an array of proinflammatory cytokines that drive IR, and the concomitant decrease in M2-

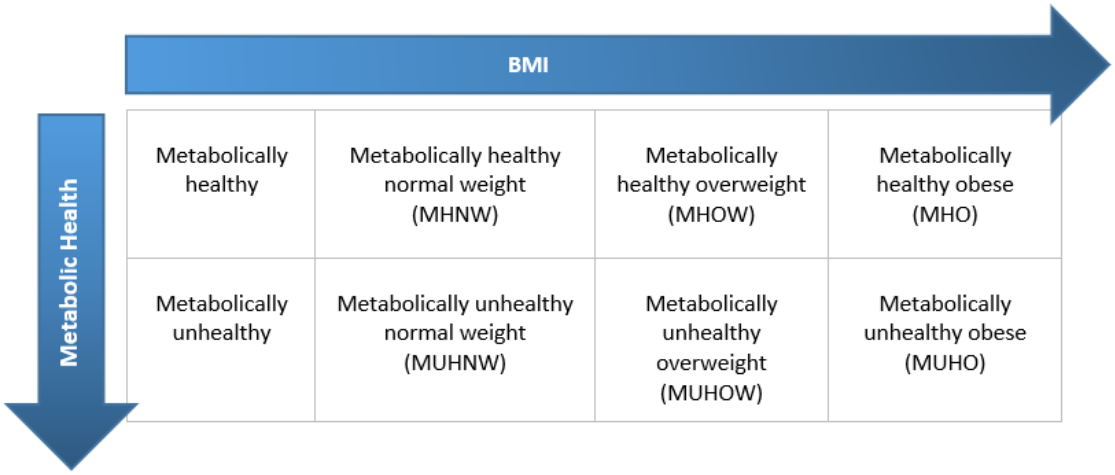
subtypes leads to a reduction in the production of anti-inflammatory mediators (such as TGF-β and IL-10 signals) that would assist with the resolution of inflammation and restore insulin sensitivity (Chawla et al., 2011; Lumeng et al., 2007).

Overall, despite the presence of incongruent results from different studies which assessed the relationship between mtDNA bioenergetics, insulin metabolic signalling and obesity, there is no doubt that a degree of functional dysfunction of the mitochondrion plays a pivotal role in obesity pathophysiology as well as in obesity-associated metabolic diseases. This ensures that quantification of mtDNA CN still holds a considerable interest as a minimally invasive biomarker in clinical and population studies. Furthermore, this investigation expands on the spectrum of established association between mtDNA and metabolic phenotypes in different populations. Thus, irrespective of whether mitochondrial dysfunction represents a primary defect in metabolic disease, preserving mitochondrial function remains an important strategy in the protection against IR and associated cardio-metabolic diseases. In conclusion, future longitudinal studies should be undertaken with the aim of characterising cell-type and cross-tissue profiles of mtDNA CN across various ethnic populations to better understand the direction of causality as well as to elucidate further the clinical and therapeutic relevance of this easily measured biomarker.

1-7 Aims and Objectives of research project

It is now universally acknowledged that an individual's risk of CVD does not solely depend on body size but also on their metabolic profile. Thus, individuals with similar body mass index may exhibit different cardiometabolic risk parameters leading to variations in CVD risk. This has resulted in the emergence of different body composition phenotypes, whereby body size (as expressed by the BMI) and presence or absence of certain metabolic parameters are incorporated together to create a spectrum of different body composition phenotypes.

At one end is the metabolically healthy normal weight individual (MHNW). This subset of individuals is characterised by a normal BMI (18.5 to 25 kg/m^2) and absence of an adverse cardiometabolic risk profile (including hypertension, dyslipidaemia and dysglycaemia). A second body composition phenotype is the individual with normal weight but who is also metabolically abnormal. This phenotype is termed as metabolically unhealthy normal weight (MUHNW). Similarly, individuals who are overweight or obese by BMI criteria may or may not harbour these adiposity-associated cardiometabolic abnormalities and thus lead to the occurrence of another four body composition phenotypes: metabolically healthy overweight, metabolically unhealthy overweight, metabolically healthy obese (MHO) and metabolically unhealthy obese phenotypes (MUHO) as shown in **figure 1.6.**



Classification according to body size (as defined by the BMI) and metabolic health. Absence and presence of major cardiometabolic risk factors allows stratification of normal weight, overweight and obese individuals into metabolically healthy and metabolically unhealthy (Source: Stefan *et al.*, 2013).
BMI-body mass index

Figure 1.6: The six different body composition phenotypes

Up till now the epidemiological aspect in terms of prevalence rates and sex differences of the six different body composition phenotypes has not been fully explored within the Maltese Islands. Moreover, the data on lifestyle factors, biochemical and anthropometric parameters and how they relate with MH is either conflicting or scarce and not available for the Maltese general population (Calori et al., 2011; Goday et al., 2016; Hajian-Tilaki and Heidari, 2018; Hankinson et al., 2013; Lee, 2009; Phillips, 2013a; Wildman et al., 2008) Secondly, little is known about the molecular processes involved the development of these body composition phenotypes. Over the last two decades a growing body of evidence has implicated the important role of mitochondrial bioenergetics in metabolic disturbances including IR and the Met S, obesity, and T2DM (Johannsen and Ravussin, 2009; Ren et al., 2010). Quantification of mitochondrial DNA copy number (mtDNA CN) is being increasingly employed as a surrogate biomarker of

mitochondrial function and which reflects the degree of mitochondrial DNA damage with recent observations demonstrating that a decreased mtDNA CN in several tissues to be associated with visceral adiposity, BMI, Met S, CVD, and mortality (Huang et al., 2011; Koller et al., 2020; Lee et al., 2014a; Skuratovskaia et al., 2018). However, no study has been specifically conducted to assess how this relates with the six different body composition phenotypes described above and within the Maltese population. It is hypothesized that individuals with the unhealthy metabolic phenotype have a lower peripheral blood leukocyte mtDNA CN.

Therefore, the overall objectives of this research were two-fold: the first centred round *epidemiological analyses* primarily aimed at determining the prevalence and characteristics of the different body composition phenotypes; and the second focussed on *molecular analyses* and sought to investigate the associations between peripheral blood leucocyte mitochondrial DNA copy number, the Met S and the different body composition phenotypes in a high prevalence population for both obesity and Met S.

The specific endpoints for each of the two main objectives are as follows:

Epidemiological studies

(1) To determine the prevalence, characteristics (in terms of the lifestyle, anthropometric and biochemical parameters) and the associations of each of the six different body composition phenotypes described above (MHNW, MUHNW, MHOW, MUHOW, MHO and MUHO) using either the NCEP ATP III definition of the Met S in the first instance or HOMA-IR to define MH, and to assess how these two definitions vary between each other.

(2) To further assess the characteristics associated with the metabolically healthy phenotype among individuals with overweight and obesity and how they vary between each other when using the NCEP ATP III definition of the Met S to define the metabolically healthy status.

(3) Since there are sex differences in the distribution of fat and in the prevalence of overweight and obesity this study also explored the sex differences in the prevalence of the different body composition phenotypes as well as in anthropometric measures and cardiometabolic parameters and in the relationship between BMI categories and MH when using the NCEP ATP III criteria to define MH.

(4) To compare the prevalence of the different body composition phenotypes this time defining MH according to eight different and frequently used criteria as proposed by Wildman *et al*., Doumatey *et al*., Hamer et al., Meigs *et al.,* Lynch *et al*., Augilar-Salinas *et al.,* Karelis *et al*., the harmonization criteria proposed by Lavie *et al.,* in addition to the NCEP ATP III definition for Met S. Furthermore, this objective also aimed to evaluate which definition mentioned above was the strongest predictor of IR (as defined by HOMA-IR) and how this varied between the two sexes.

(5) To compare the discriminatory power of the various anthropometric and biochemical parameters in predicting IR and to determine their optimal cut-offs.

Molecular studies:

(6) To investigate the associations between peripheral blood leukocyte mtDNA CN and the different body composition phenotypes.

(7) To evaluate which of the different definitions of MH and their constituent components are associated with reduced leukocyte mitochondrial DNA copy number.

Each of these endpoints will be described in the following chapters.

Chapter 2 – Research design and methods of epidemiological studies

2-1 Study design, study population and recruitment

This was an observational cross-sectional single-centre study carried out between January 2018 and June 2019, involving the recruitment of a middle-aged sample of Maltese Caucasian noninstitutionalized, civilian adults. A convenience type of sampling similar to that used in the ABCD study by Buscemi et al. was adopted (Buscemi et al., 2017).

2-2 Eligibility criteria

2-2.1 Inclusion criteria

1. Maltese Caucasian ethnicity
2. Aged 41 (±5) years
3. BMI ≥18.5 kg/m^2

2-2.2 Exclusion criteria

1. Presence of Type 1 Diabetes
2. Known underlying genetic or endocrine cause of overweight and obesity (apart from controlled thyroid disorders)
3. Terminal illness
4. Active malignancy
5. Individuals unable to give own voluntary informed consent
6. Pregnant females

2-3 Assessment of demographic, anthropometric and cardiometabolic (biochemical) parameters

Following acceptance to participate in the study, eligible participants were invited to attend for a one time visit at Mater Dei Teaching Hospital to undergo a 3-part detailed face-to-face evaluation involving: i) assessment of demographic, health behaviour and lifestyle factors via the use of a structured questionnaire especially designed for the survey**;** ii) a physical examination to measure specific anthropometric parameters; and iii) blood sampling for measurement of pertinent cardiometabolic (biochemical) components. All parts of the assessments were carried out by the same assessor, (the candidate R. A.), to minimize any form of observer bias.

A total of 521 participants accepted to participate in the study and fit the eligibility criteria for recruitment.

2-3.1 Assessments of demographic, health behaviour and physical factors

A dedicated questionnaire (composed in both English and Maltese languages and using a composite of a number of validated tools) was used to capture baseline demographic data relating to age, sex, area of residence, education level, specific occupation, consumption of tobacco and alcohol, physical activity as well as past medical and surgical history and a detailed drug history **(Appendix 1C).** Level of education was stratified as either completing primary, secondary, or tertiary education. Occupation was coded into nine major categories as per the 1994 Spanish National classification of Occupations and thereafter re-classified into either white collar (nonmanual) workers

or blue collar (manual) workers as previously described in the study by Sanchez-Chaparro *et al*. Workers in the first four categories were deemed white collar workers whereas those falling in the last five categories were grouped as blue-collar workers (Sánchez-Chaparro et al., 2008). Participants' smoking status was categorised as never smokers if they had smoked less than 100 cigarettes in their lifetime; current smokers if they had smoked more than 100 cigarettes in their lifetime and answered 'yes' to the question "Do you smoke now?; former smokers if they smoked more than 100 cigarettes in their lifetime but were not presently smoking (as used in the NHANES study by Wildman *et al.,* 2008 (Wildman et al., 2008)). Alcohol intake was assessed by asking the participants about the number of units of alcohol consumed per week. One standard unit was defined as a glass of wine, a bottle of beer or a shot of spirits. Non-drinkers were classified as those who reported consuming less than 12 alcoholic beverages in their lifetime (Wildman et al., 2008). Physical activity was assessed by asking the participants if they engaged in any type of exercise. Participants were considered as taking regular exercise if they reported exercising more than once per week. If they did, then they were asked what type of sport / activity they engaged in, the duration (in minutes) of each activity and the number of times the activity was performed per week (Wildman et al., 2008). A detailed medical and pharmacological history was also captured particularly in reference to prior diagnoses of T2DM, hypertension, hypercholesterolemia and CVD as well as use of any other prescribed and self-prescribed medications.

2-3.2 Anthropometric measurements

The physical examination was carried out with the participants dressed in light clothing and without shoes. Anthropometric measurements were recorded using validated measurement equipment at Mater Dei Teaching Hospital and which were calibrated in accordance with WHO regulations. Body weight was measured in kilograms to the nearest 0.1kg and height was measured in centimetres to 1 decimal place using a calibrated stadiometer with a vertical backboard and a movable headboard. Body mass index (BMI) was thereafter calculated as a ratio of the weight (in kg) divided by the square of the height (in meters). Participants were defined as having normal weight if the BMI value was < 25 kg/m^2; overweight if the BMI fell between the values 25.0-29.9 kg/m^2; and obese if the BMI was ≥30 kg/m^2. Waist and hip circumferences (WC and HC respectively) were measured to the nearest 0.1 cm with a non-stretchable measuring tape over the abdomen halfway between the bottom of the rib cage and superior iliac crest for WC and over the widest diameter around the buttocks for the HC with the participants standing with their feet together such that weight was evenly distributed over both feet and after full expiration. Other anthropometric parameters measured were neck circumference (NC), mid upper arm circumference (MUAC) and thigh circumference (TC). For the NC the measuring tape was placed around the mid-cervical spine to mid-anterior neck in order to obtain the mid-neck height to the nearest 1mm (Ben-Noun and Laor, 2003). The MUAC was identified by asking the participant to bend the elbow at a 90-degree angle, with the arm held parallel to the side of the body. Thereafter the midpoint of the distance between the acromion and olecranon process was identified and marked and the measuring tape was placed around this identified

point. This was done for both right and left arms. Upper TC was measured by placing the tape over the largest portion of the thigh (at the level of the gluteal fold) with the thigh muscles fully relaxed and placed either directly over the skin or over very light clothing. All circumferences were taken with the participants standing upright, with shoulders and thighs relaxed, facing the investigator (Ge et al., 2014). Blood pressure was measured according to the European Society of Hypertension Guidelines using a clinically validated digital sphygmomanometer with an appropriately sized cuff for each participant after a 5-minute rest and in the seated position. The average of the second and third readings was used for analyses (Han and Lean, 2001; Parati et al., 2014; Phillips et al., 2013)(Han and Lean, 2003; Phillips, Dillon, *et al.*, 2013a; Parati *et al.*, 2014).

2-3.3 Cardiometabolic (biochemical) parameters

All participants were asked to undergo blood sampling after a 10 hour overnight fast. Plasma and serum samples were prepared from whole blood for the measurement of several laboratory parameters including:

- Fasting Plasma Glucose (FPG)
- Serum Total Cholesterol (TChol)
- Serum High Density Lipoprotein Cholesterol (HDL-C)
- Serum Low density Lipoprotein Cholesterol (LDL-C)
- Serum Triglycerides (TG)
- Full Blood Count (FBC)
- Uric Acid (UA)

- HBA_{1c}
- Serum Creatinine, Estimated Glomerular Filtration Rate (eGFR), Potassium, Sodium
- Serum Free tri-iodothyronine (T_3), free tetra-iodothyronine (T_4) and thyroid stimulating hormone (TSH)
- Serum Vitamin D
- Serum Liver Profile including: Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Gamma glutamate transferase (GGT) and Bilirubin levels

These were sent to the main biochemistry laboratory at Mater Dei Teaching Hospital for analysis using automated and quality-controlled analysers which utilized standard clinical chemistry methods as already described in previous studies (Cuschieri et al., 2016c; Magri et al., 2018).

The assessments of FPG levels and lipid profiles were performed using COBAS INTEGRA® Analysers machines. The blood samples used to measure FPG levels were collected in fluoride-containing tubes in order to inhibit glycolysis. The FPG levels were measured using hexokinase and glucose oxidase enzyme reactions. A serum clot activator tube was used to collect blood samples for the lipid profile assessment. This involved assessing each participant's total serum cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and TG.

Haemoglobin A_{1c} was measured using high performance liquid chromatography (Variant II by BioRad). Creatinine was measured by a kinetic colorimetric test using the Jaffe reaction (Roche Diagnostics).

Serum insulin samples were collected after an overnight fast and transferred to the laboratory where they were centrifuged within two hours. The serum was isolated by centrifugation at 2000rcf x 10 minutes, and the serum supernatant pipetted into a separate storage tube, which was then frozen and stored at -80^{0}C.

Insulin was measured using a solid-phase sandwich enzyme linked immunosorbent assay (ELISA) (Kit: Diagnostic Automation, USA) according to the manufacturer's instructions and using a Mithras® microplate reader for absorbance determination. Samples were assayed in duplicate using 50µL of serum. The homeostatic model assessment (HOMA) was used as an indirect measure to evaluate IR using the formula: Fasting Serum Insulin (microunits per mililiter) x Fasting Plasma Glucose (milimoles per Liter) /22.5 (Matthews *et al.*, 1985; Wildman *et al.*, 2008). In this project, fasting serum insulin was successfully determined in 509 out of 521 samples, as 12 samples could not be analysed due to severe haemolysis affecting assay readability. Overall, serum insulin ELISA assays met standard QC parameters, with an intra-assay coefficient of variation (CV) of 6.4% and inter-assay CV of 9.3%.

Serum hsCRP was also measured using a sold phase sandwich ELISA (Kit: Diagnostic Automation, Inc, California) as per the manufacturer's instructions and using a Mithras® microplate reader for absorbance determination. Samples were assayed in duplicate. Inter-assay and intra-assay CV were 8.6% and 6.3% respectively.

An EDTA (ethylenediaminetetraacetic acid) bottle was frozen and stored at -20^{0}C at the Laboratory of Molecular Genetics, University of Malta for future genetic testing.

2-3.4 Calculation of derived indices

From the above anthropometric and biochemical parameters, the investigator was able to derive other indices of obesity as described earlier in chapter 1. These indices were selected because they can be rapidly ascertained in the clinical setting based on simple data such as weight, height, WC and HC. They have already been applied and validated in many other epidemiological studies. The following indices were derived:

Waist to height ratio (WHtR) = WC (cm) / height (cm): This ratio which is based on WC and height of an individual has been reported as being strongly associated with cardiovascular risk in certain ethnic populations and has also been shown to be the best indicator for hypertension in Chinese adults. Moreover, in the meta-analysis by Lee *et al,* it was shown to be a superior discriminator for detecting cardiovascular risk factors in both sexes (Lee et al., 2008). It is calculated by the following formula: WC (cm) / height (cm) (Ho et al., 2003).

Waist to hip ratio (WHR) = WC (cm) / HC (cm): This index gives an indication of fat distribution particularly abdominal adiposity and has been shown to be a robust independent predictor of cardiovascular morbidity and mortality, to have good discriminatory capability for T2DM and also to have the strongest correlation with cIMT (carotid intima media thickness) which is a validated marker of sub clinical atherosclerosis (Goh et al., 2014; Yan et al., 2009).

Waist to thigh ratio (WTR) = WC (cm) / TC (cm): This ratio is used as an index for fat distribution and is influenced by abdominal fat, muscle and bone mass of the thigh which

can give an indication of abdominal fat accumulation as well as information about skeletal muscle (such as muscle wasting) (Han and Lean, 2001).

Waist Index (WI) = This is sex specific and is calculated as WC (cm) / 94 for men; and WC (cm) / 80 for females for Caucasians as per the study by Magri *et al* (Magri et al., 2016)**.**

Conicity index (CI) = This was formulated by Valdez in 1991 to estimate abdominal fat and is derived from the following equation: waist / (0.109x √weight (kg)/height (m) (Valdez, 1991).

Body Adiposity index (BAI) = [HC /Height$^{2/3}$] − 18; The BAI has been shown to directly reflect percentage body fat in adult males and females of differing ethnicities without numerical correction. It is calculated from hip circumference and height only and thus can be used in the clinical setting (Bergman et al., 2011).

Abdominal volume index (AVI) = The AVI is another anthropometric-based tool used for estimation of overall abdominal volume (between the symphysis pubis and the xiphoid appendix) which theoretically includes intrabdominal (visceral) fat and adipose tissue volumes. It has been shown to be a reliable index for estimation of obesity and also exhibits a higher relationship with impaired fasting glucose and diabetes than other anthropometric indices. The AVI formula is based on the volume formulas for cylinder ($V = \pi r^2 h$) and vertical cone ($V = (1/3)\pi r^2 h$). The resultant formula is as follows:

AVI = [2 cm $(\text{waist})^2$ + 0.7 cm $(\text{waist-hip})^2$]/1000, where both waist and hip measurements are in centimetres. A cut-off value of 24.5L has been shown to be the best value to estimate obesity (Guerrero-Romero and Rodríguez-Morán, 2003).

A Body shape index (ABSI) = This parameter was developed by Krakauer *et al*. in 2012 and is based on WC (m) adjusted for height (m) and weight (kg) and is measured using the following formula: WC / BMI $^{2/3}$ x height ^{½}. According to the authors, a high ABSI corresponds with a greater fraction of abdominal (visceral) adipose tissue and predicts premature mortality risk independent of age, sex and weight. Other studies have also suggested that ABSI is able to predict onset of T2DM (He and Chen, 2013; Krakauer and Krakauer, 2012).

Body roundness Index (BRI) = This index was developed in 2013 by Thomas *et al.* It is a geometrical index based on height (*m*) and WC (*m*) and which aims to quantify body girth in relation to height (body roundness). It allows for estimation of the shape of the human body figure as an ellipse or oval. It was thus developed initially to predict the percentage of total and visceral body fat and to evaluate health status. It is measured using the following formula: BRI = 364.2-(365.5 x Ɛ) where Ɛ stands for eccentricity and is calculated using this formula:

$$\varepsilon = \sqrt{1 - \left(\frac{(WC/(2\pi))^2}{(0.5 \times height)^2} \right)}$$

Values closer to 1 are related to leaner individuals, whereas larger values are associated with rounder individuals. The authors found that this new shape measure was able to predict % body fat and % VAT better than other traditional metrics such as BMI, WC or HC (Maessen et al., 2014; Thomas et al., 2013). In other studies, the BSI also had the potential to improve the detection, evaluation, and progression of CVD and CVD risk factors implying that the BRI is capable of mathematically modelling the human body

shape to give an adequate impression of the cardiovascular health status (Maessen et al., 2014).

Visceral adiposity index (VAI) = a sex-specific index based on WC, BMI, TG and HDL-C. VAI estimates visceral adipose dysfunction associated with cardiometabolic risk, and VAI values are calculated as described in the literature using the following equations (Amato et al., 2014):

VAI female = [WC /36.58 − 1.89BMI] x [TG /0.81][1.52/HDL-C]

VAI male = [WC /39.68 − 1.88BMI] x [TG /1.03][1.31/HDL-C];

(WC in cm; TG and HDL in mmol/l)

Lipid accumulation product (LAP) = this is a sex specific parameter, and it is based on the combination of WC and triglyceride levels: [WC -65]x [TG] in males, and [WC -58]×[TG] in females. (WC in cm and TG in mmol/l). This has been recently described as an index of central lipid accumulation and visceral obesity and has been used to predict the risk of Met S and subclinical atherosclerosis and cardiovascular risk (Li et al., 2017; Namazi Shabestari et al., 2016). (Kahn, 2005; Li *et al.*, 2017).

Atherogenic index of plasma (AIP) = This is calculated by the logarithmic transformation of the ratio of plasma TG concentration to HDL-C concentration [log(TG/HDL-C)] (Hermans et al., 2012).

Neutrophil lymphocyte ratios (NLR) and **platelet-lymphocyte ratios (PLR)**. These two indices which are derived from blood counts have been described as surrogate markers of chronic subclinical inflammation in the context of cardiometabolic disease (Lou et al., 2015).

2-4 Body composition phenotype definitions

Body composition phenotypes were generated based on the combined consideration of each participants' BMI category (normal weight [BMI ≥18.5 and <25kg/m^2]; overweight [BMI between ≥25 and <29.99 kg/m^2]; and obesity [BMI ≥30 kg/$m^{2)}$]) and MH. Metabolic health was defined using several of the current different definitions available and cited in the literature:

a) In the first instance MH was defined according to the Met S definition according to the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) framework as already done in previous studies (Arnlöv et al., 2011; Durward et al., 2012; Meigs et al., 2006; NCEP, 2001; Song et al., 2007; Twig et al., 2014; Voulgari et al., 2011). This consisted of the following cardiometabolic (CM) parameters: WC >102cm in males and >88 cm in females; systolic/diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglyceride level ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in males and < 1.29 mmol/L in females or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents. Individuals were classified as being metabolically healthy if they exhibited 1 or less CM abnormalities from the above parameters in accordance with previous studies (Elías-López et al., 2021; Hinnouho et al., 2015; L. Li et al., 2018; Zhou et al., 2021).

In initial analyses, participants with overweight and obesity were analysed together as one entity thus generating four body composition phenotypes: metabolically healthy normal weight (MHNW); metabolically unhealthy normal weight (MUHNW); metabolically healthy overweight or obese (MHOW/O); metabolically unhealthy overweight or obese (MUHOW/O) **(Table 2.1).** For prevalence purposes, participants

with overweight and obesity were analysed as separate entities thus generating two other body composition phenotypes in addition to the previous ones described above: metabolically healthy overweight (MHOW) and metabolically unhealthy overweight (MUHOW) thus creating a total of six different body composition phenotypes.

b) Additionally, as part of an exploratory analysis, further sensitivity studies for the prevalence of the different body composition phenotypes were performed using definitions with less stringent or more stringent criteria of the NCEP ATPIII classification to define the metabolically healthy phenotype: i.e., having either ≤2 CM abnormalities or having 0 cardiometabolic abnormalities, that is individuals who did not meet any criteria of the NCEP-ATPIII guideline, respectively.

c) IR as measured by HOMA-IR is another criterion used to define the metabolically healthy status and to assess the prevalence of the different body composition phenotypes in this study as per previous studies (Calori et al., 2011; Kuk and Ardern, 2009). A cut-off value of <2.5 was used to identify the metabolically healthy phenotype. This cut-off value was chosen as it has already been validated in previous longitudinal studies which looked at both cardiovascular and all-cause mortality (Bo et al., 2012; Calori et al., 2011; Durward et al., 2012; Kuk et al., 2006) **(Table 2.1).**

d) Since there is yet no unified definition for the metabolically healthy phenotype, a number of authors have proposed several different criteria and cut-offs to identify the metabolically healthy state. In fact, one systematic review identified 30 different definitions of MH. A few of them stand out for their popularity and thus have been used extensively in contemporary studies which looked at prevalence rates and

characteristics of the different body composition phenotypes in various populations (Rey-López et al., 2014). Therefore, this study also aimed to compare the prevalence of the six body composition phenotypes using the classifications as proposed by Wildman *et al*., Doumatey *et al.,* Hamer *et al.,* Lynch *et al.,* Augilar-Salinas *et al.,* Karelis *et al.,* Meigs *et al*., the harmonization criteria proposed by Lavie *et al*., in addition to the NCEP ATPIII definition of the Met S (incorporating the presence of either 0, 1, or 2 abnormal parameters) to define the metabolically healthy status (Aguilar-Salinas et al., 2008; Doumatey et al., 2012; Hamer and Stamatakis, 2012; Karelis et al., 2004a; Lavie et al., 2018; Lynch et al., 2009; Meigs et al., 2006; NCEP, 2001; Wildman et al., 2008) **(Table 2.2).**

e) In 2021, Zembic *et al*. proposed an empirical definition for MH based on the risk of cardiovascular and total mortality from the NHANES III and UK Biobank dataset. They proposed that a metabolically healthy status can be identified in individuals if they meet the following three criteria: systolic blood pressure <130 mmHg and not on antihypertensive medications; waist -to-hip ratio <0.95 in females and <1.03 in males; and absence of diabetes (Zembic et al., 2021). This definition was used as one of the criteria needed to ascertain the metabolically healthy phenotype in the study population when investigating the associations between peripheral blood mitochondrial DNA copy number and the different body composition phenotypes.

Table 2.1: Definitions of the different body composition phenotypes according to BMI and metabolic status

MHNW (BMI <25 kg/m²)	MUHNW (BMI <25 kg/m²)	MHOW/O (BMI ≥25kg/m²)	MUHOW/O (BMI ≥25 kg/m²)
NCEP ATPIII: ≤1 CM abnormalities	NCEP ATPIII: ≥2 CM abnormalities	NCEP ATPIII: ≤1 CM abnormalities	NCEP ATPIII: ≥2 CM abnormalities
or	or	or	or
HOMA-IR <2.5	HOMA-IR ≥2.5	HOMA-IR < 2.5	HOMA-IR ≥2.5

MHNW, metabolically healthy normal weight; MUHNW, metabolically unhealthy normal weight; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese; BMI, body mass index; CM, cardiometabolic; NCEP ATPIII, National Cholesterol Education Program Adult Treatment Panel III; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance

Table 2.2: The different classifications used to define a metabolically healthy phenotype

	NCEP-ATPIII (2001)	Karelis *et al.* (2004)	Aguilar-Salinas *et al.* (2008)	Wildman *et al.* (2008)	Lynch *et al.* (2009)	Doumatey *et al.* (2012)	Hamer *et al.* (2012)	Harmonisation criteria (Lavie *et al.*) (2018)	Zembic *et al.* (2021)
BP (mmHg)	SBP ≥130 or DBP ≥ 85 or on Rx		SBP >140 or DBP >90 or on Rx	SBP ≥130 or DBP ≥ 85 or on Rx	SBP >130 or DBP >85 & not on Rx	SBP >130 or DBP >85 or not on Rx	SBP >130 or DBP > 85 or on Rx	SBP ≥130 or DBP ≥85 or on Rx	SBP <130 & not on Rx
TG (mmol/L)	≥1.69	≥1.70		≥ 1.70				≥ 1.70	
HDL-C (mmol/L)	HDL-C <1.03 M or < 1.29 in F or on Rx	<1.30 or on Rx	< 1.00	<1.04 M or <1.30 F or on Rx		<1.03 M or <1.29 F	<1.03 M or <1.30 F	<1.0 M or <1.30 F or on Rx	
TG/HDL ratio					>1.65 M or >1.32 F & not on Rx				
LDL-C (mmol/L)		≥2.60 or on Rx							
T. Chol (mmol/L)		≥5.20							
FPG (mmol/L)	≥ 5.6 or on Rx		≥7.0 or on Rx	≥ 5.55 or on Rx		>7.0	Presence of diabetes	≥ 5.6 or on Rx	Absence of diabetes
WC (cm)	>102 M or >88 F						>102 M or >88 F		WHR: <1.03 M or <0.95 F
HOMA-IR		>1.95		>5.13					
hsCRP (ng/L)				>0.1			≥3.0		
Other					No history of CVD, respiratory or metabolic disease				
Criteria to define the metabolically healthy phenotype	0 (NCEP-0) ≤ 1 (NCEP-1) or ≤ 2 (NCEP-2)	≤1 of the above	None of the above	<2 of the above	None of the above	None of the above	<2 of the above	None of the above	All of the above

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; F, females; hsCRP, high sensitivity C-reactive protein; HLD-C, high density lipoprotein cholesterol; HOMA-IR, homeostatic Model Assessment of Insulin Resistance; LDL-C, low density lipoprotein cholesterol; M, Males; NCEP-ATPIII, National Cholesterol Education Program-Adult Treatment Panel criteria; Rx, treatment; T. Chol. Total Cholesterol; TG, triglycerides; WC, waist circumference

2.5 Statistical analysis

A minimum sample size was calculated using the one proportion formula for cross-sectional studies. The WHO age-standardised prevalence of obesity (BMI>30 kg/m^2 =28.9%) was used since at the population level, obesity is a robust predictor of cardiometabolic risk. Considering a power of 90%, precision of 0.05, significance of 0.05, and an expected response rate of 90%, a minimum sample size of n=352 was obtained (Dhand and Khatkar 2014).

Normality of distribution of continuous data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. All continuous variables showed a skewed non-normal distribution and hence the data is presented as medians and interquartile range and non-parametric tests were used for comparisons. To evaluate differences in quantitative variables between groups, Kruskal-Wallis ANOVA was used for comparison between three or more categories, followed by Dunn's post-hoc test for pairwise comparison between subgroups. The independent samples Mann-Whitney U test was used for comparison between two categories. Bonferroni adjustment of *p*-values for multiple comparisons was applied. The χ^2 test was used to compare categorical variables.

To evaluate factors associated with MH, binary logistic regression was performed and the metabolically healthy or unhealthy phenotype was inputted as the dependent variable in separate analyses. Several demographic and lifestyle characteristics (which could be readily ascertained in a routine clinical setting, are not included in the definition of MH, and which do not exhibit multicollinearity) were incorporated into the regression model as the independent (explanatory) variables. Unadjusted ratios were calculated

initially followed by multivariate-adjusted models for all the demographic and behavioural factors simultaneously.

To identify the determinants of HOMA-IR in males and females, generalized linear modelling was applied with HOMA-IR entered as the continuous response variable. Simple parameters that can be easily ascertained at the bedside were included as scale-independent variables (such as age, WHR, BMI, and neck circumference). Lifestyle factors (including smoking, physical activity and alcohol consumption) were also combined into the regression model as categorical independent determinants . Generalized linear modelling specifying gamma as the distribution and Log as the link function was applied, in view of the positively skewed distribution of parameters being investigated. Multicollinearity diagnostics revealed no dependency between independent variables, with variance inflation factors <2.5 and tolerance statistic values >0.7, thus indicating they could be reliably used as determinants in the model.

Furthermore, to investigate the discriminatory value of the various definitions of MH outlined in point 'e' of **section 2.4** of this chapter, in predicting IR, a logistic regression analysis was performed with HOMA-IR ≥2.5 as the dependent variable and a metabolic unhealthy phenotype as the independent variable for each of the definitions of MH, except for those by Wildman *et al.* and Karelis *et al.* These two were not entered in the logistic regression analysis since HOMA-IR is a criterion used to define MH in these definitions. Further, logistic regression was repeated this time using each of the above-mentioned definitions as the independent (predictor) variables adjusted for BMI.

For the final analyses receiver operator characteristics (ROC) curves were constructed to compute the area under curve (AUC) to determine the discriminatory power of several anthropometric and biochemical parameters (and indices derived therefore), to detect IR (as defined by HOMA-IR using a cut-off value of ≥2.5 to denote insulin resistance). Furthermore, the highest Youden index (sensitivity + specificity -1) was used to determine the respective cut-off points for each of the variables of interest.

All analyses were performed using IBM SPSS version 26. ROC analysis was performed using the easyROC R application, and cut-off values were determined using the OptimalCutpoints R package (R v.3.4.2). A *p* value of <0.05 was considered significant.

Chapter 3A – Results of epidemiological studies

3-1 Prevalence and characteristics of the different body composition phenotypes

3-1.1 Characterization of the different body composition phenotypes using the NCEP ATPIII criteria of the Met S to define MH

A total of 521 individuals of Maltese ethnicity were assessed and provided the data for all the parameters required to define MH status in this study. 330 participants (63.3%) were female, and the median age was 41 years (range 30 -51 years). The prevalence of the different BMI categories in the studied population was as follows: normal weight – 29.9%, overweight – 36.7%, obese – 33.3%. Overall, 70% of the study participants were either overweight or obese. The median weight was 78kg, median BMI 27.5 kg/m^2, and the median WC was 89cm. With respect to lifestyle characteristics, 22.5% were active smokers (median 10 cigarettes per day) and 47.8% regularly consumed alcohol (median 2 units per day). Just under a half of the participants (42.8%) were physically active and 50% achieved a tertiary level of education. Upon recruitment, 22% (n = 115) had a known medical comorbidity. These included T2DM (4.78%), hypertension (7.84%), hypothyroidism (4.2%) and dyslipidaemia (6.11%).

Tables 3.1a-d show the demographic, biochemical and anthropometric characteristics of the study population according to the four different body composition phenotypes: MHNW, MUHNW, MHOW/O and MUHOW/O (incorporating the presence of ≤1 CM abnormalities of the NCEP ATPIII criteria to define the metabolically healthy phenotype).

In this cohort of middle-aged Maltese participants the prevalence of the unhealthy phenotype was 32.8% (n=171) being composed of 30.7% (n=160) MUHOW/O and 2.1% (n=11) MUHNW individuals. Overall, the population prevalence of MHOW/O was 39.3%

(n=205). Among the total overweight/obese participants 56.1% were MHOW/O while 7.0% of the total normal weight individuals exhibited the MUHNW phenotype. Although a significant difference in age was not found between the healthy and unhealthy normal weight cohorts, a difference in age between the healthy and unhealthy overweight/obese participants was noted with the MHOW/O phenotype being slightly younger (p=0.02).

Significant differences in baseline characteristics between participants with overweight/obesity were found according to the presence or absence of Met S. With respect to lifestyle factors, individuals with the MHOW/O phenotype were more likely to drink alcohol, engage in regular physical activity and have a higher level of education when compared to the metabolically unhealthy overweight/obese counterparts. On the other hand, the MHOW/O phenotype had lower values for indices of obesity measurement including BMI (p<0.01), WHR (p=0.01), WI (p<0.01), WHtR (p<0.001), WTR (p<0.001), VAI (p<0.001), BAI (p<0.001), CI (p<0.001), AVI (p<0.001), BRI (p<0.001) ABSI (p<0.001) and lower values for certain cardiometabolic risk factors (including FBG, LDL-C, TG, HBA1c and HOMA-IR and ferritin) but a higher HDL-C value as expected **(Tables 3.1c-d).**

With respect to normal weight participants those with the MUHNW phenotype were more likely to have a non-manual (white collar) occupation and exhibit a current medical comorbidity. However, there were no other significant differences in terms of lifestyle characteristics when compared to their healthy counterparts. With respect to anthropometric parameters, MUHNW participants were heavier (BMI 24 vs 22.4 kg/m^2 p=0.016), had higher values for WC (p=0.002) and HC (p=0.021) as well as for indices of

central obesity measurement compared to their healthy normal weight counterparts (including VAI [p<0.1], WI [p=0.01], WHR [p=0.019], WHtR [p<0.01], WThR [p=0.005], CI [p=0.01], AVI [p=0.002], BRI [p=<0.001] and ABSI [p=0.013]), but not for BAI (p=0.112). Moreover, while the MUHNW participants had significantly higher values for all lipid parameters and markers of inflammation (ferritin) there were no differences between values for glucose homeostasis (FPG [p=0.234], HbA_{1c} [p=0.054] and HOMA-IR [p=0.56]) when compared with the MHNW participants (**Tables 3.1a-d**).

The MHOW/O participants were comparable to their healthy non-obese counterparts (MHNW) for several lifestyle variables including age, smoking and alcohol consumption, physical activity, presence of an underlying comorbidity as well as level of education and occupation. Moreover, both had similar proportions of individuals exhibiting any of the parameters of the Met S except for TG and WC (p=<0.01, p=0.001 respectively). However, the MHO phenotype displayed higher values for all indices of obesity measurement, had higher values for parameters of glucose and lipid metabolism and inflammation (ferritin) and were more insulin resistant than the healthy nonobese individuals.

On the other hand, when the MHOW/O phenotype was compared with the unhealthy normal weight phenotype, individuals within the MUHNW phenotype were less likely to drink alcohol but more likely to have a concomitant medical problem than MHOW/O participants Notably, such individuals had higher values for total cholesterol and TG but comparable values for markers of glucose metabolism and insulin resistance as well as inflammation (including FPG, HOMA-IR, HBA_{1c} and ferritin). **(Tables 3.1a-d).**

Table 3.1a: Demographic characteristics of study participants as stratified into the four body composition phenotypes

Demographic parameters	MHOW/O* n=205 (39.3%)		MUHOW/O† n = 160 (30.7%)		MHNW* n= 145 (27.8%)		MUHNW† n= 11 (2.11%)		p value [a]	p value [b]	p value [c]	p value[d]
Age (median + IQR)	40.00	5.00	42.00	7.00	41.00	6.00	42.00	9.00	0.02	0.453	0.74	0.50
% Alcohol drinkers	53.2		37.5		53.8		18.2		0.007	0.06	0.484	0.03
% Smokers	17.6		26.9		24.1		27.3		0.025	0.554	0.235	0.258
% Regular physical activity	45.4		33.1		50.3		36.4		0.012	2.92	0.209	0.758
% White collar occupation	67.3		60		71.7		54.5		0.286	0.003	0.144	0.212
% PMH	11.7		40		13.1		54.5		0.01	0.003	0.408	0.001
% Tertiary education	53.7		42.5		57.2		36.4		0.037	0.152	0.290	0.356

Data are expressed as percentages, number or median +IQR

*MHOW/O & MHNW: individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)

†MUHOW/O & MHOW/O: individuals having ≥2 NCEP ATPIII criteria

NCEP ATPIII- national cholesterol education program adult treatment panel III;

MHOW/O- metabolically healthy overweight/obese; MUHOW/O – metabolically unhealthy overweight/obese; MHNW- metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight

[a]p-value: MHOW/O vs MUHOW/O, [b]p-value: NHNW vs MUHNW, [c]p-value: MHNW vs MHOW/O, [d]p-value: MUHNW vs MHOW/O

Table 3.1b: Percentage of Met S components among study participants as stratified into the four body composition phenotypes

Metabolic syndrome components	MHOW/O* n=205 (39.3%)	MUHOW/O† n = 160 (30.7%)	MHNW* n= 145 (27.8%)	MUHNW† n= 11 (2.11%)	p value [a]	p value [b]	p value [c]	p value[d]
%WC >102cm (M) or >88cm (F)	27.8	78.8	2.1	72.7	0.001	0.005	0.01	0.543
% FPG ≥ 5.6mmol/l or on Rx	7.3	54.4	4.8	54.5	0.001	0.001	0.237	< 0.01
% SBP ≥ 130mmHg or DBP ≥ 85mmHg or on Rx	36.1	57.5	30.3	54.5	0.001	0.096	0.157	0.336
% TG ≥ 1.7 mmol/l or on Rx	7.8	51.2	0.7	63.6	<0.001	0.001	0.001	0.01
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or on Rx	8.3	63.1	9.7	63.6	<0.001	0.001	0.398	< 0.01

Data are expressed as percentages.

*MHOW/O & MHNW: individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)

† MUHOW/O & MHOW/O: individuals having ≥2 NCEP ATPIII criteria

MHOW/O-metabolically healthy overweight/obese; MUHOW/O-metabolically unhealthy overweight/obese; MHNW-metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight; NCEP ATPIII- national cholesterol education program adult treatment panel III; M-male; F-female; WC-waist circumference; FPG, fasting plasma glucose; SBP-systolic blood pressure; DBP-diastolic blood pressure; TG- triglycerides; HDL-C – high density lipoprotein cholesterol; Rx - treatment

[a]p-value: MHOW/O vs MUHOW/O, [b]p-value: NHNW vs MUHNW, [c]p-value c: MHNW vs MHOW/O, [d]p-value: MUHNW vs MHOW/O

Table 3.1c: Anthropometric parameters and indices of obesity measurement of study participants as stratified into the four body composition phenotypes

	MHOW/O* n=205 (39.3%)		MUHOW/O† n = 160 (30.7%)		MHNW* n= 145 (27.8%)		MUHNW† n= 11 (2.11%)		p value [a]	p value [b]	p value [c]	p value[d]
BMI (kg/m²)	27.80	4.20	32.95	7.55	22.40	2.60	24.00	1.60	<0.01	0.016	<0.01	<0.01
Waist circumference (cm)	89.00	13.00	103.00	13.25	74.00	11.00	82.00	15.00	<0.01	0.002	<0.01	0.19
Hip circumference (cm)	101.00	10.50	109.00	17.00	91.00	8.00	96.00	10.00	<0.01	0.021	<0.01	0.06
Neck circumference (cm)	36.00	5.00	38.30	6.50	31.00	2.50	33.00	8.00	<0.01	0.158	<0.01	0.08
Mean arm circumference (cm)	30.00	3.50	33.00	4.25	26.00	3.50	28.00	5.00	<0.01	0.031	<0.01	0.08
Mean Thigh circumference (cm)	53.50	5.20	56.00	9.00	49.00	4.10	48.50	9.50	<0.01	0.479	<0.01	0.03
Left thigh circumference (cm)	53.00	5.50	56.00	9.00	49.00	4.10	48.50	9.50	<0.01	0.476	<0.01	0.03
SBP (mmHg)	120.00	10.00	120.00	15.00	120.00	15.00	125.00	20.00	0.01	0.098	0.01	0.29
DBP (mmHg)	80.00	5.00	80.00	5.00	80.00	10.00	80.00	10.00	0.00	0.611	0.23	0.31
Indices of obesity measurement												
Visceral adiposity index	0.94	0.58	2.15	1.46	0.74	0.44	2.28	0.99	<0.001	<0.01	<0.01	<0.01
Waist hip ratio	0.89	0.11	0.93	0.11	0.82	0.10	0.88	0.11	<0.01	0.019	<0.01	0.76
Waist Height Ratio	0.53	0.07	0.62	0.09	0.45	0.05	0.52	0.04	<0.001	<0.001	<0.01	0.28
Waist Thigh Ratio	1.69	0.28	1.83	0.31	1.50	0.21	1.61	0.33	<0.001	0.005	<0.01	0.86
Mean Arm Height ratio	0.18	0.02	0.19	0.03	0.16	0.02	0.17	0.04	<0.01	0.073	<0.01	0.22
Waist Index	1.03	0.15	1.15	0.2	0.88	0.1	1.01	0.13	<0.01	0.001	<0.01	0.5
Body Adiposity Index	28.89	7.41	32.52	9.82	25.30	4.30	29.14	8.70	<0.001	0.112	<0.01	0.32
Conicity Index	1.18	0.13	1.26	0.10	1.12	0.12	1.22	0.12	<0.001	0.001	<0.01	0.03
Abdominal Volume Index	15.98	4.37	21.50	5.80	11.13	2.78	13.72	4.96	<0.001	0.002	<0.01	0.17
Body Roundness Index	3.84	1.40	5.77	1.94	2.53	0.91	3.72	0.87	<0.001	<0.001	<0.01	0.28
A Body Shaped Index	0.12	0.02	0.13	0.02	0.12	0.02	0.13	0.03	<0.001	0.013	0.18	0.05

Data are expressed as median + IQR.
*MHOW/O & MHNW: individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)
†MUHOW/O & MUHNW individuals having ≥2 NCEP ATPIII criteria. NCEP ATPIII- national cholesterol education program adult treatment panel III; HOMA-IR, homeostatic model assessment of insulin resistance MHOW/O-metabolically healthy overweight/obese; MUHOW/O-metabolically unhealthy overweight/obese; MHNW-metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight; BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; [a]p-value: MHOW/O vs MUHOW/O, [b]p-value: NHNW vs MUHNW, [c]p-value: MHNW vs MHOW/O, [d]p-value: MUHNW vs MHOW/O

Table 3.1d: Biochemical parameters of study subject as stratified into the four body composition phenotypes

	MHOW/O* n=205 (39.3%)		MUHOW/O† n = 160 (30.7%)		MHNW* n= 145 (27.8%)		MUHNW† n= 11 (2.11%)					
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	p value [a]	p value [b]	p value [c]	p value[d]
Biochemical parameters												
T Chol (mmol/l)	4.80	0.97	5.00	1.24	4.62	1.09	5.60	1.01	0.09	0.004	0.11	0.01
LDL-C (mmol/l)	2.81	0.95	3.14	1.17	2.61	1.02	3.65	0.73	0.01	<0.01	0.01	0.00
HDL-C (mmol/l)	1.51	0.43	1.19	0.32	1.65	0.50	1.19	0.09	<0.01	<0.01	<0.01	0.00
Triglycerides (mmol/l)	0.92	0.50	1.49	0.92	0.75	0.33	1.59	0.87	<0.01	<0.01	<0.01	<0.01
Uric Acid (umol/l)	282.0	95.0	311.0	112.5	241.0	85.0	273.0	138.0	<0.01	0.222	<0.01	0.71
FPG (mmol/l)	5.07	0.46	5.64	0.86	4.93	0.53	5.60	1.37	<0.01	0.234	0.00	0.50
HbA_{1c} (%)	5.20	0.40	5.50	0.65	5.20	0.30	5.30	0.20	<0.01	0.054	0.01	0.47
HOMA-IR	1.62	0.82	2.34	1.14	1.13	0.91	1.13	0.92	<0.01	0.563	<0.01	0.26
% HOMA-IR ≥2.5	6.6		45.6		3.4		22.2		<0.001	0.05	0.148	0.132
Vitamin D (ng/L)	18.0	9.0	17.0	6.5	19.0	10.00	15.0	4.00	0.03	0.032	0.26	0.05
ALP (U/l)	63.0	20.0	69.0	19.0	55.0	20.00	63.0	13.00	0.00	0.097	<0.01	0.83
GGT (U/l)	18.0	16.0	27.0	21.0	14.0	9.00	17.0	46.00	<0.01	0.037	<0.01	0.63
ALT (U/l)	16.0	13.0	22.0	16.0	14.0	9.00	18.0	30.00	<0.01	0.355	<0.01	0.92
Ferritin (ng/mL)	52.0	107.0	83.5	149.5	28.0	55.00	85.0	120.0	0.02	0.012	<0.01	0.47
TSH (micIU/mL)	1.48	0.88	1.51	1.07	1.49	1.17	1.29	1.32	0.15	1.000	0.50	0.86
FT4 (pmol/L)	14.7	2.45	14.50	2.66	14.65	2.27	14.80	2.92	0.18	0.563	0.37	0.33
Birth weight (g)	3.19	1.10	3.30	0.80	3.10	0.70	3.70	1.00	0.48	0.237	0.84	0.25
LAP	26.24	19.52	63.42	44.72	11.57	8.97	34.41	30.65	<0.01	<0.01	<0.01	0.06
log (TG/HDL-C)	-0.21	0.30	0.12	0.36	-0.35	0.23	0.12	0.26	<0.01	<0.01	<0.01	<0.01
PLR	134.5	54.4	122.9	63.8	142.8	54.78	121.74	78.68	0.03	0.085	0.10	0.22
NLR	1.96	0.85	2.04	0.89	2.06	0.97	1.89	1.03	0.36	0.063	0.30	0.92

Data are expressed as median + IQR or percentages.

*MHOW/O & MHNW: individuals having ≤1 NCEP ATPIII criteria (waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)

†MUHOW/O & MUHNW: individuals having ≥2 NCEP ATPIII criteria.

MHOW/O-metabolically healthy overweight/obese; MUHOW/O-metabolically unhealthy overweight/obese; MHNW-metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight; T Chol – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; FPG – fasting plasma glucose; HBA_{1c}- haemoglobin A1c; HOMA-IR – homeostatic model assessment of insulin resistance; HBA1c ALP – alkaline phosphatase; GGT - Gamma glutamyl transferase; ALT- alanine transaminase; TSH – thyroxine stimulating hormone; FT4- free thyroxine; LAP – lipid accumulation product; PLR – platelet lymphocyte ratio; NLR-neutrophil lymphocyte ratio

[a]p-value: MHOW/O vs MUHOW/O, [b]p-value: NHNW vs MUHNW, [c]p-value: MHNW vs MHOW/O, [d]p-value: MUHNW vs MHOW/O

Tables 3.2a-e shows the prevalence, demographic and metabolic characteristics of the study population stratified by the three different BMI categories (normal weight [BMI <25 kg/m^2], overweight [BMI 25-29.9 kg/m^2] and obese [BMI ≥30 kg/m^2]) and MH (adopting the presence of ≤1 NCEP ATP III criteria to characterise the metabolically healthy phenotype), thus generating the six different body composition phenotypes: MHNW, MHOW, MHO, MUHNW, MUHOW, MUHO. The population prevalence of each combination of BMI and metabolic phenotype are as follows: MHNW-27.8%, MHOW-28.6%, MHO-10.7%, MUHNW-2.1%, MUHOW-8.1%, MUHO-22.6% **(Table 3.2a).**

Table 3.2a: Population prevalence of the six body composition phenotypes when adopting the presence of ≤1 NCEP ATP III criteria to define MH

	Normal weight	Overweight	Obese
Metabolically unhealthy	2.1%	8.1%	22.6%
Metabolically healthy	27.8%	28.6%	10.7%

NCEP ATP III, national cholesterol education program adult treatment panel III

72.6% (n=149) of the total healthy overweight/obese cohort were characterized as MHOW, whereas the majority (73%, n= 118) of the total unhealthy overweight and obese cohort consisted of individuals with obesity and only 26% (n=42) fell within the overweight BMI category. Thus, more than three quarters of the total overweight population was metabolically healthy (77.6%), whereas 67.8% of the total population with obesity consisted of the metabolically unhealthy subtype.

Overall, the metabolically healthy phenotype was more prevalent in females, in those with a tertiary level of education and in those holding a white-collar (non-manual) occupation. There was a lower proportion of individuals within the overweight and obese BMI categories who engaged in some regular form of physical activity **(Table 3.2b).** As expected, there was a trend towards increasing values for most anthropometric parameters and indices of obesity measurement (WC, BMI, NC, HC, WI, WTR, WHtR, BAI, CI, AVI, BRI and ABSI) as well as certain biochemical parameters (TC, LDL-C, HDL-C) from healthy normal weight to obesity state **(Tables 3.2d-e).**

Within the unhealthy group, obesity was also associated with the female sex and lower likelihood of engaging in physical activity. Similarly, there was also a significant trend towards an increase in values of a number of anthropometric parameters and indices of obesity measurement from normal weight to obese BMI categories (including WC, HC, NC, AC, TC, WI, WHR, WHtR, AVI and BRI), however, this trend was not observed within certain biochemical parameters including lipid profile and fasting glucose levels **(Tables 3.2b,d-e).**

Of note 7.7% and 3.7% of metabolically healthy participants with overweight and obesity respectively could be defined as being insulin resistant as evident by the proportion of individuals having a HOMA-IR value of ≥2.5. On the other hand, 22% of normal weight participants with Met S were insulin resistant and approximately half of the participants with obesity and Met S were insulin resistant **(Table 3.2e).** Moreover, while the overall prevalence of most Met S components increased with increasing BMI categories in both metabolically healthy and unhealthy participants (except for FPG and TG), the prevalence for increasing risk factors was higher across BMI categories in the metabolically unhealthy group **(Table 3.2c)**.

Table 3.2b: Demographic characteristics of the study participants as stratified into the six body composition phenotypes.

			Metabolically healthy*					Metabolically unhealthy‡				
	Overall (n = 521)		Normal weight n = 145 (27.8%)	Overweight n = 149 (28.6%)	Obese n = 56 (10.7%)	p trend[a]	Normal weight n = 11 (2.1%)		Overweight n = 42 (8.1%)	Obese n = 118 (22.6%)		p trend[b]
Age (median + IQR)	41.0	6.0	41.0 6.0	40.0 6.0	40.0 5.5	NS	42.0	9.0	43.00 6.0	41.0	6.0	<0.05
% Males	36.7		17.9	45.6	32..1		27.3		57.1	44.1		
% Alcohol drinkers	47.8		53.80	57.00	42.90	NS	18.20		47.60	33.90		<0.05
% Smokers	22.6		24.10	15.40	23.20	NS	27.30		28.60	26.30		<0.05
% Regular physical activity	42.8		50.30	49.00	35.70	<0.05	36.40		45.20	28.80		<0.05
% White collar occupation	66		71.70	69.80	60.70	NS	54.50		61.90	59.30		<0.05
% PMH	21.7		13.10	12.10	10.70	NS	54.50		35.70	41.50		<0.05
% Tertiary education	50.9		57.20	53.00	55.40	NS	36.40		45.20	41.50		<0.05

Data are expressed as percentages, or median +IQR

*Metabolically healthy – individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)

[T] Metabolically unhealthy – individuals having ≥2 NCEP ATPIII criteria

[a]p trend across BMI categories within the metabolically healthy category

[b]p trend across BMI categories within the metabolically unhealthy category

NCEP ATPIII- national cholesterol education program adult treatment panel III; PMH- past medical history

Table 3.2c: Percentage of Met S components of study participants as stratified into the six body composition phenotypes.

		Metabolically healthy				Metabolically unhealthy			
	Overall	Normal weight n = 145 (27.8%)	Overweight n = 149 (28.6%)	Obese n = 56 (10.7%)	p trend[a]	Normal weight n = 11 (2.1%)	Overweight n = 42 (8.1%)	Obese n = 118 (22.6%)	p trend[b]
Metabolic syndrome components									
%WC >102cm (M) or >88cm (F)	36.3	2.10%	14.80	62.50	<0.05	27.30	45.20	90.70	<0.05
% FPG ≥ 5.6mmol/l or on Rx	22.1	4.80%	9.40%	1.80%	<0.05	54.50	59.50	52.50	<0.05
% BP ≥130/85mmHg or on Rx	41.5	30.30	34.90	39.30	<0.05	54.50	57.10	57.60	<0.05
% TG ≥ 1.7 mmol/l or on Rx	20.3	0.70%	9.40%	3.60%	<0.05	63.60	59.50	48.30	<0.05
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or on Rx	26.7	9.70%	8.70%	7.10%	<0.05	63.60	52.40	66.90	<0.05

Data are expressed as percentages.
*Metabolically healthy – individuals having ≤1 NCEP ATPIII criteria from the following: consisting of waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)
†Metabolically unhealthy – individuals having ≥2NCEP ATPIII criteria
[a]p trend across BMI categories within the metabolically healthy category
[b]p trend across BMI categories within the metabolically unhealthy category
NCEP ATPIII- national cholesterol education program adult treatment panel III;
WC- waist circumference; M-male; F-female; FPG- fasting plasma glucose BP- blood pressure; TG- triglycerides; HDL-C – high density lipoprotein cholesterol; Rx -treatment

Table 3.2d: Anthropometric parameters and indices of obesity measurement of study participants as stratified into the six body composition phenotypes

	Overall (n=521)		Metabolically healthy*							Metabolically unhealthy‡						
			Normal weight n= 145 (27.8%)		Overweight n= 149 (28.6%)		Obese n= 56 (10.7%)		P trend[a]	Normal weight n=11 (2.1%)		Overweight n=42 (8.1%)		Obese n=118 (22.6%)		P trend[b]
	Median	IQR	Median	IQR	Median	IQR	Median	IQR		Median	IQR	Median	IQR	Median	IQR	
Anthropometric parameters																
BMI (kg/m²)	27.50	7.80	22.40	2.60	27.10	2.10	33.00	4.05	<0.05	24.00	1.60	28.20	2.20	34.70	6.70	<0.05
WC (cm)	89.00	20.00	74.00	11.0	86.50	11.00	96.00	12.00	<0.05	82.00	15.00	92.00	10.0	106.00	12.50	<0.05
HC (cm)	99.00	16.00	91.00	8.00	98.00	10.00	110.50	13.00	<0.05	96.00	10.00	101.50	7.00	114.00	16.00	<0.05
NC (cm)	35.00	6.00	31.00	2.50	35.60	5.00	36.00	5.50	<0.05	33.00	8.00	38.00	8.00	40.00	6.00	<0.05
Mean AC (cm)	30.00	5.50	26.00	3.50	29.20	3.00	31.00	2.70	<0.05	28.00	5.00	30.50	4.00	33.00	4.00	<0.05
Mean TC (cm)	52.00	7.50	49.00	4.10	52.00	7.00	56.00	5.20	<0.05	48.50	9.50	51.00	6.50	58.00	7.00	<0.05
Mean Arm to Height Ratio	0.2	0.00	0.2	0.02	0.2	0.10	0.19	0.02	<0.05	0.17	0.04	0.2	0.02	0.20	0.03	<0.05
SBP (mmHg)	120.00	10.00	120.00	15.0	120.00	10.00	122.50	8.50	<0.05	125.00	20.00	125.00	10.0	120.00	15.00	NS
DBP (mmHg)	80.00	10.00	80.00	10.0	80.00	10.00	80.00	5.00	NS	80.00	10.00	80.00	5.00	80.00	5.00	NS
Indices of obesity measurement																
VAI	1.09	1.10	0.74	0.44	0.97	0.65	0.91	0.40	<0.05	2.28	0.99	2.10	1.36	2.17	1.48	NS
WHtR	0.53	0.11	0.45	0.05	0.52	0.05	0.59	0.06	<0.05	0.52	0.04	0.56	0.04	0.63	0.08	<0.05
WTR	1.68	0.35	1.50	0.21	1.69	0.28	1.71	0.27	<0.05	1.61	0.33	1.83	0.29	1.83	0.35	<0.05
BAI	27.89	8.11	25.30	4.30	27.08	6.42	35.04	10.07	<0.05	29.14	8.70	27.95	6.51	35.58	9.83	<0.05
Conicity Index	1.20	0.14	1.12	0.12	1.18	0.12	1.20	0.13	<0.05	1.22	0.12	1.26	0.11	1.26	0.10	<0.05
AVI	16.04	7.29	11.13	2.78	15.15	3.29	19.07	4.45	<0.05	13.72	4.96	17.19	3.55	22.83	5.13	<0.05
BRI	3.85	2.22	2.53	0.91	3.67	0.87	5.13	1.45	<0.05	3.72	0.87	4.52	0.89	6.24	1.94	<0.05
ABSI	0.12	0.02	0.12	0.02	0.13	0.02	0.12	0.02	<0.05	0.13	0.03	0.13	0.02	0.13	0.02	<0.05
WHR	0.88	0.12	0.82	0.10	0.89	0.10	0.89	0.13	<0.05	0.88	0.11	0.93	0.09	0.93	0.15	NS
Waist Index	1.02	0.22	0.88	0.10	1.00	0.13	1.15	0.16	<0.05	1.01	0.13	1.04	0.15	1.20	0.18	<0.05

Data are expressed as median +IQR.

*Metabolically healthy – individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents;

‡ Metabolically unhealthy – individuals having ≥2 NCEP ATPIII criteria

[a]P trend across BMI categories within the metabolically healthy category; [b]P trend across BMI categories within the metabolically unhealthy category

BMI-body mass index; WC- waist circumference; HC-hip circumference, NC-neck circumference; AC-arm circumference; TC-thigh circumference; SBP- systolic blood pressure; DBP- diastolic blood pressure; VAI-visceral adiposity index, WHtR-waist height ratio; WTR- waist thigh ratio; BAI-body adiposity index; AVI-abdominal volume index; BRI-body roundness index; ABSI-a body shape index; WHR-waist hip ratio.

Table 3.2e: Biochemical parameters of study participants as stratified into the six body composition phenotypes

	Metabolically healthy*									Metabolically unhealthy[T]						
	Overall		Normal weight		Overweight		Obese			Normal weight		Overweight		Obese		
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	P trend[a]	Median	IQR	Median	IQR	Median	IQR	P trend[b]
Biochemical parameters																
TChol (mmol/l)	4.87	1.10	4.62	1.09	4.79	1.00	4.91	0.88	NS	5.60	1.01	5.18	1.53	4.91	1.21	NS
LDL-C (mmol/l)	2.85	1.06	2.61	1.02	2.81	0.93	2.86	0.94	<0.05	3.65	0.73	3.12	1.23	3.14	1.15	NS
HDL-C (mmol/l)	1.41	0.49	1.65	0.50	1.48	0.42	1.56	0.43	<0.05	1.19	0.09	1.23	0.40	1.17	0.30	NS
Triglycerides (mmol/l)	1.01	0.71	0.75	0.33	0.92	0.56	0.92	0.38	<0.05	1.59	0.87	1.62	1.26	1.48	0.82	<0.05
Uric Acid (umol/l)	280.00	95.00	241.00	85.0	280.00	90.00	286.00	94.00	<0.05	273.00	138.0	308.50	120.0	311.50	113.0	NS
FPG (mmol/l)	5.13	0.66	4.93	0.53	5.10	0.49	5.04	0.36	<0.05	5.60	1.37	5.64	0.82	5.61	0.89	NS
HbA1c (%)	5.30	0.40	5.20	0.30	5.20	0.40	5.25	0.30	<0.05	5.30	0.20	5.40	0.50	5.58	0.70	<0.05
%HOMA-IR ≥2.5	18.1		3.40		7.70		3.70		<0.05	22.20		40.50		47.40		<0.05
HOMA-IR	1.65	1.17	1.13	0.91	1.64	0.92	1.53	0.92	<0.05	1.13	0.92	2.20	1.60	2.41	1.07	<0.05
Vitamin D (ng/L)	18.00	8.00	19.00	10.0	18.00	10.00	18.00	7.50	NS	15.00	4.00	17.00	6.00	18.00	7.00	NS
ALP (U/l)	63.00	21.00	55.00	20.0	63.00	22.00	63.50	15.50	<0.05	63.00	13.00	67.50	15.00	70.00	18.00	NS
GGT (U/l)	19.00	16.00	14.00	9.00	18.00	16.00	18.00	14.50	<0.05	17.00	46.00	29.00	26.00	25.00	18.00	NS
ALT (U/l)	17.00	13.00	14.00	9.00	17.00	13.00	16.00	14.50	<0.05	18.00	30.00	23.00	23.00	22.00	16.00	NS
Ferritin (ng/ml)	53.00	105.00	28.00	55.00	52.00	112.0	52.00	84.50	<0.05	85.00	120.00	77.50	175.0	85.00	147.0	NS
TSH (micIU/mL)	1.49	1.00	1.49	1.17	1.42	0.82	1.66	1.21	<0.05	1.29	1.32	1.51	0.92	1.51	1.09	NS
FT4 (pmol/L)	14.62	2.48	14.65	2.27	15.06	2.63	14.38	2.06	<0.05	14.80	2.92	14.70	2.19	14.40	2.79	NS

Table 3.2d: (Continued)

LAP	27.50	36.46	11.57	8.97	23.69	18.40	33.40	22.12	<0.05	34.41	30.65	52.04	35.64	65.92	45.87	<0.05
log (TG/HDL-C)	-0.16	0.42	-0.35	0.23	-0.20	0.33	-0.25	0.26	<0.05	0.12	0.26	0.14	0.44	0.12	0.28	NS
PLR	134.39	62.25	142.86	54.78	135.16	56.70	127.61	54.13	NS	121.74	78.68	127.78	70.45	119.79	65.25	NS
NLR	2.02	0.89	2.06	0.97	1.93	0.81	1.97	0.95	NS	1.89	1.03	1.92	0.60	2.10	0.99	NS

Data are expressed as median +IQR

*Metabolically healthy – individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents)

[†] Metabolically unhealthy – individuals having ≥2 NCEP ATPIII criteria

[a] P trend across BMI categories within the metabolically healthy category

[b] P trend across BMI categories within the metabolically unhealthy category

NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III;

TChol-total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; FPG- fasting plasma glucose; HBA_{1c}- haemoglobin A1c; HOMA-IR- homeostatic model assessment of insulin resistance; ALP- alkaline phosphatase; GGT- Gamma glutamyl transferase; ALT- alanine transaminase; TSH- thyroxine stimulating hormone; FT4- free thyroxine; LAP – lipid accumulation product; PLR – platelet lymphocyte ratio; NLR – neutrophil lymphocyte ratio;

3-1.2 Sensitivity analysis assessing the prevalence of the six different body composition phenotypes using more stringent and less stringent criteria of the NCEP ATPIII definition and HOMA-IR to define metabolic health

Figure 3.1 compares the population prevalences of the six different body composition phenotypes - MHO, MUHO, MHOW, MUHOW, MHNW and MUHNW using less stringent *i.e.* the presence of ≤2 cardiometabolic parameters of the NCEP ATPIII definition [NCEP-ATPIII 2 criteria] and more stringent criteria i.e. the presence of 0 cardiometabolic parameters of the NCEP ATPIII definition [NCEP-ATPIII Zero criteria] in addition to the standard criteria used previously (i.e. the presence of ≤1 cardiometabolic parameters of the NCEP ATPIII) as well the presence of IR as defined by HOMA-IR using a cut-off of <2.5 to denote the metabolically healthy status.

The population prevalence of MHO varied from 3.1% to 22.8% with the highest prevalence observed when categorising MH utilising HOMA-IR <2.5 classification (which is equivalent to the 81st percentile in this cohort of participants); the prevalence of MHOW ranged from 18% to 34% with the highest prevalence attained when adopting the presence of ≤2 NCEP- ATPIII criteria. The MUHNW phenotype ranged from 0.4% to 6.9% with the highest prevalence detected when utilizing the presence of NCEP-ATPIII zero criteria.

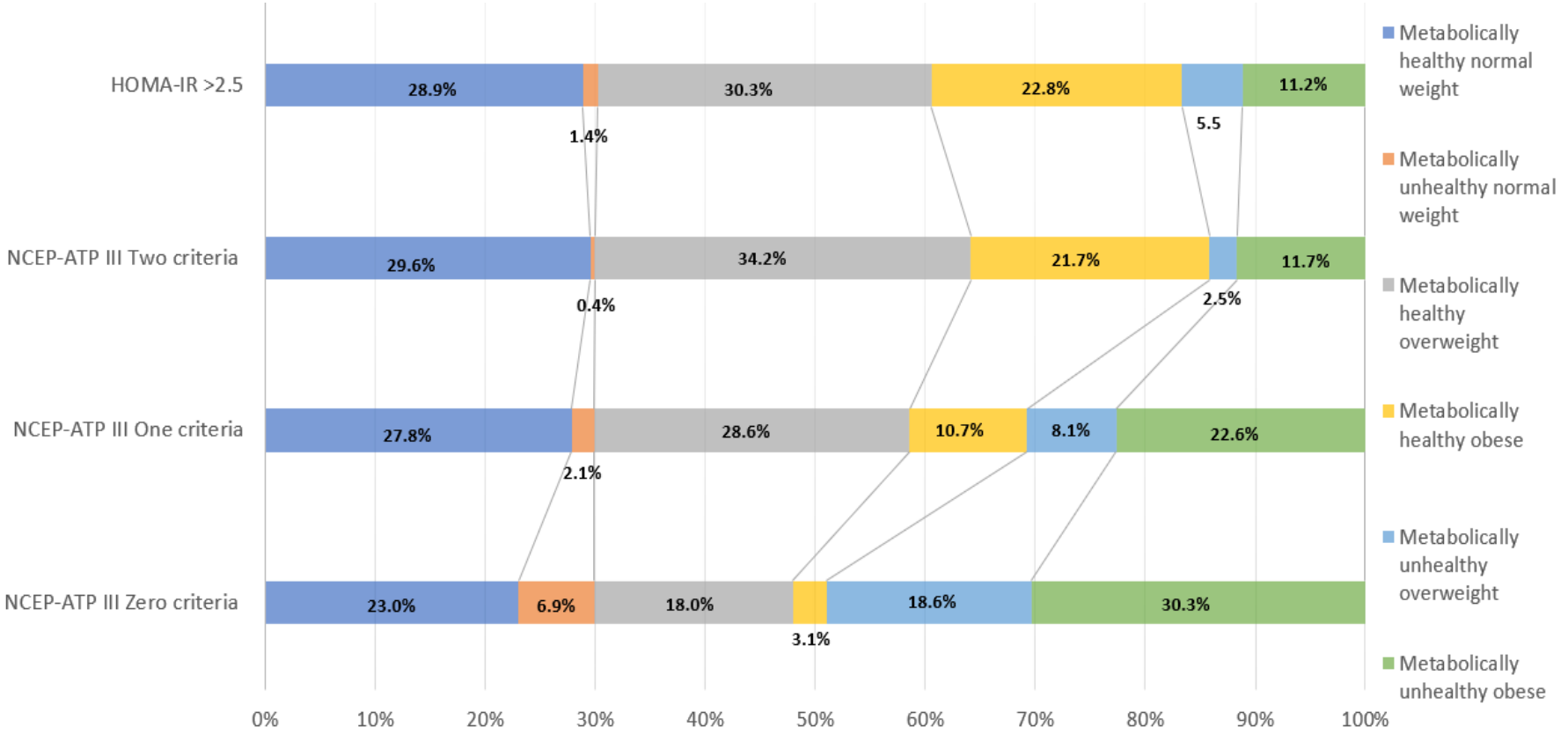


Figure 3.1: Prevalence of the six body composition phenotypes according less stringent and more stringent criteria of the NCEP ATPIII* and HOMA-IR**

*Less stringent criteria implies the presence of ≤2 features of the NCEP ATPIII criteria (NCEP-ATPIII two criteria); more stringent criteria implies the presence of zero features of the NCEP ATPIII criteria (NCEP-ATPIII Zero criteria) to categorise the metabolically healthy phenotype ** A cut-off value of <2.5 denotes the metabolically healthy phenotype
NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III; HOMA-IR, homeostatic model assessment of insulin resistance

3-1.3 Characteristics associated with the metabolically healthy phenotype among individuals with overweight and obesity

When the overweight and obese BMI categories were considered, participants with metabolically healthy overweight or obesity were less likely to have a past medical history (12.1% vs 35.7% for overweight and 10.7% vs 41.5% for obese categories; p = 0.001) than their unhealthy overweight or obese counterparts **(Table 3.3a)**. However, there were no other significant differences in lifestyle characteristics between the healthy and unhealthy overweight and obese BMI categories. There was a significant difference in prevalence of Met S components between the healthy and unhealthy overweight and obese cohorts with higher proportions in the obese phenotypes **(Table 3.3b).** Compared to their unhealthy counterparts, participants exhibiting either the healthy overweight or obese phenotype had lower values for indices of obesity measurement (implying lower amounts of visceral fat) while higher value for thigh circumference (reiterating the fact that these individuals preferentially accumulated fat in lower gluteofemoral subcutaneous areas) compared to individuals with MUHO or MUHOW **(Table 3.3c)**. Furthermore, MHO and MHOW individuals presented with an overall better cardiometabolic profile: they had lower values for makers of glucose metabolism, IR, inflammation (ferritin) and TG, higher values for HDL-C but similar LDL and TC values when compared to their unhealthy counterparts. **(Table 3.3d).** On comparing participants with healthy overweight to those with healthy obesity, no significant differences in lifestyle characteristics were noted. However, the MHOW phenotype had lower values for most anthropometric parameters except for NC, WHR and VAI (p=0.283; p=0.827 and p=0.704 respectively). On the other hand, there were

no differences in lipid and glucose profiles between the two categories. Moreover, there was no difference in prevalence of Met S components between the two categories except for WC (p=0.001). There was a higher but nonsignificant proportion of MHOW individuals having a HOMA-IR value ≥2.5 when compared to MHO (7.7% vs 3.7% p=0.256). Similarly, when comparing the unhealthy overweight and obese BMI cohorts together no significant differences were noted for lifestyle factors but significant differences for most anthropometric measurements of obesity were observed with the unhealthy overweight cohort having lower values except for the WHR. There was no difference in prevalence of Met S components between the two categories (except for WC). Finally, no differences were noted in the glucose and lipid profiles of both categories **(Tables 3.3a-d).**

Table 3.3a: Demographics of participants with overweight and obesity stratified by metabolic health status

	Metabolically healthy overweight (MHOW)*	Metabolically unhealthy overweight (MUHOW)†	Metabolically healthy obese (MHO)*	Metabolically unhealthy obese (MUHO)†	P value[a]	p value[b]	p value[c]	p value[d]
Age (median +IQR)	40.00 6.00	43.00 6.00	40.00 5.50	41.00 6.00	0.003	0.168	0.485	0.009
% Males	45.60%	57.10%	32.10%	44.10%	0.126	0.237	0.055	0.101
% Alcohol drinkers	57.00%	47.60%	42.90%	33.90%	0.182	0.091	0.049	0.254
% Smokers	15.40%	28.60%	23.20%	26.30%	0.085	0.427	0.378	0.935
% Regular physical activity	49.00%	45.20%	35.70%	28.80%	0.4	0.386	0.061	0.041
% White collar occupation	69.80%	61.90%	60.70%	59.30%	0.377	0.228	0.21	0.662
% PMH	12.10%	35.70%	10.70%	41.50%	0.001	0.001	0.501	0.319
% Tertiary education	53.00%	45.20%	55.40%	41.50%	0.237	0.166	0.444	0.659

Data are expressed as median +IQR or percentages.
*MHOW / MHO –individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting plasma glucose ≥5.6mmol/L or on antihyperglycemic agents
†MUHOW / MUHO – individuals having ≥2 NCEP ATPIII criteria
NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III; PMH, past medical history;
[a]p value: MHOW vs MUHOW; [b]p value: MHO vs MUHO; [c]p value: MHO vs MHOW; [d]p value: MUHO vs MUHOW

Table 3.3b: Percentage of Met S components among participants with overweight and obesity stratified by metabolic health status

	Metabolically healthy overweight (MHOW)*	Metabolically unhealthy overweight (MUHOW)†	Metabolically healthy obese (MHO)*	Metabolically unhealthy obese (MUHO)†	P value[a]	P value[b]	P value[c]	P value[d]
%WC >102cm (M) or >88cm (F)	14.80%	45.20%	62.50%	90.70%	<0.05	<0.05	<0.01	<0.01
% FPG ≥ 5.6mmol/l	9.40%	59.50%	1.80%	52.50%	<0.05	<0.05	0.049	0.275
% BP ≥130/85mmHg or on Rx	34.90%	57.10%	39.30%	57.60%	<0.05	0.018	0.335	0.549
% TG ≥ 1.7 mmol/l or on Rx	9.40%	59.50%	3.60%	48.30%	<0.05	<0.05	0.135	0.142
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or on Rx	8.70%	52.40%	7.10%	66.90%	<0.05	<0.05	0.482	0.068

Data are expressed as percentages.
*MHOW / MHO–individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting plasma glucose ≥5.6mmol/L or on antihyperglycemic agents
†MUHOW / MUHO – individuals having ≥2 NCEP ATPIII criteria.
NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III; WC, waist circumference; FPG, fasting plasma glucose; M, male; F, female; BP, blood pressure; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; Rx, treatment.
[a]p value: MHOW vs MUHOW; [b]p value: MHO vs MUHO; [c]p value: MHO vs MHOW; [d]p value: MUHO vs MUHOW

Table 3.3c: Anthropometric parameters and indices of obesity measurement among participants with overweight and obesity stratified by metabolic health status

	Metabolically healthy overweight (MHOW)*		Metabolically unhealthy overweight (MUHOW) †		Metabolically healthy obese (MHO)*		Metabolically unhealthy obese (MUHO)†		P value[a]	p value[b]	p value[c]	p value[d]
	Median	IQR	Median	IQR	Median	IQR	Median	IQR				
Anthropometric parameters:												
BMI (kg/m²)	27.10	2.10	28.20	2.20	33.00	4.05	34.70	6.70	0.001	0.003	0.001	0.001
Waist circumference (cm)	86.50	11.00	92.00	10.00	96.00	12.00	106.00	12.50	0.002	0.001	0.001	0.001
Hip circumference (cm)	98.00	10.00	101.50	7.00	110.50	13.00	114.00	16.00	0.054	0.028	0.001	0.001
Neck circumference (cm)	35.60	5.00	38.00	8.00	36.00	5.50	40.00	6.00	0.006	0.001	0.283	0.010
Mean Arm circumference (cm)	29.20	3.00	30.50	2.00	31.00	2.50	33.00	4.50	0.006	0.001	0.001	0.001
Mean thigh circumference (cm)	52.00	7.00	51.30	6.50	56.50	5.00	58.00	7.50	0.375	0.477	0.001	0.001
SBP (mmHg)	120.00	10.00	125.00	10.00	122.50	8.50	120.00	15.00	0.024	0.544	0.142	0.573
DBP (mmHg)	80.00	10.00	80.00	5.00	80.00	5.00	80.00	5.00	0.090	0.361	0.070	0.586
Birth weight (g)	3.17	0.70	3.40	1.00	3.20	1.30	3.30	0.80	0.316	0.958	0.973	0.537
Indices of obesity Measurement												
Visceral adiposity index	0.97	0.65	2.10	1.36	0.91	0.40	2.17	1.48	0.001	0.001	0.704	0.831
Waist hip ratio	0.89	0.10	0.93	0.09	0.89	0.13	0.93	0.15	0.001	0.001	0.827	0.739
Waist index	1.00	0.13	1.04	0.15	1.15	0.16	1.20	0.18	0.005	0.001	0.001	0.001
Waist Height Ratio	0.52	0.05	0.56	0.04	0.59	0.06	0.63	0.08	<0.01	<0.01	<0.01	<0.01
Waist Thigh Ratio	1.69	0.28	1.83	0.29	1.71	0.27	1.83	0.35	<0.01	<0.01	0.552	0.887

Table 3.3c: (Continued)

Body Adiposity Index	27.08	6.42	27.95	6.51	35.04	10.07	35.58	9.83	0.916	0.854	<0.01	<0.01
Conicity Index	1.18	0.12	1.26	0.11	1.20	0.13	1.26	0.10	<0.01	<0.01	0.206	0.664
AVI	15.15	3.29	17.19	3.55	19.07	4.45	22.83	5.13	<0.01	<0.01	<0.01	<0.01
Body Roundness Index	3.67	0.87	4.52	0.89	5.13	1.45	6.24	1.94	<0.01	<0.01	<0.01	<0.01
A Body Shaped Index	0.126	0.021	0.133	0.018	0.118	0.016	0.126	0.02	<0.01	<0.01	0.012	0.015

Data are expressed as median +IQR.

*MHOW / MHO–individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting plasma glucose ≥5.6mmol/L or on antihyperglycemic agents

[†]MUHOW / MUHO – individuals having ≥2 NCEP ATPIII criteria

NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III; SBP, systolic blood pressure; DBP, diastolic blood pressure; AVI, abdominal volume index

[a]p value: MHOW vs MUHOW; [b]p value: MHO vs MUHO; [c]p value: MHO vs MHOW; [d]p value: MUHO vs MUHOW

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Table 3.3d: Biochemical parameters among participants with overweight and obesity stratified by metabolic health status

	Metabolically healthy overweight (MHOW)*		Metabolically unhealthy overweight (MUHOW)†		Metabolically healthy obese (MHO)*		Metabolically unhealthy obese (MUHO)†		P value[a]	P value[b]	P value[c]	P value[d]
	Median	IQR	Median	IQR	Median	IQR	Median	IQR				
Biochemical parameters:												
Total cholesterol (mmol/l)	4.79	1.00	5.18	1.53	4.91	0.88	4.91	1.21	0.033	0.605	0.720	0.167
LDL-C (mmol/l)	2.81	0.93	3.12	1.23	2.86	0.94	3.14	1.15	0.128	0.177	0.671	0.957
HDL-C (mmol/l)	1.48	0.42	1.23	0.40	1.56	0.43	1.17	0.30	0.002	0.001	0.315	0.230
Triglycerides (mmol/l)	0.92	0.56	1.62	1.26	0.92	0.38	1.48	0.82	0.002	0.001	0.573	0.479
Uric Acid (µmol/l)	280.0	90.0	308.5	120.0	286.0	94.0	311.5	113.0	0.13	0.12	0.13	0.32
FPG (mmol/l)	5.10	0.49	5.64	0.82	5.04	0.36	5.61	0.89	0.002	0.001	0.270	0.618
HbA_{1c} (%)	5.20	0.40	5.40	0.50	5.25	0.30	5.58	0.70	0.002	0.001	0.561	0.241
%HOMA-IR ≥ 2.5	7.7%		40.5%		3.7%		47.4%		<0.05	<0.05	0.256	0.277
HOMA-IR	1.64	0.92	2.20	1.60	1.53	0.92	2.41	1.07	0.002	0.001	0.158	0.424
Vitamin D (ng/L)	18.00	10.00	17.00	6.00	18.00	7.50	18.00	7.00	0.084	0.350	0.528	0.796
ALP (U/l)	63.00	22.00	67.50	15.00	63.50	15.50	70.00	18.00	0.305	0.018	0.756	0.248
GGT (U/l)	18.00	16.00	29.00	26.00	18.00	14.50	25.00	18.00	0.001	0.004	0.463	0.178
ALT (U/l)	17.00	13.00	23.00	23.00	16.00	14.50	22.00	16.00	0.002	0.004	0.727	0.546
Ferritin (ng/ml)	52.00	112.0	77.50	175.00	52.00	84.50	85.00	147.00	0.393	0.032	0.533	0.606
TSH (micIU/mL)	1.42	0.82	1.51	0.92	1.66	1.21	1.51	1.09	0.232	0.572	0.028	0.855
FT4 (pmol/L)	15.06	2.63	14.70	2.19	14.38	2.06	14.40	2.79	0.397	0.756	0.026	0.585
LAP	23.69	18.40	52.04	35.64	33.40	22.12	65.92	45.87	0.001	0.001	0.001	0.001
log (TG/HDL-C)	-0.20	0.33	0.14	0.44	-0.25	0.26	0.12	0.28	0.001	0.001	0.498	0.895
NLR	1.93	0.81	1.92	0.60	1.97	0.95	2.10	0.99	0.979	0.799	0.285	0.199
PLR	135.16	56.70	127.78	70.45	127.61	54.13	119.79	65.25	0.327	0.328	0.224	0.438

Data are expressed as median +IQR.

*MHOW/MHO– individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

†MUHOW/MUHO– individuals having ≥2 NCEP ATPIII criteria.

NCEP ATPIII- National Cholesterol Education Program Adult Treatment Panel III; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HBA_{1c}, haemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; ALP, alkaline phosphatase; GGT, Gamma glutamyl transferase; ALT, alanine transaminase; TSH, thyroxine stimulating hormone; FT4, free thyroxine; LAP, lipid accumulation product; NLR, neutrophil lymphocyte ratio; PLR, platelet lymphocyte ratio;

[a]p value: MHOW vs MUHOW; [b]p value: MHO vs MUHO; [c]p value: MHO vs MHOW; [d]p value: MUHO vs MUHOW

3-1.4 Comparison of the Met S and IR definition for metabolic health on the prevalence and characteristics of the different body composition phenotypes

When taking into consideration both the Met S and IR criteria to define MH, only physical activity was significantly different between the healthy and unhealthy overweight/obese groups with respect to lifestyle characteristics. On the other hand, significant differences in anthropometric and biochemical parameters were noted between the MHOW/O and MUHOW/O cohorts when considering both criteria. MHOW/O individuals differed from MUHOW/O individuals for the following cardiometabolic variables: Weight, BMI, WC, HC, NC, indices of central obesity measurement, prevalence of Met S components, FBG, HBA1c, HDL-C, TG, and HOMA-IR values **(Tables 3.4a-d).** Therefore, overall, the MHOW/O participants presented a better metabolic profile when compared to MUOW/O individuals using both the Met S and IR definition criteria for MH. Interestingly there were higher proportions of MHOW/O individuals defined by IR criteria who exhibited components of the Met S and higher overall values for indices of obesity measurement and anthropometric parameters when compared to MHOW/O individuals defined by the Met S criteria. Thus, overall, the IR definition for MHOW/O presented a worse cardiometabolic picture when compared to the NCEP ATPIII definition **(Tables 3.4a-d).**

With respect to the normal weight individuals, the MHNW cohort had significant differences for several anthropometric and biochemical variables as well as for indices of obesity measurement (including BMI, WC, WHR, WI, VAI, WHtR, WTR, CI, AVI, BRI, ABSI, LDL-C, TG, HDL-C) compared to MUHNW participants when using the NCEP ATPIII

criteria to categorise MH, but no such differences were observed when utilising the IR criteria. Interestingly, while the MUHNW cohort as defined by the IR definition had a higher proportion of individuals exhibiting components of the Met S when compared with MHNW participants , these did not reach statistical significance (except for HDL-C). However, significant differences in proportions were observed when MHNW and MUHNW were defined by the NCEP ATPIII criteria **(Tables 3.5a-d)**. Therefore, overall, individuals with MUHNW presented a worse cardiometabolic profile to MHNW when using the NCEP ATPIII definition of the Met S **(Tables 3.5a-d).**

Table 3.4a: Demographic characteristics of the MHOW/O and MUHOW/O phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using HOMA-IR criteria					Metabolic health using NCEP-ATPIII criteria				
	MHOW/O* n=270		MUHOW/O** n = 85		p value	MHOW/O† n = 205		MUHOW/O†† n = 160		p value
Age (years) (median +IQR)	41.00	6.00	42.00	6.00	0.11	40.00	5.00	42.00	7.00	0.02
% Males	43		50.6		0.26	42		47.5		0.170
% Alcohol drinkers	46.7		43.5		0.19	53.2		37.5		0.007
% Smokers	20.4		25.9		0.08	17.6		26.9		0.025
% Physical activity	44.1		30.6		0.03	45.4		33.1		0.012
% White collar occupation	65.2		58.8		0.50	67.3		60		0.286
% PMH	18.1		44.7		<0.01	11.7		40		0.01
% Tertiary education	51.1		42.4		0.25	53.7		42.5		0.037

Data are expressed as median +IQ or percentages.
*MHOW/O–individuals having a HOMA-IR value <2.5; **MUHOW/O-individuals having a HOMA-IR value ≥2.5.
†MHOW/O-individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents
††MUHOW/O–individuals having ≥2 ATPIII criteria.
ATP III, adult treatment panel III; HOMA-IR, homeostatic model of insulin resistance; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese; PMH, past medical history

Table 3.4b: Percentage of Met S components among MHOW/O and MUHOW/O phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using HOMA-IR criteria			Metabolic health using NCEP ATP III criteria		
	MHOW/O* n=270	MUHOW/O**	P value	MHOW/O† n-205	MUHOW/O†† N=160	P value
Metabolic Syndrome components (%)						
%WC >102cm (M) or >88cm (F)	45.6	65.9	<0.01	27.8	78.8	0.001
% FPG ≥ 5.6mmol/l	19.6	57.6	<0.01	7.3	54.4	<0.001
% BP ≥130/85mmHg or Rx	41.9	58.8	0.01	36.1	57.5	< 0.001
% TG ≥ 1.7 mmol/l or Rx	17.8	58.8	<0.01	7.8	51.2	<0.001
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or Rx	21.1	70.6	<0.01		63.1	<0.001

Data are expressed as percentages.
*MHOW/O–individuals having a HOMA-IR value <2.5; **MUHOW/O-individuals having a HOMA-IR value ≥2.5.
†MHOW/O-individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents
††MUHOW/O–individuals having ≥2 ATPIII criteria.
ATP III, adult treatment panel III; HOMA-IR, homeostatic model of insulin resistance; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese; WC, waist circumference; FPG, fasting plasma glucose; M, male; F, female; BP, blood pressure; TG, triglycerides; HDL-C, high density lipoprotein cholesterol;

Table 3.4c: Anthropometric parameters and indices of obesity measurement among MHOW/O and MUHOW/O phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using HOMA-IR criteria					Metabolic health using NCEP ATPIII criteria				
	MHOW/O* n=270		MUHOW/O** n=85			MHOW/O† n=205		MUHOW/O†† n=160		
	Median	IQR	Median	IQR	p value	Median	IQR	Median	IQR	p value
Anthropometric parameters										
Body mass index (kg/m²)	29.10	5.70	32.70	7.10	<0.01	27.80	4.20	32.95	7.55	<0.01
Waist circumference (cm)	92.00	15.00	102.0	17.00	<0.01	89.00	13.00	103.00	13.25	<0.01
Hip circumference (cm)	104.00	14.00	107.0	16.00	0.00	101.00	10.50	109.00	17.00	<0.01
Neck circumference (cm)	36.00	7.00	38.0	5.00	<0.01	36.00	5.00	38.30	6.50	<0.01
Mean arm circumference (cm)	31.00	4.00	33.0	5.00	0.00	30.00	3.00	33.00	4.23	<0.01
Mean thigh circumference (cm)	54.50	7.00	5.00	10.00	0.36	53.00	5.50	56.00	9.00	<0.01
Systolic blood pressure (mmHg)	120.00	10.00	120.00	15.00	0.77	120.00	10.00	120.00	15.00	0.01
Diastolic blood pressure (mmHg)	80.00	5.00	80.00	5.00	0.64	80.00	5.00	80.00	5.00	0.00
Indices of obesity measurement										
Visceral adiposity index	1.11	0.95	2.51	2.04	<0.01	0.94	0.58	2.15	1.46	<0.001
Waist Height Ratio	0.56	0.09	0.61	0.09	<0.01	0.53	0.07	0.62	0.09	<0.001
Waist Thigh Ratio	1.71	0.27	1.83	0.34	<0.01	1.69	0.28	1.83	0.31	<0.001
Body Adiposity Index	29.65	9.03	31.65	9.20	0.01	28.89	7.41	32.52	9.82	<0.001
Conicity Index	1.21	0.12	1.26	0.10	<0.01	1.18	0.13	1.26	0.10	<0.001
Abdominal Volume Index	17.28	5.40	20.88	6.40	<0.01	15.98	4.37	21.50	5.80	<0.001
Body Roundness Index	4.45	1.77	5.56	2.12	<0.01	3.84	1.40	5.77	1.94	<0.001
A Body Shaped Index	0.12	0.02	0.13	0.02	0.04	0.12	0.02	0.13	0.02	<0.001
Waist hip ratio	0.90	0.11	0.93	0.10	<0.01	0.89	0.11	0.93	0.11	<0.01
Waist index	1.07	0.19	1.14	0.25	<0.01	1.03	0.15	1.15	0.20	<0.01

Data are expressed as median +IQR

*MHOW/O – individuals having a HOMA-IR value <2.5; †MUHOW/O – individuals having a HOMA-IR value ≥2.5

*MHOW/O – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents ††MUHOW/O – individuals having ≥2 ATPIII criteria

ATP III, adult treatment panel III; HOMA-IR, homeostatic model of insulin resistance; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese

Table 3.4d: Biochemical parameters among MHOW/O and MUHOW/O phenotypes considering the ATPIII and HOMA-IR definitions for metabolic health

	Metabolic health using HOMA-IR criteria					Metabolic health using NCEP ATPIII criteria				
	MHOW/O* n=270		MUHOW/O** n=85			MHOW/O† n=205		MUHOW/O†† n=160		
	Median	IQR	Median	IQR	P value	Median	IQR	Median	IQR	P value
Biochemical parameters										
Total cholesterol (mmol/l)	4.86	1.04	5.09	1.29	0.07	4.80	0.97	5.00	1.24	0.09
LDL-C (mmol/l)	2.88	1.01	3.14	1.20	0.13	2.81	0.95	3.14	1.17	0.01
HDL-C (mmol/l)	1.39	0.40	1.09	0.31	<0.01	1.51	0.43	1.19	0.32	<0.01
Triglycerides (mmol/l)	1.07	0.64	1.70	1.11	<0.01	0.92	0.50	1.49	0.92	<0.01
Uric Acid (µmol/l)	290.00	96.00	307.00	106.00	0.06	282.00	95.00	311.00	112.50	<0.01
Fasting plasma glucose (mmol/l)	5.13	0.61	5.70	1.61	<0.01	5.07	0.46	5.64	0.86	<0.01
HbA1c (%)	5.30	0.49	5.60	1.30	<0.01	5.20	0.40	5.50	0.65	<0.01
%HOMA-IR ≥ 2.5	0		100		<0.01	6.6		45.6		<0.001
HOMA-IR	1.64	0.86	3.09	0.97	<0.01	1.62	0.82	2.34	1.14	<0.01
Vitamin D (ng/L)	18.00	8.00	17.00	7.00	0.39	18.00	9.00	17.00	6.50	0.03
ALP (U/l)	65.00	19.00	71.00	22.00	0.00	63.00	20.00	69.00	19.0	0.00
GGT (U/l)	20.00	18.00	29.00	22.00	<0.01	18.00	16.00	27.00	21.0	<0.01
ALT (U/l)	19.00	14.00	22.50	17.50	0.01	16.00	13.00	22.00	16.0	<0.01
Ferritin (mg/ml)	59.50	114.00	86.00	158.0	0.02	52.00	107.0	83.50	149.5	0.02
TSH (micIU/ml)	1.50	1.03	1.45	0.74	0.09	1.48	0.88	1.51	1.07	0.15
FT4 (pmol/l)	14.49	2.50	15.13	2.58	0.25	14.70	2.45	14.50	2.66	0.18
Birth weight (g)	3.30	1.00	3.20	0.68	0.81	3.19	1.10	3.30	0.80	0.48
Lipid accumulation product	33.70	30.44	70.98	48.73	<0.01	26.24	19.52	63.42	44.7	<0.01
log (TG/HDL-C)	-0.14	0.35	0.20	0.33	<0.01	-0.21	0.30	0.12	0.36	<0.01
Platelet lymphocyte ratio	129.90	57.36	125.30	57.66	0.42	134.47	54.36	122.90	63.7	0.03
Neutrophil lymphocyte ratio	1.96	0.96	2.09	0.80	0.37	1.96	0.85	2.94	0.89	0.36

Data are expressed as median +IQR.
*MHOW/O – individuals having a HOMA-IR value <2.5; †MUHOW/O – individuals having a HOMA-IR value ≥2.5
*MHOW/O – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and <1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents
††MUHOW/O – individuals having ≥2 NCEP ATPIII criteria.
ATP III, adult treatment panel III; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese
LDL-C , low density lipoprotein cholesterol; HDL-C , high density lipoprotein cholesterol; HBA_{1c}, haemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; ALP, alkaline phosphatase; GGT, Gamma glutamyl transferase; ALT, alanine transaminase; TSH , thyroxine stimulating hormone; FT4, free thyroxine

Table 3.5a: Demographic characteristics of the MHNW and MUHNW phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using ATP III criteria					Metabolic health using HOMA-IR criteria				
	Metabolically healthy normal weight (MHNW)* n= 145		Metabolically unhealthy normal weight (MUHNW)** n= 11		*p* value	Metabolically healthy normal weight (MHNW)† n = 147		Metabolically unhealthy normal weight (MUHNW)†† n = 7		*p* value
age (median + IQR)	41.00	6.0	42.00	9.0	0.453	41.00	7.0	39.00	5.0	0.38
% Males	17.9		27.3		0.334	19.70%		0.00%		UTC
% Alcohol drinkers	53.8		18.2		0.06	53.10%		28.60%		0.425
% Smokers	24.1		27.3		0.554	23.10%		42.90%		0.389
% Regular physical activity	50.3		36.4		2.92	50.30%		28.60%		0.442
% White collar occupation	71.7		54.5		0.003	70.70%		85.70%		0.019
% PMH	13.1		54.5		0.003	15.00%		28.60%		0.299
% Tertiary education	57.2		36.4		0.152	55.10%		71.40%		0.465

Data are expressed as median +IQR or percentages

*MHNW – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents;

**MUHNW– individuals having ≥2 ATPIII criteria

†MHNW– individuals having a HOMA-IR value <2.5; ††MUHNW – individuals having a HOMA-IR value ≥2.5

ATPIII- adult treatment panel III, HOMA-IR- homeostatic model assessment of insulin resistance; PMH, past medical history

Table 3.5b: Percentage of Met S components among MHNW and MUHNW phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using ATPIII criteria			Metabolic health using HOMA-IR criteria		
	Metabolically healthy normal weight (MHNW)* n= 145	Metabolically unhealthy normal weight (MUHNW)** n= 11	P Value	Metabolically healthy normal weight (MHNW)† n = 147	Metabolically unhealthy normal weight (MUHNW)†† n = 7	P value
Metabolic syndrome components						
%WC >102cm (M) or >88cm (F)	2.1	72.7	0.005	2.70	14.30	0.21
% FPG ≥ 5.6mmol/l	4.8	54.5	< 0.001	7.50%	14.30	0.44
% BP ≥ 130/85mmHg or Rx	30.3	54.5	0.096	31.30%	42.90	0.68
% TG ≥ 1.7 mmol/l or on Rx	0.7	63.6	< 0.001	4.10%	14.30	0.283
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or Rx	9.7	63.6	< 0.001	11.60%	57.1	0.007

Data are expressed as percentages.

*MHNW – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents; **MUHNW– individuals having ≥2 ATPIII criteria

†MHNW– individuals having a HOMA-IR value <2.5; ††MUHNW – individuals having a HOMA-IR value ≥2.5

ATPIII- adult treatment panel III, HOMA-IR- homeostatic model assessment of insulin resistance

WC, waist circumference; M, male; F, female; FPG, fasting plasma glucose; BP, blood pressure; TG, triglycerides; HDL-C , high density lipoprotein cholesterol

Table 3.5c: Anthropometric parameters and indices of obesity measurement among MHNW and MUHNW phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using ATPIII criteria					Metabolic health using HOMA-IR criteria				
	Metabolically healthy normal weight (MHNW)* n= 145		Metabolically unhealthy normal weight (MUHNW)** n= 11			Metabolically healthy normal weight (MHNW)† n = 147		Metabolically unhealthy normal weight (MUHNW)†† n = 7		
	Median	IQR	Median	IQR	p value	Median	IQR	Median	IQR	p value
Anthropometric parameters										
Body Mass Index (kg/m^2)	22.40	2.60	24.00	1.6	0.016	22.40	2.70	24.00	1.80	0.10
Waist circumference (cm)	74.00	11.00	82.00	15.0	0.002	74.00	11.00	76.00	16.00	0.79
Hip circumference (cm)	91.00	8.00	96.00	10.0	0.021	91.00	7.70	89.00	9.00	0.50
Neck circumference (cm)	31.00	2.50	33.00	8.0	0.158	31.50	2.50	30.50	3.00	0.38
Mean Arm circumference (cm)	26.00	3.50	28.00	5.0	0.031	26.00	3.50	28.00	6.00	0.48
Mean thigh circumference (cm)	49.00	4.10	48.50	9.50	0.479	49.00	4.10	49.00	7.50	0.56
Systolic BP (mmHg)	120.00	15.00	125.00	20.00	0.098	120.00	15.00	120.00	20.00	0.42
Diastolic BP (mmHg)	80.00	10.00	80.00	10.00	0.611	80.00	10.00	80.00	10.00	0.52
Indices of obesity measurement										
Visceral adiposity index	0.74	0.44	2.28	0.99	<0.01	0.75	0.51	0.70	1.93	0.71
Waist Height Ratio	0.45	0.05	0.52	0.04	<0.001	0.45	0.06	0.49	0.08	0.35
Waist Thigh Ratio	1.50	0.21	1.61	0.33	0.005	1.51	0.21	1.51	0.17	0.76
Body Adiposity Index	25.30	4.30	29.14	8.70	0.112	25.11	4.39	26.39	3.10	0.43
Conicity Index	1.12	0.12	1.22	0.12	0.001	1.14	0.14	1.17	0.18	0.93
Abdominal Volume Index	11.13	2.78	13.72	4.96	0.002	11.27	3.06	11.79	4.14	0.83
Body Roundness Index	2.53	0.91	3.72	0.87	<0.001	2.54	0.98	3.13	1.46	0.35
A Body Shaped Index	0.12	0.02	0.13	0.03	0.013	0.12	0.02	0.12	0.02	0.35
Waist hip ratio	0.82	0.10	0.88	0.11	0.019	0.82	0.09	0.85	0.11	0.76
Waist index	0.88	0.10	1.01	0.13	0.001	0.89	0.09	0.95	0.20	0.30

Data are expressed as median +IQR.

*MHNW – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents;**MUHNW– individuals having ≥2 ATPIII criteria;

†MHNW– individuals having a HOMA-IR value <2.5; ††MUHNW – individuals having a HOMA-IR value ≥2.5

ATPIII- adult treatment panel III, HOMA-IR- homeostatic model assessment of insulin resistance; BP- blood pressure

Table 3.5d: Biochemical parameters among MHNW and MUHNW phenotypes considering the ATPIII and HOMA-IR definitions of metabolic health

	Metabolic health using ATPIII criteria					Metabolic health using HOMA-IR criteria				
	Metabolically healthy normal weight (MHNW)* n= 145		Metabolically unhealthy normal weight (MUHNW)** n= 11			Metabolically healthy normal weight (MHNW)† n = 147		Metabolically unhealthy normal weight (MUHNW)†† n = 7		
Biochemical parameters	Median	IQR	Median	IQR	P value	Median	IQR	Median	IQR	P value
Total cholesterol (mmol/l)	4.62	1.09	5.60	1.01	0.004	4.64	1.14	5.71	1.68	0.14
LDL-C (mmol/l)	2.61	1.02	3.65	0.73	<0.01	2.62	1.07	3.49	1.20	0.01
HDL-C (mmol/l)	1.65	0.50	1.19	0.09	<0.01	1.63	0.50	1.29	0.74	0.08
Triglycerides (mmol/l)	0.75	0.33	1.59	0.87	<0.01	0.77	0.38	0.58	1.05	0.62
Uric Acid (µmol/l)	241.00	85.0	273.00	138.0	0.222	242.00	91.00	276.00	96.00	0.57
Fasting plasma glucose (mmol/l)	4.93	0.53	5.60	1.37	0.234	4.94	0.56	4.87	0.48	0.68
HbA_{1c} (%)	5.20	0.30	5.30	0.20	0.054	5.20	0.30	5.10	0.60	0.45
HOMA-IR	1.13	0.91	1.13	0.92	0.563	1.12	0.85	2.67	0.20	<0.01
% HOMA-IR ≥ 2.5	3.4		22.2		0.05	0.00%		100.00		<0.01
Vitamin D (ng/L)	19.00	10.0	15.00	4.00	0.032	19.00	10.00	18.00	9.00	0.70
ALP (U/l)	55.00	20.0	63.00	13.00	0.097	56.00	20.00	64.00	23.00	0.39
GGT(U/l)	14.00	9.00	17.00	46.00	0.037	14.00	9.00	17.00	18.00	0.47
ALT (U/l)	14.00	9.00	18.00	30.00	0.355	14.00	9.00	13.00	7.00	0.23
Ferritin (ng/mL)	28.00	55.0	85.00	120.0	0.012	29.00	65.00	19.00	64.00	0.29
TSH (micIU/L)	1.49	1.17	1.29	1.32	1.000	1.46	1.16	1.98	2.30	0.74
FT4 (pmol/L)	14.65	2.27	14.80	2.92	0.563	14.65	2.31	15.04	2.71	0.76
Birth weight (g)	3.10	0.70	3.70	1.00	0.237	3.10	0.70	4.20	0.00	0.11
Lipid Accumulation Product	11.57	8.97	34.41	30.65	<0.01	11.70	10.89	10.08	31.93	0.95
log (TG/HDL-C)	-0.35	0.23	0.12	0.26	<0.01	-0.32	0.26	-0.35	0.67	0.68
Platelet-lymphocyte ratio	142.86	54.78	121.74	78.68	0.085	142.17	53.64	104.72	95.86	0.40
Neutrophil-lymphocyte ratio	2.06	0.97	1.89	1.03	0.063	2.06	0.99	1.89	0.73	0.96

Data are expressed as median +IQR.

*MHNW – individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents; **MUHNW– individuals having ≥2 ATPIII criteria

†MHNW– individuals having a HOMA-IR value <2.5; ††MUHNW – individuals having a HOMA-IR value ≥2.5

ATPIII, Adult treatment panel III; HOMA-IR- homeostatic model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL-C , high density lipoprotein cholesterol; HBA_{1c} haemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; ALP, alkaline phosphatase; GGT, Gamma glutamyl transferase; ALT, alanine transaminase; TSH , thyroxine stimulating hormone; FT4, free thyroxine

3-1.5 Lifestyle determinants of the MHOW/O and MUHNW phenotypes

To determine the factors which are independently associated with the prevalence of different body composition phenotypes, multivariate logistic regression techniques were applied to estimate the unadjusted and adjusted odds ratio (OR) and its 95% confidence interval for different demographic and lifestyle characteristics on MH in participants with normal weight and overweight/obesity . Body composition phenotype was inputted as the dependent variable whilst the demographic and lifestyle factors of interest were incorporated into the regression model as the independent (predictor) variables. The two body composition phenotypes of interest were the metabolically healthy overweight/obese phenotype (MHOW/O) and the metabolically unhealthy normal weight phenotype (MUHNW) as defined by either the NCEP ATPIII criteria of the Met S or by HOMA-IR.

A) Lifestyle determinants of the MHOW/O phenotype

A multinomial regression analysis was carried out to estimate the odds ratio (and its 95% CI) of expressing ≤1 CM abnormalities associated with demographic and behavioural characteristics among individuals with overweight/obesity . Lifestyle characteristics which could be rapidly ascertained in the clinical setting, and which bear no relation to the definition of MH were incorporated into the regression model. Unadjusted prevalence ratios were calculated initially followed by multivariate-adjusted models for all demographic and behavioural factors simultaneously.

Table 3.6: Lifestyle determinants of the MHOW/O phenotype as defined by the ATPIII criteria*

Variable		Exp(B)	95% CI	P value
Physical activity	Yes	1.737	1.1-2.74	**0.018**
	No	1(Ref)		
Alcohol	Drinker	2.359	1.47-3.77	**<0.01**
	Non-Drinker	1(Ref)		
Gender	Male	0.638	0.397-1.026	0.064
	Female	1(Ref)		
Smoking	Non-smokers	2.06	1.278-3.321	**0.003**
	Smokers	1(Ref)		
Age	<40 years	1.85	1.186-2.877	**0.007**
	>40 years	Ref		

*Defined as the presence of ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents;
MHOW/O, metabolically healthy overweight/obese; ATPIII, Adult Treatment Panel III

Among individuals with overweight and obesity , physical activity, alcohol consumption, non-smoking status, and age <40 years were associated with higher odds of the metabolically healthy phenotype **(Table 3.6).**

In comparison, when the metabolically healthy phenotype was defined using the HOMA-IR criteria (using a cut-off of <2.5 to define MH) only physical activity and non-smoking status were independently associated with the MHO state. No association with age, sex or alcohol consumption was observed **(Table 3.7)**.

Table 3.7 Lifestyle determinants of the MHOW/O as defined by HOMA-IR*

Variable		Exp(B)	95% CI	P value
Physical activity	Yes	1.906	1.11-3.267	**0.019**
	No	1(Ref)		
Alcohol	Drinker	1.288	0.758-2.191	0.35
	Non-Drinker	1(Ref)		
Gender	Male	0.672	0.391-1.152	0.148
	Female	1(Ref)		
Smoking	Non-smokers	1.695	1.003-2.864	**0.049**
	Smokers	1(Ref)		
Age	<40 years	1.53	0.9-2.5	0.12
	>40 years	Ref		

Table 3.8 Lifestyle determinants MUHNW as defined by the ATPIII criteria*

Variable		Exp(B)	95% CI	P value
Physical activity	Yes	0.649	0.174-2.421	0.52
	No	1(Ref)		
Alcohol	Drinker	0.163	0.32-0.815	0.027
	Non-Drinker	1(Ref)		
Gender	Male	1.826	0.394-8.46	0.442
	Female	1(Ref)		
Smoking	Non-smokers	0.465	0.123-1.759	0.26
	Smokers	1(Ref)		
Age	<40 years	0.669	0.176-2.541	0.555
	>40 years	Ref		

*Defined as the presence of ≥2 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents; MUHNW- metabolically unhealthy normal weight; ATPIII, Adult Treatment Panel III

B) Lifestyle determinants of the MUHNW phenotype

When a multinomial regression analysis was carried out to assess the independent determinants of the metabolically unhealthy normal weight phenotype (as defined by the presence of ≥2 cardiometabolic abnormalities of the NCEP ATPIII criteria) no significant associations were observed between behavioural characteristics (including physical activity, sex, smoking and age) and the metabolically unhealthy lean state **(Table 3.8).** Furthermore, when the analysis was repeated using HOMA-IR as the definition of MH, no significant association between any of the lifestyle parameters to MH was similarly observed (data not shown).

3-2 Sex differences in the prevalence of the different body composition phenotypes

Within the context of MH, the presence of visceral adiposity (as opposed to peripheral subcutaneous adiposity) is associated with an unhealthy metabolic phenotype, predisposing individuals within this metabolic category at higher risk for CVD (Lapidus et al., 1984; Neeland et al., 2019). WC is widely regarded as being a surrogate marker of visceral adiposity and is also directly linked with increased CVD (Pouliot et al., 1994; Stefan, 2020a; Yusuf et al., 2004). Furthermore it is also acknowledged that there are significant sex differences in the prevalence of overweight and obesity and in the distribution of fat (Chang et al., 2018). Thus, another objective of this study was to explore the sex differences in the prevalence of each of the different body composition phenotypes as well as to investigate sex differences in anthropometric measurements and in CM parameters and in the relationship between the different BMI categories and MH. For this analysis participants were identified as having the metabolically healthy phenotype if they exhibited ≤1 of the cardiometabolic abnormalities of the NCEP ATPIII criteria for the Met S as described in **chapter 2**. Furthermore, participants with overweight and obesity were analysed separately in initial analysis thus generating the 6 different body composition phenotypes. Thereafter they were analysed together as one entity and thus generating four body composition phenotypes (MHNW; MUHNW; MHOW/O; MUHOW/O).

When stratifying the population by sex, BMI and MH, an increase in BMI was associated with an increase in the prevalence of metabolic abnormalities in both males and females **(Figure 3.2a**). Overall males were more likely to exhibit the metabolically unhealthy phenotype (41.3% vs 27.8%; p<0.001) **(Figure 3.2b).** While the majority of normal

weight participants were categorised as healthy, a higher percentage of males (10.3%) exhibited the MUHNW phenotype and only 6.3% of normal weight females were categorised as unhealthy. On the other hand, 73.9% and 82% of male and female overweight participants respectively exhibited the MHOW phenotype but only 25.7% of males and 36.5% of females had the MHO phenotype **(Figure 3.2a).**

A summary of the phenotypic differences observed between the two sexes in the entire study population is provided in **Table 3.9 and Figure 3.3**. Overall, males had a less favourable metabolic profile: they had higher FPG, HBA_{1c}, HOMA-IR, TC, LDL-C, and TG but lower HDL-C when compared to their female counterparts. Males also exhibited higher median hip, neck, and arm circumferences. On the other hand, females had larger median thigh circumference and overall lower values for most indices of obesity measurements (such as WHR, WHtR and WThR AVI, CI, VAI and BRI). Furthermore, despite males having a significantly higher median BMI than females (28.1 vs 26.8 kg/m^2 respectively), a lower proportion of males exhibited an abnormally high WC (i.e. >102 cm) (31.4% vs 39.1% p=0.048). When considering the total population, a higher percentage of males exhibited the MUHOW phenotype (4.6% vs 3.5%), however a higher percentage of females had the MHO phenotype (12.7% vs 10.0%).

Finally, when sex stratification was analysed for each of the six categories of MH, a significant difference in sex distribution was noted for each of the body composition phenotypes **(Figure 3.4).**

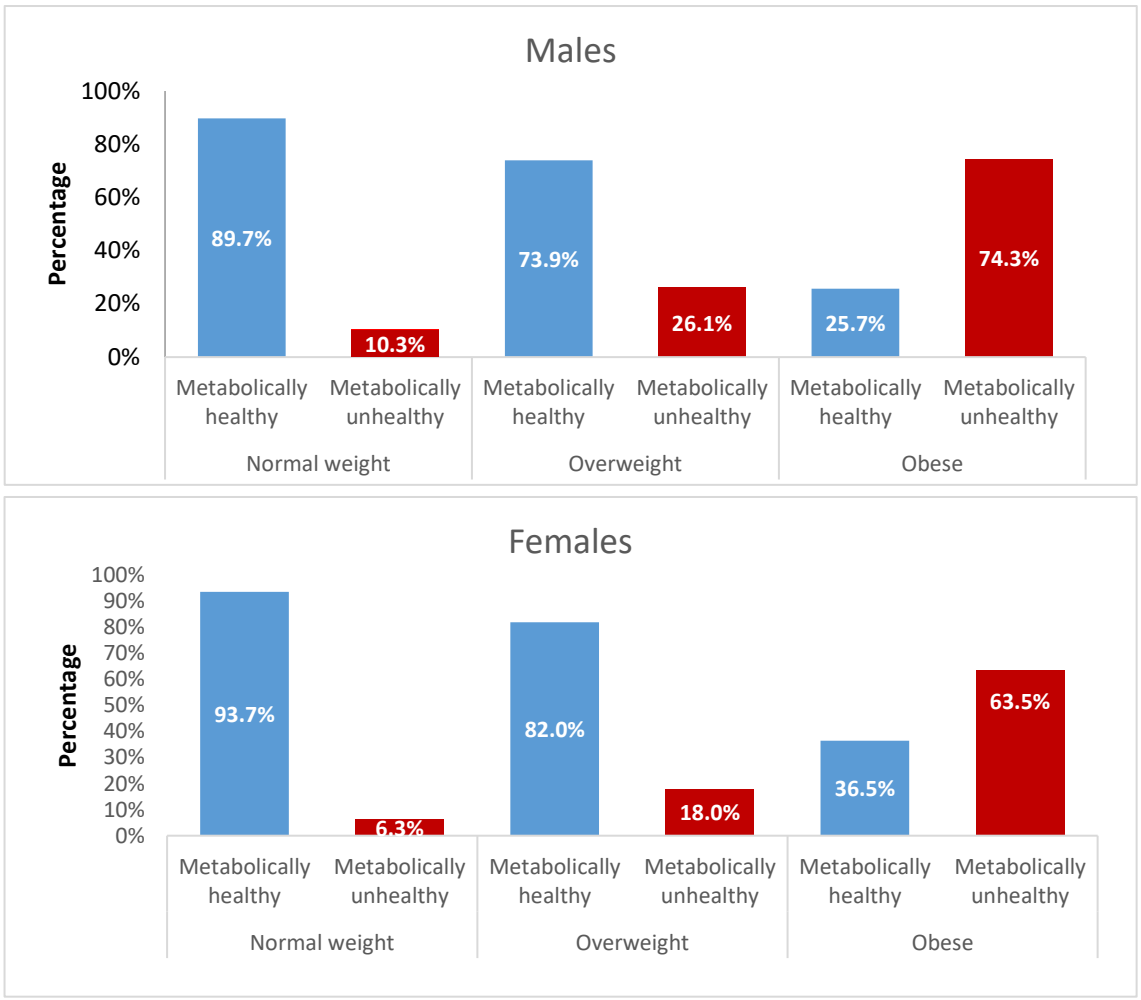


Figure 3.2a: Percentages of metabolically unhealthy and healthy phenotype within each BMI category stratified by sex

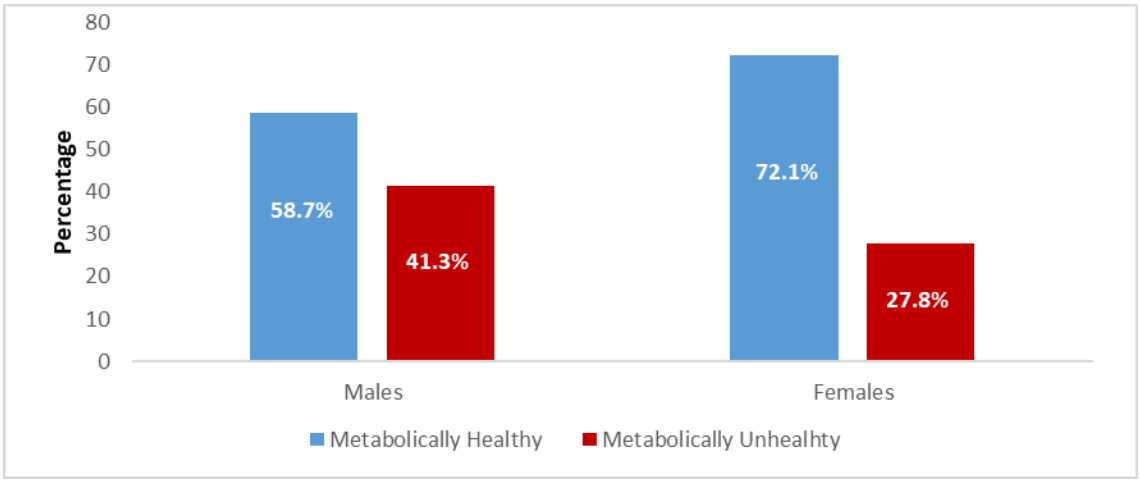


Figure 3.2b: Percentage of metabolically unhealthy and healthy phenotypes for the entire male and female population

Table 3.9: Sex differences in demographic, anthropometric and biochemical parameters, and indices of obesity measurement in the entire population

	Male n = 191 (36.7%)	Female n = 330 (63.3%)	p-value
MHNW n (%)*	26 (5.0)	119 (22.8)	<0.05
MUHNW n (%)†	3 (0.5)	8 (1.5)	NS
MHOW n (%)*	67 (12.8)	81 (15.5)	<0.05
MUHOW n (%)†	24 (4.6)	18 (3.5)	<0.05
MHO n (%)*	18 (3.5)	38(7.3)	NS
MUHO n (%)†	52 (10)	66 (12.7)	NS
Demographic parameters			
Age (IQR)	42.0 (6.0)	40.0 (7.0)	0.024
% Alcohol drinkers	63.5	38.7	<0.01
% Smokers	25.7	20.6	NS
% Physical activity	48.7	39.4	0.024
% White collar occupation	57.1	71.2	<0.01
% PMH	23.0	20.9	NS
% Tertiary education	43.5	55.2	0.036
Metabolic syndrome components			
% WC >102cm (M) or >88cm (F)	31.4	39.1	0.048
% FPG ≥ 5.6mmol/l	33.0	15.8	<0.01
% SBP ≥ 130mmHg or DBP ≥85mmHg or Rx	46.6	38.5	0.043
% TG ≥ 1.7 mmol/l or on statins	36.1	11.2	<0.01
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or Rx	27.2 (26.4)	26.4	NS
Anthropometric parameters			
Body Mass Index (kg/m^2)	28.1 (5.2)	26.8 (9.1)	0.001
Waist circumference (cm)	96.0 (16.0)	83.0 (21.0)	<0.001
Hip circumference (cm)	101.0 (11.0)	97.8 (18.0)	0.008
Neck circumference (cm)	39.0 (5.0)	33.0 (4.50)	<0.001
Mean Arm circumference (cm)	31.0 (4.0)	28.0 (5.0)	<0.001
Mean thigh circumference (cm)	51.0 (8.0)	53.0 (8.0)	0.021
SBP (mmHg)	120.0 (13.0)	120.0 (10.0)	NS
DBP (mmHg)	80.0 (5.0)	80.0 (15.00)	0.029
Biochemical parameters			
Total cholesterol (mmol/l)	4.98 (1.32)	4.76 (1.11)	0.012
LDL-C (mmol/l)	3.07 (1.11)	2.73 (1.09)	<0.001
HDL-C (mmol/l)	1.26 (0.41)	1.54 (0.53)	<0.001
Triglycerides (mmol/l)	1.25 (0.99)	0.89 (0.61)	<0.001
Uric Acid (umol/l)	329.0 (90.0)	249.0 (82.0)	<0.001
FPG (mmol/l)	5.33 (0.82)	5.03 (0.55)	<0.001
HbA1c (%)	5.40 (0.50)	5.20 (0.40)	<0.001
HOMA-IR	1.85 (1.21)	1.57 (1.16)	0.001
Vitamin D (ng/L)	18.0 (9.0)	18.0 (8.0)	NS
ALP (U/l)	65.0 (19.0)	61.5 (22.0)	0.001
GGT (U/l)	28.0 (24.0)	15.0 (11.0)	<0.001
ALT (U/l)	26.0 (17.0)	15.0 (8.0)	<0.001
Ferritin (ng/ml)	153.0 (141.0)	27.0 (41.0)	<0.001

Table 3.9: (Continued)

%HOMA-IR ≥ 2.5	22.9	15.3	0.022
TSH (micIU/mL)	1.45 (0.86)	1.49 (1.15)	NS
fT4 (pmol/L)	15.20 (2.38)	14.43 (2.23)	<0.001
Birth weight (Kg)	3.20 (1.28)	3.20 (0.86)	0.966
Lipid Accumulation Product	41.14 (46.93)	21.81 (26.87)	<0.001
log (TG/HDL-C)	0.00 (0.42)	-0.25 (0.35)	<0.001
Platelet-lymphocyte ratio	117.7 (46.5)	143.3 (60.8)	<0.001
Neutrophil-lymphocyte ratio	1.91 (0.77)	2.09 (1.03)	0.001
Indices of obesity measurement			
Visceral Adiposity Index	1.28 (1.28)	0.99 (0.89)	<0.001
Waist Height Ratio	0.54 (0.09)	0.52 (0.13)	<0.001
Waist Thigh Ratio	1.85 (0.31)	1.58 (0.28)	<0.001
Body Adiposity Index	25.68 (5.03)	30.65 (9.93)	<0.001
Conicity Index	1.25 (0.11)	1.16 (0.16)	<0.001
Abdominal Volume Index	18.44 (6.18)	14.15 (7.22)	<0.001
Body Roundness Index	4.24 (1.90)	3.71 (2.56)	<0.001
'A' Body Shaped Index	0.14 (0.01)	0.12 (0.01)	<0.001
Waist Index	1.02 (0.17)	1.02 (0.26)	NS

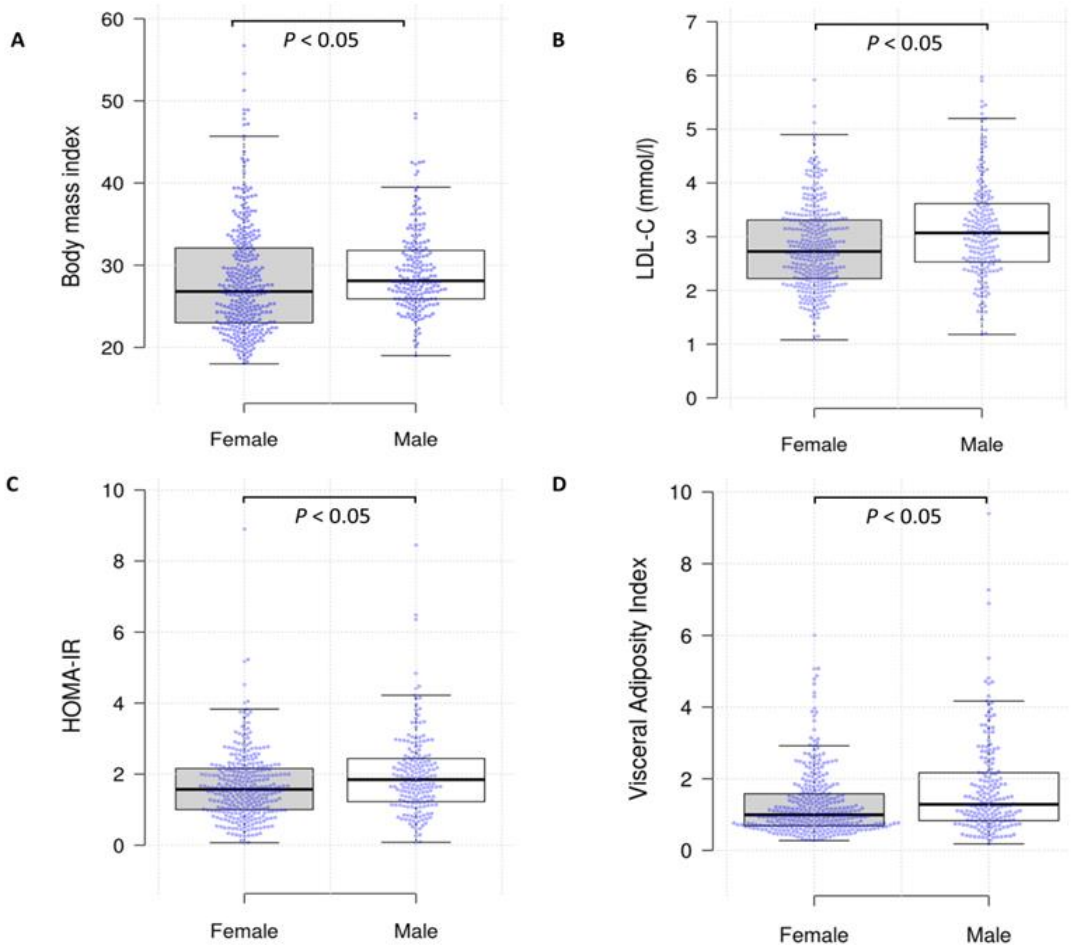
Data are expressed as number and percentage, or median +IQR

* Individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglyceride level ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

† Individuals having ≥2 NCEP ATPIII criteria

MHNW- metabolically healthy normal weight; MUHNW- metabolically unhealthy normal weight; MHOW- metabolically healthy overweight; MUHOW- metabolically unhealthy overweight; MHO – metabolically healthy obese; MUHO – metabolically unhealthy obese; PMH- past medical history; WC- waist circumference; FPG- fasting plasma glucose; HOMA-IR- homeostatic model assessment of insulin resistance; Rx – treatment; SBP- systolic blood pressure; DBP- diastolic blood pressure; TG- triglycerides; HDL-C–high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; HBA1c- haemoglobin A1c; ALP – alkaline phosphatase; GGT- Gamma glutamyl transferase; ALT- alanine transaminase; TSH – thyroxine stimulating hormone; FT4- free thyroxine; NS – not significant.

Categorical variables are compared using the Chi-square test, and continuous variables are compared using the independent-samples Mann-Whitney U test. A p-value of <0.05 is considered significant.



Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; individual data points are plotted as blue circles. A statistically significant difference in these four parameters across sexes was observed (Mann-Whitney U-test).

Figure 3.3: Box and whiskers plot showing distribution of (A) BMI, (B) LDL-C, (C) HOMA-IR, and (D) VAI stratified by sex

Within the MHNW group, males had a higher BMI, neck, hip, and arm circumference, as well as WHR, WThR, WHtR, AVI, CI, BRI, and ABSI but similar WI and thigh circumference to female participants . Furthermore, although being categorised as metabolically healthy, male participants had a less favourable cardiometabolic profile compared to their female counterparts. They, in fact had higher values for FPG, LDL-C, TG, HOMA-IR, LAP, and a lower HDL-C **(Table 3.10).** Due to the small number of participants within the MUHNW category it wasn't possible to derive any meaningful statistical comparisons in sex differences within this category (data not shown).

When considering the MHOW/O group, males exhibited higher neck and arm circumferences as well as higher WHR, WThR, AVI, CI, and ABSI; however, they had a lower thigh circumference and waist index compared to their female counterparts. Like the MHNW group, males had an overall worse cardiometabolic profile than females, even though they were categorised as being metabolically healthy. In fact, they had higher values for FPG, HBA1c, LDL-C, TG, HOMA-IR, LAP, and a lower HDL-C. **Tables 3.11** and **3.12** compare the clinical and biochemical characteristics in males and females in the MHOW/O and MUHOW/O categories respectively.

Subsequently, a generalised linear model was constructed to evaluate the clinical determinants of HOMA-IR. Within the male cohort, BMI was the only significant predictor of HOMA-IR (β=0.092, 95% CI 0.046-0.119, $p<0.01$). In females both the BMI (β=0.047, 95% CI 0.362-0.062, p=0.016) and WHR (β=1.91, 95% CI 0.362-3.45, p=0.016) were identified as significant determinants of HOMA-IR. Effect size estimates of BMI and WHR under different models are presented in **Table 3.13**.

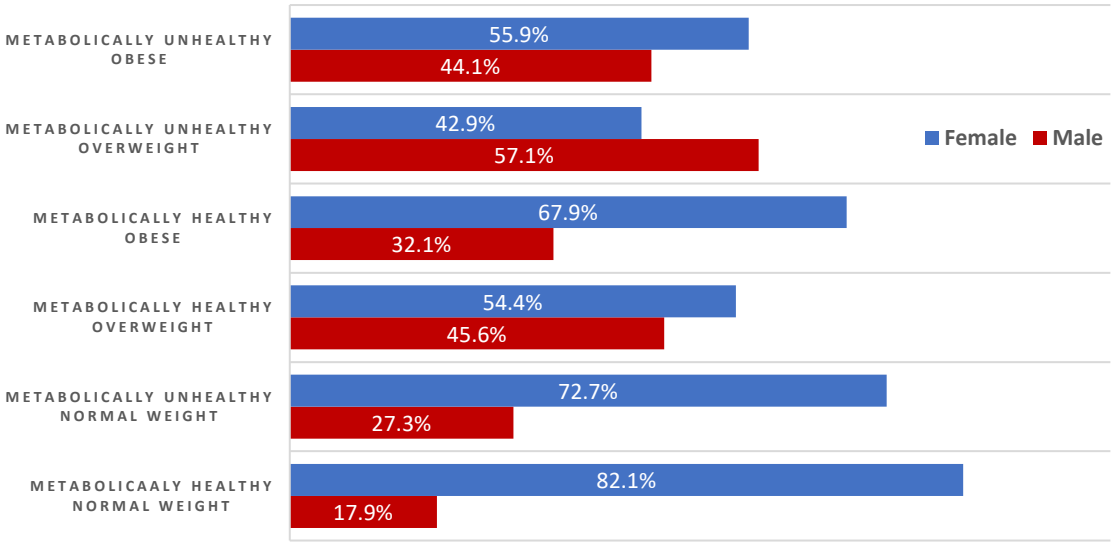


Figure 3.4: Percentage of males and females within each of the six body composition phenotypes

Table 3.10: Sex differences in demographic, anthropometric, and biochemical parameters and indices of obesity measurement in the MHNW* phenotype

	Male n = 26 (17.9%)	Female n = 119 (82.1%)	p-value
Demographic parameters			
Age (IQR)	41.5 (8.0)	40 (6.0)	NS
% Alcohol drinkers	69.2	50.4	0.015
% Smokers	26.9	23.5	NS
% Physical activity	42.3	52.1	NS
% White collar occupation	53.0	75.6	0.045
% PMH	0	16.0	0.018
% Tertiary education	50.0	58.8	NS
Metabolic syndrome components			
% WC >102cm (M) or >88cm (F)	0	2.5	NS
% FPG ≥ 5.6mmol/l	7.7	4.2	NS
% HOMA-IR ≥ 2.5	0	4.2	NS
% SBP ≥ 130mmHg or DBP ≥85mmHg or Rx	26.9	31.1	NS
% TG ≥ 1.7 mmol/l or Rx	0	0.8	NS
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or Rx	3.8	10.9	NS
Anthropometric parameters			
Body Mass Index (kg/m^2)	23.7 (2.0)	22.2 (2.5)	0.01
Waist circumference (cm)	83 (7.0)	71.5 (7.5)	<0.001
Hip circumference (cm)	94 (6.0)	89 (7.0)	<0.001
Neck circumference (cm)	36 (2.0)	31 (3.0)	<0.001
Mean arm circumference (cm)	28 (1.5)	25.5 (3.0)	<0.001
Mean thigh circumference (cm)	48 (5.0)	49 (4.0)	NS
SBP (mmHg)	120.0 (10)	115 (15.0)	NS
DBP (mmHg)	80.0 (0)	80.0 (10.0)	NS
Biochemical parameters			
Total cholesterol (mmol/l)	4.96 (0.64)	4.48 (1.21)	0.025
LDL-C (mmol/l)	2.97 (0.57)	2.44 (0.99)	0.002
HDL-C (mmol/l)	1.48 (0.36)	1.72 (0.51)	0.011
Triglycerides (mmol/l)	0.89 (0.43)	0.73 (0.33)	0.02
Uric Acid (µmol/l)	300 (74)	230 (83)	<0.001
FPG (mmol/l)	5.03 (0.37)	4.92 (0.55)	0.044
HbA1c (%)	5.2 (0.4)	5.1 (0.3)	NS
HOMA-IR	1.1 (0.69)	1.18 (0.91)	NS
Vitamin D (ng/L)	21.5 (11)	19 (9.0)	NS
ALP (U/l)	64 (13.0))	52 (20.0)	0.003
GGT (U/l)	21.5 (7.0)	13 (7.0)	<0.001
ALT (U/l)	19 (9.0)	13 (7.0)	0.001
Ferritin (ng/ml)	134 (138)	25 (30)	<0.001
TSH (micIU/l)	1.47 (1.03)	1.49 (1.22)	NS

Table 3.10: (Continued)

fT4 (pmol/L)	15.77 (2.9)	14.55 (2.11)	0.005
Birth weight (Kg)	4.0 (0.0)	3.1 (0.75)	NS
Lipid Accumulation Product	16.22 (8.53)	10.4 (8.19)	0.001
log (TG/HDL-C)	-0.26 (0.27)	-0.37 (0.24)	0.004
Platelet-lymphocyte ratio	141.2 (48.0)	145.4 (55.4)	NS
Neutrophil-lymphocyte ratio	2.02 (0.66)	2.09 (1.11)	NS
Indices of obesity measurement			
Visceral adiposity index	0.74 (0.54)	0.74 (0.44)	0.734
Waist Hip Ratio	0.9 (0.06)	0.81 (0.08)	<0.001
Waist Index	0.88 (0.07)	0.88 (0.1)	0.345
Waist Height Ratio	0.49 (0.03)	0.45 (0.05)	<0.001
Waist Thigh Ratio	1.7 (0.26)	1.48 (0.19)	<0.001
Body Adiposity Index	22.81 (2.06)	25.98 (4.06)	<0.001
Conicity Index	1.21 (0.07)	1.11 (0.11)	<0.001
Abdominal Volume Index	14.09 (2.34)	10.82 (2.17)	<0.001
Body Roundness Index	3.09 (0.57)	2.42 (0.83)	<0.001
'A' Body Shape Index	0.135 (0.014)	0.117 (0.014)	<0.001

Data are expressed as percentages or median +IQR.

*MHNW individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglyceride level ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

MHNW- metabolically healthy normal weight; PMH- past medical history; WC- waist circumference; FPG- fasting plasma glucose; SBP- systolic blood pressure; DBP- diastolic blood pressure; Rx- treatment; TG- triglycerides; HDL-C- high density lipoprotein cholesterol; LDL-C- low density lipoprotein cholesterol; HOMA-IR- homeostatic model assessment of insulin resistance; HBA_{1c}- haemoglobin A1c; ALP – alkaline phosphatase; GGT- Gamma glutamyl transferase; ALT- alanine transaminase; TSH- thyroxine stimulating hormone; FT4- free thyroxine; NS- not significant

Table 3.11: Sex difference in demographic, anthropometric, and biochemical parameters and indices of obesity measurement in the MHOW/O* phenotype

	Male n = 85 (41.7%)	Female n = 119 (58.3%)	p-value
Demographic parameters			
Age (IQR)	41 (6)	40 (6)	NS
% Alcohol drinkers	70	40.3	<0.01
% Smokers	19.8	16.0	NS
% regular physical activity	57.0	37.0	0.003
% White collar occupation	65.1	68.9	NS
% PMH	9.3	13.4	NS
% Tertiary education	53.5	53.8	NS
Metabolic syndrome components			
%WC >102cm (M) or >88cm (F)	14.0	37.8	<0.01
% FPG ≥ 5.6mmol/l	12.8	3.4	0.011
% HOMA-IR ≥ 2.5	7.1	6.2	NS
% SBP ≥ 130mmHg or DBP ≥85mmHg or on Rx	32.6	38.7	NS
% TG ≥ 1.7 mmol/l or on statins	75.0	25.0	0.006
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or on Rx	42.0	58.0	NS
Anthropometric parameters			
Body Mass Index (kg/m²)	27.5 (3.0)	28.2 (4.9)	NS
Waist circumference (cm)	92 (11.0)	85.0 (14.0)	<0.001
Hip circumference (cm)	99.0 (8.0)	102.0 (13.5)	NS
Neck circumference (cm)	38.0 (3.0)	33.0 (3.5)	<0.001
Mean Arm circumference (cm)	30.5 (4.0)	29.5 (3.0)	.002
Mean thigh circumference (cm)	51.0 (6.5)	55.8 (7.0)	<0.001
SBP (mmHg)	120 (10)	120 (10)	NS
DBP (mmHg)	80 (5)	80 (10)	NS
Biochemical parameters			
Total cholesterol (mmol/l)	4.91 (1.3)	4.77 (0.88)	NS
LDL-C (mmol/l)	3.05 (1.01)	2.71 (0.95)	.016
HDL-C (mmol/l)	1.32 (0.34)	1.6 (0.49)	<0.001
Triglycerides (mmol/l)	1.09 (0.58)	0.85 (0.44)	<0.001
Uric Acid (µmol/l)	322 (74)	246 (72)	<0.001
Fasting plasma glucose (mmol/l)	5.16 (0.58)	5.02 (0.38)	0.019
HbA1c (%)	5.3 (0.5)	5.2 (0.3)	<0.001
HOMA-IR	1.63 (0.95)	1.61 (0.84)	NS
Vitamin D (ng/L)	18 (11)	18 (8)	NS
ALP (U/l)	65.5 (20.0)	61 (21)	NS
GGT (U/l)	27 (22)	14 (9)	<0.001
ALT (U/l)	26.0 (15.0)	14	<0.001
Ferritin (ng/ml)	134.5 (126.0)	27	<0.001
TSH (micIU/l)	1.48 (0.91)	1.48 (0.91)	NS
fT4 (pmol/L)	15.23 (2.07)	14.38 (2.32)	0.002
Birth weight (Kg)	3.2 (1.1)	3.1 (1.1)	NS

Table 3.11: (Continued)

Lipid Accumulation Product	**30.6 (22.2)**	**23.0 (18.7)**	**0.002**
log (TG/HDL-C)	-0.09 (0.28)	-0.3 (0.24)	<0.001
Platelet-lymphocyte ratio	121.8 (44.0)	147.0 (64.1)	<0.001
Neutrophil-lymphocyte ratio	1.91 (0.76)	1.98 (0.92)	NS
Indices of obesity measurement			
Visceral adiposity index	1.04 (0.73)	0.90 (0.52)	NS
Waist Hip Ratio	0.92 (0.07)	0.83 (0.1)	<0.001
Waist Index	1.0 (0.13)	1.06 (0.16)	<0.001
Waist Height Ratio	0.52 (0.07)	0.53 (0.08)	NS
Waist Thigh Ratio	1.79 (0.23)	1.6 (0.31)	<0.001
Body Adiposity Index	25.2 (3.5)	32.3 (7.21)	<0.001
Conicity Index	1.22 (0.09)	1.15 (0.16)	<0.001
Abdominal Volume Index	17.0 (4.1)	15.0 (4.34)	<0.001
Body Roundness Index	3.84 (1.37)	3.84 (1.65)	NS
'A' Body Shaped Index	0.134 (0.012)	0.114 (0.014)	<0.001

Data are expressed as percentages or median +IQR.

*MHOW/O individuals having ≤1 NCEP ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglyceride level ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

PMH- past medical history; WC- waist circumference; FPG- fasting plasma glucose; HOMA-IR- homeostatic model assessment of insulin resistance; SBP- systolic blood pressure; DBP- diastolic blood pressure; Rx – treatment; TG- triglycerides; HDL-C– high density lipoprotein cholesterol; LDL-C– low density lipoprotein cholesterol; HBA1c- haemoglobin A1c; ALP- alkaline phosphatase; GGT- Gamma glutamyl transferase; ALT- alanine transaminase; TSH- thyroxine stimulating hormone; FT4- free thyroxine; NS- not significant;

Categorical variables are compared using the Chi-square test, and continuous variables are compared using the independent-samples Mann-Whitney U test. A p-value of <0.05 is considered significant.

Table 3.12: Sex difference in demographic, anthropometric, and biochemical parameters, and indices of obesity measurement in the MUHOW/O* phenotype

	Male n = 76 (47.5%)	Female n = 84 (52.5%)	p-value
Demographic parameters			
Age (IQR)	42 (5)	41.5 (7)	NS
% Alcohol drinkers	53.9	22.6	<0.01
% Smokers	32.9	21.4	0.027
% Physical activity	42.1	25.0	0.017
% White collar occupation	50.0	69.0	0.001
% PMH	44.7	35.7	NS
% Tertiary education	30.3	53.6	0.012
Metabolic syndrome components			
%WC >102cm (M) or >88cm (F)	61.8	94.0	<0.01
% FPG ≥ 5.6mmol/l	63.2	46.4	0.025
%HOMA-IR ≥ 2.5	49.3	42.2	NS
% SBP ≥ 130mmHg or DBP ≥85mmHg or on Rx	69.7	46.4	0.002
% TG ≥ 1.7 mmol/l or on Rx	71.1	33.3	<0.01
% HDL-C ≤1.29mmol/l (F) or ≤1.02mmol/l (M) or on Rx	57.9	67.9	NS
Anthropometric parameters			
Body Mass Index (kg/m²)	31.9 (5.5)	34.2 (8.4)	0.003
Waist circumference (cm)	106 (14.5)	100.0 (15.5)	0.012
Hip circumference (cm)	106.3 (13.0)	113.5 (19)	0.001
Neck circumference (cm)	41.0 (3.75)	36.0 (3.5)	<0.001
Mean Arm circumference (cm)	33.0 (3.0)	33.0 (5.5)	NS
Mean thigh circumference (cm)	53.5 (5.6)	58.0 (8.0)	<0.001
SBP (mmHg)	120 (14.5)	124.5 (13)	NS
DBP (mmHg)	85 (10)	80 (5)	0.025
Biochemical parameters			
Total cholesterol (mmol/l)	5.12 (1.4)	4.89 (1.1)	NS
LDL-C (mmol/l)	3.16 (1.23)	3.13 (1.01)	NS
HDL-C (mmol/l)	1.06 (0.31)	1.25 (0.29)	<0.001
Triglycerides (mmol/l)	1.91 (1.14)	1.43 (0.71)	<0.001
Uric Acid (µmol/l)	349 (84)	273 75)	<0.001
Fasting blood glucose (mmol/l)	5.75 (1.07)	5.52 (0.92)	0.016
HbA1c (%)	5.7 (0.7)	5.4 (0.5)	0.001
HOMA-IR	2.47 (1.36)	2.27 (1.3)	NS
Vitamin D (ng/L)	17 (5.5)	17.5 (7)	NS
ALP (U/l)	67.5 (22.5)	70.5 (17)	NS
GGT (U/l)	33.5 (28.5)	21 (12.5)	<0.001
ALT (U/l)	27 (22)	19 (10)	<0.001
Ferritin (ng/ml)	178 (151.5)	29.5 (60)	<0.001
TSH (micIU/l)	1.46 (0.61)	1.6 (1.42)	NS

Table 3.12: (Continued)

fT4 (pmol/l)	14.94 (2.53)	14.25 (2.45)	NS
Birth weight (Kg)	3.0 (0.8)	3.55 (0.72)	NS
Lipid Accumulation Product	70.6 (42.62)	57.74 (48.73)	0.014
log (TG/HDL-C)	0.24 (0.37)	0.07 (0.25)	<0.001
Platelet-lymphocyte ratio	110.9 (47.42)	140.99 (67.19)	<0.001
Neutrophil-lymphocyte ratio	1.9 (0.82)	2.22 (0.91)	<0.001
Indices of obesity measurement			
Visceral adiposity index	2.23 (1.96)	2.07 (1.2)	NS
Waist Hip Ratio	0.98 (0.09)	0.88 (0.08)	<0.001
Waist Index	1.1 (0.14)	1.21 (0.2)	<0.001
Waist Height Ratio	0.6 (0.08)	0.63 (0.08)	0.026
Waist Thigh Ratio	1.97 (0.25)	1.71 (0.22)	<0.001
Body Adiposity Index	27.86 (5.77)	37.25 (10.38)	<0.001
Conicity Index	1.28 (0.08)	1.24 (0.09)	<0.001
Abdominal Volume Index	22.47 (6.01)	20.4 (6.68)	0.05
Body Roundness Index	5.44 (1.85)	6.17 (2.0)	0.025
'A' Body Shaped Index	0.137 (0.012)	0.12 (0.012)	<0.001

Data are expressed as percentages or median +IQR.

*MUHOW/O individuals having ≥2 NCEP ATPIII criteria from the following: consisting of waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglyceride level ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and <1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

PMH- past medical history; WC- waist circumference; FPG- fasting plasma glucose; HOMA-IR- homeostatic model assessment of insulin resistance; SBP- systolic blood pressure; DBP- diastolic blood pressure; Rx - treatment TG- triglycerides; HDL-C– high density lipoprotein cholesterol; LDL-C– low density lipoprotein cholesterol; HBA1c- haemoglobin A1c; ALP- alkaline phosphatase; GGT- Gamma glutamyl transferase; ALT- alanine transaminase; TSH- thyroxine stimulating hormone; FT4- free thyroxine; NS- not significant;

Categorical variables are compared using the Chi-square test, and continuous variables are compared using the independent-samples Mann-Whitney U test. A p-value of <0.05 is considered significant.

Table 3.13 Clinical determinants of HOMA-IR stratified by sex

	Males	Females		
	Model 1	Model 1	Model 2	Model 3
Variable	β (95% CI), p value	β1 (95% CI), p value	β2 (95% CI), p value	β3 (95% CI), p value
Age	0.007 (-0.032 - 0.046), p = 0.74	0.008 (-2.988-0.018), p = 0.06		
BMI	**0.082 (0.046 - 0.119), p < 0.01**	**0.047 (0.032-0.061), p < 0.01**	**0.048 (0.033-0.062), p < 0.01**	
Neck circumference	-0.016 (-0.04 - 0.008), p = 0.202	-0.003 (-0.017 - 0.012), p = 0.702		
Waist: Hip Ratio	0.299 (-2.33 - 2.99), p = 0.824	**1.91 (0.362 - 3.459), p = 0.016**		**2.18 (0.547-3.82), p= 0.009**
Physical activity	0.058 (-0.245- 0.362) p = 0.708	0.077 (-0.127 - 0.281), p = 0.458		
Smoking	-0.197 (-0.514 - 0.121) p = 1.47	0.013 (-0.203 - 0.23), p = 0.903		
Alcohol consumption	-0.216 (-0.515 - 0.118) p = 0.206	-0.109 (-0.315 - 0.097), p = 0.299		

Generalized linear modelling specifying gamma as the distribution and log as the link function was applied to identify significant predictors of HOMA-IR separately in males and females. Simple bedside clinical parameters were included as scale (age, waist: hip ratio, BMI and neck circumference) or categorical independent variables (smoking, physical activity, alcohol consumption). In males, BMI emerged as the only significant predictor of HOMA-IR, whereas in females, both BMI and WHR exceeded significance thresholds.

*θ*1 total effect of BMI and WHR on HOMA-IR in females, *θ*2 total effect of BMI on HOMA-IR in females, *θ*3 total effect of WHR on HOMA-IR in females

BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance

3-3 Prevalence of the body composition phenotypes according to several different and commonly used definitions of MH and their association with insulin resistance

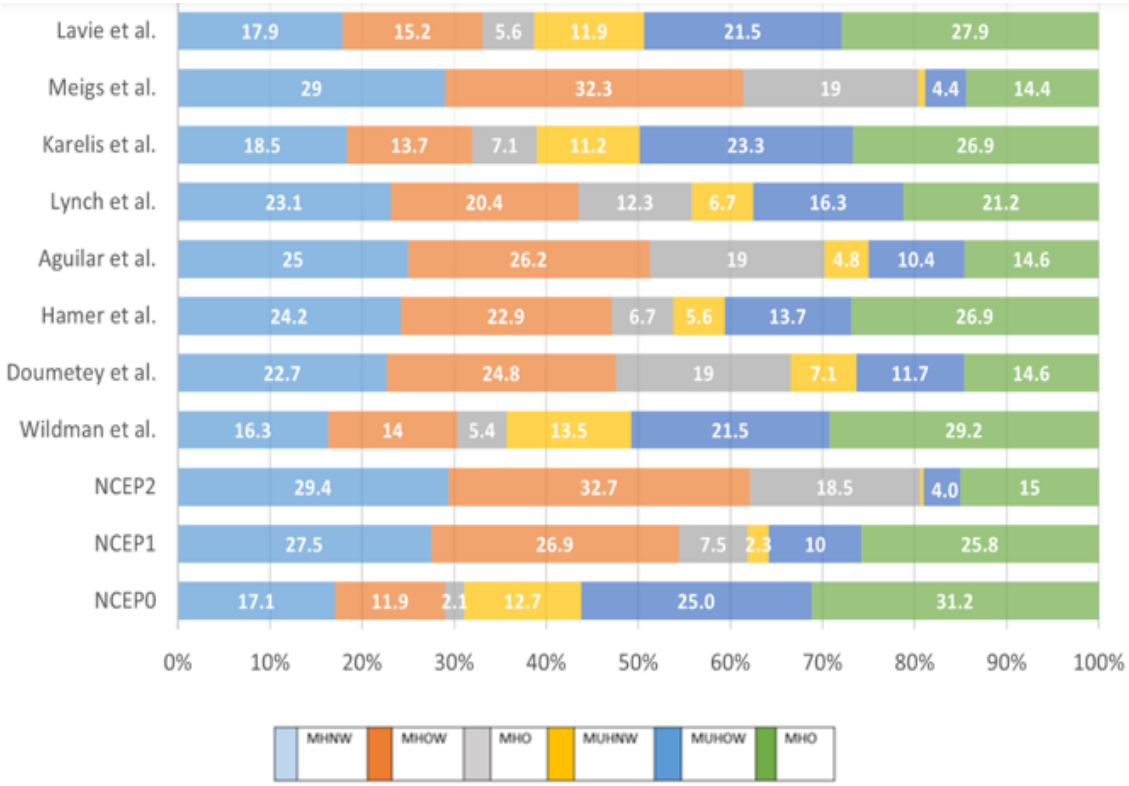
To date there is no universally accepted definition for MH, and thus different authors have proposed several definitions each varying in terms of choice of parameters, their respective cut-offs as well as in the number of abnormal parameters needed to characterize a subject as being metabolically unhealthy. Therefore, the aim of this study was to compare the prevalence of the different body composition phenotypes in the studied population according to several commonly used definitions of MH. The definitions included were those proposed by Wildman *et al.,* Doumatey *et al.,* Meigs *et al.,* Hamer *et al.,* Aguilar-Salinas *et al.,* Lynch *et al*., Karelis *et al.,* and the harmonisation criteria by Lavie *et al*. Additionally the NCEP ATPIII criteria for the Met S was also included. With respect to NCEP ATPIII, participants were classified as metabolically healthy when they met none of the criteria (NCEP0), if they exhibited a maximum of one abnormal parameter (NCEP1), or if they had a maximum of two abnormal criteria (NCEP2). Thereafter, sex differences in the relationship of the different body composition phenotypes to MH and IR was also investigated and as a final analysis, logistic regression analysis was used to assess which of the above definition of MH was the strongest predictor of IR and how this varied between the sexes. A description of the criteria for each of the above-mentioned definitions is found in **chapter 2, Table 2-2**.

Complete data was available for 520 individuals of the sample population and thus 520 participants were included in this analysis of whom 36% were males. The prevalence of the various body composition phenotypes according to the different definitions is shown

in **Figure 3.5.** The prevalence of the metabolically healthy normal weight (MHNW) ranged from 16.3 to 29.4%; metabolically healthy overweight (MHOW) from 11.9 to 32.7%; metabolically healthy obese (MHO) from 2.1 to 19.0%; metabolically unhealthy normal weight (MUHNW) from 0.6 to 13.5%; metabolically unhealthy overweight (MUHOW) from 4.0 to 25.0% and metabolically unhealthy obese (MUHO) from 14.4 to 31.2%.

Thereafter each of the definitions used to categorise the metabolically healthy and unhealthy individuals was compared to HOMA-IR. In males, the metabolically unhealthy phenotype was associated with higher median HOMA-IR values for all definitions used ($P<0.001$ for all definitions) as shown in **Table 3.14**. Within the female sex, a metabolically unhealthy phenotype was also associated with a higher median HOMA-IR value for all definitions, except for the Doumatey *et al.* criteria **(Table 3.14**). On applying logistic regression, the metabolically unhealthy phenotype was consistently associated with the presence of IR (defined as a HOMA-IR ≥2.5) across all definitions and in both sexes. However, there were notable sex differences in the performance of the various definitions of the metabolically unhealthy phenotype in the ability to predict IR, as evidenced by the odds ratios shown in **Table 3.15** and **Figure 3.6.** In females the strongest observed association was for the NCEP-2 definition (i.e., having ≤2 abnormal NCEP-ATPIII parameters to characterize the metabolically healthy phenotype), as evidenced by an odds ratio (OR) of 19.7. On the other hand, the Doumatey *et al*. criteria had lowest predictive ability in the female cohort (OR of 2.6). Within the male sex, the strongest association was for the Aguilar-Salinas *et al*. definition for the metabolically healthy phenotype as evidenced by an OR of 18.7, followed by the Lynch *et al.* definition

(OR of 16.6) and the NCEP-2 (OR 13.1) definitions (**Table 3.15 and Figure 3.6).** Furthermore, the Doumatey *et al.* definition performed better in males than in females (OR of 12.2 vs 2.6 in males and females respectively). When considering BMI category as the sole independent predictor of HOMA-IR ≥2.5, a lower predictive performance relative to a metabolically unhealthy phenotype (using any definition) was observed, with an OR of 1.90 in females and 2.07 in males ($p<0.001$). Additionally, even after adjusting for BMI category, the metabolically unhealthy phenotype was associated with a higher prevalence of having a HOMA-IR ≥2.5 for all definitions used and in both sexes **(Table 3.15 and Figure 3.6**). After adjusting for BMI category, the metabolically unhealthy phenotype as defined by the NCEP2 criteria retained the strongest association with IR in females (adjusted OR of 16.1), whilst in males, a metabolically unhealthy phenotype as defined by the Aguilar-Salinas *et al*. criteria was again observed to have the strongest association with IR (adjusted OR 15.3).



NCEP, National Cholesterol Education Program, Adult Treatment Panel III; NCEP0, zero parameters of the NCEP ATPIII for the diagnosis of metabolic health; NCEP 1: presence of ≤ 1 parameters of the NCEP ATPIII for the diagnosis of metabolic health; NCEP2; presence of ≤2 parameters of the NCEP ATPIII criteria for the diagnosis of metabolic health; MHNW, metabolically healthy normal weight; MHOW, metabolically healthy overweight; MHO, metabolically healthy obese; MUHNW, metabolically unhealthy normal weight; MUHOW, metabolically unhealthy overweight; MUHO, metabolically unhealthy obese.

Figure 3.5: Prevalence (%) of the six body compositon phenotypes according to the different definitions of MH

Table 3.14: Comparison of HOMA-IR values for the metabolically healthy and unhealthy subgroups as classified by the different definitions and stratified by sex

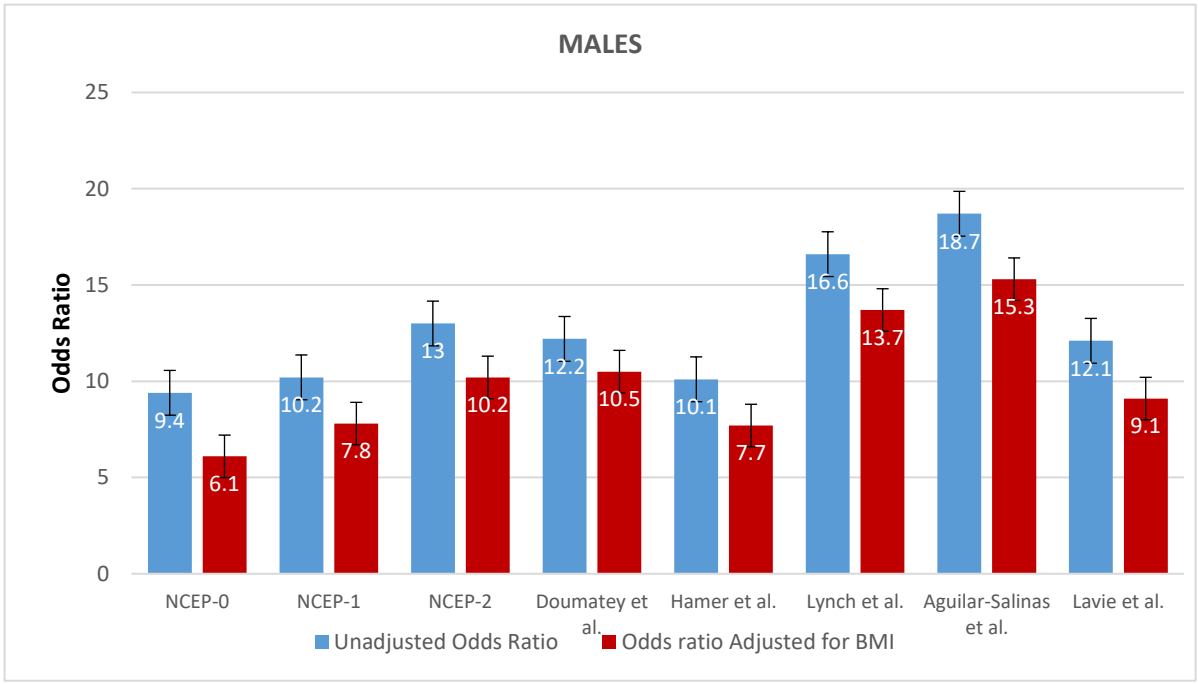
	Metabolically healthy	Metabolically unhealthy	
	Males HOMA-IR median (IQR)		
NCEP0	1.32 (0.89-1.64)	2.12 (1.49-2.71)	<0.001
NCEP1	1.48 (1.08-1.95)	2.31 (1.90-3.01)	<0.001
NCEP2	1.61 (1.13-2.19)	2.89 (2.19-3.45)	<0.001
Doumatey *et al.*	1.52 (1.07-1.95)	2.35 (1.95-3.02)	<0.001
Hamer *et al.*	1.51 (1.07-1.95)	2.28 (1.65-2.96)	<0.001
Aguilar-Salinas *et al.*	1.53 (1.08-1.97)	2.51 (1.96-3.08)	<0.001
Karelis *et al.*	1.27 (0.84-1.65)	2.13 (1.53-2.73)	<0.001
Lavie *et al.*	1.29 (0.83-1.66)	2.13 (1.57-2.80)	<0.001
	Females HOMA-IR median (IQR)		
NCEP0	1.24 (0.78-1.79)	1.71 (1.09-2.27)	<0.001
NCEP1	1.31 (0.89-1.84)	2.13 (1.51-2.77)	<0.001
NCEP2	1.45 (0.95-1.96)	2.72 (2.18-3.19)	<0.001
Doumatey *et al.*	1.52 (0.98-2.07)	1.73 (1.02-2.47)	0.115
Hamer *et al.*	1.36 (0.91-1.85)	1.96 (1.30-2.54)	<0.001
Aguilar-Salinas *et al.*	1.51 (0.96-2.05)	1.88 (1.11-2.66)	0.039
Karelis *et al.*	1.28 (0.82-1.71)	1.97 (1.27-2.55)	<0.001
Lavie *et al.*	1.29 (0.89-1.82)	1.83 (1.20-2.42)	<0.001

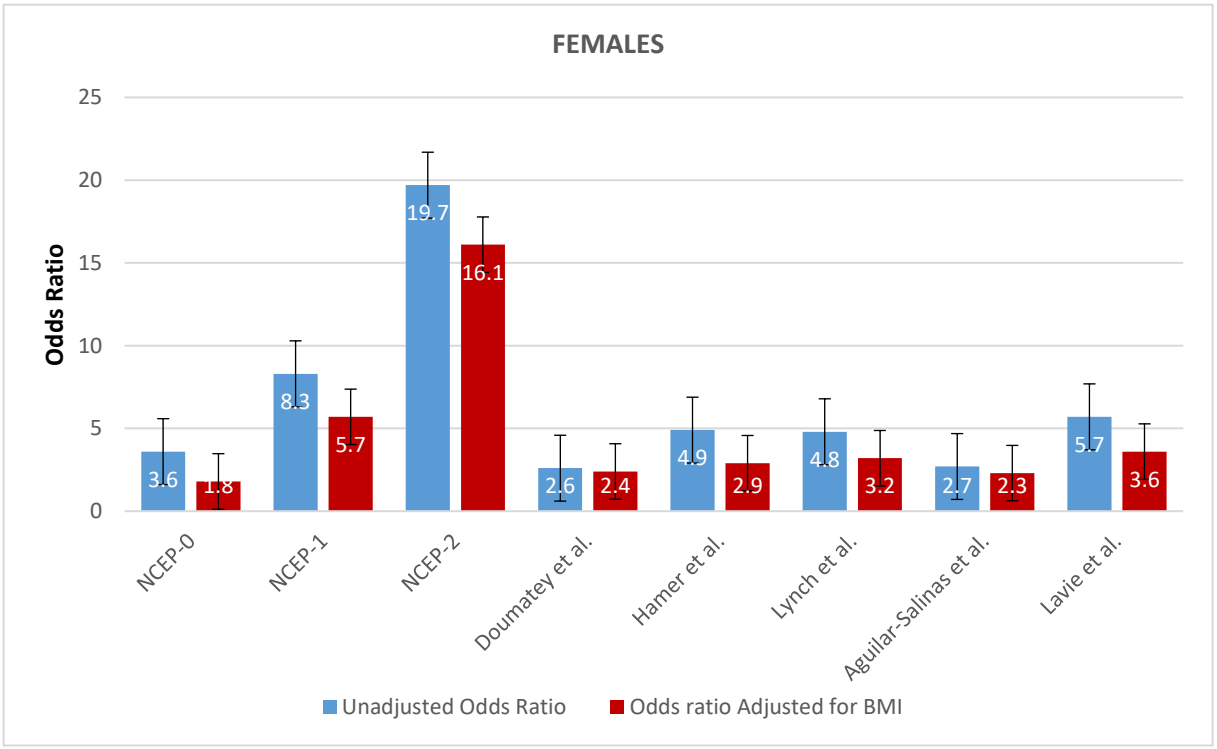
Data are expressed as median (+interquartile range)
HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; NCEP, National Cholesterol Education Program Adult Treatment Panel III
NCEP0: presence of zero criteria of the metabolic syndrome; NCEP1: presence of ≤ 1 criteria of the metabolic syndrome; NCEP2; presence of ≤2 criteria of the metabolic syndrome.

Table 3.15: Performance of the different definitions of MH in predicting insulin resistance defined as HOMA-IR ≥2.5

	Crude Odds Ratio (p value)	Odds Ratio (p value) adjusted for BMI
Males		
NCEP0	9.4 (6.1-14.3. p=0.03)	6.1 (5.5- 13.3 p=0.017)
NCEP1	10.2 (8.6-12.3 p<0.001)	7.8 (7.3-12.6 p<0.001)
NCEP2	13.1 (12.5 – 13.6 p<0.001)	10.2 (5.5- 12.8 p<0.001)
Doumatey *et al.*	12.2 (10.3 – 14.6 p<0.001)	10.5 (8.3 – 12.9 p<0.001)
Hamer *et al.*	10.1 (8.6 –15.5 p<0.001)	7.7 (6.5 – 8.3 p<0.001)
Lynch *et al.*	16.6 (9.6 – 21.3 p<0.001)	13.7 (12.6 -16.7 p<0.001)
Aguilar-Salinas *et al.*	18.7 (12.3 – 21.9 p<0.001)	15.3 (12.6 – 18.3 p<0.001)
Lavie *et al.*	12.1 (10.6 – 15.6 p= 0.001)	9.1 (8.6 –15.5 p=0.003)
Females		
NCEP0	3.6 (2.5- 5.6 p=0.002)	1.8 (0.6 – 3.5 p=0.22)
NCEP1	8.3 (7.6 – 12.3 p<0.001)	5.7 (2.5 – 6.6 p<0.001)
NCEP2	19.7(16.6 – 22.3 p<0.001)	16.1 (11.5 – 18.6 p<0.001)
Doumatey *et al.*	2.6 (2.3- 5.36 =0.003)	2.4 (1.5 – 9.8 p=0.008)
Hamer *et al.*	4.9 (2.6 - 6.6 p<0.001)	2.9 (1.3 – 4.5 p=0.008)
Lynch *et al.*	4.8 (2.6 – 6.1 p<0.001)	3.2 (1.6 – 4.9 p=0.001)
Aguilar-Salinas *et al.*	2.7 (2.3 – 7.3 p=0.002)	2.3 (1.1- 3.5 p=0.014)
Lavie *et al.*	5.7 (3.5 – 7.66 p<0.001)	3.6 (1.9 -6.7 p=0.001)

NCEP, National Cholesterol Education Program/Adult Treatment Panel III
NCEP0: presence of zero criteria of the metabolic syndrome; NCEP1: presence of ≤ 1 criteria of the metabolic syndrome; NCEP2; presence of ≤2 criteria of the metabolic syndrome.
HOMA, Homeostatic Model Assessment for Insulin Resistance





Error bars indicate the standard error
HOMA-IR, Homeostatic Model Assessment for Insulin Resistance
NCEP, National Cholesterol Education Program/Adult Treatment Panel

Figure 3.6: Odds ratios of the different definitions of MH in determining HOMA-IR ≥2.5

3-4 Anthropometric and biochemical determinants of IR

Insulin resistance and hyperinsulinemia are both associated with the presence of the unhealthy metabolic phenotype and are both independent predictors of CVD as well as all-cause, cardiovascular and cancer morality (Després et al., 1996; Facchini et al., 2001; Pan et al., 2020; Perseghin et al., 2012; Pyörälä et al., 2000). However, measures of IR may not be readily available in clinical practice. On the other hand, relatively simple and easily available anthropometric and biochemical parameters known to be associated with increased cardiovascular risk may be used as surrogate markers of IR (Gaziano et al., 1997; Jeppesen et al., 2001; Laws and Reaven, 1992; McLaughlin et al., 2003). However, data comparing the discriminatory power of these cardiometabolic parameters is lacking. Besides, the cut-off for each parameter is uncertain, with different bodies citing different cut-off values. Additionally, these cut-offs were developed more than two decades ago; thus secular changes might have contributed to a change in the optimal cut-offs of the various parameters used to predict IR in clinical practice. Given the current epidemic of obesity and the concomitant increase in burden of cardiometabolic disease as well as the limited therapeutic resources available, identifying those individuals who are insulin resistant and therefore at greatest risk for CVD is paramount. Furthermore, as discussed in the previous sections, not all individuals with overweight/obesity tend to be insulin resistant while some normal weight individuals may be hyperinsulinemic as well as insulin resistant and thus at increased cardiometabolic risk. Therefore, early identification of these high-risk individuals with the use of simple and readily available markers of IR would allow for the channelling of timely and successful therapeutic interventions towards those who are most likely to

benefit from them. Thus, the final objective of this section aimed to compare the discriminatory power of the various easily accessible anthropometric and biochemical parameters and indices derived thereof in predicting IR (defined as a HOMA-IR ≥2.5) and to determine their optimal cut-offs using receiver operator characteristics analysis (ROC). ROC analyses were used to compute the area under curve (AUC) to assess the performance of the several cardiometabolic variables in discriminating individuals with IR. Thereafter the highest Youden index (sensitivity + specificity -1) was used to determine the optimal cut-off points. (All analyses were performed using IBM SPSS version 26 and ROC analysis was performed using the easyROC R application, and cut-off values were determined using the OptimalCutpoints R package R v.3.4.2) (Goksuluk et al., 2016; López-Ratón et al., 2014).

521 individuals were included in this analysis. IR was defined as the homeostatic model assessment-insulin resistance (HOMA-IR) ≥2.5. This cut-off was chosen since it has been linked to increased mortality in large population-based studies as stated in previous chapters (Calori et al., 2011; Kuk and Ardern, 2009). In view of the sex differences in the relationship of anthropometric and biochemical parameters with IR observed in **section 3-2** of this chapter, males and females were studied separately.

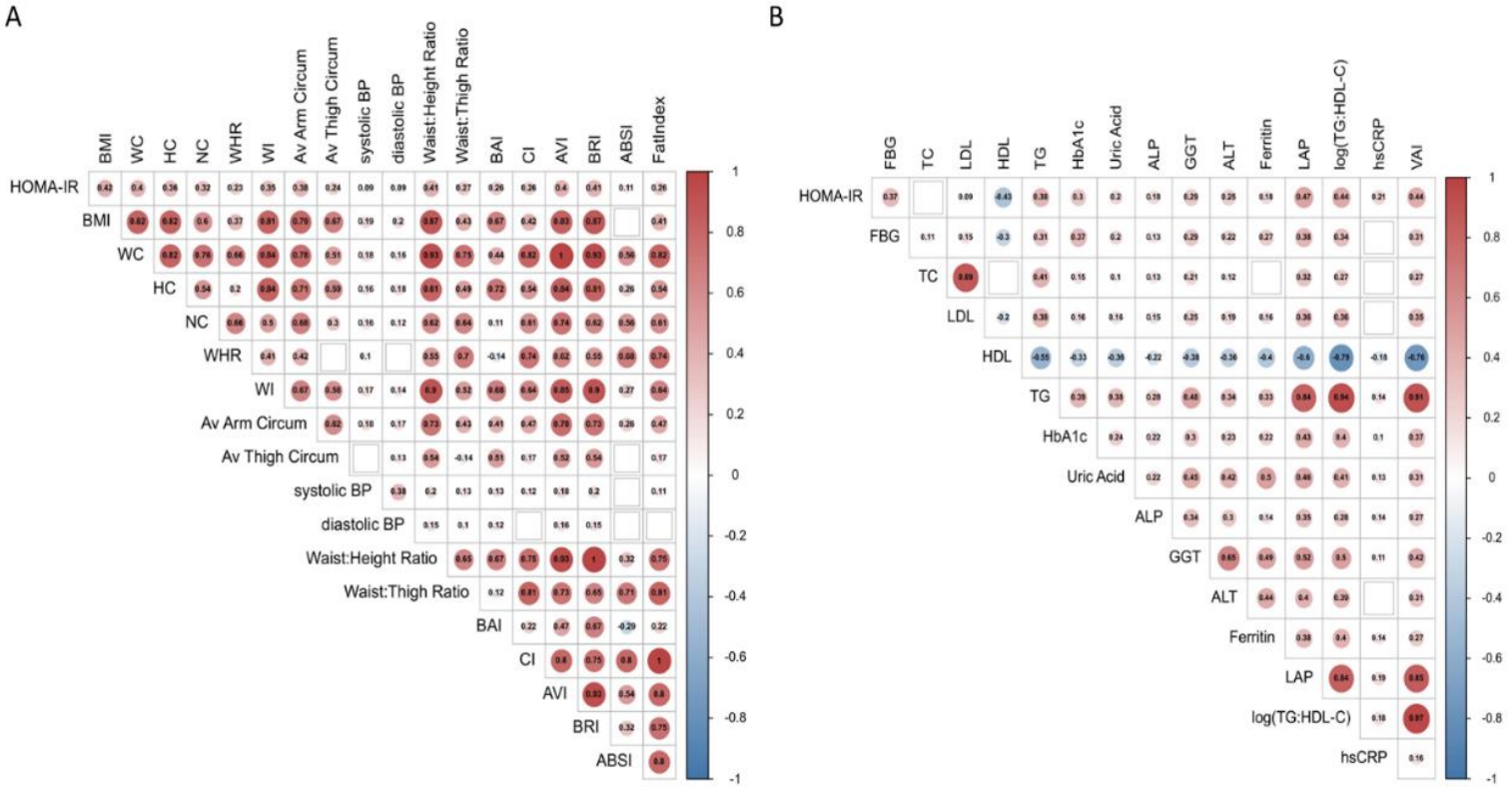
The relationship between HOMA-IR and several anthropometric, clinical, and biochemical indices of adiposity measures were investigated by Spearman's correlation. A correlation matrix of HOMA-IR with quantitative anthropometric and biochemical indices respectively is provided in **figures 3.7A-B.** As expected, significant positive correlations were observed between HOMA-IR and anthropometric or biochemical indices of adiposity measures.

Table 3.16 shows the AUC of the receiver operator characteristics curve. In males the lipid accumulation product was observed to have the best discriminatory power to predict IR as evidenced by an AUC of 0.79. Furthermore, the highest Youden index for LAP corresponded to a value of 42.5 with a sensitivity of 86% and a specificity of 63% (**Figure 3.8(A)).** The visceral adiposity index (VAI), TG/HDL ratio and serum TG also had good discriminatory power (AUC of 78.4%, 78.6% and 75% respectively) **(Table 3.16).** A value of VAI of 1.44 was associated with a sensitivity of 86% and a specificity of 65.8% **(Figure 3.8(B))**, whilst a triglyceride level of 1.35 mmol/L had a sensitivity of 76.2% and a specificity of 63.7%.

On the other hand, in females, the VAI, lipid accumulation product and the TG/HDL ratio had equivalent discriminatory power to detect IR as evidence by an AUC of 82% for VAI and TG/HDL ratio and 81% for LAP **(Table 3.16).** A value of the lipid accumulation product of 36.2 had a sensitivity of 75.5% and a specificity of 80.4% to detect IR, while a value of VAI of 1.41 had 79.6% sensitivity and 77.8% specificity and a TG/HDL ratio of 0.78 was associated with a sensitivity of 77.6% and a specificity of 76.9% **(Figure 3.9 (A&B)).** Of note, serum TG level was also observed to have good discriminatory power (AUC of 78.1%), and a value of 1.33 had a sensitivity of 65.3% and a specificity of 85.9%.

When looking at the discriminatory power of the anthropometric variables, within the female sex the WC emerged as being the best discriminator with an AUC of 76%, followed closely by the body mass index (AUC 74%) **(Table 3.16).** The optimal cut-off for WC to predict IR was 82 cm with a sensitivity of 85.7% and a specificity of 53.3% **(Figure 3.9(C)).** The optimal BMI cut-off for females corresponded to a value of 31.9 kg/m^2 with a sensitivity of 59% and a specificity of 80%.

Within the male sex, both BMI and WC were strongly associated with IR (AUC of 73% and 70% respectively) **(Table 3.16).** The optimal cut-off for WC in predicting IR was 96.5cm with a sensitivity of 72.1% and a specificity of 60.3% **(Figure 3.8(C))**, while the optimal cut-off for BMI corresponded to a value of 29.1 kg/m^2 with a sensitivity of 74.4% and a specificity of 64.4%. Of note, the WHR, BAI, AVI, FI, HDL-C, serum uric acid, liver transaminase and weight adjusted thigh circumference all had poor discriminatory power, whereas ferritin level, systolic and diastolic blood pressure and 'A' body shape index did not even exceed significant thresholds in ROC analysis.



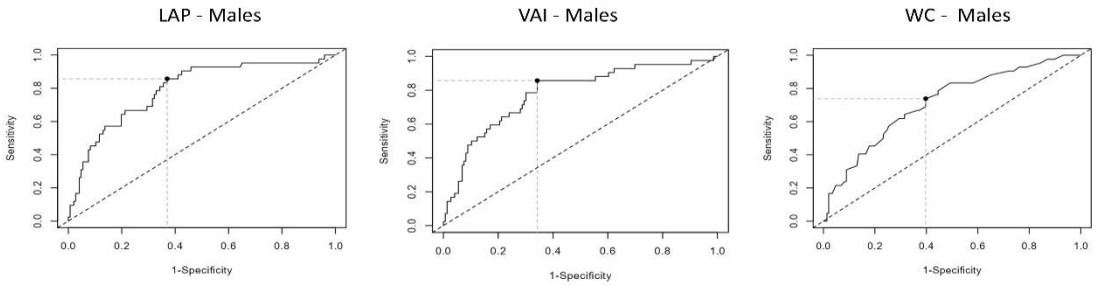
Colour depicts Spearman's rank order correlation coefficient; circle size and colour intensity indicate the magnitude of the correlation coefficient. Significant corelation coefficients are labelled, empty cells represent insignificant correlation between indices

Figure 3.7: Correlation matrix between HOMA-IR and anthropometric/clinical indices of adiposity (A) and biochemical parameters (B)

Table 3.16: Comparison of the Area under the ROC Curves for all anthropometric and biochemical determinants of IR,* stratified by sex

	Males (n=129)				Females (n=328)			
Parameter	**AUC**	**SE**	**P value**	**95%CI**	**AUC**	**SE**	**P value**	**95% CI**
Lipid Accumulation Product	0.79	0.45	<0.01	0.71-0.87	0.81	0.04	<0.01	0.73-0.89
TG:HDL-C Ratio	0.78	0.04	<0.01	0.70-0.86	0.82	0.04	<0.01	0.74-0.90
Visceral Adiposity Index	0.78	0.04	<0.01	0.7—0.86	0.82	0.04	<0.01	0.73-0.90
Triglycerides (mmol/l)	0.74	0.05	<0.01	0.66-0.83	0.77	0.04	<0.01	0.68-0.86
Body Mass Index (kg/m2)	0.73	0.04	<0.01	0.65-0.81	0.74	0.04	<0.01	0.67-0.82
Waist:Height Ratio	0.72	0.04	<0.01	0.63-0.81	0.76	0.04	<0.01	0.70-0.84
Waist:Thigh Ratio	0.71	0.04	<0.01	0.63-0.81	0.66	0.04	<0.01	0.58-0.75
Wasit circumference (cm)	0.70	0.05	<0.01	0.61-0.79	0.76	0.04	<0.01	0.69-0.84
Abdominal Volume Index	0.70	0.05	<0.01	0.61-0.79	0.76	0.04	<0.01	0.68-0.83
Body Adiposity Index	0.69	0.05	<0.01	0.59-0.77	072	0.04	<0.01	0.65-0.80
Hip circumference (cm)	0.68	0.05	<0.01	0.59-0.77	0.72	0.04	<0.01	0.63-0.79
Neck circumference	0.66	0.05	<0.01	0.57-0.75	0.72	0.04	<0.01	0.63-0.79
Waist:Hip Ratio	0.65	0.05	<0.12	0.56-0.75	0.67	0.04	<0.01	0.59-0.75
Fat index	0.63	0.05	<0.01	0.53-0.71	0.70	0.04	<0.01	0.62-0.77
Mean arm circumference (cm)	0.62	0.05	0.02	0.53-0.71	0.73	0.04	<0.01	0.65-0.81
GGT (U/l)	0.62	0.05	0.02	0.53-0.71	0.69	0.04	<0.01	0.62-0.77
ALP (U/l)	0.57	0.05	0.16	0.47-0.68	0.68	0.04	<0.01	0.60-0.76
Uric Acid (mol/l)	0.57	0.05	0.20	0.47-0.68	0.63	0.04	<0.01	0.54-0.71
ALT (U/l)	0.54	0.05	0.44	0.44-0.64	0.63	0.04	<0.01	0.55-0.72
Mean thigh circumference (cm)	0.52	0.05	0.65	0.42-0.63	0.68	0.04	<0.01	0.59-0.76
Weight-adjusted thigh circumference (cm)	0.26	0.04	<0.01	0.18-0.34	0.33	0.05	<0.01	0.24-0.41
HDL-C (mmol/l)	0.23	0.04	<0.01	0.15-0.31	0.20	0.04	<0.01	0.130.27

*Insulin resistance defined as HOMA-IR ≥2.5
TG, Triglyceride; HDL-C, high density lipoprotein cholesterol; ALP, alkaline phosphatase; GGT, Gamma glutamyl transferase; ALT, alanine transaminase



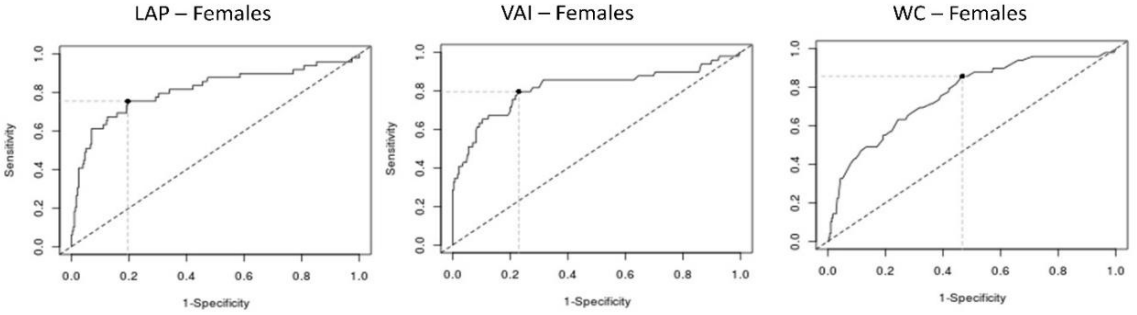
Cut-off Results			
Optimal cut-off point : 42.56			
Optimal criterion : 0.48			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.86	0.72	0.95
Specificity	0.63	0.55	0.71
PPV	0.40	0.32	0.66
NPV	0.94	0.87	0.96
PLR	2.32	1.81	2.96
NLR	0.23	0.11	0.48

Cut-off Results			
Optimal cut-off point : 1.44			
Optimal criterion : 0.51			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.86	0.72	0.95
Specificity	0.66	0.58	0.73
PPV	0.42	0.34	0.68
NPV	0.94	0.87	0.96
PLR	2.50	1.94	3.23
NLR	0.22	0.10	0.46

Cut-off Results			
Optimal cut-off point : 96.5			
Optimal criterion : 0.34			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.74	0.58	0.86
Specificity	0.60	0.52	0.68
PPV	0.35	0.28	0.54
NPV	0.89	0.80	0.92
PLR	1.86	1.42	2.43
NLR	0.44	0.26	0.73

LAP, Lipid accumulation product; VAI, visceral adiposity index; WC, waist circumference; HOMA-IR, homeostatic model for insulin resistance

Figure 3.8: ROC Curves for LAP, VAI and WC in determining a HOMA-IR 2.5 in males



Cut-off Results			
Optimal cut-off point : 36.25			
Optimal criterion : 0.56			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.76	0.61	0.87
Specificity	0.80	0.75	0.85
PPV	0.41	0.34	0.60
NPV	0.95	0.90	0.96
PLR	3.85	2.88	5.14
NLR	0.31	0.19	0.50

A

Cut-off Results			
Optimal cut-off point : 1.4			
Optimal criterion : 0.56			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.80	0.66	0.90
Specificity	0.77	0.72	0.82
PPV	0.39	0.32	0.59
NPV	0.95	0.91	0.97
PLR	3.47	2.67	4.50
NLR	0.27	0.15	0.46

B

Cut-off Results			
Optimal cut-off point : 82			
Optimal criterion : 0.39			
Performance Measures			
	Value	Lower Limit	Upper Limit
Sensitivity	0.86	0.73	0.94
Specificity	0.53	0.47	0.59
PPV	0.25	0.21	0.47
NPV	0.95	0.90	0.96
PLR	1.84	1.55	2.18
NLR	0.27	0.13	0.54

C

LAP, Lipid accumulation product; VAI, visceral adiposity index; WC, waist circumference; HOMA-IR, homeostatic model for insulin resistance

Figure 3.9: ROC Curves for LAP, VAI and WC in determining a HOMA-IR ≥2.5 in females

Chapter 3B – Discussion of epidemiological studies

3-5 Prevalence, characteristics, and determinants of the different body composition phenotypes

Overall, this study shows that within a middle-aged Maltese population there was a high proportion of participants with overweight and obesity, furthermore whilst the prevalence of all six body composition phenotypes was common, this varied based on definition and gender. Interestingly, when adopting the presence of zero cardiometabolic parameters of the NCEP ATP III definition the prevalence of MHO was observed to be lower (3.1%) than that of the MUHNW (6.9%). This could be due to the presence of a normal waist circumference in the MHO cohort. This study also found that younger age, alcohol consumption, physical activity and being a non-smoker to be associated with the metabolically healthy phenotype in participants living with overweight and obesity .

3-5.1 Different prevalence rates when using the NCEP ATPIII or the HOMA-IR definitions of MH

In this cross-sectional analysis more than two-thirds of the participants were found to be within the overweight or obese BMI category and approximately one third were metabolically unhealthy when adopting the presence of at least 1 NCEP ATP III criteria as the cut-off. The high prevalence of the metabolically unhealthy phenotype in a working-age population would be expected to result in an increased future cardiovascular disease burden in the Maltese population. . The prevalence of obesity is in accordance with that reported in the Maltese general population (Cuschieri et al.,

2016a). Here the authors observed that 69.75% (95% CI: 68.32–71.18) of the adult Maltese population suffered from excessive body weight and that the majority of patients living with T2 DM were overweight or obese.

The population prevalence rates for MHO , MHOW , and MUHNW observed in this study are in keeping with two previous Spanish studies which also analysed a well characterized Mediterranean cohort as the study population but used different criteria to define MH: either the presence of Met S as per NCEP ATP III (as per the study of Goday et al.) or having 0-1 cardiometabolic abnormalities including elevated SBP (≥130 mm Hg) and/or elevated DBP ≥85 mm Hg and/or antihypertensive medication use; elevated triglycerides (≥150 mg/dL); low HDL-C (<40 mg/dL in men and <50mg/dL in women and/or lipid lowering medication use); elevated glucose (100mg/dL and/or antidiabetic medication use); insulin resistance (HOMA-IR >4.05, the 90th percentile); and elevated hsCRP (>0.74 mg/dL, the 90th percentile) as per the study of Lopez-Garcia et al., (Goday et al., 2016; Lopez-Garcia et al., 2017).

However, other Mediterranean studies report much lower prevalence rates for MHO: for example the Cremona study by Calori *et al*., observed a population prevalence of healthy obesity among 2,074 individuals from the Lombardia region in Italy to be only 2.1% when using the presence of insulin resistance as defined by HOMA-IR and a cut-off of <2.5 to denote MH (Calori et al., 2011). Conversely, using this definition of MH in the current study resulted in the highest prevalence for MHO (22.8%) . Another cross-sectional study from Spain found a population prevalence of metabolically healthy overweight and obesity to be 16.4% (less than half the 39.3% observed in this study) when MH was defined as the presence of ≤1 cardiometabolic abnormalities of the Met

S according to the harmonized criteria proposed by the IDF and the American Heart Association (Gomez-Huelgas et al., 2013). On the other hand, while the Sicilian study by Buscemi *et al*., found an overall prevalence of overweight and obesity to be similar to that found in this study (71.1%), only 19% out of the total cohort where metabolically healthy overweight or obese when MH was defined as 0-1 conditions from the following: prediabetes/T2D, hypertension, hypertriglyceridemia or low HDL-C, and hypercholesterolemia. Furthermore, they noted an MUHNW prevalence of 9.5% among the normal weight population which is reminiscent to the 7% noted in this study (Buscemi et al., 2017). Such discrepancies in prevalence rates for the healthy overweight/obese phenotypes could be attributed to the differences in the size and age range of the studied populations, the criteria used to define MH, differences in lifestyle and behavioural factors such as variations of adherence to the Mediterranean diet as well as variation in population genetics (da Silva et al., 2009; Moorjani et al., 2011; Vilarnau et al., 2019).

Notably the prevalence of MHO among subjects living with obesity observed in this study (32%) is in keeping with that reported in the literature (which ranged between 9 to 34% in older studies but even up to 75% in a recent systematic review) (Blüher, 2012; Phillips, 2013a; Rey-López et al., 2014). Over the past 10 years or so the prevalence of this unique obesity phenotype has been well studied and documented in different ethnic groups including Caucasian, Asian and African-American populations (Bonora et al., 1998; Cherqaoui et al., 2012; Geetha et al., 2011; Hwang et al., 2012; Pajunen et al., 2011; Shea et al., 2011; Wildman et al., 2008). For example, the study by Wildeman *et al*., (which looked at 5440 American civilians of different ethnicities including non-

Hispanic blacks, non-Hispanic whites and Mexican Americans) observed a higher prevalence of MHO within non-Hispanic black participants and that after adjusting for several other confounding factors (including age, sex, smoking status and physical activity) people of this ethnicity were more likely to express the healthy phenotype (Wildman et al., 2008). In the Canadian study by Brochu et al., which looked at 43 postmenopausal females with obesity (determined by the percentage of body fat rather than BMI), the authors identified 17 (39.5%) individuals who had high levels of insulin sensitivity (as assessed by the hyperinsulinemic- euglycemic clamp technique) despite having nearly half of their weight as body fat (Brochu et al., 2001). An African American cohort study of 126 predominantly female individuals living with obesity found a total of 38.5% participants to have the MHO phenotype and in the Chinese study by Zheng et al. (who investigated adults aged over 20 years from seven geographically representative areas in China) the reported prevalence of MHO was of 27.9% (Cherqaoui et al., 2012; Zheng et al., 2015b). The main reason that has been attributed to this wide range in prevalence of MHO is due to heterogeneity between the studies notably in the criteria used to define MH both in terms of adiposity assessment and choice of risk factor parameters and their respective cut-off values (Phillips et al., 2013; Velho et al., 2010).

Moreover, in the comprehensive systematic review by Rey-Lopez *et al.* the prevalence of MHO was noted to range from 6% to 75%. Notably, they identified 30 different definitions of MH predominantly based on different combinations of blood pressure, HDL-C, TG and glucose levels. They also noted that MHO was inversely related to age and a higher proportion of MHO prevalence was found in Asian populations then in

Caucasian or individuals of multi-ethnic origins. They ascribed these differences to marked variations in definition of MHO (less strict vs more strict criteria as in the studies of Meigs et al. and Karelis et al. respectively), differences in sociodemographic variables between the populations, selection bias from poorly described target populations and differences in response rates (Rey-López et al., 2014). In a recent metanalysis by Wang et al. the authors aimed to summarise the prevalence of MHO and MUHNW worldwide. They observed that American populations had the highest MHO prevalence and in contrast European populations had the highest MONW prevalence. They also noted that prevalence of MHO and MUHNW were also influenced by sex, age, smoking and alcohol consumption and criteria used to define MH (Wang et al., 2015).

The highest prevalence for MHO in this study was seen when MH was categorised using HOMA-IR with a cut-off of <2.5 to denote the healthy phenotype (22.8%). This translated to around 67% of all individuals living with obesity . However, understandably, only 9.2% of individuals with obesity exhibited zero cardiometabolic parameters of the NCEP ATPIII criteria. When less stringent criteria were applied (that is the presence of ≤2 features of the NCEP ATPIII criteria) the prevalence of MHO among individuals with obesity was 64.9%. Kuk and Arden also utilised the presence of one or zero Met S criteria according to NCEP ATPIII or IR (as defined by HOMA) to characterize the MHO phenotype within their sample population (selected from the NHANES III cohort). However, in their study they found the prevalence of MHO among individuals living with obesity to be higher when using the Met S criteria than when using HOMA-IR on its own (38.4% vs 30.2% respectively) while only 6.0% of individuals living with obesity were free from any Met S parameters and IR implying there is minimal overlap between the

two definitions (Kuk and Ardern, 2009). Although the authors used the same cut-off value of HOMA-IR to classify MH (HOMA-IR ≥2.5) and defined Met S according to NECEP-ATPIII criteria, they did not include WC as part of the definition of Met S. This could, in part, explain the discrepancy in results with respect to the present study.

In this study, a percentage of participants with metabolically healthy obesity/overweight and normal weight, were found to be insulin resistant (as defined by HOMA-IR) while only 22% of MUHNW individuals were insulin resistant implying that sole use of the ATPIII or HOMA-IR definitions to categorise MH may be insufficient to truly identify metabolically healthy individuals and therefore a combined definition may be more sensitive. Similar findings were also observed in the study by Meigs *et al.,* which also reported the prevalences of MHO and MUHNW when using either IR or the Met S to define MH in the same cohort. Accordingly, they found that 32% of individuals with normal weight and Met S (as defined by the NCEP ATPIII criteria) to be insulin resistant, 34% of participants with obesity but *without* Met S to be insulin resistant, 14.9% of their metabolically healthy overweight participants to be also insulin resistant, whereas only 68% of participants living with obesity and with Met S were insulin resistant (Meigs et al., 2006). These proportions are considerably higher when compared to those observed in this study and could well be due to different cut -off values used to define HOMA-IR. In this study a cut-off of <2.5 was taken to imply insulin sensitivity whereas in the study by Meigs *et al.,* the authors defined insulin sensitivity as the three lower quartiles of HOMA-IR (<75th percentile). This cut-off has the disadvantage that the proportion of a population with IR is fixed even if it is changing with time. It also means that different

populations would have the same overall prevalence of IR, even though the mean measure of IR is different (Meigs *et al.*, 2006).

In addition to this, they also observed that the presence of either Met S or IR (regardless of level of BMI), conferred similar risk in terms of magnitude for incident CVD (about a 2-fold increased risk) or diabetes (4- to 11- fold increased multivariable risk) after approximately 11 years of follow-up. They also observed that individuals who were obese and insulin-sensitive were associated with a 3-fold increased risk of developing T2DM when compared with insulin-sensitive normal weight individuals but on the other hand were at lesser risk when compared to their obese insulin-resistant counterparts. Furthermore, the presence of the metabolically unhealthy normal weight phenotype conferred a higher risk for developing T2DM when compared to individuals who were obese but insulin sensitive. Thus, this lends support to the notion that the presence of the MHO phenotype is not totally benign (as was previously thought) and its risk is intermediate between that of the MHNW and MUHNW phenotypes (Meigs et al., 2006). On the same thought was the meta-analysis by Kramer *et al.,* which found that both the presence of the healthy obese state and the metabolically unhealthy phenotype (irrespective of the BMI) to be at increased risk of all-cause and cardiovascular mortality when compared to healthy normal weight individuals. Moreover, the MUHNW phenotype conferred the same risk for these events as the metabolically unhealthy obese phenotype (Kramer et al., 2013). All things conserved, this reiterates that both the MUHNW and MHO phenotypes are not without risk and the risks conferred by the MUHNW phenotype equate to those of the highest risk group (i.e., the MUHO

phenotype), implying that attention should also be given to this subgroup in terms of risk factor control.

Thus, the definition of what constitutes 'metabolically healthy' clearly makes an impact on the prevalence of the different BMI–metabolic risk phenotypes. However caution must be exercised when categorising individuals as being 'metabolically healthy' since individuals with excess adiposity (irrespective of metabolic status) are also at increased risk of several other debilitating health conditions such as metabolic dysfunction-associated steatotic liver disease (MASLD), obstructive sleep apnoea, osteoarthritis, urinary incontinence and some forms of cancer which are also associated with higher morbidity and mortality and therefore necessitating the treating clinician to make a timely diagnosis and institute treatment for these conditions even in people who are otherwise 'healthy' from a metabolic stand point.

3-5.2 Characteristics and associations of the MHO and MUHNW phenotype

In this study substantial differences in demographic and behavioural/ lifestyle characteristics were observed between the different body composition phenotypes. With respect to *sex,* significant sex differences were seen across all six body composition phenotypes. Overall females were more likely to exhibit the healthy metabolic phenotypes and whilst out of the total population there was a higher percentage of females with MUHNW, a greater proportion of normal weight males had the MUHNW phenotype.

With respect to *lifestyle and behavioural* factors, metabolically healthy individuals were more likely to consume alcohol, engage in some form of physical activity, occupy a white-collar occupation and have a higher education level when compared to unhealthy individuals irrespective of BMI. Moreover, MHO and MHNW individuals had comparable lifestyle and behavioural characteristics.

As expected, metabolically healthy participants presented better profiles for most *anthropometric* and *biochemical* parameters, compared to their metabolically unhealthy counterparts irrespective of whether they were of normal weight or overweight/obese. For example, they had higher HDL-C and lower TG and LAP. The latter is an index of central lipid accumulation and of visceral obesity and has been used to predict the risk of Met S, subclinical atherosclerosis, and cardiovascular risk (Kahn, 2005; Li et al., 2017). On the other hand, while total cholesterol was lower within the metabolically healthy overweight/obese group, the opposite was true in the normal weight group. This may be partly because total cholesterol reflects both subcutaneous fat and muscle mass (Park et al., 2012b). Metabolically healthy subjects (irrespective of BMI) were also observed to have lower serum ferritin levels. Ferritin is an acute phase protein and is regarded to be a marker of subclinical inflammation which in turn is thought to be responsible for the cardiometabolic complications observed in obese states (Cho et al., 2022). Interestingly, some studies demonstrate a lower inflammatory profile (including lower circulating levels of C-reactive protein, plasminogen activator inhitor-1 [PAI-1], TNF-α and IL-6) as being one of the mechanisms to explain the apparently healthy metabolic profile among individuals with MHO (Kloting et al., 2010;

Karelis et al., 2005; Jung et al., 2015). Furthermore, even though BMI was used to categorise individuals into being either of normal weight or overweight/obese, metabolically healthy individuals still had a lower BMI within their respective categories. Metabolically healthy individuals also had lower values for most obesity indices compared to the unhealthy cohort across all BMI categories. These included the BAI, VAI, CI, AV, BRI, and ABSI. The body adiposity index is a marker of total body fat (Bergman et al., 2011), whist the other indices are markers of visceral fat (Guerrero-Romero and Rodríguez-Morán, 2003; Maessen et al., 2014; Thomas et al., 2013). These anthropometric markers have been shown to predict CVD (Park et al., 2012b; Snijder et al., 2003).

When individuals with MHOW/O are compared to those with MHNW, the former were observed to have a worse cardiometabolic profile (including higher values for markers of subclinical inflammation, IR and lipid metabolism) and this was despite them being classified as metabolically healthy. This finding is in agreement with other studies which observed the MHO status to be associated with a worse micro metabolic milieu (including higher levels of visceral fat, oxidative stress and chronic inflammation) compared to healthy controls and which is not being picked up by current screening criteria (Su et al., 2022). Furthermore, there are also studies which observed no significant differences in markers of inflammation between MHO individuals and those with metabolic obesity leading authors to speculate that subtle abnormalities in these micro indicators could well explain the increased cardiometabolic disease risk observed in MHO individuals (Du et al., 2015; van Wijk et al., 2016; Dong et al., 2019). Therefore, this implies that current screening criteria cannot truly identify a cohort of individuals

with obesity who are healthy. This consolidates the notion that MHO is an intermediate-risk state between that of MHNW and MUHO and due to the dynamic nature of disease risk, the MHO phenotype may be regarded as a transient state such that given enough time it will degenerate into a metabolically unhealthy state with the consequent increase in cardiometabolic disease risk.

When comparing the MHOW/O to the MUHNW phenotypes using the Met S definition, a lower proportion of individuals with MHOW/O exhibited one or more metabolic syndrome components , despite having a higher BMI. These findings are not unexpected since these parameters are used to categorise such individuals. However, they also had a lower visceral adiposity index and higher thigh circumference, both of which are known to be associated with decreased cardiovascular risk. On the other hand, they had similar waist, hip and neck circumferences, waist-hip ratio, IR (as measured by HOMA-IR) and serum ferritin levels. These findings again underscore that the MHO phenotype also carries its own risks which probably lies somewhere between that of metabolically healthy normal weight and that of metabolically unhealthy normal weight categories.

When looking at the determinants for MHO phenotypes within the studied population, regression analysis revealed several demographic and behavioural characteristics to be associated with being overweight/obese but metabolically healthy. It was observed that *physical activity, alcohol consumption, non-smoking status* and younger age to be independently associated with the metabolically healthy overweight/obese phenotype according to either the Met S or IR definitions. On the other hand, within the normal weight cohort no significant associations were observed between lifestyle characteristics and the unhealthy state when MH was defined by either Met S or IR

criteria. However, there was a trend for participants with an unhealthy metabolic phenotype to have increasing age (over 40 years) and not to participate in any physical activity or consume alcohol.

Some of the findings in this study were echoed in several other American, Asian and European studies. For example, the Sicilian study by Buscemi *et al.,* found that older age, male sex, and an inactive lifestyle to be characteristic of the unhealthy phenotypes whereas the female sex, younger age and participation in physical activity to be more closely associated with the healthy obese phenotype (Buscemi *et al.*, 2017). In the US NHANES 1994-2004 study by Wildman et al*.,* the authors noted that younger age, modest alcohol intake, non-Hispanic black ethnicity, and greater physical fitness to be associated with the MHO phenotype while older age, lower physical activity and a larger WC to be associated with the MUHNW phenotype (Wildman *et al.*, 2008). Similar results were also observed in an Iranian study where the authors also observed increasing age and abdominal obesity to be inversely associated with the healthy obese state whereas increasing age and smoking to be associated with an unhealthy metabolic state in normal weight individuals (Hajian-Tilaki and Heidari, 2018). The Spanish study by Goday et al*.* found that out of a number of lifestyle factors (including age, sex, occupation, smoking and alcohol intake, BMI and physical exercise), the parameters most strongly associated with the unhealthy metabolic phenotype were BMI and age (Goday *et al.*, 2016). Furthermore, another Spanish study which assessed the MHO phenotype using different definitions noted that low physical activity was associated with the unhealthy obese state irrespective of the criteria used to define MH, but smoking and alcohol intake habits were not different when comparing MHO with MUHO under any of the

criteria used (Martínez-Larrad et al., 2014). This contrasts with the study by Phillips *et al.,* which found that physical activity was similar between the healthy and unhealthy states regardless of BMI value in a cohort of Irish individuals (Phillips et al., 2013).

While studies have consistently shown that several biological and genetic factors to be implicated in the pathogenesis of the MHO phenotype, when it comes to sociodemographic and other modifiable behavioural and lifestyle characteristics, only a handful of studies are available and these showed conflicting results (Iacobini et al., 2019; Phillips, 2013a; Stefan et al., 2013). Moreover, the determinants of the MUHNW phenotype have been even less well-studied. Most studies carried out in different populations and across different ethnicities concur that in terms of demographic characteristics the MHO state occurs more frequently in the female sex and younger individuals and that increasing age and being male to be associated with the unhealthy metabolic phenotype (Wildman *et al*., 2008; Goday *et al.,*2016; Buscemi *et al*., 2017; Iacobini *et al.*, 2019). With respect to lifestyle and behavioural factors, most studies focussed on the role of diet, fitness, tobacco smoking and alcohol intake as being a few of the potential contributors towards MH.

Clearly, certain behavioural characteristics (which are modifiable) have contributed significantly towards the obesity epidemic and may also partly explain the heterogeneity of metabolic abnormalities observed among individuals with obesity. Notably, the dietary environment has changed drastically in the last century to one which is energy dense and high in fat content, and in tandem with the global rise in urbanisation and sedentary behaviours, an increased state of positive energy imbalance follows which leads to increased accumulation of adipose tissue and the onset of overt obesity (Camhi

et al., 2015b, 2013). The study by Camhi *et al.,* assessed whether physical activity, sedentary behaviour and diet preferences differ between healthy and unhealthy young females with obesity by using a combination of questionnaires and an accelerometer to calculate level of physical activity. They noted that MHO females had healthier overall lifestyle habits: they spent less time doing sedentary activities, more time doing light physical activity and preferred healthier dietary fats and fibre then MUHO females (Camhi et al., 2015a). With regards to dietary composition, some studies show no differences between dietary macronutrient intake and overall total calorie intake between MHO and MUO individuals (Phillips, 2017; Phillips et al., 2013). On the other hand, one study which used data from the NHANES cohort noted superior dietary quality scores as assessed by the Healthy Eating Index 2005 scores among healthy female adolescents and adults living with obesity but not in the male counterparts. These findings might prove pivotal when instituting dietary intervention targets especially starting from an earlier time in life. The Mediterranean style diet (MSD) has long been known to have beneficial effects on cardiovascular risk factors (Camhi et al., 2015b; Estruch et al., 2018; Phillips, 2017). In a study by Park *et al*. which also looked at participants from the NHANES III cohort noted that individuals with MHO and who adhered to the MSD had lower risk of all-cause mortality but such observation was not seen among individuals with MUHO (Park et al., 2016). A recent comprehensive cross sectional study by Philips *et al.* which investigated the role of dietary composition and quality, food pyramid compliance, physical activity, alcohol and smoking behaviour in a cohort of 2047 individuals found no differences between in macronutrient composition, dietary quality and total calorie intake between MHO and MUO but moderate and high

levels of physical activity and compliance with food pyramid recommendations increase the likelihood of MHO (Phillips et al., 2013).

This present study found higher alcohol consumption in the metabolically healthy group compared with the metabolically unhealthy group among participants with both overweight/obesity and normal weight. Furthermore, on regression analysis, alcohol intake was found to be associated with the healthy overweight/obese phenotype in this study. This was also noted in the survey by Wildman *et al.* (Wildman, 2008;). Several studies have also reported the beneficial effects of modest alcohol intake on both glucose and lipid metabolism with various authors reporting alcohol consumption to be associated with increased HDL-C levels as well as decreased prevalence of the Met S. Others also observed an association between alcohol intake and lower LDL-C levels. Such effects on lipid parameters would be expected to improve on cardiovascular risk although this is not captured by current definitions of MH (Muga et al., 2019; Rosoff et al., 2019). Furthermore, the finding of an association between alcohol consumption and improved metabolic health in participants with overweight and obesity in this study can also be in part explained by the French Paradox - a term coined in 1992 to describe the epidemiological observation of a low incidence of cardiovascular disease in the French population, despite a diet relatively rich in saturated fats, a phenomenon potentially attributed to the moderate consumption of red wine (Renaud et al., 1992; Haseeb et al., 2017) . Red wine is rich in flavonoid phenolics such as resveratrol, the presence of which have been linked to vascular protection possibly mediated by an increased in plasma antioxidant activity and consequent inhibition of LDL-C oxidation which is an important event in the formation of the atherosclerotic plaque (Goldfinger, 2003).

Furthermore, Justice et al., demonstrated that exposure of Wistar rats to alcohol for 2 months not only led to higher HDL-C and lower total cholesterol and oxidised LDL levels but also to a significant reduction in expression of hydroxymethylglutaryl-coenzyme A reductase (the rate-determining step in cholesterol synthesis) and in sterol regulatory element-binding protein-2 (a transcription factor in cholesterol synthesis) together with up-regulation of paraoxonase-1 (known to inhibit LDL oxidation). These observations collectively suggest that alcohol may improve lipid profile via down-regulation of genes involved in cholesterol synthesis and up-regulation of genes that protect against LDL oxidation and thus the onset of atherogenesis (Justice et al., 2019). Adding to these findings is the observation that the Mediterranean diet (which also includes moderate consumption of red wine) has been consistently found to be associated with improved cardiometabolic outcomes and all-cause mortality in different populations (Estruch et al., 2018; Di Daniele et al., 2017).

On the other hand the detrimental effects include raised TG levels and increased abdominal obesity which may partly reflect the lack of consistent relationship between MHO and alcohol intake found in other studies (Kroenke et al., 2003; Martínez-Larrad et al., 2014; Velho et al., 2010). The relationship between alcohol intake and MH may therefore be bimodal.

Smoking was observed to be more prevalent in individuals with metabolically unhealthy overweight/obesity than in their metabolically healthy counterparts in the current study. However, no relationship between smoking and an adverse metabolic profile was found. In the US NHANES study, smoking also was not associated with the unhealthy metabolic phenotype both in normal weight and in individuals living with obesity

(Wildman, 2008); this is in contrast to a Russian study which found that smoking was associated with the unhealthy phenotype in lean individuals (Rotar et al., 2017). While no associations were also observed in this study between smoking status and the MUHNW phenotype, most studies found smoking status to be a potential contributor towards the unhealthy normal weight phenotype. In fact, in the metanalysis by Wang et al., the prevalence of the MUHNW phenotype was higher in participants who smoked and consumed alcohol (Wang et al., 2015). This could potentially be due to a decrease in insulin sensitivity through both a direct acute effect of smoking as well as smoking-associated decrease in muscle mass and central fat distribution (Attvall et al., 1993; Canoy et al., 2005; Lee and Choi, 2019). Smoking is also associated with metabolic derangement such as an adverse effect on lipid profile (Marano et al., 2015). Further research into the effects of smoking and alcohol intake are required for better understanding of their role in MH.

The beneficial effects of physical activity on overall weight status is indisputable and recent reports also confirmed that in individuals living with overweight and obesity , 20 minutes of physical activity among other lifestyle factors can lead to the same mortality risk as in normal weight people (Iacobini et al., 2019; Matheson et al., 2012). Physical activity is known to improve insulin sensitivity, lower blood pressure and improve lipid profile (Che and Li, 2017; Conn et al., 2014). However, the favourable effects of exercise on MHO and MUHNW have not always been consistent. In this study metabolically healthy individuals were more likely to be physically active than their metabolically unhealthy counterparts in indiviuals with both overweight/obesity and normal weight . Furthermore, physical activity remained an independent determinant for the MHO state

after adjustment for other lifestyle characteristics. This could relate to the underlying fact that concurrent physical activity in MHO individuals leads to increased fatty acid oxidation and higher fat utilization than in MUHO individuals (Iacobini et al., 2019; Pujia et al., 2016). However, this finding has not been reproduced in other studies (Pajunen et al., 2011; Phillips et al., 2013). Within the MUHNW cohort one study noted that physical activity and energy expenditure were lower when compared to a control group (Dvorak et al., 1999). Conus et al. attempted to identify the metabolic, behavioural and lifestyle phenotypes that could distinguish MUHNW females from the MHNW. They found that despite similar BMI values between these two groups the MUHNW females exhibited lower physical activity energy expenditure and lower peak oxygen uptake vs MHNW females. Furthermore, while both MHNW and MUHNW females had similar eating behaviours in terms of energy intake and measures of disinhibition and hunger, the MUHNW individuals showed less dietary restraint than MHNW females which also happened to be an independent predictor of insulin sensitivity (Conus et al., 2004).

Whether the healthy phenotype transitions into an unhealthy state over time has been debated over the past decade. The importance of identifying potential underlying behavioural and other modifiable lifestyle characteristics stems from the fact that they could be key factors in preventing degeneration of the metabolically healthy state to the unhealthy state. Dietary, and other lifestyle factors such as physical activity have all been shown to play an important role in the development of IR, T2DM and CVD (Manson et al., 2002; Phillips, 2013b; Tuomilehto et al., 2001). The study by Schroder et al. showed that increases in certain anthropometric surrogate markers of abdominal obesity (including BMI, WC and WHR) predicted the degeneration into the unhealthy

obese state whereas a healthy lifestyle (including participation in physical activity, non-smoking and eating a healthy diet) decreased the risk for this transition (Schröder et al., 2014). The current obesity epidemic entails that better improvements in obesity diagnosis and management particularly in those at higher cardiometabolic risk is paramount. Other factors apart from modifiable lifestyle characteristics such as assessing adiposity by body fat percentage together with BMI and assessing of inflammatory status has been shown to help identify both obese and lean individuals at greater cardiometabolic risk. Thus, taking into account metabolic risk in people living with obesity or normal weight might help not only in identification of individuals at greatest risk but also in ascertaining appropriate interventional strategies (Phillips and Perry, 2013).

3-6 Sex differences in prevalence and in cardiometabolic abnormalities among the different body composition phenotypes

This objective sought to provide an overview of the sex differences in the prevalence of the various phenotypes of interest when stratifying MH by the presence of ≤1 cardiometabolic abnormalities of the NCEP ATPIII criteria. This criterion was chosen since it has been linked with cardiovascular events and all-cause mortality in various studies as well as meta-analyses (Eckel et al., 2015; Hinnouho et al., 2015; Kuk and Ardern, 2009; Jung et al., 2014; Dalzill et al., 2014; Kim et al., 2016; Eckel et al., 2015; Park et al., 2016). Within the female population living with obesity, 36.5% were observed to be MHO whereas within the male population living with obesity, 25.7% were observed to be MHO; however when looking at the population prevalence there was no significant difference in MHO prevalence between male and females 3.5% vs 7.3% respectively. The potential explanation for this could be due to the differences in fat distribution patterns in both sexes by virtue of the different effects of sex hormones on fat deposition (such that males are more prone to android obesity [central /visceral fat deposition] and females are linked to gynaecoid obesity [lower body / gluteofemoral fat]). The latter is considered protective with respect to metabolic disturbances whilst the former is associated with increased cardiometabolic disease risk. Furthermore, males have a higher degree of visceral and ectopic adiposity for a given BMI (and WC) compared to females which may also in part explain the lower prevalence of MHO in males.

Males were more likely to be unhealthy despite higher levels of alcohol consumption and higher reported physical activity. The beneficial effect of physical activity on several

CM diseases is well-known and documented in the literature. While there is still limited and somewhat conflicting data with respect to the effect of physical exercise in MHO, it is generally observed that physical (cardiorespiratory) fitness is associated with improvement and maintenance of MH in individuals living with obesity (Al-Rashed et al., 2020; Sattelmair et al., 2011; Shabkhiz et al., 2021).

Although alcohol consumption is associated with a rise in serum TG, it has also been linked to improvements in MH by some authors (Enríquez Martínez et al., 2019; Muga et al., 2019) but not by others (Würtz et al., 2016). Additionally, there was a higher proportion of males who were classified as insulin resistant (as evidenced by a ≥HOMA-IR 2.5) when compared to females.

Thus, these data suggest that contemporary middle-aged Maltese males to be inherently more metabolically unhealthy than their middle-aged female counterparts. One of the reasons may be due to a secular decline in serum testosterone in males over the last few decades as has been reported by various authors (Mazur et al., 2013; Perheentupa et al., 2013). Testosterone is associated with the improvement of several metabolic parameters, such as serum lipids and insulin sensitivity (Kelly and Jones, 2013). The reasons for this secular decline in serum testosterone is largely unknown, however, it cannot be solely explained by increasing male obesity (Mazur et al., 2013). Other putative contributing mechanism have included dietary factors (Fantus et al., 2020) and the presence of environmental pollutants (Scinicariello and Buser, 2016).

Interestingly, since even normal weight males were more often observed to be metabolically unhealthy than normal weight females in this population, then this might

suggest the need to introduce sex-specific BMI cut-offs to better characterise MH. Furthermore, there was a lower proportion of males exhibiting an abnormally high WC when compared to females, and this was despite the fact that they had a less favourable metabolic profile (in terms of glycaemic, lipid and liver enzyme parameters) and despite being metabolically unhealthy more frequently.

Thus, these data also suggest that the currently used cut-off for WC as per the NCEP ATPIII criteria (which were developed in 2001) and subsequently adopted by the American Heart Association and the National Heart, Lung and Blood Institute (AHA/NHLBI) in 2004 may be too high in males at least in some populations (Grundy et al., 2004; NCEP, 2001). The decline in serum testosterone over the last decades could have also altered the relation between WC and visceral fat. The WC is a maker of intra-abdominal adiposity which in itself takes into consideration both subcutaneous as well as visceral fat depots, however it should be noted that males have a higher degree of visceral adiposity for a given WC (Camhi et al., 2011; Kuk et al., 2006). In animal studies, testosterone was associated with preferential reduction in visceral rather than subcutaneous adipocyte size (Abdelhamed et al., 2015). Furthermore, androgen deprivation in humans has been reported to cause a greater increase in visceral fat area than in subcutaneous fat area (Hamilton et al., 2011). In these patients, a significant and negative association between total testosterone and visceral but not subcutaneous fat area was observed (Hamilton *et al*., 2011). It may therefore be possible for the secular decline in serum testosterone in males over the last decades to result in a lower WC being predictive of IR. In this study there was no difference in the waist index between males and females, however this is a derived index based on current cut-offs. Thus, if a

lower cut-off for WC were to be used, the median waist index would be higher in males, which would be consistent with their worse metabolic parameters, again suggesting that the current cut-offs may be too high for males in this population. In keeping with the notion that males have more visceral fat for a given WC , this study also observed them to have higher values for indices of central obesity measurements such as WHR, WHtR and WTR.

Females, on the other had exhibited higher neutrophil-lymphocyte (NLR) and platelet-lymphocyte ratios (PLR), both of which have been associated with increased CVD risk (Dentali et al., 2018; Horne et al., 2005). The divergence of metabolic and haematological risk factors observed between the two sexes may be related to sex differences in the pathogenesis of CVD. Both the NLR and PLR are associated with microvascular disease (Fawwad et al., 2018; Okyay et al., 2015), which is thought to be a more importance pathogenic mechanism in females (Seidelmann et al., 2016; Wong et al., 2002).

These data also suggest that the anthropometric determinants for IR may be differ according to sex. In this analysis, BMI was found to be the only significant and independent determinant of HOMA-IR in males, while both BMI and WHR were significant independent determinants in females. This is in keeping with the fact that for a given BMI, males tend to accrue a higher proportion of fat in visceral and ectopic areas than females. Conversely, females tend to have more subcutaneous fat (Camhi *et al.,* 2011) which has been reported to be associated with increased leptin expression (Montague et al., 1997). Leptin has been shown to improve insulin sensitivity (D'souza et al., 2017; Levi et al., 2011). Since WC in females is a stronger marker for abdominal

subcutaneous fat rather than for visceral fat, and since subcutaneous fat in the gluteofemoral regions contribute to the hip circumference, the WHR may be a better overall marker of visceral adiposity than the uncorrected WC in females.

In conclusion males were generally observed to be more metabolically unhealthy and more insulin resistant than females in this contemporary sample of middle-aged individuals. Furthermore, a divergence in metabolic and haematological risk factors between the two sexes was observed. Namely, males were observed to have an abnormal WC less frequently than females despite having a higher median BMI suggesting that currently used cut-offs for WC should be revised downwards in males. Similarly, normal weight males were more like to be metabolically unhealthy than normal weight females, implying that BMI cut-offs may also need to be lowered in males. Conversely, females were more likely to exhibit higher neutrophil-lymphocyte and platelet-lymphocyte ratios which is consistent with established sex differences in the pathogenesis of CVD. Thus, these results merit further investigation. Furthermore, since the relationships between anthropometric parameters and MH are likely to vary across different age and ethnic groups, future studies should be conducted aimed at replicating and validating this study using revised cut-offs within other contemporary populations. Furthermore, longitudinal prospective studies should be carried out in order to further assess the role of these cardiometabolic alterations in the causal trajectory to CVD.

3-7 Variation in prevalence rates and in prediction of insulin resistance when using different criteria to define metabolic health

Since currently there are no standardised criteria for the diagnosis of the metabolically healthy phenotype, several definitions have been proposed and replicated in studies by other authors (Velho *et al.*, 2010). The most widely cited criteria are those proposed by Karelis *et al.* (2004), Meigs *et al.* (2006), Aguilar-Salinas *et al.* (2008), Wildman *et al.* (2008) and Lynch *et al.* (2009). Essentially these criteria incorporate different combinations of Met S parameters and/or measures of IR and/or the use of inflammatory markers (CRP) to define the healthy metabolic status (Aguilar-Salinas et al., 2008; Karelis et al., 2004a; Lynch et al., 2009; Meigs et al., 2006; Wildman et al., 2008). Thus, the objective of this study aimed to assess the prevalence of the different body composition phenotypes when using the above definitions (and others) to define MH.

The findings demonstrate that within a contemporary middle-aged Maltese population, there are considerable differences in the prevalence of each of the body composition phenotypes when using different diagnostic criteria. Overall, these results are reminiscent to those observed in previous studies (Liu et al., 2019; Phillips et al., 2013; Velho et al., 2010). Furthermore, the present study reinforces the need to adopt a population-specific approach in the definition of MH, since the criteria applied to this Mediterranean population were developed for Northern European / American Caucasians and may not be generalisable. While most definitions incorporate the presence of certain cardiometabolic abnormalities (such as dysglycaemia, hyperlipidaemia or hypertension), inflammatory and immune biomarkers (such as hs-

CRP) and / or the presence of IR to categorise MH, these diagnostic criteria need to be reproduced and validated in specific populations to account for the regional differences in genetic admixture, demographics, background prevalence of obesity as well as variation in anthropometric characteristics. In fact, the prevalences of the different body size phenotypes reported in this study are markedly different to those quoted when the same definitions were used in other populations. For example, when using the Aguilar-Salinas et al. criteria to define the metabolically healthy status in an Irish population, Phillips and Perry et al. observed a much lower prevalence of the MHOW/O phenotype (2.2% compared to 45.2% in this study when applying the same definition) and of MHNW phenotype (8.8% vs 25.0% in this cohort) (Phillips and Perry *et al.,* 2013). However, one should note that direct comparison between studies is limited by population-specific differences in life-style factors, variable patient ascertainment criteria, the impact of genetic factors on adiposity and fat distribution patterns as well as temporal changes in the prevalence of the body size phenotypes.

Additionally, all definitions of MH were found to have a higher predictive value with respect to IR than BMI alone in both males and females. This is in-keeping with the importance of incorporating MH as opposed to the use of simple BMI-based classifier when assessing CM risk. Notably, the metabolically unhealthy phenotype remained a strong predictor of IR for all the definitions used even after adjusting for BMI category within both sexes.

Although over the last two decades many studies have been conducted which aimed to assess the prevalence and characteristics of the different body composition phenotypes, few have compared the strength of the association of MH with IR when using the

different definitions applied in this study. Therefore, this data is important and novel as it shows which definition has the strongest association with IR.

This objective also brings out important sex-specific effects in the ability of the various definitions of MH to predict IR. As already explained before, a cut-off value of HOMA-IR of ≥2.5 was chosen since this threshold was associated with increased mortality in previous studies (Kuk and Arden, 2009; Calori *et al*., 2011; Durward *et al*., 2012). Within the female sex, the NCEP2 definition was the single strongest predictor of IR compared to the other definitions even after adjusting for BMI category (OR 19.7) On the other hand, the definition by Aguilar-Salinas et al. proved to be the strongest predictor of IR in males (OR of 18.7), followed by that of Lynch *et al*. and NCEP-2 even after adjusting for BMI. Interestingly, the Doumatey et al., definition also performed much better in males than in females (an OR of 12.2 vs 2.6 respectively). There are possible physiological mechanisms that underlie the observed sex-specific differences in associations. Females are known to exhibit greater blood pressure variability than males (Boubouchairopoulou et al., 2021). This may be mediated by greater baroreceptor sensitivity and by greater sensitivity to changes in dietary sodium in females (Sacks et al., 2001; Veiz et al., 2021). Thus, the increased blood pressure variability would be expected to create greater inaccuracies when characterising the different body composition phenotypes especially for those definitions requiring only one abnormal criterion to classify an individual as metabolically unhealthy (as are those by Aguilar-Salinas *e*t al., Lynch et al*.,* Doumatey et al., NCEP0 and Lavie *et* al). On the other hand, the NCEP-ATPIII criteria use a higher cut-off for WC in males than in females and as demonstrated earlier this cut-off may be too high in males. This may thus explain the

stronger association of the NCEP2 definition of MH with IR in females compared to males. The Aguilar-Salinas *et al*. definition uses identical cut-offs for HDL-C in males and females as opposed to the other definitions, thus it is significantly lower in females than the one used by NCEP ATPIII (1.0 vs 1.3mmol/L respectively). The former may thus be too low, and which may in part explain why the NCEP-2 definition performed better than that proposed by Aguilar-Salinas *et al.* In females. Females are known to have inherently higher HDL-C (Cho and Kim, 2021; Palmer and Toth, 2019). In fact data from the US National Health and Nutrition Examination Survey indicates that the optimal HDL-C cut-off to predict CVD to be 1.45 mmol/L in females and 1.06 mmol/L in males (Moon et al., 2015). However, these cut-offs are likely to differ among populations such that in Koreans the optimal HDL-C cut-offs were observed to be 1.24 mmol/L in females and 1.11 mmol/L in males (Moon *et al*., 2015).

Thus, the various currently used definitions of MH carry important caveats whose interpretation may have different impacts within a clinical context. The present MH definitions are based on findings from investigations carried out in different ethnicities. Furthermore, there is extensive between-study heterogeneity, with the use of different sample sizes and different sex proportions.

These factors might have contributed to the observed variation between males and females reported in this study. Additionally, while most definitions categorise individuals as metabolically healthy or unhealthy according to the presence or absence of specific cardiometabolic abnormalities, most were not derived from studies which assessed the association of MH with IR or CVD (Karelis *et al*., 2004a; Aguilar-Salinas *et al.,* 2008; Wildman *et al*., 2008; Lynch *et al.,* 2009; Doumatey *et al*., 2012). Of note an

additional definition of the metabolically healthy phenotype proposed by Meigs et al. is based solely on HOMA-IR values below the 75th centile (Meigs *et al.,* 2006). In the present study, this definition was not explored since one of the objectives was to investigate which classification is most predictive of IR hence obviating the need to determine HOMA-IR values (which may be cumbersome and not readily available for use in clinical practice). Furthermore, using this definition will result in a fixed prevalence of the metabolically unhealthy phenotype (i.e. 25%) in all populations and at all times and hence does not account for the dynamic nature of IR based on population-specific differences in cardiometabolic risk. On the other hand, while the definitions proposed by Wildman *et al.,* and Karelis *et al*., also incorporate similar cut-offs for HOMA-IR, they also incorporate additional biochemical and anthropometric criteria. Hence although these definitions were used to calculate the prevalence rates of the various body size phenotypes in this study, they were not entered in the logistic regression analyses to predict IR using HOMA-IR.

Thus, these results demonstrate that the prevalence of the various body size phenotypes is highly dependent on the definition criteria used to categorise MH, thereby highlighting the need for standardization of definitions. Nonetheless, irrespective of which definition was used, the metabolically unhealthy phenotype was more strongly associated with IR than when using BMI as the sole classifier to define at-risk groups. Furthermore, the metabolically unhealthy phenotype using any of the definitions available, was associated with IR even after adjusting for BMI category. Thus, this study informs on the importance of incorporating MH in patient stratification since this offers additional information on cardioembolic risk compared to BMI alone. The

sex-differences observed in the predictive value of the various definitions used to predict IR (as measured by HOMA-IR) also suggests the need for sex-specific definitions of MH. Thus, future studies should strive to replicate these findings in other age groups and across different populations as well as to evaluate the longitudinal relationship of these different definitions with long term cardiometabolic outcomes.

3-8 Differences in discriminatory power of several cardiometabolic parameters in determining insulin resistance

The final objective of the epidemiological studies aimed to compare the discriminatory power of the various anthropometric and metabolic parameters known to be associated with IR and increased cardiovascular risk in predicting IR as well as to determine their optimal cut points. The results demonstrate that several routinely available parameters can be used to predict IR in clinical practice. With respect to biochemical markers, the lipid accumulation product (LAP) and/or visceral adiposity index (VAI) in both males and females was observed to be of clinical utility in the prediction of IR. On the other hand the BMI and the WC were both observed to be the anthropometric parameters with the highest discriminatory power to predict IR in males and females.

At the forefront of the CM parameters was the LAP, which was observed to have the highest discriminatory power in males and a similar discriminatory power to both VAI and TG/HDL ratio in females. This parameter incorporates both the WC and serum TG in its calculation, both of which are independently associated with increased CVD risk (Hokanson and Austin, 1996; Huxley et al., 2010). Furthermore, in-keeping with this observation, both the WC and serum TG (which are components of the LAP) were found to have good discriminatory power in both sexes. The WC is a well-established marker of visceral adiposity, which in turn is strongly associated with IR. In fact, studies which looked at individuals with both normal weight or obesity observed that those with metabolic abnormalities have higher intrabdominal visceral fat deposition and were at highest CM risk irrespective of BMI (Kramer et al., 2013; Meigs et al., 2006; Ruderman et al., 1998; Sahakyan et al., 2015). Moreover, Eckel and colleagues found that normal

weight individuals who developed T2DM had higher WC values compared to normal weight counterparts without incident diabetes despite both falling within a range considered to be 'normal', and despite falling below the recommended thresholds for the Met S criteria (Eckel *et al.,* 2015). Although it also incorporates abdominal subcutaneous fat, which is thought to be less detrimental than visceral fat, the WC was a strong predictor of IR in both sexes in this analysis and performed better than the BMI, which is consistent with previous data (Grundy et al., 2013; Magri et al., 2016). Additionally, WC has also been shown to predict incident T2DM (Wei et al., 1997) and CVD independent of BMI (Dagenais et al., 2005; Zhang et al., 2008). VAI also emerged to be closely related to IR in both males and females. The VAI is a sex-specific index and it, too, incorporates measures of the WC, serum TG and HDL-C values as well as the BMI. VAI is an indicator of visceral adipose dysfunction and has been shown to be independently associated with both cardiovascular and cerebrovascular events as well as to have a negative correlation with insulin sensitivity under clamp studies (Amato *et al.,* 2014).

Interestingly, the TG/HDL ratio was not significantly better than TG levels on their own in predicting IR in both sexes, whereas HDL-C had poor discriminatory power in both males and females. HDL-C exhibits higher heritability than other lipids (Robertson et al., 1980). It also has much higher hereditability when compared to IR (Montali et al., 2015), implying that environmental factors that affect IR have much less impact on overall HDL-C values. Furthermore, many genetic polymorphisms that have been shown to affect HDL-C concentrations would not be expected to affect IR (Liu et al., 2021a; Rozhkova et al., 2021; Vitali et al., 2017). While epidemiological data show that low HDL-C values are

negatively associated with CVD, most known genetic variants that affect HDL-C levels are not associated with the risk of CVD (Kawashiri et al., 2018; Rosenson et al., 2018; Vitali et al., 2017). Thus, dysfunctional HDL-C may be more important in identifying IR and is not captured by simply measuring serum HDL-C levels (Kappelle et al., 2011; Riwanto et al., 2015). In keeping with these results is the study by McLaughlin *et al.* which also found fasting plasma TG concentration and plasma TG/HDL-C ratio to have similar and an equally good discriminatory power to fasting serum insulin concentration in identifying IR (defined by insulin-mediated glucose disposal during an insulin suppression test) in participants with overweight (McLaughlin *et al*., 2003). Furthermore, they also observed HDL-C to have less diagnostic utility in identifying insulin resistant individuals. However, they did not find any interaction between the sexes and the predicative ability of these markers for IR. They observed cut-off values of 1.8 or greater for the TG/HDL-C ratio and a triglyceride concentration of 1.47mmol or greater to identify insulin resistant individuals with reasonably similar sensitivity and specificity to the ATPIII criteria. Thus, they argue that since low HDL-C is associated with increased CVD risk, the TG/HDL-C ratio makes for an appealing clinical marker to identify insulin resistant individuals at high cardiovascular risk.

In this current study, the optimal cut-off for serum TG to predict IR was 1.35 and 1.33 mmol/L for males and females respectively. These are much lower than the 1.7 mmol/L recommended by the NCEP ATPIII criteria and many others (including the IDF). There is surprisingly little data to support the use of the 1.7 mmol/L cut-off. Serum TG are strongly and independently associated with IR, T2DM and CVD (Hokanson and Austin, 1996; Laws and Reaven, 1992). However, the risk

for CVD starts to increase at much lower levels, for example a TG level of >0.68 mmol/L was associated with increased risk for CVD in Korean individuals (Kim et al., 2022). Recently, Imano and co-workers reported that the best cut-off for non-fasting TG to predict ischemic heart disease in Japanese individuals to be 1.24 mmol/L (Imano et al., 2023). Lipoprotein lipase activity is known to be impaired in insulin resistant states resulting in elevated TG levels (Maheux et al., 1997; Panarotto et al., 2002). Since circulating FFAs are the major determinant of hepatic TG production and packaging into very low-density-lipoprotein, serum TG levels may be a marker of FFA levels (also known as non-esterified fatty acids [NEFAs]) (Adiels et al., 2006; Vatner et al., 2015). The latter are thought to be causally related to IR and are also known to inhibit lipoprotein lipase activity thereby resulting in a further increase in circulating TG levels (Liang et al., 2013; Randle et al., 1963; Saxena et al., 1989). Prospective contemporary and longer-term studies assessing serum triglyceride levels on hard outcomes such as CVD and T2DM are required to re-evaluate optimal cut-points for serum TG levels in different populations.

With respect to anthropometric parameters, the WC emerged as being the best predictor for IR in females and of similar discriminatory power to the BMI in predicting IR in males. For both parameters the optimal cut-points were observed to be lower (96.5 cm and 88 cm in males and females respectively) than the currently recommended cut-offs by the NCEP ATPIII criteria (102cm in males and 88cm in females). Importantly, the ATPIII definition was designed in 2002 to facilitate the diagnosis of the Met S in clinical practice in comparison to the previously existing definitions (those proposed by WHO and the European Group for the study of Insulin Resistance [EGIR]), notably by excluding the presence of insulin resistance as an obligatory component for the

diagnosis to be made (Balkau et al., 2002; NCEP, 2001; World Health Organization, 1999). Furthermore, it had lower diagnostic thresholds for certain characteristics (such as HDL-C and hypertension) and higher thresholds for others (WC). In fact, both the EGIR (developed in 2002) and, subsequently, the International Diabetes Federation (IDF) definitions (developed in 2005) of the Met S use lower cut-offs for WC for both males and females. The EGIR definition, which introduced the WC as a measure of central obesity (in contrast to the waist-hip ratio purported by the WHO criteria) was a major conceptual advance since although still a crude measure of abdominal fat, the WC was found to correlate better with visceral adipose depots (as measured by computed tomography) and with IR (Balkau et al., 2002; Pouliot et al., 1994). The cut-points proposed were ≥94 cm in males and ≥80 cm in females, albeit without introducing ethnic-specific cut-points. The more recent IDF classification aimed to introduce a unifying world-wide definition as well as to update levels and cut points in the diagnosis of the syndrome to reflect the growing obesity epidemic as well as to better predict CVD risk. Indeed, the IDF definition considers obesity to be one of the main drivers for the development of the Met S and its constituent components as well as increasing CVD risk. Thus, for persons to be identified as having the Met S they must have evidence of central obesity as defined by country/ethnic-specific values for WC (**Appendix 2** gives a summary of the different definitions of the Met S as proposed by the different organizations). The cut points proposed for Europids (which is the ethnic group pertaining to the population in this study) were ≥94 cm for males and ≥80 cm for females, which are by far lower than the ATPIII cut points used in this study. These values were based on cross-sectional data which showed them to be associated with an

adverse cardiovascular risk profile (Alberti et al., 2006). In fact, these cut-offs have been subsequently adopted by both WHO and EGIR. Thus, the finding of a lower optimal cut-off for WC in males and females in this population is both in accordance with the newer IDF criteria cut points and with the previous finding in this study that males were more likely to be metabolically unhealthy and more insulin resistant than females despite exhibiting an abnormal WC less frequently. This may also suggest that changes in fat distribution patterns, a decrease in muscle mass and the secular decline in serum testone levels in males may have resulted in a lower cut-points for WC to be predictive of IR (Guimarey et al., 2014; Sedlak et al., 2020; Żegleń et al., 2022). Furthermore, cut-offs for WC to define high-risk groups are likely to be population specific especially since there is data which shows clear differences across ethnic populations in the relationship between overall adiposity, abdominal obesity and visceral fat accumulation reinforcing the notion that cut-offs may need to be revised downwards at least for this population. In conclusion this data shows that within a Maltese Caucasian middle-aged population both the LAP and the VAI constitute relatively simple metabolic markers which can help identify those individuals who are sufficiently insulin resistant and therefore at higher risk for adverse cardiovascular outcomes. While serum TG and WC were also observed to have relatively good discriminatory power in predicting IR in both males and females, the optimal cut-offs for both TG and WC were lower than those currently recommended by the NCEP ATPIII criteria in both sexes. This thus calls for replication of the study in other populations of European descent as well as in other racial groups and in different age ranges in order to be able to update cut-offs to ones which reflect the contemporary

population as well as to evaluate their longitudinal relationship with longer-term outcomes.

3-9 Study strengths and limitations

This study has several strengths. Primarily it involved the recruitment of a well-characterised, homogenous, and adequately sized representative sample of middle-aged adult participants across the Maltese Islands. The preferential selection of a middle-age population was such so that the participants would have lived long enough for phenotypic expression while eliminating the potential of survival bias which could have led to an underestimation of effect size. Additionally, sarcopenic obesity, which is defined as the age-related decline in muscle mass coupled with higher adiposity and IR is uncommon in this age group. Loss of muscle bulk results in a lower BMI for the degree of adiposity supporting the notion that prevalence of MHO decreases with increasing age (Velho et al., 2010). Thus, other age groups should be studied separately since relationships between anthropometric parameters and MH are likely to vary across different age groups because of the age-related changes in muscle mass and function as well as in fat distribution.

Standard methods for data collection and for definition of MH were used, as already validated in previous studies. Furthermore, data collection, measurements of the body composition parameters and blood makers were carried out prospectively using robust measurement techniques in a controlled setting and by the same investigator (R. A.) rather than relying on retrospectively collected data or yet still, self-reported data, thus ensuring accuracy of all the information collected and the avoidance of potential sources of bias or interobserver variability. Biochemical parameters were centrally analysed under appropriate quality control.

This study carries some limitations. While standard definitions of Met S and IR were used to characterize the metabolically healthy from unhealthy phenotypes in the initial analyses, this study lacks information pertaining to adipokines, cardiorespiratory fitness, or dietary intake. Several studies have found the MHO phenotype to be associated with a better adipocytokine profile (including high adiponectin and low leptin levels) compared to the unhealthy obese state (Aguilar-Salinas *et al*., 2008; Mauriége *et al*., 2020). Additionally, one large study found that MH (when defined as the presence of ≤1 cardiometabolic abnormalities) to be associated with increased cardiorespiratory fitness (Ortega *et al.,* 2013). Therefore, inclusion of these criteria to differentiate the healthy from unhealthy phenotypes could have resulted in different prevalence rates. Furthermore, metabolic health was defined as the presence of ≤1 components of the NCEP ATPIII criteria. This definition was chosen since it has already been validated in several prospective studies which investigated cardiovascular disease and mortality risk in different populations and thereafter cited in the literature by several other researchers (Hinnouho et al., 2015; Kuk and Ardern, 2009; Jung et al., 2014; Dalzill et al., 2014; Kim et al., 2016). Whilst acknowledging that this definition allows for the presence of one metabolic abnormality and hence does not truly reflect a 'healthy' obese state as observed by Lavie and co-workers (Lavie et al., 2018), this study also looked at how prevalence varied when metabolic health was defined as meeting zero of the five criteria of the Met S.

BMI was used as an index of obesity measurement and thus it could have misclassified individuals with short stature or muscular build. It is acknowledged that the use of convenience sampling as opposed to stratified random sampling may have led to a

higher proportion of females than males being recruited (due to the voluntary nature of recruitment), and which may thus explain the skewed sex ratios observed in this study. While this study investigated a cohort of participants whose characteristics were reminiscent to that of the local population, this modality of cohort recruitment does not affirm that the cohort is fully representative of the general population and therefore limits extrapolation of the results within the general Maltese population and globally. However, the results obtained were consistent with a previous population study reported by another group of Maltese authors (Cuschieri et al., 2016a).

Furthermore, in this study, participants were categorized according to currently used definitions of adiposity and metabolic health, however it is acknowledged that cut-offs for each cardiometabolic parameter used in the definition of metabolic health is arbitrary and risk is likely to increase progressively with each unit change in risk factor parameter and BMI. Therefore, it will be useful for future studies to investigate such risk using each cardiometabolic parameters as a continuous variable.

The cross-sectional design of this study precludes the direct evaluation of the interaction between the different body composition phenotypes and sex and in the consequent development of cardiometabolic disease. This study was specifically conducted in Maltese individuals of Caucasian ethnicity, thus these findings are limited to this population may not be extrapolated to other ethnic groups, especially since there are racial differences in the relationship of IR to anthropometric and biochemical parameters. Therefore, it is important that other authors replicate these findings in other age-matched ethnic/racial groups.

Up until now this is the first study in Malta to evaluate the prevalence and characteristics of the different body composition phenotypes in such a well-characterized population. So far little is known about the characteristics and determinants of the different phenotypic traits in the Maltese Islands. Familiarity with potential predictors of adverse MH will help identify those patients with higher metabolic and cardiovascular risk and who may benefit most from preventive measures or interventional treatment.

In conclusion the findings from this study show that although the prevalence of overweight and obesity is high, the prevalence of the MHO and MUHNW phenotypes are comparable to other European studies. However, the wide range in prevalence rates observed when using different criteria to define MH emphasises the pressing need for a unified and standardised definition. Furthermore, currently used cut-offs for certain anthropometric parameters may need to be revised downwards to reflect a more contemporary population at least in middle-aged individuals. Based on the results obtained using the Youden index, the optimal cutoff levels in this population for waist circumference and BMI were observed to be 82cm and 31.9 kg/m^2 respectively in women and 96.5cm and 29.1 kg/m^2 respectively in men. Further prospective analyses are anticipated aiming to assess the influence of genetic predisposition factors and early life / maternal characteristics on the different body composition phenotypes in the Maltese Islands. This would in turn, allow for better risk stratification and the tailoring of customised preventive and cost-effective treatment paradigms when managing the different types of 'obesities' rather than adopting the traditional 'one size fits all' approach.

Chapter 4 – Mitochondrial DNA copy number and metabolic health

4-1. Mitochondrial DNA copy number and metabolic health

Mitochondria are subcellular organelles located within the cytoplasm of eukaryotic cells and contain their own double-stranded circular DNA genome that primarily encodes for proteins involved in oxidative phosphorylation and ATP generation. They are thus primarily responsible for cellular energy generation and for maintaining metabolic homeostasis but also have essential roles associated with cellular proliferation, differentiation, and apoptosis, free radical production, and calcium homeostasis (Kiefel et al., 2006; Kim et al., 2013c). Effectively, mitochondria convert ingested nutrients into useable energy via a highly regulated electron transport chain whereby a proton gradient leads to the synthesis of ATP from ADP and phosphate via the process of oxidative phosphorylation. Despite their key role in cellular energy production and in the dissipation of reactive oxygen species (ROS), mitochondria are sensitive to oxidative stress and the resultant oxidative damage and dysfunction particularly in tissues involved in nutrient metabolism such as adipose, liver and skeletal muscle has been postulated to contribute towards the development of several chronic metabolic disorders such as T2DM and CVD (Rani et al., 2016). During conditions of nutrient excess, the mitochondrial substrate load increases resulting in a surge in ROS production within the mitochondrial milieu promoting oxidative stress – a state where there is loss of balance between oxidative and anti-oxidative processes within cells. These changes lead to damage of cellular proteins, lipids and nucleic acids causing cell damage and dysfunction of key cellular pathways within the mitochondria including abnormalities in fatty acid beta oxidation and glucose oxidation which in turn have been associated with

abnormal lipid accumulation as NEFA and the downstream onset of IR and other metabolic disorders (Bournat and Brown, 2010; Hirabara et al., 2009; Lin et al., 2005).

Studies have demonstrated that abnormalities of mitochondrial bioenergetics specifically a reduction in the abundance of cellular mitochondria are linked to disorders associated with cardiometabolic risk, such as IR, T2DM, obesity, hypertension, and atherosclerotic CVD (Johannsen and Ravussin, 2009). Over the past few years, quantification of mitochondrial DNA copy number (mtDNA CN), a surrogate index of mitochondrial content, has emerged to be a potential biomarker of mitochondrial dysfunction in clinical practice (Castellani et al., 2020; Malik and Czajka, 2013). Mitochondrial dysfunction is associated with lower mtDNA CN and numerous clinical and population studies have confirmed an association between lower mtDNA CN in different tissues (including leukocytes, skeletal myocytes, hepatocytes and white adipose tissue) and several chronic health conditions (including the Met S, hypertension, hyperlipidaemia, T2DM, CVD and mortality) (Koller et al., 2020; Huang et al., 2011; Fazzini et al., 2021; Liu et al., 2021b). However only a limited number of epidemiological studies have assessed the relationship between mtDNA CN and obesity. A few cross-sectional studies found a relationship between mtDNA CN and BMI. While the direction of the association varied depending on the tissue studied, the majority showed a lower mtDNA content in peripheral blood leukocytes or subcutaneous adipose tissue in individuals with obesity. However, Lindinger and co-workers found that mtDNA CN in human omental tissue was significantly higher in patients with a BMI of ≥30 kg/m^2, which led the authors to speculate that the higher mitochondrial count could be a compensatory mechanism resulting from mitochondrial dysfunction which occurs in the

setting of excess adiposity (Kaaman et al., 2007; Lindinger et al., 2010; Meng et al., 2016; Skuratovskaia et al., 2018).

Obesity is often considered as a single trait, however it is increasingly recognised that there exist a subset of individuals with obesity who are protected from the risk of metabolic and CVD. These individuals are termed as being metabolically healthy obese and typically demonstrate a healthy metabolic profile in terms of preserved insulin sensitivity and absence of cardiometabolic risk factors. So far there is no universally accepted definition for this trait (Blüher, 2010; Karelis, 2008). On the other hand, a subset of normal weight individuals may harbour metabolic disturbances which are characteristic of obesity and are thus termed metabolically unhealthy normal weight (Schulze, 2019; Stefan et al., 2017). Thus, the presence or absence of MH generates different adiposity-related body composition phenotypes with metabolically healthy normal weight at one end of the spectrum and metabolically unhealthy obesity at the opposite end with MUHNW and MHO lying somewhere in between. Interestingly, despite a growing interest on this topic even less studies have been conducted to assess the relationship between mtDNA CN and MH and in relation to the different body composition phenotypes described above. It is hypothesized that individuals with the metabolically unhealthy body composition phenotype have a lower leukocyte mtDNA CN.

4-1.2 Aim and objectives

This research seeks to explore peripheral blood leukocyte mtDNA CN across the different adiposity-associated body composition phenotypes using several definitions of MH within a middle-aged cohort from a Mediterranean island population having a high prevalence of obesity and cardiometabolic disease. Furthermore, this study also evaluated which of the different definitions of MH and their constituent components are associated with reduced leukocyte mtDNA CN. Peripheral blood leukocytes were selected for mtDNA CN determination in view of their ease of accessibility as a minimally invasive disease biomarker. In this context, several studies have demonstrated that mtDNA CN is a potential biomarker of mitochondrial dysfunction (Castellani et al., 2020).

4.2 Research design and methods

4-2.1 Study population and study design

A sample consisting of 521 individuals aged 41±5 years of Maltese-Caucasian ethnicity were enrolled in this study through a method of convenience sampling as detailed in chapter 2. These were the same participants who participated in the observational cross-sectional study carried out between January 2018 and June 2019 which was aimed at characterising the different body composition phenotypes within a Mediterranean island population as part of the epidemiology studies described in Chapter 2. Similar inclusion and exclusion criteria applied, such that enrolled individuals had to be of Maltese Caucasian descent, aged 41±5 years and a BMI ≥18.5 kg/m^2. The exclusion criteria were a history of type 1 diabetes, individuals with known underlying genetic or endocrine conditions causing overweight or underweight (apart from treated or controlled thyroid disorders), individuals with a terminal illness or active malignancy, those who were unable to give their own voluntary informed consent as well as pregnant females. Participants were invited for a one-time visit whereby baseline anthropometric, demographic, and clinical parameters (including lifestyle and medical comorbidities) were captured by the use of a structured questionnaire especially designed for this survey, with blood sampling also being carried out on the same day of the visit.

4-2.2 Anthropometry, body composition assessment and biochemical analysis

Anthropometric measurements were recorded with the participants dressed in light clothing and without shoes, using validated equipment which was calibrated in accordance with WHO recommendations (detailed in Chapter 2).

Body composition phenotypes were generated based on the combined consideration of each participants' BMI category and MH status. Participants were defined as having normal weight if the BMI was <25kg/m^2 and overweight/obese if the BMI was ≥30kgm^2.

Three definitions of MH were used to cross-classify the study participants:

i) The Met S components based on the National Cholesterol Education Program (NCEP) Adult treatment Panel III (ATPIII) criteria but excluding WC (due to its collinearity with BMI): serum TG ≥1.69 mmol/l or on lipid lowering agents; HDL-cholesterol <1.03mmol/l in males and <1.29in females or on treatment aimed to increase HDL-C; systolic or diastolic blood pressure ≥130/85mmHg or use of antihypertensive medication; and fasting plasma glucose ≥5.6mmol/l or on antihyperglycemic treatment (NCEP, 2001). Participants having ≤1 of the above criteria were considered to be metabolically healthy.

ii) Normal insulin sensitivity as defined by a HOMA-IR <2.5 (this cut-off value has been validated in other studies) (Bo et al., 2012; Durward et al., 2012)

iii) An empirical definition of MH based on the risk of cardiovascular and total mortality recently described by Zembic and colleagues (Zembic et al., 2021). The criteria included the following parameters: systolic blood pressure <130mmHg and on no antihypertensive agents, a WHR of <0.95 in females and <1.03 in males, and absence of

diabetes. All the above criteria had to be present to be classified as metabolically healthy.

This thus led to the generation of four body composition phenotypes: metabolically healthy normal weight (MHNW), metabolically unhealthy normal weight (MUHNW), metabolically healthy overweight/obese (MHOW/O) and metabolically unhealthy overweight /obese (MUHOW/O). Additionally, in secondary analysis, MH was further characterised by the presence or absence of the Met S(defined as the presence of three or more components as established by the NCEP ATPIII: WC >102cm in males and >88 cm in females; Systolic/diastolic blood pressure ≥130/85 mmHg or use of antihypertensive agents, serum triglyceride level ≥1.69 mmol/l or on lipid lowering agents, HDL-C <1.20 mmol/l in females or <1.03 in males or on treatment aimed to raise HDL-C, fasting plasma glucose ≥ 5.6mmol/l or on antihyperglycemic agents.

Blood samples were drawn from the participants after an overnight fast. Haematologic and biochemical parameters (including HbA_{1c}, fasting plasma glucose, liver and lipid profiles) were determined using standard automatic biochemical analysers as described in Chapter 2.

Fasting insulin and high sensitivity CRP were measured at baseline by sandwich ELISA (Diagnostic Automation, USA). Thereafter fasting insulin and fasting plasma glucose were used to calculate the homeostasis model assessment of IR (HOMA-IR) according to the formula developed by Matthews and colleagues (Matthews et al., 1985):

HOMA-IR = Fasting Insulin (μIU/mL) x Fasting Glucose (mmol/l)/22.5.

4.3 Determination of mtDNA copy number

4-3.1 DNA extraction and quality control

Genomic DNA was extracted from peripheral blood leukocytes collected in K2-EDTA tubes as described next. 1 ml of anti-coagulated blood was used for extraction using a QIAamp DNA extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). An overview of the extraction process is provided in Appendix 3. The integrity of genomic DNA was assessed via agarose gel electrophoresis, using 1% agarose gels stained with 1.0µg/mL ethidium bromide against a 100bp DNA ladder (Solis BioDyne, Estonia). Electrophoretic separation in 1x TAE buffer was performed to visualise intact high-molecular weight DNA using a UV transilluminator. DNA concentration and purity was evaluated by NanodropTM 2000C spectrophotometry (ThermoFisher Scientfic USA). The purity of DNA was assessed as the ratio of absorbance at 260nm to the absorbance at 280nm (A260/A280). DNA samples with concentrations in excess of 20 ng/µL and A260/A280 ratios between 1.7 - 2.0 were considered of good quality for downstream analysis. Any extracted sample not meeting these parameters was re-extracted and re-analysed using the same process. Following extraction, the eluted DNA was stored in coded 0.5 mL screw-capped tubes in 96-well storage format (Micronic®). These were kept in a refrigerator till further processing. The extracted DNA samples were than frozen at -20oC until future use without repeated freeze-thawing cycles.

4-3.2 mtDNA quantification by qPCR

Relative leukocyte mtDNA CN was determined by estimating the relative ratio of mtDNA to nuclear DNA (nDNA) using a fluorescence-based quantitative polymerase chain reaction (qPCR). The mitochondrial gene *MT-CYB* encoding cytochrome b, and the nuclear gene encoding hemoglobin subunit β (*HBB*) as the single copy reference were used for relative quantification as described by Xu et al (Xu et al., 2012).

PCR primers (artificially synthesized oligonucleotide sequences) complementary to the two genes of interest were used to enable the selective amplification of the target region during a polymerase chain reaction.

Oligonucleotide primer sequences specific to *MT-CYB* (target gene) were as follows:

forward: 5'-CCA ACA TCT CCG CAT GAT GAA AC-3'and

reverse: 5'-TGA GTA GCC TCC TCA GAT TC-3' and these amplified a 434bp amplicon.

The primers specific to the sequences of the *HBB* gene were as follows:

forward: 5′-GAA GAG CCA AGG AGA GGTAC- 3' and

reverse: 5' – CAA CTT CAT CCA CGT TCA CC-3' and these were used to amplify a 268-bp product as the nuclear single copy reference.

The oligonucleotide sequences were ordered from Macrogen Inc, Seoul, Republic of Korea. The primers were received in lyophilized form. Thus, they were reconstituted using the indicated volume of molecular biology grade water and made into a stock solution with a concentration of 100 pM/µL. The primer solutions were then diluted to

a working concentration of 10 µM in a separate labelled tube. The prepared primer solutions were stored in a freezer at a temperature of -20 ˚C.

The polymerase chain reaction (PCR) technique enables efficient and rapid amplification of target genomic regions using sequence specific oligonucleotides, heat stable DNA polymerase and thermal cycling. Quantification of mtDNA CN was performed using quantitative PCR, which incorporates a fluorescence-based reporter dye into the reaction chemistry. In qPCR, fluorescence signal intensity is an indirect measure of the quantity of nucleic acid present in each step of the amplification cycle. A double-stranded DNA binding flurophore, EvaGreen® was used for quantification as outlined below.

Reactions were carried out in optical 96-well plates, using 4µL of 5x HOT FIREpol® EvaGreen® qPCR Mix (12.5mM MgCl2, dNTPs, EvaGreen® dye and ROX dye – Solis BioDyne, Estonia), 20ng of genomic DNA, and 0.5µL of each of the forward and reverse oligonucleotides at 10µM concentration, and molecular biology grade water to make up a reaction volume of 20µL. qPCR amplification was performed under the following conditions: initial denaturation at 95°C for 300 seconds, followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 56°C for 90 seconds and extension at 72°C for 120 minutes. Fluorescence signal acquisition was carried out in the extension phase. All assays were carried out in triplicate, and a no-template control was included in each run. Analysis was carried out in a blinded manner with regards to case-control and disease status. Post-amplification melt curve analysis was performed to check for primer-dimer artifacts and to ensure reaction specificity. For melt curve analysis, the

temperature was increased from 65 °C to 95 °C at 0.5 degree increments every 5 seconds.

Data collection was carried out using the CFX Maestro® software. Baseline subtracted curve fitting with fluorescent drift correction was applied to generate a horizontal baseline. For each amplicon, melt curves (relative fluorescence units [RFU] per temperature for each well) and melt peaks (negative derivative of the RFU data per temperature for each sample) were displayed to confirm reaction specificity.

The ratio of mtDNA/nDNA was calculated using the Pfaffl method, which is the method best suited for the interpretation of qPCR data where primer efficiency is not identical (Pfaffl, 2001) :

$$\text{mtDNA/nDNA ratio} = \frac{E^{\Delta \text{CtCytB}}}{E^{\Delta \text{CtHBB}}}$$

Where ΔCt is Ct_{HBB}-$Ct_{MT\text{-}CYB}$ and E stands for primer efficiency.

4-3.3 PCR efficiency and amplification specificity

To assess qPCR efficiency, standard curves were constructed by 10-fold serial dilutions of PCR products of target gene *MT-CYB* and the nuclear reference gene *HBB* (range: 5.05 $x10^{10}$ – 5.05 $x10^{5}$ copies/µl). The Ct values (cycles to threshold) for each reaction represents the number of PCR cycles required in order to detect a signal over background fluorescence and is inversely proportional to the amount of DNA. The $\log_{10}$ of template copy number (x-axis) was plotted against the corresponding Ct value (y-axis) value which represents the number of PCR cycles required to detect a signal over background fluorescence) and linear regression analysis was applied **(Figure 4.1).** The PCR efficiency of each reaction, *E,* was calculated using the standard curve points in the exponential phase using the equation $E = 10^{-1/slope}$. The calibrator was a mixed DNA sample pooled from six randomly selected normal weight metabolically healthy controls.

The specificity of the amplification reaction was confirmed by melt curve analysis (dissociation curve) during temperature ramping, and by resolution of amplicons during agarose gel electrophoresis. **Figure 4.2** depicts melting curve (A) and melt peak (B) analysis of amplicons generated during qPCR. Both *MT-CYB* and *HBB* amplicons demonstrated single melting peaks at 86.5^{o}C and 88^{o}C respectively. Furthermore, specificity was also ascertained with electrophoretic separation of PCR products using 2% agarose gels which demonstrated prominent bands with the expected sizes **(Figure 4.2C).** The standard curves for both *HBB* and *MT-CYB* were linear over the serial dilution range (R^2=0.99), and all Ct values of unknown samples fell within the linear range. The

gradients (slopes) of the standard curves for *HBB* and *MT-CYB* were -3.62 and -3.42 respectively, with the amplification efficiencies being 88.9% and 95% respectively.

4-3.4 Coefficient of variation values

The inter-assay and intra-assay coefficient of variation for replicates in different batches were 2.1% and 6.5% for *HBB* and 1.8% and 3.5% for *MT-CYB* respectively. The acceptable standard deviation for the triplicate threshold **c**ycle (ΔCt) was set at 0.5, indicating that the reactions have an acceptable degree of repeatability and reproducibility.

4-3.5 Corrected mtDNA copy number

A corrected leukocyte mtDNA CN was calculated in order to adjust for possible contamination of leukocyte genomic DNA by mitochondrial DNA in platelet fractions from whole blood. This was calculated as outlined by Hurtado-Roca and colleagues (Hurtado-roca et al., 2016). Since platelets contain only mtDNA and no nuclear DNA the variation in platelet levels could confound relative mtDNA CN estimates from whole blood. A correct count was calculated using the following formula:

$$mtDNA\ CN\ leukocytes = mtDNA\ CN\ whole\ blood - K\frac{Platelet\ count}{Leukocyte\ count}\ (K = 1.1)$$

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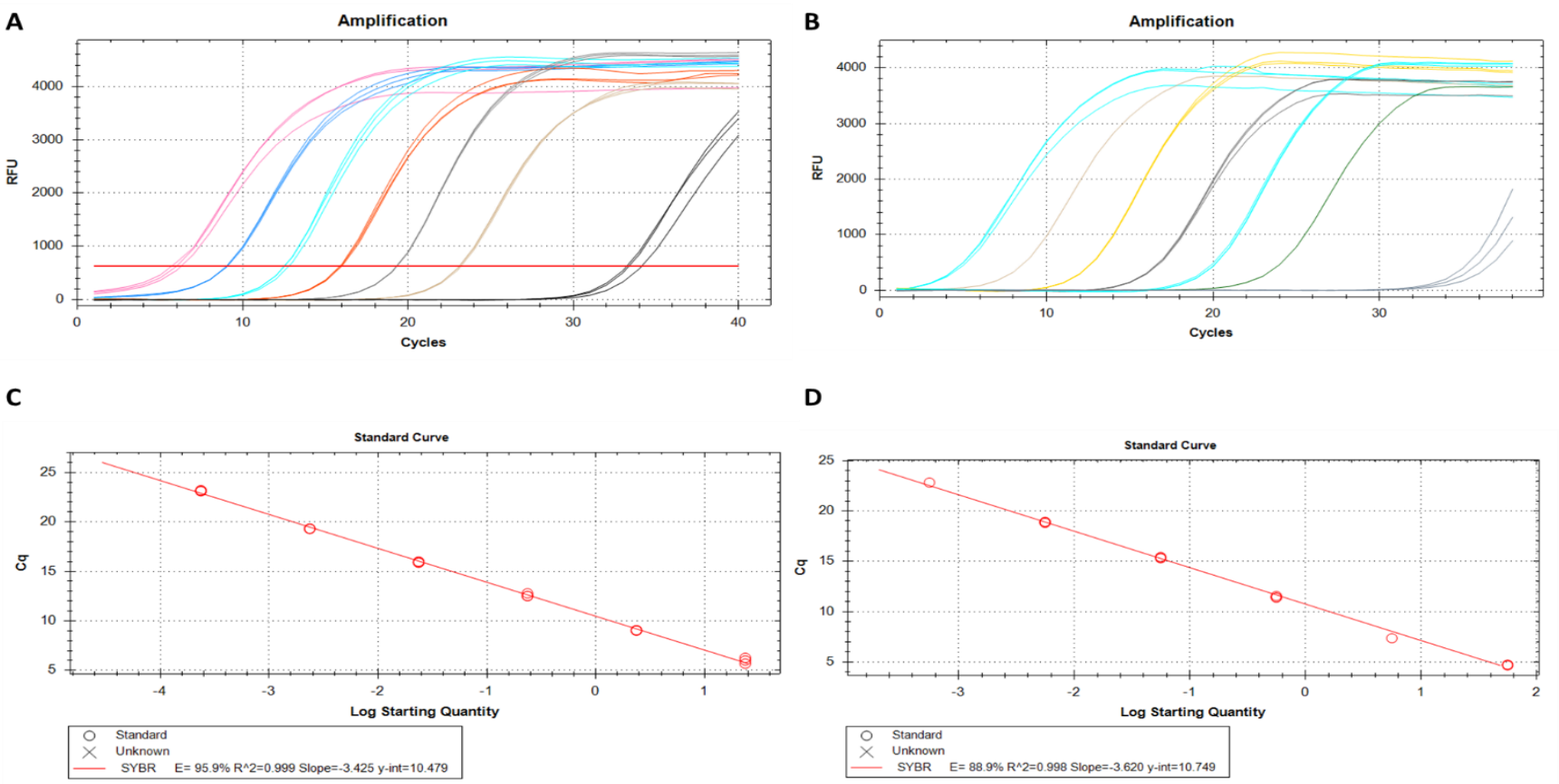


Figure 4.1: Amplification plots and standard curves for *MT-CYTB* and *HBB*

A and **B** – amplification plots for MT-CYTB and HBB respectively**.** Plots **C** and **D** show standard curves for *MT-CYTB* and *HBB* respectively, represented as a semi-log plot of Ct against starting concentration. The Ct values of unknown samples fell within the linear range. Standard curves were constructed using six 10-fold serial dilutions of PCR products, and each standard dilution was amplified by real-time quantitative PCR using the *HBB* and *MT-CYTB* primer sets. A no-template control is included. *MT-CYTB,* mitochondrial gene encoding cytochrome b; *HBB*- nuclear gene coding haemoglobin subunit ß (single copy reference)

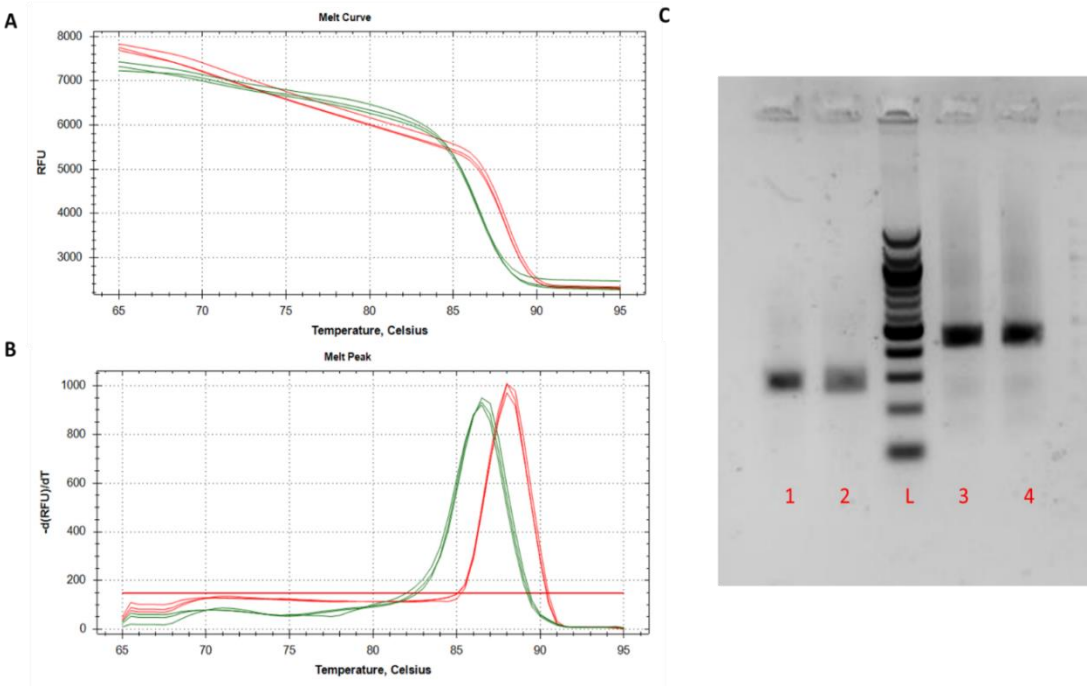


Figure 4.2: Melt curves and melt peaks of *MT-CYTB* (green) and *HBB* (red)

Melt curve (A) and melt peak (B) analysis of *MT-CYTB* (green) and *HBB* (red) triplicate amplicons. The dissociation curve charts the reduction in fluorescence observed during temperature ramping. The melt peak analysis is a plot of the negative first derivative of the dissociation curve and shows a characteristic peak for each product (the derivative is the negative of the rate of change in fluorescence as a fraction of temperature). **C** shows 2% agarose gel electrophoresis of PCR amplicons. Lanes 1 and 2 show the 268 bp *HBB* amplicon, and lanes 3 and 4 the 434 bp *MT-CYTB* amplicon. Lane L shows a 100bp DNA ladder (Solis Biodyne, Estonia).
MT-CYTB, mitochondrial gene encoding cytochrome b; *HBB*- nuclear gene coding haemoglobin subunit ß (single copy reference)

4.4 Statistical Analyses

Normality of distribution of continuous variables was assessed using the Shapiro-Wilk and Kolmogrov-Smirnov tests. Since all continuous parameters exhibited a skewed non-normal distribution, non-parametric statistics using medians and interquartile ranges (IQR) and the chi-squared test was applied to compare dichotomous outcomes. Categorical variables are presented as percentages. To evaluate differences in qualitative variables between groups the Kruskal-Wallis ANOVA was used for comparison between three or more categories, followed by Dunn's post hoc test for pairwise comparison between subgroups. The independent samples Mann-Whitney U test was used for comparison between two categories. Spearman's rank-order coefficient was used to explore the strength and direction of association between quantitative variables. To assess the effect of mtDNA CN on Met S, binary logistic regression models were constructed and adjusted for age, with mtDNA CN as the independent predictor and Met S as the dependent response variable. Binary logistic regression modelling adjusted for age, was also used to assess the association between mtDNA CN and single components of the Met S. Since some definitions of Met S components included the use of drugs for the management of hypertension, dyslipidaemia and hyperglycaemia each component was considered as a binary response variable. Furthermore, to account for the unequal representation of the sexes in the study population, regression analysis was additionally stratified by sex.

To further refine the association between adiposity, Met S components and mtDNA CN principal components analysis (PCA) was applied to reduce the dimensionality of the dataset. PCA was performed on nine standardized inter-correlated quantitative

variables (including WC, fasting plasma glucose, HDL-C, TG, BMI, systolic BP, diastolic BP, HOMA-IR and hs-CRP) using the FactoMineR package (Lê, Josse and Husson, 2008). This enabled the construction of a scree plot of eigenvalues such that eigenvalues>1 were used to determine the number of selected factors. Orthogonal rotation (varimax) was taken to force variables strongly with a single component. Subsequently, the derived principal components were used as the dependent response variable in regression modelling, with mtDNA CN as the independent predictor adjusted for age and sex.

Odds ratios and 95% confidence intervals (CI) are reported for a decrease in 10mtDNA copies. Statistical analysis was performed using SPSS v26 and R v.3.4.2. A p value of <0.05 was considered statistically significant.

Chapter 5A – Results of molecular studies

5-1 Clinical and biochemical characteristics of the study participants

Table 5.1 depicts the anthropometric, biochemical, and clinical characteristics of the study cohort stratified according to the four different body composition phenotypes and according to the three different classifications of MH described above: MHNW; MUHN; MHOW/O; MUHOW/O. No significant difference in leukocyte count, platelet counts and in the proportion of leukocyte subpopulations was detected across all body composition phenotypes or across Met S categories (data not shown). As expected, the metabolically unhealthy participants displayed a less favourable anthropometric and metabolic indices compared to their healthy counterparts including higher WHR, total cholesterol and LDL-C, uric acid and hs CRP values.

5-2 Relationship between peripheral blood leukocyte mtDNA copy number and metabolic indices

The relationship between relative mtDNA CN and metabolic parameters was investigated using Spearman's correlation. A correlation matrix of mtDNA CN with quantitative anthropometric and metabolic parameters is provided in **figure 5.1(A).** Significant negative correlations between mtDNA CN and BMI, WC, WHR, triglyceride levels, fasting plasma glucose, HbA1c, HOMA-IR and hs-CRP were observed, along with a positive correlation with HDL-C levels. However, no significant correlation was observed with age (r_s= 0.03, p= 0.497). Furthermore, no difference in mtDNA CN between sexes was detected (Mann-Whitney U test, p= 0.065).

5-3 Relationship between peripheral blood leukocyte mtDNA copy number and the different body composition phenotypes using different definitions of metabolic health, the Met S and obesity

Subsequently, relationship between leukocyte mtDNA CN and the different body composition phenotypes was evaluated. When the study population was categorized according to the different MH definitions (metabolic health as defined either by the presence of ≤1 NCEP-ATPIII criteria or when defined by HOMA-IR <2.5 or as defined according to the empirical definition proposed by Zemibc and colleagues), a significantly lower median mtDNA CN was present in both the MHOW/O and MUHOW/O categories, compared to MHNW participants (Kruskal-Wallis test, p <0.001) **(Figure 5.1 (B-D))** Interestingly no significant difference in relative mtDNA CN between the MHOW/O and MUHOW/O phenotypes was observed by any of the definitions used to denote MH. Likewise, no significant differences in mtDNA CN were detected between the MHNW and MUHNW phenotypes across all definitions of MH, although the small number of participants within the MUHNW category restricts this comparison. Additionally, the mtDNA CN in participants with the Met S was similarly observed to be lower than in those without the syndrome (Mann-Whitney U test, p<0.001) **(Figure 5.1(E)).** As expected, a significant decrease in median mtDNA CN with an increase in Met S components was observed (Kruskal-Wallis test, p <0.001) **(figure 5.2). Table 5.2** summarises mtDNA CN values across definitions of MH based on different cross-classifications, with pairwise comparisons between the categories.

Binary logistic regression analysis was applied to evaluate the association between mtDNA CN and 1) Met S 2) each of the NCEP-ATPIII components as outcome variables 3) the metabolically unhealthy state as per the empirical definition by Zembic and colleagues and 4) HOMA-IR ≥2.5. In the age-adjusted models, a 10-fold reduction in mtDNA CN was associated with a marginally higher odds of the Met S in both sexes, increased TG in males and increased WC in females. A reduction in mtDNA CN was also associated with a minor (but significantly higher) increased risk of having both the metabolically unhealthy phenotype (in both sexes) and of having a HOMA-IR ≥2.5 in females. The results of the regression analyses in the overall cohort and when stratified by sex are presented in **table 5.3.**

5-4 Principal components analysis

Since several adiposity/cardiometabolic risk parameters are known to be interrelated and to converge physiologically to determine the causal trajectory to cardiometabolic disease, the method of principal component analysis (PCA) was used in order to reduce the dimensionality of the dataset and explore its relationship with mtDNA. PCA with orthogonal (varimax) rotation was conducted on 9 inter-correlated variables as outlined earlier. Bartlett's test of sphericity indicated that the correlations were sufficiently large to undertake PCA ($\chi 2 = 1299$, $p < 0.01$). The initial analysis centred around obtaining eigenvalues for each data components. Three principal components (PC) had eigenvalues >1 and in combination could explain 62.5% of the data, and a scree plot justified retaining 3 factors in the final analysis **(figure 5.3).** The rotated component matrix showed that PC-1 had a high loading for WC and BMI; PC-2 for systolic and diastolic blood pressure and PC-3 for fasting plasma glucose, HOMA-IR and TG **(Table 5.4).** Subsequently, the three PC were incorporated as response variables within regression models to test their association with mtDNA CN as the predictor. Allowing for age and sex, an inverse association between PC-1 (adiposity parameters), PC-3 (insulin resistance parameters) and mtDNA CN was detected. The other PC defined by systolic and diastolic blood pressure, showed no significant association with mtDNA CN **(Table 5.5).** These analyses inform further on the association between excess adiposity and IR with reduced mtDNA CN obtained from univariate analysis.

Table 5.1: Anthropometric, clinical, and biochemical characteristics of the study cohort, stratified according to the different metabolic health definitions

	Metabolic health defined by ≤1 NCEP-ATPIII criteria				Metabolic health defined by Zembic *et al.*				Metabolic health defined by HOMA-IR <2.5			
	MHNW (n=131)	MUHNW (n=25)	MHOW/O (n=217)	MUHOW/O (n=148)	MHNW (n=143)	MUHNW (n=13)	MHOW/O (n=268)	MUHOW/O (n=97)	MHNW (n=159)	MUHNW (n=7)	MHOW/O (n=270)	MUHOW/O (n=85)
Female (N/%)	109/83.2%	18/72%	144/66.3%	59/39.8%	116/81.1%	11/84.6%	161/60.1%	42/43.3%	118/74.2%	7/100%	154/57%	42/49.4%
Physical activity (N/%)	63/48.1%	14/56%	93/42.9%	53/35.8%	68/47.6%	9/69.2%	115/42.9%	31/32%	74/46.5%	2/28.6%	119/44.1%	26/30.6%
Smokers (N/%)	32/24.4%	6/24%	48/22.1%	31/20.9%	35/24.5%	3/23.1%	54/20.1%	25/25.8%	34/21.3%	3/42.9%	55/20.4%	22/25.9%
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**
Age (years)	41(7)	41(4)	40(6)	42(5)	41(7)	39(5)	41(5)	42(6)	41(7)	39(5)	41(6)	42(6)
BMI (kg/m²)	22.5(2.5)	21.8(3.3)	29(6.1)	31.1(7)	22.4(2.8)	23.5(2.5)	28.9(5.9)	32.3(7.2)	22.4(2.7)	24(1.8)	29.1(5.7)	32.7(7.1)
SBP (mmHg)	115(15)	125(10)	120(10)	122(14)	118(15)	125(15)	120(10)	125(10)	120(15)	120(20)	120(10)	120(15)
DBP (mmHg)	80(10)	80(0)	80(10)	80(5)	80(10)	80(0)	80(10)	80(5)	80(10)	80(10)	80(5)	80(5)
WHR	0.82(0.1)	0.85(0.1)	0.89(0.11)	0.93(0.1)	0.83(0.1)	0.85(0.1)	0.89(0.1)	0.97(0.12)	0.82(0.1)	0.85(0.1)	0.9(0.1)	0.93(0.1)
TChol (mmol/l)	4.58(1.2)	5.08(1.13)	4.79(0.89)	5.1(1.53)	4.63(1.2)	5.08(1.1)	4.89(1.01)	4.9(1.39)	4.64(1.2)	5.71(1.7)	4.86(1.04)	5.09(1.29)
LDL-C (mmol/l)	2.6(0.98)	3.2(1.06)	2.84(0.94)	3.15(1.3)	2.66(1.2)	3.27(1.4)	2.91(1)	3.03(1.2)	2.62(1.1)	3.49(1.2)	2.88(1.01)	3.14(1.2)
HDL-C (mmol/l)	1.66(0.52)	1.5(0.58)	1.41(0.46)	1.27(0.38)	1.63(0.5)	1.82(0.6)	1.4(0.4)	1.16(0.3)	1.63(0.5)	1.29(0.7)	1.39(0.4)	1.09(0.3)
TG (mmol/l)	0.75(0.4)	0.86(0.51)	0.97(0.59)	1.53(0.97)	0.77(0.4)	0.76(0.7)	1.07(0.6)	1.44(0.9)	0.77(0.4)	0.58(1.1)	1.07(0.6)	1.7(1.11)
FPG (mmol/l)	4.92(0.5)	5.24(1.1)	5.06(0.5)	5.69(0.8)	4.94(0.6)	4.92(0.4)	5.17(0.6)	5.45(1.3)	4.94(0.6)	4.87(0.5)	5.13(0.6)	5.7(1.61)
HbA1c (%)	5.2(0.4)	5.2(0.2)	5.2(0.4)	5.5(0.55)	5.2(0.3)	5.2(0.29)	5.3(0.4)	5.6(1.3)	5.2(0.3)	5.1(0.6)	5.3(0.49)	5.6(1.3)
UA (mmol/l)	242(83)	255(103)	280(85)	315(123)	242(91)	255(77)	289(92)	314(123)	242(91)	276(96)	290(96)	307(106)
HOMA-IR	1.13(0.9)	1.13(0.96)	1.69(1.1)	2.2(1.3)	1.13(0.9)	1.39(1)	1.75(0.9)	2.44(1.5)	1.12(0.8)	2.67(0.2)	1.64(0.8)	3.09(0.9)
hs CRP (mg/l)	3.5(4)	4.2(3.6)	4.6(4.4)	5.1(4.6)	3.5(3.8)	6.1(4.1)	4.7(4.5)	5.15(4.3)	3.6(3.9)	4.2(4.2)	4.6(4.3)	6.2(4.1)

IQR, interquartile range; NCEP-ATPIII National Cholesterol Education Program – Adult Treatment Panel III; MHNW, Metabolically healthy normal weight; MUHNW, metabolically unhealthy normal weight; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, Metabolically unhealthy overweight/obese; BMI, body mass index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; WHR, waist to hip ratio; TChol, total Cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, Triglycerides; FPG, Fasting plasma glucose; HbA1c, glycated haemoglobin; UA, Uric acid; HOMA-IR, homeostatic model assessment of insulin resistance; hs CRP, high sensitivity C-reactive protein

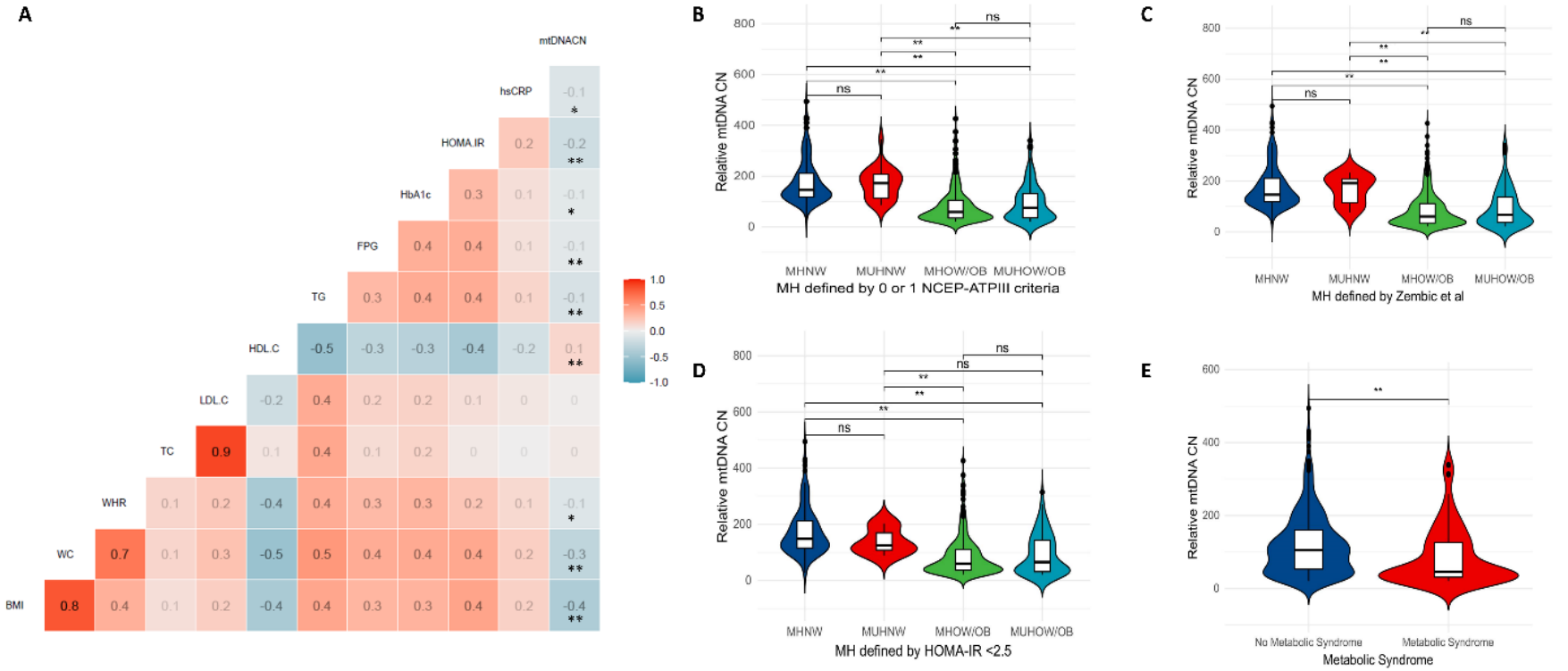


Figure 5.1: Correlation matrix and Violin plots

A: Correlation matrix between relative mtDNA copy number and several key metabolic parameters. Colour scale depicts Spearman's rank-order correlation coefficient. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). **B-E**: Violin plots depicting mtDNA copy number differences between body composition phenotypes and across different definitions of metabolic health and the metabolic syndrome. A significantly lower mtDNA copy number was observed in individuals with metabolic syndrome (Mann-Whitney U test, $p < 0.05$). A significantly lower mtDNA copy number was present in both the MHOW/O and MUHOW/O categories, compared to MHNW participants (Kruskal-Wallis test, $p < 0.001$), across different metabolic health definitions. The violin plots reflect data distribution. The centre line in the box plot illustrates the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. ** significant difference at $p < 0.01$. NS, not significant; MH, metabolic health; MHNW; metabolically healthy normal weight; MUHNW, metabolically unhealthy normal weight; MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese; NCEP ATPIII, national cholesterol education program adult treatment panel III; HOMA-IR, homeostatic model assessment of insulin resistance

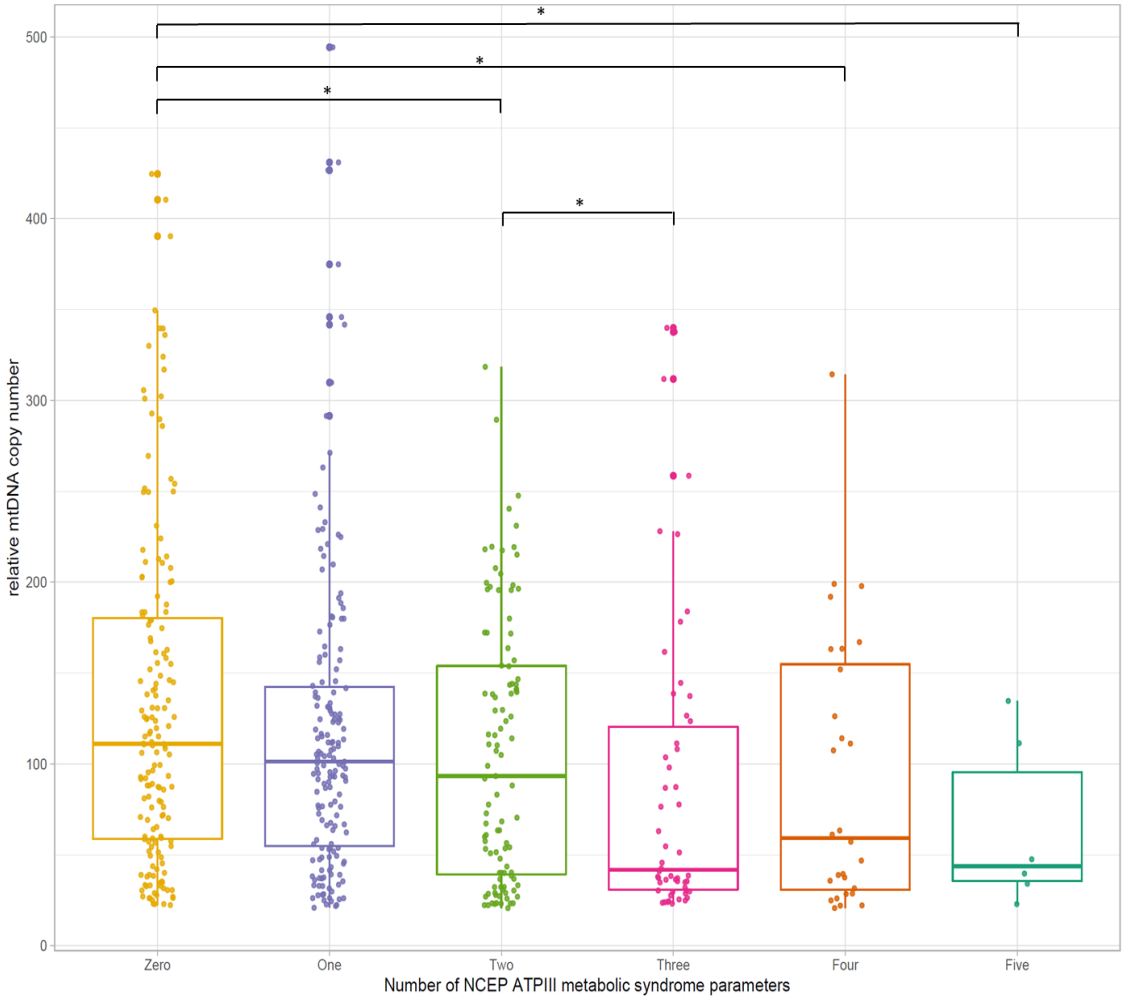


Figure 5.2: Box plot showing relative mtDNA copy number against number of diagnostic components of Met S as per NCEP-ATP III

A significant reduction in mtDNA copy number with increasing metabolic syndrome components was observed (independent samples Kruskal-Wallis ANOVA, $p < 0.001$). Pairwise comparison using Dunn's post-hoc tests revealed significant differences between individual categories, indicated by * symbol.

Table 5.2: Relative mtDNA copy number across definitions of metabolic health

Metabolic health definition		Relative mtDNA copy number										
		n	Median	Q75	Q25	p value[a]	p value[b]	p value[c]	p value[d]	p value [e]	p value [f]	p value [g]
Metabolic health defined by 0 or 1 NCEP-ATPIII parameters	MHNW	131	145.5	211.1	117.7	<0.01	<0.01	NS	NS	<0.01	<0.01	-
	MUHNW	25	172.3	207.0	114.0							
	MHOW/O	217	59.2	105.2	34.9							
	MUHOW/O	148	76.0	133.0	35.4							
Metabolic health defined by HOMA-IR < 2.5	MHNW	147	152.1	212.7	118.9	<0.01	<0.01	NS	NS	NS	0.018	-
	MUHNW	7	125.0	199.6	107.4							
	MHOW/O	270	59.9	110.2	36.4							
	MUHOW/O	85	65.3	143.5	31.5							
Metabolic health defined by Zembic *et al.*	MHNW	143	146.1	211.1	117.7	<0.01	<0.01	NS	NS	<0.01	<0.01	-
	MUHNW	13	191.3	207.0	115.1							
	MHOW/O	268	60.4	111.5	34.9							
	MUHOW/O	97	67.2	137.3	37.1							
Metabolic Syndrome	Absent	433	105.2	160.1	53.2	-	-	-	-	-	-	<0.01
	Present	88	46.2	126.4	31.0							

p value [a] MHNW *vs* MUHOW/O
p value [b] MHNW *vs* MHOW/O
p value [c] MHOW/O vs MUHOW/O
p value [d] MHNW vs MUHNW
p value [e] MUHNW vs MUHOW/O
p value [f] MUHNW vs MHOW/O
p value [g] Metabolic syndrome vs No metabolic syndrome
MHNW, metabolically healthy normal weight; MUHNW, metabolically unhealthy normal weight MHOW/O, metabolically healthy overweight/obese; MUHOW/O, metabolically unhealthy overweight/obese. Q75: Upper quartile. Q25: lower quartile; NCEP-ATPIII: National Cholesterol Education Program – Adult Treatment Panel III; NS = not significant

Table 5.3: Binary logistic regression analysis between mtDNA copy number and Met S, its individual components, the metabolically unhealthy phenotype* and HOMA-IR

	Overall			Males			Females		
Dependent variable**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Metabolic syndrome	1.05	1.02-1.09	**0.002**	1.04	1.01-1.09	**0.040**	1.06	1.01-1.12	**0.011**
Increased triglycerides	1.04	1.01-1.08	**0.015**	1.02	1.00-1.05	**0.010**	1.05	0.99-1.11	0.075
Increased FPG	1.02	1.00-1.05	0.094	1.02	0.98-1.06	0.258	1.02	0.98-1.06	0.287
Hypertension	1.02	1.00-1.04	0.102	1.03	0.99-1.07	0.111	1.01	0.99-1.04	0.113
Increased waist circumference males	-			1.01	0.97-1.05	0.561	-		
Increased waist circumference females	-			-			1.07	1.04-1.11	**0.010**
Reduced HDL-C males	-			1.03	0.98-1.08	0.257	-		
Reduced HDL-C females	-			-			1.03	0.99-1.06	0.184
Metabolically unhealthy phenotype	1.04	1.02-1.09	**0.039**	1.07	1.03-1.12	**0.03**	1.04	1.02-1.20	**0.04**
HOMA-IR ≥2.5	1.03	1.01-1.07	**0.029**	1.01	0.97-1.06	**0.06**	1.06	1.01-1.10	**0.016**

Odds ratios are given for a ten-fold reduction in mtDNA copy number in the overall cohort and stratified by gender.
**Adjusted for age
*Based on the empirical definition by Zembic et al.
FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance.

This table shows the results of binary logistic regression analysis adjusted for age. mtDNA CN was inputted as the independent predictor variable and Met S / its individual components and the metabolically unhealthy phenotype were the outcome (response) measures assessed.

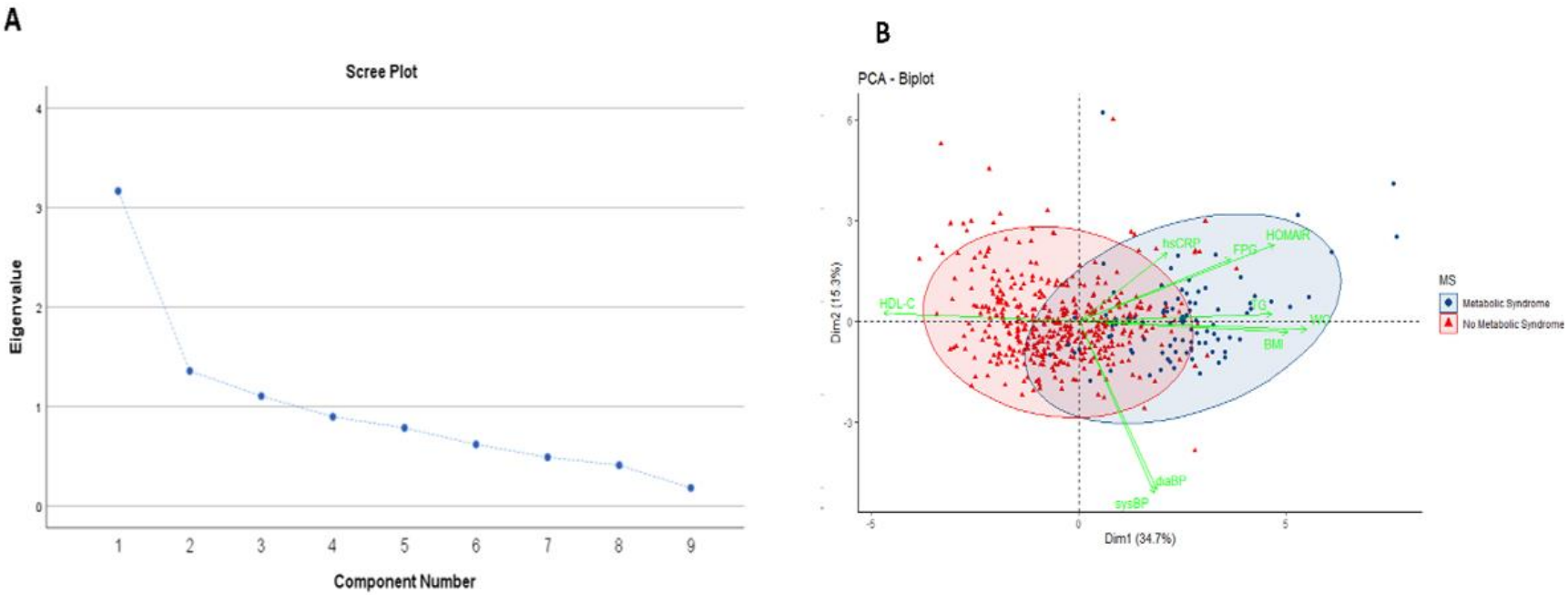


Figure 5.3: Scree plot and PCA biplot

A: Scree plot chart showing eigenvalue against all factors. Three factors have eigenvalues >1, and collectively explain 62.5% of the cumulative variance in the dataset. **B**: PCA biplot shows individual observations as datapoints, coloured according to presence or absence of metabolic syndrome. Points are plotted on a plane formed by the first two principal components. The original variables are shown as green vectors from the origin. The orientation of the vector with respect to the principal component space represents its contribution to the PC.

Table 5.4: Factor loading matrix for adiposity/metabolic parameters

Variable	PC1	PC2	PC3
Waist circumference (cm)	**0.906**	0.069	0.189
Fasting plasma glucose (mmol/l)	-0.015	0.045	**0.833**
HDL-C (mmol/l)	-0.564	-0.146	-0.384
Triglycerides (mmol/l)	0.349	0.182	**0.611**
Body Mass Index (kg/m^2)	**0.895**	0.054	0.1
Systolic BP (mmHg)	0.093	**0.806**	0.037
Diastolic BP (mmHg)	0.09	**0.801**	0.042
HOMA-IR	0.258	-0.018	**0.808**
hs CRP (mg/l)	0.222	-0.231	0.325

Rotated component matrix derived from varimax rotation with Kaiser normalisation. Rotation reduces the number factors on which the variables under investigation have high loadings. The loadings represent correlations between each factor and the PC. PC1 is characterised by BMI and WC, PC3 is characterised by FPG and HOMA-IR and PC2 by systolic and diastolic blood pressure.
PC, principal component; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; hs CRP, high sensitivity C-reactive protein; PC

Table 5.5: Regression estimates for each of the three Principal Components derived from PCA

Dependent variable*	β	95%CI	p value
PC1 (adiposity)	-0.03	(-0.04) - (-0.02)	**<0.001**
PC3 (insulin resistance)	-0.01	(-0.02) - (0.001)	**0.016**
PC2 (hypertension)	-0.006	(-0.01) - (0.01)	0.904

PC1 represents adiposity, PC3 insulin resistance and PC2 hypertension. PC1 and PC3 showed a strong significant inverse association with mtDNA CN. Regression coefficient β is reported for an increase in 10 mtDNA copies.*Adjusted for age and gender.

PC, principal component

Chapter 5B – Discussion of molecular studies

5-5 Reduced peripheral blood leukocyte mitochondrial DNA copy number in Met S and MHO

This cross-sectional study explores the relationship between mtDNA CN, obesity, the Met S as well as its constituent components within an island population that bears a high burden for obesity, T2DM and cardiometabolic disease (Cuschieri et al., 2016a; Cuschieri et al., 2016b). Effectively, the findings of this research show that after adjustments for traditional clinical covariates and blood cell composition as potential confounders a significant inverse association between peripheral blood mtDNA abundance and Met S was observed. More importantly, the obese state was associated with a reduced mtDNA CN compared to the healthy normal weight participants , and this reduction was present in both metabolically healthy and unhealthy subtypes of obesity thus implying that mtDNA CN may have limited utility as a biomarker in stratifying these two subtypes of obesity. These findings are relevant and new to a regional Southern European Island population and to date is the first study that directly aimed to evaluate the association between mtDNA CN and metabolically healthy and unhealthy subtypes of obesity as outcomes.

5-5.1 Association between mtDNA CN, Met S, and its constituent components

In this study, peripheral blood mtDNA CN was found to be inversely correlated with cardiometabolic risk parameters including adiposity (BMI, WHR, WC), glycaemic (HBA_{1c}, FPG), and lipid (TG) indices as well as HOMA-IR (a marker of insulin resistance). Furthermore, the leukocyte mtDNA CN decreased as the number of diagnostic

components of the Met S increased, and a reduced mtDNA CN was an independent predictor of prevalent Met S and the metabolically unhealthy phenotype.

Broadly, the outcomes of this study are consistent in direction and magnitude with other investigations which report a similar association between reduced mtDNA CN and the presence of several cardiometabolic risk factors and CVD supporting the concept of mtDNA CN as a promising biomarker both in the prevention as well as in monitoring of metabolic and cardiovascular health status in humans (Ashar et al., 2017; Bordoni et al., 2021; Castellani et al., 2020). Moreover, other smaller-scale studies similarly reported a depleted leukocyte mtDNA CN in individuals with the Met S (Huang et al., 2011; Kim et al., 2012). A recent study which included just over four hundred thousand participants of multiple ancestries from the TOPMed Consortium and UK Biobank, found that reduced mtDNA CN levels to be an independent predictor for obesity ($p = 5.6 \times 10^{-238}$), hypertension ($p = 2.8 \times 10^{-50}$), T2DM ($p = 3.6 \times 10^{-7}$) and dyslipidaemia ($p = 6.3 \times 10^{-5}$) (Liu et al., 2021b). The authors thus postulate that since these cardiometabolic diseases are associated with the presence of the Met S and the development of atherosclerotic CVD disease, for which IR is a fundamental pathophysiological abnormality, then, a decrease in mtDNA quantity may be the core contributing mechanism leading towards the onset of IR in these individuals. In accordance with this reasoning, the authors also observed a lower mtDNA CN to be predictive of a HOMA-IR value >2.5. On the other hand, and contradicting these findings is the study by Guyatt et al., which included approximately five thousand participants recruited from two large cohorts of European females. Whilst they were able to adjust for the presence of a wide range of confounding variables (including age at sampling, sociodemographic factors, laboratory covariates and

haematological parameters) the authors acknowledged they could not find any consistent evidence to support a relationship between mtDNA CN and the presence of several cardiometabolic traits save for an inverse association with insulin in older participants ($p = 0.002$). This prompted the investigators to speculate that mtDNA CN may not be as important a predictor for cardiometabolic risk as previously thought (Guyatt et al., 2018).

Insulin resistance is the hallmark for diseases such as T2DM and the Met S (Khan et al., 2006; Meigs et al., 2007; Fahed et al., 2021). However some authors have suggested that hyperinsulinemia may be the primary abnormality. For example, when studying a human liposarcoma cell line, Fernandez –Velodo et al., found that hyperinsulinemia deregulates adipocyte secretion pattern, producing insulin resistance in adipocytes and myocyte (Fernandez-Veledo et al., 2008). Furthermore, van Vliet et al. reported that basal and postprandial insulin secretion rates were greater in people with obesity than lean people even though insulin sensitivity was not different between groups and that weight loss decreased insulin secretion in the absence of changes insulin sensitivity (van Vliet et al., 2020). On the other hand, data from a study in high fat diet-fed Sprague-Dawley rats suggests that although obesity induces compensatory hyperinsulinemia, it is the hyperinsulinemia rather than insulin resistance that causes blood pressure elevation (Wang et al., 2020). The relationship between mitochondrial dysfunction and IR has been extensively investigated over the last two decades with several studies showing a directionally consistent relationship between lower peripheral blood mtDNA CN and markers of IR and glycaemic control (Fazzini et al., 2021; Huang et al., 2011; Kim et al., 2012; Xu et al., 2012). Chronic hyperglycaemic states induce excessive production

of ROS which cause damage to mtDNA, proteins and lipids leading to decreased activity of respiratory chain complexes and increased susceptibility to oxidative damage, further ROS production and impaired fuel oxidation. Defective fatty acid beta oxidation leads to the accumulation of intracellular lipotoxic lipid metabolites (including ceramides and diacylglycerol) in non-adipose cells and several researchers agree that this may be the mechanistic link between mitochondrial dysfunction and abnormalities of insulin signalling in classical insulin target tissues (Gao et al., 2010; Schrauwen et al., 2010; Sergi et al., 2019).

Another important observation to consider is that while the Met S (as defined by NCEP-ATPIII) consists of five interrelated metabolic parameters which essentially exhibit collinearity due to overlap in their aetiopathological mechanisms, yet they should not be considered as mutually exclusive traits. Insulin resistance is a core component of the Met S and considered to be one of the main drivers for this syndrome as well as for its individual components, the presence of which has been associated with increased cardiovascular risk (Mottillo et al., 2010). However, the strength of the link between IR and the Met S has been questioned in the face of studies showing that the Met S itself does not predict cardiovascular risk better than the sum of its individual components (Cheal et al., 2004; Kahn, R., Buse, J., Ferrannini, E., & Stern, 2005). Furthermore, stratifying obesity phenotypes based on the number of NCEP-ATPIII components assumes arbitrarily that the risk factors are metabolically equivalent. This generalisation may be inaccurate since even mild elevations in plasma glucose lead to secondary effects via alterations in lipid metabolism and in the generation or ROS and proinflammatory cytokines which may explain the increased risk of CVD observed in

these individuals (Ganda et al., 1985; Stentz and Kitabchi, 2005). Thus, the presence of abnormal values for any one of the five components should be an impetus to institute the relevant therapeutic intervention regardless of whether the NCEP ATPIII diagnostic criteria have been met or not. In fact, some studies detected subtle but significant differences in cardiometabolic risk factor parameter values (such as larger WC and higher blood pressure, insulin, and triglyceride levels) between metabolically healthy obese and metabolically healthy normal weight participants despite both being categorised as healthy using the same criteria and cut-off points (Calori et al., 2011; Karelis, 2008; Marini et al., 2007). Furthermore, the Met S was only able to predict incident CVD in 20% of normal weight individuals, clearly showing that it may be inadequate to accurately stratify disease risk within this cohort of patients and that its absence does not automatically imply cardiovascular protection (Lassale et al., 2018). In the present study, out of all the metabolic components, a reduced mtDNA CN was only predictive of increased TG in males and increased WC in females, lending further support to the notion that each individual Met S component varies both in terms of utility in establishing a diagnosis of the Met S, in their relationship with IR and there are sex differences in these associations. Additionally, the notion of MH as being a static concept may also underestimate the long-term adverse effects of weight gain from baseline and does not provide any insight into obesity-related comorbidities nor their prognosis (Alley and Chang, 2010; Neeland et al., 2018).

Moreover, some researchers observed associations of mtDNA CN levels with age. For example, Xu et al. found that age of onset of diabetes to be a predictor of mtDNA content; furthermore, the recent study by Liu and colleagues observed a threshold

effect of age on mtDNA CN. They found that in individuals who were 65 years or older, increasing age was associated with larger declines in mtDNA CN, whereas those who were 65 or younger, increasing age was associated with higher levels of mtDNA CN (Liu et al., 2021b; Xu et al., 2012). Ageing is known to be associated with a decline in mtDNA content and function in several tissues which may contribute to the development of common chronic diseases typically associated with ageing (Castellani et al., 2020). In this study no significant correlation with age was observed. Thus, the findings from this research merit critical interpretation in the context of the study cohort characteristics. The population within this study comprised a carefully phenotyped and homogeneous cohort of middle-aged adult individuals. This thus contrasts with the older and broader age range reported in the literature on mtDNA CN and Met S. The choice for middle-aged individuals as a selection criterion for this research was such so that the population would have lived long enough for phenotypic expression while minimizing the risk of survival bias and therefore underestimation of effect size. Furthermore, sarcopenic obesity, defined as the age-related decline in muscle mass coupled with increased adiposity and IR is less common in this age group (Roh and Choi, 2020). These factors underscore the clinical relevance of utilising a carefully selected age group when evaluating associations between mtDNA CN and metabolic outcomes.

5-5.2 Relationship between mtDNA CN and the different body composition phenotypes

A novel finding from this investigation is that the difference in relative mtDNA CN between the MHOW/O and MUHOW/O phenotypes did not exceed the statistical significance threshold, therefore this data suggests that mtDNA CN is related to overall

adiposity. While no difference in mtDNA CN was observed between the 2 subtypes of obesity, the difference in mtDNA CN observed between individuals with the metabolic syndrome vs those without could be in part explained by how the metabolic syndrome is defined: According to the NCEP ATPIII criteria an individual requires the presence of 3 or more metabolic risk factors to be present in order to be categorised as having Met S. This research thus provides additional perspective into obesity-associated mitochondrial dysfunction and reinforces the challenges of risk stratification of this complex trait.

As detailed previously, individuals with metabolically healthy obesity characteristically demonstrate preserved insulin sensitivity as well as a favourable cardiometabolic profile, reduced incidence of T2DM compared to individuals with unhealthy obesity and similar risks for CVD compared to MHNW individuals (Karelis, 2008; Meigs et al., 2006; Stefan et al., 2013). In accordance with this, some large prospective studies which used a strict definition of MH (absence of all components of the Met S) and more recently the study by Zembic *et al*., (which characterised individuals with MHO based on an a priori definition composed of cardiometabolic parameters known to be associated with total and CVD mortality) observed no increased risk in CVD and total morality (Al-khalidi et al., 2018; Eckel et al., 2016; Zembic et al., 2021). However, contradicting these findings is the landmark study by Caleyachetty et al., which investigated the relationship between MHO and incident cardiovascular disease in over 3.5 million individuals. They observed that the presence of obesity (even in the absence of metabolic abnormalities) was associated with higher risk of cardiovascular disease (including coronary heart disease, cerebrovascular disease and heart failure) when compared to healthy normal weight individuals (Caleyachetty et al., 2017).

Therefore , this model is not without its controversies, and a strong unresolved debate centres round whether 'obese but healthy' individuals are truly free of adverse cardiovascular outcomes (Loprinzi and Frith, 2017). This study shows that obesity, regardless of whether it is accompanied by metabolic abnormalities or not, is associated with reduced mtDNA CN thus lending further support to the notion that a degree of mitochondrial dysfunction is present even when obesity is separated from its usual metabolic consequences. This finding is broadly congruent with evidence from meta-analysis showing that even in the absence of metabolic abnormalities, individuals living with obesity are still at increased risk of adverse long term clinical outcomes (Caleyachetty et al., 2017; Eckel et al., 2018; Kramer et al., 2013). Clinically, the observations from this study keep underscoring the importance of regulating body weight even in the absence of the Met S. Furthermore, the binary characterization of obesity into metabolically healthy vs unhealthy subtypes bypasses important but confounding factors including cardiorespiratory fitness, which has shown to explain both all-cause and cardiovascular mortality in obesity (Barry et al., 2014; Ortega et al., 2013).

More importantly, one must also consider that body composition phenotypes based on anthropometric indices do not identify the location of fat depots which requires imaging-based assessment (Neeland et al., 2013, 2012). Differences in fat distribution patterns (for example subcutaneous vs visceral fat depots) are associated with different cardiometabolic disease risk across the BMI continuum. Visceral or central fat deposition is known to be more detrimental to MH compared to peripheral fat depots (Stefan, 2020a). In fact, increased adipose tissue distribution within peripheral subcutaneous

(gluteo-femoral) regions is associated with lower cardiometabolic risk independent of precisely measured markers of visceral fat mass particularly in lean individuals (Schulze, 2019; Stefan, 2020b; Stefan et al., 2017). One meta-regression analysis found both WC and the WHR (which are indices reflecting central adiposity) to be associated with increased incidence of CVD in both sexes (De Koning et al., 2007). The Brazilian Longitudinal Study of Adult Health found an association between a higher lower limb to trunk ratio and a reduced 10-year cardiovascular risk. This association was found to be mediated by lower systolic blood pressure and total cholesterol, and increased HDL-C levels (Christiansen et al., 2021; Oliveira et al., 2011). Central abdominal (visceral) fat depots are composed of dysfunctional hyperplastic adipocytes characterised by a pro-inflammatory adipokine secretion pattern, and a higher turnover of bioactive lipids. On the other hand, lower subcutaneous (gluteo-femoral) fat depots expand through hyperplasia (rather than hypertrophy) during periods of positive energy balance to accommodate fat undergoing redistribution and thereby offer protection against overspill of fat into ectopic sites (Badoud et al., 2015; Teixeira et al., 2015). Gluteo-femoral fat depots are also associated with a lower rate of lipid turnover and are less inflammatory, and thus are deemed to have a lower detrimental effect on MH (Karpe and Pinnick, 2015; Schulze, 2019). Therefore, the clearcut categorisation of body phenotypes as being either metabolically healthy or unhealthy obese based on BMI cut-offs and presence/absence of Met S can create a false dichotomy, since both cardiometabolic disease and obesity are dynamic states along the pathophysiological continuum. For example the risk for CVD increases in a stepwise manner with increasing fasting glucose values within the prediabetes range or with increasing blood pressure

measurements within a range considered to be normal (Emerging Risk Factors Collaboration et al., 2010; Vasan et al., 2001).

In keeping with this research's findings are a handful of other studies which also found levels of mtDNA CN to be similarly reduced in peripheral blood and adipocytes from individuals living with obesity. Furthermore, mtDNA CN was found to be inversely associated with several adiposity-related anthropometric and body composition variables (such as BMI, WC, WHR, WHtR, visceral fat area, body cellular mass [BCM] and phase angle [PhA]) (Bordoni et al., 2022, 2019; Kaaman et al., 2007; Lee et al., 2014a; Meng et al., 2016; Mengel-From et al., 2014; Zheng et al., 2015a). For example, Zheng and co-workers found that levels of mtDNA CN in peripheral blood to be 6.9-fold lower compared to normal weight individuals while Meng and colleagues also observed a bi-directional and inverse association between mtDNA CN and weight gain suggesting a bi-directional relationship between oxidative stress and weight change (Meng et al., 2016; Zheng et al., 2015a). Building on the dynamic nature of mtDNA is one study which found a sex-specific variation in peripheral blood mtDNA CN in individuals before and after bariatric surgery (Skuratovskaia et al., 2019a). However, directionally inconsistent associations between obesity and mtDNA CN within various adipose tissue depots have also been observed. Lindinger and colleagues demonstrated a significantly higher mtDNA content in the omental tissue of individuals with a BMI >30 kg/m^2 compared to normal weight individuals, and it was not associated with either basal metabolic rate or fat oxidation rate. On the other hand, the study of Skuratovskaia and co-workers did not find any significant differences in mtDNA CN in omental or mesenteric adipose tissue in people with obesity versus healthy controls, but BMI was positively correlated with

mtDNA abundance in SAT and negatively with mtDNA copies in peripheral blood (Lindinger *et al.*, 2010; Skuratovskaia *et al.*, 2018). Thus, the high variability in mtDNA CN observed in several tissues and the dependence of the BMI on specific fat depots imply that the multidirectional dynamics of mtDNA content may be a reflection of the unique inherent pathological processes in each type of tissue studied. Another study showed that participants with abdominal obesity and T2DM, mtDNA CN was higher in various fat depots compared to normal weight controls or to individuals with obesity but without T2DM, but obesity (irrespective of whether accompanied by T2DM or not) was positively associated with levels of leptin and proinflammatory cytokines (IL-6, IL-8, and TNF-α) (Litvinova et al., 2019). Recently, Bordoni and colleagues found that after adjusting for age, sex, and diagnosis of diabetes an inverse association between BMI and mtDNA CN in omental adipose cells was observed (Bordoni et al., 2022; Lindinger et al., 2010; Skuratovskaia et al., 2018).

Apart from changes in mtDNA count, increasing evidence suggests that accumulation of body fat is also associated with abnormalities of mitochondrial oxidative function in subcutaneous adipose tissue. Furthermore, other studies were able to demonstrate that *in-vitro* depletion of mtDNA reduces expression of several mitochondrial proteins involved in oxidative pathways including fatty acid oxidation, TCA cycle, ketolysis and ketogenesis as well as branched chain amino acid degradation (Jeng et al., 2008; Lee et al., 2019). Interestingly, one study found abnormalities of mitochondrial enzyme activity such as reduced citrate synthase activity (a citric acid cycle enzyme) but a higher cytochrome *c* oxidase (COX) activity (a respiratory chain enzyme) in lymphocytes of individuals with obesity compared to healthy controls. This may be due to obesity-

associated changes in proportions of respiratory chain and Krebs cycle enzymes supporting the view that mitochondrial oxidative capacity may be increased in obese states favouring energy conservation via the generation of ATP rather than energy dissipation in the form of heat. Such metabolic efficiency may further propagate weight gain in response to positive energy balance and may negatively affect weight loss in response to energy deficit (Čapková et al., 2002). On the other hand, studies which looked at the relationship of BMI with several variables associated with respiratory control in mitochondria from subcutaneous adipose tissue found an inverse association of BMI with ATP-linked mitochondrial respiratory capacity. Furthermore, this relationship was potentially mediated by a reduced mtDNA CN and a decreased protein expression of complex I and IV components of the electron transport chain in individuals living with obesity. This led authors to hypothesize that while adipocyte dysfunction in VAT is notoriously associated with the presence of obesity-related metabolic complications, human white SAT may also be contributing towards adipocyte dysfunction by impairing mitochondrial respiratory capacity (Fischer et al., 2015). Additionally, another study showed that high-fat diet-fed mice developed obesity, hepatic steatosis and IR and concomitantly also displayed reduced hepatic mitochondrial respiratory capacity and increased oxidative stress and efficiency, suggesting that alterations within the mitochondrial compartment could occur in response to long term high-fat feeding and which could potentially be responsible for the development of obesity and the other metabolic sequalae observed (Raffaella et al., 2008).

Abnormalities of mitochondrial biogenesis have also been demonstrated from both human and mouse adipose tissue with acquired obesity. Accordingly, a decrease in mitochondrial mass was observed in white adipose tissue of genetic mice models of obesity (Rong et al., 2007). Furthermore, a widespread reduction in the expression of both mitochondrial-and nuclear-encoded genes associated with mitochondrial biogenesis was observed in SAT of people living with obesity s compared to their leaner co-twins. Concomitantly a downregulation of mitochondrial proteins associated with oxidative ATP production and oxidative catabolic functions were also observed (Heinonen et al., 2015). Similar findings were also detected from VAT depots in studies from both humans with obesity as well as in animal models. One study found that a high-fat diet in rats led to the down regulation of adipose tissue mitochondrial proteins (including reductions in cytochrome *c*, COX IV and PGC-1α expression) which are indicative of mitochondrial dysfunction and which subsequently led to the development of glucose intolerance (Gómez-Serrano et al., 2017; Sutherland et al., 2008).

Furthermore, recent genome wide association scans have identified several independent loci that regulate mtDNA CN; thus it is also important to take into account the population genetic element within the context of mtDNA CN (Guyatt et al., 2019; Longchamps et al., 2022). Moreover, mtDNA CN also correlates with environmental factors including fine particulate matter ($PM_{2.5}$) and components of the built environment (Z. Li et al., 2018; Zhao et al., 2020). The Maltese population has an alarmingly high prevalence of obesity that is compounded by an 'obesogenic' environment and a limited infrastructure for active living (Cauchi et al., 2015). It is thus essential that when undertaking molecular epidemiological studies to take into

consideration these environmental factors and how they impact on mtDNA CN and disease risk.

Collectively, these studies keep providing additional evidence of the tight association between aberrations of mitochondrial bioenergetics and obesity. Yet, despite the well documented associations, a direct cause-effect relationship between mitochondrial function and obesity still remains a matter of debate and further studies are required to understand the underlying mechanisms responsible for impaired mitochondrial function in the context of obesity and its associated metabolic disorders. Future studies should also explore the directionality of any causal relationship.

5-6 Strengths and limitations

The findings from this research are novel and for the first time demonstrate that mtDNA CN does not differ between metabolically healthy and unhealthy individuals with obesity . This lends further support to the idea that mitochondrial function (as assessed by a reduction in mtDNA CN) is impaired in obesity even when it is separated from its usual metabolic consequences. Furthermore, these findings are consistent across several definitions of MH and thus reiterate that there is no healthy pattern of weight gain. This study is strengthened by the use of a well-phenotyped and adequately sized representative cohort of middle-aged adults encompassing a narrow age-range. This thus excludes the potential of survival bias as well as age-related changes in muscle mass (such as sarcopenic obesity) and function as well as changes in fat distribution patterns (Kuk et al., 2009). Standard methods for data collection and for defining MH were used as already validated in previous studies. Furthermore, the same DNA extraction method was used in all participants. This is relevant since it has been shown that DNA isolation methods influence mtDNA content measurement (Fazzini et al., 2018). The analysis was also adjusted to correct for differences in both amplification efficiency and blood cell type composition. Importantly, the abundant quantities of platelet mitochondria are known to artificially skew mtDNA CN, thus correcting for cell composition is considered essential for interpretation (Knez et al., 2016; Urata et al., 2008). All measurements were performed by the author in a single laboratory, with random allocation of participants to minimize batch effects. To further limit the risk for possible pre-analytic effects, due care was taken to harmonise phlebotomy, sample transport as well as storage conditions.

This research also acknowledges a number of limitations. Primarily its cross-sectional design limits the evaluation of the interaction between mtDNA CN and body composition phenotypes along the developmental trajectory to CVD endpoints. mtDNA CN was inputted as an exposure variable driving metabolic outcomes, an approach which has also been adopted by other researchers (Memon et al., 2021). Thus, it is understood that no causal direction can therefore be robustly inferred from this analysis, and that a reverse causation could also be a possibility. Fazzini and co-workers applied a mediation analysis approach which showed that a major proportion of the effect of mtDNA CN on T2 DM was accounted for by obesity parameters (Fazzini et al., 2021). While the association between IR, obesity and defects in mitochondrial function and structure are now well recognised, it is still unclear whether these changes are mechanistically primary or secondary and thus future longitudinal studies are required to truly clarify whether mitochondria are the perpetrators of these disease states.

This research involved the quantification of mtDNA CN extracted from total peripheral blood leukocytes which are a heterogenous cell population and thus can dilute biological effect sizes. In effect, studies which investigated the relationship between mtDNA CN and white blood cell composition and platelet count found that it varied by leukocyte subtype. Accordingly, most studies found an inverse association with total WBC count and neutrophil count while positive associations were observed for proportions of lymphocytes, monocytes and platelets count implying that overall leucocyte composition may be a confounder in the association between mtDNA CN and several disease traits (Guyatt et al., 2018; Liu et al., 2021b). Although mtDNA CN was adjusted for total platelet count in this study, adjusting for white cell subpopulations was not

possible which might have been a potential source of bias. Furthermore, estimates of mtDNA CN levels obtained from peripheral blood leukocytes may not be directly extrapolated to other more physiologically relevant target tissues such as adipocytes, skeletal myocytes, and hepatocytes. However, obtaining tissue samples such as myocytes or adipocytes requires an invasive approach which makes it less feasible for epidemiological studies. On the other hand, peripheral blood has the advantage of being easily accessible and entails a more acceptable approach for analysis. Nonetheless, the study by Huang et al. showed that in participants with heart failure , peripheral blood mtDNA CN correlated strongly with human cardiac myocytes (Pearson's r = 0.718, p = 0.019) and therefore is more likely to reflect MH across other human tissues of interest.

Additionally, this investigation centred only on mtDNA CN as the focus of mitochondrial dysfunction and did not factor in other measures of mitochondrial activity or other molecular elements which could impact on mitochondrial bioenergetics and disease and which, in turn, could have led to different outcomes in this study. However, while mtDNA CN is not a direct measure of mtDNA damage, it is associated with mitochondrial enzyme activity and ATP production such that a reduction in mtDNA CN is associated with mitochondrial dysfunction making it a validated tool for assessing mitochondrial function (Malik et al., 2013). Furthermore, it is quantified using relatively low-cost scalable assays (such as qPCR) allowing for rapid determination of cellular mitochondrial content in a large number of samples and hence serves as a readily available biomarker of mitochondrial function in clinical practice (Jeng et al., 2008; Ashar et al., 2017). To date a variety of other methods are available to examine

mitochondrial function including changes in mRNA levels of mitochondrial markers using microarray approaches, measurements of protein subunit levels of respiratory chain complexes via immunoblotting techniques, assessment of enzymatic activity of key components of mitochondrial oxidation (such as citrate synthase activity and cytochrome c oxidase activity, changes in mitochondrial shape/size (using electron microscopy), measurement of oxygen consumption rate as well as substrate oxidation studies (calorimetry studies) (Montgomery and Turner, 2015). Accordingly, while a number of studies conducted in mice and humans with T2 DM or obesity observed impairment of mitochondrial functional capacity (as assessed by several of the different methods described above) in skeletal muscle and adipocytes others failed to find an association (Kelley et al., 2002; Choo et al., 2006; Nair et al., 2008; Litvinova et al., 2019; Phielix et al., 2008). Moreover, studies which assessed mtDNA haplotypes and sequence variation (including single-nucleotide polymorphisms [SNPs] such as the 4977bp deletion and structural variants) in relation to a variety of cardiometabolic endpoints also observed conflicting associations (Chinnery et al., 2010; Corral-Debrinski et al., 1992; Nardelli et al., 2013). Therefore, in light of the inconsistencies found in the literature regarding mitochondrial function, IR and adiposity, future larger and well-powered studies utilizing different methods for assessing mitochondrial function should be conducted in different populations in order to fully elucidate the relationship between mitochondrial function and the different body composition phenotypes.

The environmental and lifestyle determinants of mtDNA CN are poorly understood with several factors being implicated (Zhao et al., 2020). Importantly, interactions between certain medications and mtDNA content may also introduce bias for example statins

have pleiotropic immunomodulatory effects which can negatively impact mitochondrial function and mtDNA CN (Mollazadeh et al., 2021). This study also did not factor in data on pro-inflammatory cytokines, diet, imaging-based assessment of visceral adiposity or cardiorespiratory fitness. Obesity was defined by BMI thresholds which could have misclassified individuals of a short stature or muscular build. Moreover, no uniform definition of MH exists to date in the scientific literature (Rey-López et al., 2014). This study recruited premenopausal females only. The MHO phenotype is known to be highly prevalent in premenopausal females, however MHO is not a static trait and a transition to the metabolically unhealthy phenotype has been observed in longitudinal studies as part of the natural course of obesity. (Blüher, 2014; Eckel et al., 2018). Thus, future studies investigating the relationship between mtDNA CN and disease transition are required. The metabolically unhealthy normal weight phenotype was under-represented and unequal sex representation restricts interpretation of the findings within these cohorts.

Deriving meaningful comparisons to identical studies is also challenging, particularly in view of heterogeneity in patient ascertainment criteria especially in those used to define MH, variation in background prevalence of obesity and differences in study design (cross- sectional vs longitudinal). Furthermore, cohort-specific aspects such as sociodemographic factors including education level, physical activity and economic status also impact on the risk and progression of metabolic outcomes and can also potentially be a source of unaccounted for confounders. In this study mtDNA CN was determined using a qPCR technique which generates relative measures of mtDNA CN (as opposed to absolute measurements derived from other techniques such as digital

PCR) (Castellani et al., 2020). Moreover, accurate and replicable quantification of mtDNA CN and the lack of integration of mtDNA CN with multi-omic datasets which capture the genomic, proteomic and metabolomic landscape of metabolic disease further constraints comparisons across other studies.

Conclusion and future recommendations

The findings from this study reinforce the association between reduced leukocyte mtDNA CN, obesity, and Met S. While caution should be taken when making inferences on direction of causality, this study adds to the pathophysiology of obesity given that excess adiposity, even in the absence of metabolic abnormalities is associated with a reduction in mtDNA CN. Moreover, the distinction between healthy and unhealthy obesity may not be directly explained by molecular changes at the level of the mitochondria. Thus, the role of mtDNA CN in the stratification of different obese phenotypes requires further evaluation. This study should be replicated in insulin sensitive- tissues to further assess the role of this biomarker in obesity and cardiometabolic risk classification. Furthermore, future efforts should focus towards standardising the definition of MH, assessing the direction of the association between mitochondrial dysfunction and excess adiposity and metabolic disease through longitudinal studies as well as to elucidate further the underlying molecular determinants of healthy and unhealthy obese phenotypes.

Chapter 6 - Summary of main findings and clinical implications

6-1 What is already known about the topic?

Obesity is generally accompanied by a cluster of metabolic abnormalities which increase cardiometabolic disease risk. It is now evident that obesity is a heterogenous disease with diverse phenotypes, thus generating a spectrum of 'obesities'. One such phenotype is called 'metabolically healthy obese' (MHO) and is characterised by a subset of individuals who, despite exhibiting increased amounts of total fat mass, present a high level of insulin sensitivity, a favourable metabolic and inflammatory profile, and preserved adipose tissue dynamics (including selective expansion of subcutaneous gluteofemoral adipose tissue through hyperplasia, reduced levels of visceral and ectopic fat and lower adipose tissue inflammation and fibrosis) compared to the metabolically unhealthy obese (MUHO) phenotype. Thus, MHO individuals are deemed to be at lower cardiometabolic disease risk. At the other end of the spectrum are normal weight individuals who display an excess and dysfunctional adiposity level similar to that observed in obese states and are thus at higher risk for cardiometabolic diseases. These individuals are called the metabolically unhealthy normal weight (MUHNW). Notably, one of the main traits of this phenotype is the presence of a low level of gluteofemoral fat mass as opposed to an elevated amount of visceral and liver fat which is typical of the MUHO phenotype. Furthermore, the MUHNW phenotype is also associated with insulin secretion failure and IR, increased carotid intima-media thickness (cIMT) and an adverse cardiometabolic and inflammatory risk factor profile akin to individuals with unhealthy obesity **(Figure 6.1).**

Globally epidemiological studies show that as many as 35% of individuals living with obesity may be MHO while approximately 30% of normal weight adults are

metabolically unhealthy. To date, no uniform standardised criteria exist to describe MHO and MUHNW, with many authors using different combinations (and cut-points) of cardiometabolic risk factor parameters and/or insulin sensitivity to define MH. Furthermore, long term outcomes of the MHO phenotype are conflicting, particularly those relating to CVD risk and T2 DM. Several studies point towards MHO as presenting an intermediate state of risk between that of healthy normal weight and unhealthy obesity, while those studies pertaining to MUHNW show that its' risk probably lies somewhere between MHO and MUHO. Recently, analysis of the UK Biobank dataset showed that individuals with 'MHO' have modest alterations in their cardiometabolic risk factor parameters when compared to healthy normal weight people . For example, they displayed higher blood pressure and HbA_{1c} values and a worse lipid profile than MHNW individuals and this was despite the greater use of lipid-lowering and antihypertensive medications in the MHO group. Compounding this are studies which made an in-depth analysis of the micro-metabolic milieu of the MHO phenotype. These observed subtle differences in micro level indices compared to healthy normal weight individuals even though they were characterised as 'healthy' using standard screening criteria. Accordingly, abnormalities in fat distribution patterns (higher visceral adiposity levels) and lipid metabolism, oxidative stress and chronic inflammation, as well as aberrations in small molecule metabolites (omics), were observed, which could in part explain the increased CVD risk found in individuals with MHO . Moreover, the lack of a standardised definition for MH is a recurring concept surrounding the differences observed both in prevalence as well as in CVD outcomes for the MHO phenotype. This is evidenced by the fact that several definitions allowed for the presence of one (or

sometimes even more) metabolic abnormalities and thus may not truly reflect a 'healthy' obese state. Additionally, researchers did not factor in regional adiposity (most studies used BMI rather than indices of central obesity measurement or cross-sectional imaging) to assess nutritional status and moreover did not properly control for key confounding factors such as dietary quality and quantity and cardiorespiratory fitness, both of which are known to modulate adipose tissue dynamics as well as metabolic function. These observations suggest:

- Contemporary definitions for MH may be too crude and currently used cut-points for several cardiometabolic parameters may need to be revised downwards in order to identify those individuals who are truly metabolically healthy and, possibly, at a similar cardiometabolic disease risk to that of MHNW.
- MHO is generally considered to be an unstable entity and as many as 50% of MHO individuals transition to develop more severe alterations in their cardiometabolic risk profile over time.
- Evaluating outcomes relating to CVD risk and morality based on a single assessment of MHO may lead to an underestimation of the true risk associated with this phenotype.
- Given that MHO is a transient phenotype associated with increased risks for several cardiometabolic diseases, identification of individuals who are in a 'MHO' state provides a window of opportunity for the timely implementation of effective preventive strategies (via different modalities such as lifestyle, pharmacological and/or surgical interventions) which would deter from further weight gain and/or transition to a metabolically unhealthy state as well as the

institution of early treatment paradigms should metabolic abnormalities (or other complications) occur. This in turn, would be expected to result in reduced medical and seriocomic costs associated with the management of obesity and obesity-associated comorbidities from a public health standpoint as well as reduce risk for premature morbidity and mortality at an individual level.

- Metabolic health is also deemed to be unstable within the normal weight BMI range. In fact, around 27% of healthy lean individuals develop at least one metabolic abnormality over a period of 10 years, converting them into the unhealthy phenotype. Furthermore, many mechanistic studies observed the MUHNW cohort to be 'fatter' (such as having a larger WC) than their healthy lean counterparts even though both groups fell within a normal BMI range. Additionally, this phenotype has been associated with several-fold greater risk for developing cardiometabolic disease not only compared to individuals with MHNW but also to those with MHO .

From a molecular standpoint, studies have shown the important role of mitochondrial bioenergetics (as the organelle responsible for whole body energy homeostasis) in the pathophysiology of obesity, metabolic inflammation, and its associated sequelae (IR, T2 DM, Met S, and atherosclerosis). In fact, mitochondrial dysfunction (as assessed by a reduction in the abundance of cellular mitochondria [mtDNA CN] in several tissues) is directly associated with disorders linked to cardiometabolic risk such as atherosclerotic CVD, Met S, T2DM, hypertension, obesity and IR. However, to date the role of mitochondrial function in the different subtypes of obesities is still under- investigated.

6-2 What are the key questions?

This research sought to evaluate two key aims:

a) Evaluate the prevalence and characteristics of the body composition phenotypes in the Maltese population.
b) Explore the relationship between mtDNA CN as a surrogate index of mitochondrial dysfunction and different body composition phenotypes.

Rationale:

Based on existing local and international epidemiological data, it was anticipated that approximately one third of the study population to be in the obese BMI category, and that one third of participants living with obesity to classify as MHO. Furthermore, it was expected that MHO presents an intermediate state risk (in terms of cardiometabolic and inflammatory profiles) between that of MHNW and MUHO. The molecular correlates of obesity phenotypes are also complex, and the relevance of mitochondrial dysfunction to differences in body composition are not fully ascertained. This study thus sought to assess the distribution of mtDNA CN across different body composition phenotypes, in an attempt to refine the contribution of mitochondrial dysfunction across different obesity phenotypes.

Objectives:

Using a well-phenotyped middle-aged Maltese Caucasian population, this research sets out to explore, for the first time, the following objectives:

1) Establish the prevalence of the six body composition phenotypes (MHNW, MUHNW, MHOW, MUHOW, MHO and MUHO) according to multiple definitions of MH
2) Explore which definition which best predicts IR
3) Evaluate characteristics and determinants of the major phenotypes of interest (MHO and MUHNW)
4) Assess sex differences in prevalence, anthropometric and biochemical parameters of each of the body composition phenotypes and in the determinants of IR
5) Evaluate the discriminatory power and the respective cut-points of the various anthropometric and biochemical parameters in predicting IR
6) Explore the relationship between peripheral blood leukocyte mtDNA CN and the different body composition phenotypes

Parameter/characteristics		MHNW (Referent group)	MUHNW	MHO	MUHO
BMI		18.5-25 kg/m²	18.5-25 kg/m²	≥30 kg/m²	≥30 kg/m²
Population prevalence (% of BMI category)		50%	6.6-45.9%[T] (10-27%[α])	1.3-25.8%[T] (6-40%[ß])	18%
Total fat mass		<30%	>30%	>30%	>30%
Adiposopathy	**SAT expansion through hyperplasia**		↓	↑↑	↓
	Gluteofemoral sc fat		↓↓	↑↑	↓
	Visceral adipose tissue		↑	Low levels but higher than MHNW	↑↑
	Ectopic fat distribution (liver, muscle, heart, pancreas)		↑	↓↓	↑↑
	Lipotoxicity: AT lipolysis, impaired FAO, circulatory NEFAs & bioactive lipids (ceremides, DAG, FA co-A), HGO, VLDL production & ß-cell dysfunction		↑	↓	↑
	OS & AT subclinical inflammation (meta-inflammation): pro-inflammatory / immune profile (IL-6, hs CRP, TNFα, INF-γ, visfatin, resistin, M1 & CLS, T_{H1} Cells, T_c Cells)		Increased OS & inflammatory status	Subtle degree of OS & low-grade inflammation	Permanently high inflammatory status
	Anti-inflammatory (cardioprotective) adipokines (adiponectin, omentin-1)		Low levels	Preserved levels	Low levels
Appendicular skeletal muscle mass & strength			Very low skeletal muscle mass and strength	Preserved skeletal muscle mass and strength	Low muscle mass in states of sarcopenic obesity

Figure 6.1: Overview of the prevalence, characteristics, pathophysiological features and long term outcomes of the different body composition phenotypes

Cardiometabolic profile (WC, FPG, HDL-C, TG, BP)			Abnormal cardiometablic profile	Normal cardiometabolic profile but ↑than MHNW	Abnormal cardiometabolic profile
Insulin sensitivity & secretion			Insulin secretion failure & ↓Insulin-mediated glucose disposal	Preserved insulin secretion & sensitivity	Insulin resistance & Hyperinsulinemia
Genetic predisposition & early life factors			GWAS: loci regulating lipid storage and adipogenesis (IRS1, GRB14, PPARG); Adverse intrauterine milieu	GWAS: genes related to adipocyte differentiation; polymorphisms of adiponectin receptor 1 (*ADIPOR1)* and hepatic lipase *(LIPC);* Preserved intrauterine milieu	Genes regulating food intake (FTO, MCR4); Upregulation of genes related to immune and inflammatory responses in VAT; Adverse intrauterine milieu; Thrifty genotype/phenotype
Lifestyle characteristics	Demographics		↑Age, male sex, Asian ethnicity/race	↓ Age & female sex	↑Age & male sex
	Diet		↑consumption of glucose, fructose, and sat fat	↑ compliance with food pyramid	↑ adherence to a Western diet / ↑consumption of glucose, fructose, sat fat
	Cardiorespiratory fitness		Lower physical fitness levels	↑PA, ↑CRF, ↓fasting respiratory quotient & ↑FAO ↓ Sedentary behaviour	↓PA, ↑CRF, ↑fasting respiratory quotient &↓ FAO ↑ Sedentary behaviour
	Others				Abnormal sleep characteristics ?Nickel allergy ?role of IGF-1
CVD events risk			↑↑	↔/↑ risk vs MHNW if stable MHO[a,*] ↑↑Risk if transition to MUHO[a,*]	↑↑
HF risk			↑	↑[Φ,*]	↑↑
T2 DM risk			↑↑	↔ Risk vs MHNW if stable MHO ↑Risk if transition to MUHO[a,†]	↑↑
Mortality	All-cause		↑↑	↔ risk vs MHNW if stable MHO* ↑↑Risk if transition to MUHO*	↑↑
	CVD mortality		↑↑	↑[Φ,*]	↑↑

AT- adipose tissue, BP- blood pressure, CLS - crown-like structures,CRF- cardiorespiratory fitness, DAG- diacyl glycerol, FAO – fatty acid oxidation, FPG- fasting plasma glucose, FTO- fat mass and obesity-associated, GRB14 – growth factor receoptor-bound protien 14, GWAS – genome wide association sutides, HDL-C-high density lipoprotien cholesterol, HGO – hepatic gluocse output, hsCRP-high senstivity C-reactive protein, IL- interleukin, INF- Y- interferon- Y, M1 – M1 macrophage polarisation, MC4R- metalnocortin-r receptor, OS- oxidative stress, IRS1- insulin receptor substrate 1, MHNW-metbaolcially helahty nomal weight, MUHNW-metbaoclially unhelahty normal wieght, MHO-metbaoclially healthy obese, MUHO-metbaoclially unehlathy obese, PA-physcial activity, PPAR - peroxisome roliferator activated repceotr gamma, SAT- subcatuaenous adipose tissue, sc-subcutaenous, T_{H1} Cells – T helper cells, T_c Cells – cytotoxic T cell, T2DM- type 2 diabetes mellitus, TG-triglycerides, VAT- cisceral adipose tissue, VLDL – very low density lipoprotein, WC- wasit circumference

[a] Badoud et al., 2015, [β] Jung et al., 2017, [ε] Zhang 2023, [•] Zhou et al., 2021, [Φ] Wang 2022, [†] Wang et al., 2015, [ψ] Fan et al 2013

Figure 6.1: (Continued)

6-3 What are the study's main findings?

Table 6.1: Summary of main findings

Total population (n)	521		
Females, n (%)	330 (63.3%)		
Subjects with OW/O, n (%)	365 (70%)		
Subjects with the metabolically unhealthy phenotype, n (%) **	171 (32.8%)		
Subjects with normal weight, n (%)	156 (30%)		
MHNW, n (%)*	145 (27.8%)		
MHOW, n (%)*	149 (28.6%)		
MHO, n (%)*	56 (10.7%)		
MUHNW, n (%)**	11 (2.1%)		
MUHOW, n (%)**	42 (8.1%)		
MUHO, n (%)**	118 (22.6%)		
Prevalence of MHOW/O among overweight/obese subjects, n (%)	205 (56.1%)		
Prevalence of MHOW among overweight subjects, n (%)	149 (78%)		
Prevalence of MHO among obese subjects, n (%)	56 (32.1%)		
Gender differences			
	Males	**Females**	
Overall, n (%)	191 (36.7%)	330 (63.3%)	
Metabolically unhealthy phenotype, n (%)**	78 (41.3%)	92 (27.8 %)	
MHNW, n (%)*	26 (5%)	119 (22.8%)	
MUHNW, n (%)**	3 (0.5%)	8 (1.5%)	
MHOW, n (%)*	67 (12.8%)	81 (15.5%)	
MUHOW, n (%)**	24 (4.6%)	18 (3.5%)	
MHO, n (%)% *	18 (3.5%)	38 (7.3%)	
MUHO, n (%)**	52 (10%)	66 (12.7%)	
Prevalence range using different definitions of MH[±]			
MHNW, (%)*	16.3 – 27.5%		
MUHNW, (%)**	0.6% - 13.5%		
MHOW, (%)*	11.9 – 32.9%		
MUHOW, (%)**	3.8 – 25.0%		
MHO, (%)*	2.1- 19.0%		
MUHO, (%)**	14.6 – 31.2%		
Anthropometric and biochemical predictors of IR[†]			
Males **Parameter (cut-off value)**	**AUC±SE (95%CI)**	**Sensitivity% (95%CI)**	**Specificity% (95%CI)**
LAP (42.5)	0.79±0.04 (0.71-0.87)	86 (72-95)	63 (55-71)
VAI (1.44)	0.78±0.04 (0.70-0.86)	86 (72-95)	66 (58-73)
WC (96.5cm)	0.70 ±0.05 (0.61-0.79)	74 (58-86)	60 (52-68)
Females **Parameter (cut-off value)**			
VAI (1.41)	0.82±0.04 (0.73-0.90)	80 (66-90)	77 (72-82)
LAP (36.2)	0.81±0.04 (0.73-0.89)	76 (61-87)	80 (75-85)
WC (82cm)	0.76 ±0.04 (0.69-0.84)	86 (73-94)	53 (47-59)

Data are presented as number (percentage, %)
OW/O-overweight/obesity; MHNW- metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight; MHOW/O-metabolically healthy overweight/obese; MUHOW/O-metabolically unhealthy overweight/obese; MH- metabolic health; IR – insulin resistance; VAI – visceral adiposity index ; LAP – lipid accumulation product ; WC – waist circumference; AUC – area under the Receiver-operating characteristic Curve;
*Individuals having ≤1 ATPIII criteria from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents. **Presence of ≥2 NCEP ATPIII criteria for the metabolic syndrome [±]Consisting of Wildman *et al.,* Doumatey *et al.,* Meigs *et al.,* Hamer *et al.,* Aguilar-Salinas *et al.,* Lynch *et al.*, Karelis *et al.,* Lavie *et al* and NCEP ATP III (presence of either 0, 1 or 2 parameters)
[†] defined as HOMA-IR ≥2.5

- More than **2/3** of the study population was overweight or obese (in keeping to that reported in the Maltese general population). A summary of the main findings observed in this study are summarised in **table 6.1.**
- **1/3** (32.8%) of this working-age population was found to have a **metabolically unhealthy phenotype** which carries important clinical and public health implications.
- While more than ¾ of individuals with overweight were metabolically healthy (78%), the majority of individuals with obesity were metabolically unhealthy (67.9%) and only 32.1% were **MHO** (using the presence of ≤1 parameters of the NCEP ATP III criteria).
- 7.7% of **MHOW** and 3.7% of **MHO** were insulin resistant (HOMA-IR ≥2.5) while only 22%, 40.5% & 47.4% of **MUHNW, MUHOW** and **MUHO** respectively were insulin resistant when using NCEP ATPIII criteria to define MH. Furthermore, compared to the NCEP ATPIII criteria, the IR definition for MH presents a worse cardiometabolic profile in MHOW/O participants while within the **MUHNW** cohort, the NCEP ATPIII definition presents a worse cardiometabolic profile.
- Overall, the metabolically healthy phenotype was more prevalent in females, those who were physically active, those who consumed alcohol and those with a tertiary level of education or held a white-collar occupation. This is consistent with other studies.
- The prevalence of different body composition phenotypes is definition dependent. In this study the prevalence of MHO was highest when adopting the Doumatey et al., Meigs et al. and Aguilar et al. definitions (population prevalence

of 19%) whilst the lowest prevalence was observed when using the NCEP-0 criteria (2.15%). Prevalence of **MUHNW** was highest when adopting the Wildman et al. definition (13.5%), whilst the lowest was observed when using the NCEP-2 criteria (0.6%).

- In females the NCEP-2 definition had the highest odds for predicting IR (HOMA-IR ≥2.5) (OR 19.7, CI 16.6 -22.3) even after adjusting for BMI. .
- In males the Aguilar-Salinas et al definition had the highest odds for predicting IR (OR 18.7, CI 12.3 – 21.9) even after adjusting for BMI – this implies that sex-specific definitions for MH may be required.
- **MUHNW** were 'fatter' than **MHNW**, i.e., higher values for indices of central obesity measurements and higher values for several biochemical parameters were observed compared to MHNW.
- **MHOW/O** participants were comparable to **MHNW** in terms of lifestyle characteristics and in the proportion of individuals exhibiting any of the Met S criteria (except TG); however, they had higher values for anthropometric parameters and indices of central obesity measures and presented a worse cardiometabolic profile (in fact they had higher values for biomarkers of lipid and glucose metabolism, insulin resistance and inflammation).
- **MHOW/O** and **MUHNW** individuals exhibited differences in key anthropometric parameters such that the MHOW/O phenotype had higher values for measures of peripheral fat accumulation (thigh circumference) and lower values for indices of central adiposity (VAI). Furthermore, MUHNW participants were more likely

to have an associated medical comorbidity and exhibited worse values for lipid parameters.

- The major lifestyle determinants for the **MHOW/O** phenotype defined by the presence of ≤1 parameters of the NCEP ATPIII criteria were: engaging in physical activity, alcohol consumption, non-smoking status, and age <40 years.
- No significant lifestyle determinants were observed for the MUHNW phenotype
- Males were more likely to exhibit the unhealthy metabolic phenotype compared to females (41.3% vs 27.8%).
- The majority of individuals with normal weight and overweight in both sexes were metabolically healthy while the majority of males and females living with obesity were metabolically unhealthy (74.3% and 63.5%).
- Overall, males tend to exhibit a less favourable metabolic profile and have higher values for indices of obesity measurements even when classified as healthy, except for thigh circumference which was higher in females. While having higher median BMI, a lower proportion of males exhibited an abnormally high WC (>102cm) compared to females (31.4% vs 39.1%, respectively).
- BMI was the strongest determinant of HOMA-IR in males (ß=0.082, 95%CI 00.046-0.119, p<0.01). In females, both BMI and WHR were independently associated with HOMA-IR (β=0.047, 95% CI 0.362-0.062, p=0.016 and β=1.91, 95% CI 0.362-3.45, p=0.016 respectively).
- LAP, VAI and WC were observed to have the strongest discriminatory power to predict a HOMA-IR ≥ 2.5 in both sexes in ROC analysis.

- The cut-off for WC in predicting HOMA-IR in both sexes was lower than those currently recommended (NCEP ATPIII). Possibly, sex-specific WC cut-offs may need to be revised in the Maltese population.
- A comparable mtDNA CN was observed between **MUHO** and **MHO** participants (irrespective of the definition used); however, this was statistically lower than that observed in MHNW individuals ($p<0.01$ for all comparisons).
- A 10-fold reduction in mtDNA CN is associated with a higher odds of having the Met S (OR 1.05, 1.02-1.09), the metabolically unhealthy phenotype (OR 1.04, 1.02-1.09) and of IR (defined as HOMA-IR ≥2.5) (OR 1.03, 1.01-1.07) in both sexes.
- In females a 10-fold reduction in mtDNA CN was also associated with a higher WC (OR 1.07, 1.04-1.11), whilst in males it was associated with increased odds of having elevated serum TG (OR 1.02, 1.00-1.05).
- Principal components analysis shows that lower mtDNA CN is associated with adiposity (BMI, WC) and insulin resistance (FPG, HOMA-IR) – further confirming the relationship between excess adiposity, insulin resistance and reduced mtDNA CN.
- **Figure 6.2** shows the salient characteristics of the different body composition phenotypes observed in this study.

6-4 What is their novelty and how may they impact on clinical practice?

The findings from this study have direct clinical implications, as outlined below:

- A considerable proportion of the studied population were living with overweight or obesity (70%) and as many as 1 in 3 individuals carried the unhealthy metabolic phenotype across all BMI categories. These findings are expected to result in increased future CVD burden within the Maltese population.
- The MHOW/O phenotype is associated with subtle differences in several cardiometabolic risk parameters – including worse lipid, glycaemic and IR profiles compared to **MHNW** – implying that **MHOW/O** is not completely benign.
- **MUHNW** participants may be thought of as being 'fatter' than their **MHNW** counterparts and overall present a worse cardiometabolic profile than **MHOW/O** confirming this phenotype is not without its risks.
- These findings thus suggest that the cardiometabolic risk associated with **MHO** may lie somewhere between that of metabolically healthy normal weight and that of metabolically unhealthy normal weight. Additionally, the prevalence of the different body composition phenotypes is definition dependent thus highlighting the need for having standard criteria.
- The finding that only around half of **MUHO** participants were insulin resistant (i.e., having a HOMA ≥2.5) is unexpected. It is generally assumed that an abnormal metabolic phenotype would be accompanied by IR. Furthermore, a small but significant number of metabolically healthy individuals with overweight and obesity (MHOW/O) were insulin resistant. These findings may

imply that sole use of the NCEP ATPIII or HOMA-IR definitions to categorise MH may be insufficient to identify true metabolically healthy individuals. This may, in part, also explain the heterogeneity in prevalence and long-term outcomes observed in several studies.

- Males were consistently observed to have less favourable anthropometric and metabolic profiles compared to females across all BMI categories and even when classified as being metabolically healthy, suggesting that BMI cut-offs may need to be revised downwards in males. Furthermore, the fact that a lower percentage of males exhibited an abnormal WC despite a higher BMI compared to females suggests that currently used cut-offs for WC may be too high for males and should be lowered.
- Timely and aggressive management of modifiable risk factors as well as preventive strategies (both with respect to obesity and in the development of metabolic abnormalities) need to be implemented across all BMI categories.
- Several routinely available parameters can be used to predict IR in clinical practice, however some were observed to have lower thresholds than those currently recommended by ATPIII. Future longitudinal studies especially those relating to hard outcomes are required to ascertain cut-offs so as to reflect a more contemporary population and which can be used as biomarkers for CVD risk in a clinical setting.
- The presence of obesity is associated with a reduced mtDNA CN level (and thus mitochondrial dysfunction) irrespective of whether it is healthy or unhealthy and

the distinction between MHO and MUHO may not be directly explained by pathophysiological changes at the level of the mitochondrion.

Parameter/characteristics	MHNW	MUHNW	MHOW/O	MUHOW/O
Population prevalence*, % (% of BMI category)	27.8%	2.11% (7.0%)	39.3% (56.1%) MHOW: 28.6% (77.6%) MHO: 10.7% (32.2%)	30.7% MUHOW: 8.1% (21.9%) MUHO: 22.6% (67.8%)
Population prevalence range (using 11 definitions of MH)**	16.3-29.4%	0.6-13.5%	14-51.3%	19-56.2%
Males*, %	17.9%	27.3%	41.7%	47.5%
Anthropometric & central obesity indices*		↑ values for both indices vs MHNW	↑values for both indices vs MHNW	
Cardiometabolic parameters*		↑ values for lipid parameters; similar values for markers of glucose metabolism, IR & inflammation vs MHNW	↑values for all cardiometabolic parameters: glucose & lipid metabolism, IR & inflammation) vs MHNW	
Lifestyle determinants*			PA, alcohol consumption, non-smoking, age <40 years	
Mitochondrial DNA CN*	↔	NA	↓	↓

*When defining metabolic health by presence of ≤1 NCEP ATPIII parameters from the following: waist circumference >102cm in men and >88 cm in women; systolic or diastolic blood pressure ≥130/85 mmHg or on antihypertensive medication; serum triglycerides ≥1.69mmol/L or on lipid-lowering medication; HDL-C <1.03 mmol/L in men and < 1.29 mmol/L in women or on treatment aimed to increase HDL-C; fasting glucose ≥5.6mmol/L or on antihyperglycemic agents

** Consisting of Wildman *et al.*, Doumatey *et al.*, Meigs *et al.*, Hamer *et al.*, Aguilar-Salinas *et al.*, Lynch *et al.*, Karelis *et al.*, Lavie *et al* and NCEP ATP III (presence of either 0, 1 or 2 parameters)

MHNW- metabolically healthy normal weight; MUHNW-metabolically unhealthy normal weight; MHOW/O-metabolically healthy overweight/obese; MUHOW/O-metabolically unhealthy overweight/obese; BMI-body mass index; MH-metabolic health; HOMA-IR-homeostatic model assessment for insulin resistance; PA-physical activity; Mitochondrial DNA CN- deoxyribonucleic acid copy number

Figure 6.2: Salient characteristics of the body composition phenotypes in the studied population

Chapter 7 - Future studies

It will be useful to assess the relationship between the various definitions of MH and carotid-intima media thickness (cIMT), which is another well-established biomarker of atherosclerosis. Since both MH and adiposity are dynamic states, a longitudinal follow-up study should be conducted to determine transitions in between body size phenotype of the currently investigated population as well as the determinants of such changes. Furthermore, the longitudinal relation between leukocyte mtDNA CN at baseline and transitions across MH phenotypes over the length of follow up should be evaluated. In addition, longitudinal approaches would enable assessment of the incident cardiometabolic disease burden in the study cohort.

It will be worthwhile to study the prevalence of the body size phenotypes in the island of Gozo, which is generally more rural and where the population has a different lifestyle to that of the main island of Malta. Other possible studies include characterisation of the various body size phenotypes and studying sex differences in a more elderly cohort. As discussed previously, elderly individuals have different fat distribution patterns and less muscle mass and should therefore be studied separately. In addition to this another study could focus on determining the optimal cut-offs for the various cardiometabolic parameters to predict IR in this elderly cohort.

Other studies which could be conducted to evaluate further the relationship between the different body composition phenotypes and mitochondrial function would involve measures of mitochondrial activity such as citrate synthase activity and oxygen consumption rate. It would also be interesting to investigate the effect of bariatric surgery, glucagon-like peptide 1 receptor agonists and the incorporation of a very low-calorie diet (VLDL) on mitochondrial copy number and on mitochondrial function.

Molecular analysis could also incorporate additional biomarkers of oxidative stress and cell senescence and ageing. Specifically, the assessment of untargeted telomere length using qPCR approaches and how this relates to MH and different body composition phenotypes should be ascertained. Recent studies have also shown that individuals with obesity, particularly those with increased visceral adiposity, tend to have lower serum IGF-1 levels and a blunted growth hormone response on dynamic testing. Furthermore, a diminished IGF-1 level in the serum was observed to be associated with a worse metabolic profile (Miller et al., 2005). IGF-1 is a peptide hormone produced primarily by the liver and has a molecular structure similar to that of insulin. It primarily mediates the effects of growth hormone (GH) through its mitogenic and anabolic actions and preliminary studies show that it could potentially act as a novel biomarker for identifying those clinical phenotypes at highest risk of adverse cardiometabolic outcomes (Masi et al., 2022). Thus, another study could entail the measurement of serum IGF-1 levels (or its surrogate marker, the IGF-1 z Standard of Deviation Score [zSDS]), within the studied population and assess how it varies among the different body composition phenotypes and in its relationship with mtDNA CN.

Appendices

Appendix 1A: Copy of University of Matla Research Ethics Committee Study Approval.

L-UNIVERSITÀ TA' MALTA

Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA

Msida – Malta
Medical School
Mater Dei Hospital

Ref No: **06/2016**

Monday 8th August 2016

Dr Rachel Agius
4, Dwardu Cachia Street
Iklin, IKL1271

Dear Dr Rachel Agius,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Prevalence and early life determinants of metobilically healthy and unhealthy lean and obese individuals in a Maltese population

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Email: [illegible] • Web: http://www.um.edu.mt/ms

Appendix 1B: Copy of Consent Form.

I am a Maltese citizen, and I am 41 (±5) years of age

I have been asked to participate in the research study entitled:

Molecular determinants and prevalence of the different body composition phenotypes in a Maltese cohort

The reason for this study is to identify the health status of people who are around 40 years of age (i.e., whether they are normal weight, overweight or obese) by assessing their body composition characteristics as well as to find out if they have presence of any underlying condition such as diabetes, high blood pressure and high cholesterol.

This will be done by asking you to come to the **diabetes clinic** at the **outpatient department of Mater Dei Hospita**l between 7am and 8: 30am whereby initially you will be asked a couple of questions regarding general information (this will include age, DOB, gender, ethnicity, area of residence, level of education and current or past occupations, smoking and alcohol practices and if you undertake any physical activity. You will then also be asked questions regarding your current state of health including if you are known to suffer from any illnesses (such as diabetes, high blood pressure, asthma, or heart disease) or if you underwent surgical operations in the past or if you are currently taking any medications. Thereafter I will proceed to examine you. At this point I will ask you to lie on the couch with light underwear clothing so as to assess the blood pressure, the waist, neck, thigh, hip and arm circumferences. Then I will assess your weight and height using a digital scale and stadiometer. Following this I will then proceed to take a blood sample via a needle prick (roughly around 30 mls – 3 tablespoons of blood) so as to check your blood count, sugar, cholesterol, liver, kidney and thyroid levels. Blood will also be used to isolate DNA in order to carry out genetic tests.

It is envisaged that all the above will not last longer than 20-30 minutes.

Further to this, at a later stage we would also like to assess the case notes of your mother in order to allow us to gather information regarding the time she was pregnant with you. We shall be looking at any events or problems that cropped up during her pregnancy (such as diabetes or high blood pressure in pregnancy), if she took any medication, and if there were any problems during delivery. From the same notes we would also be able to capture information regarding your early life characteristics such as birth weight, weight of placenta, weight and height gained in the first year, if you were bottle or breast fed, and if you were ever exposed to cigarette smoke. Your mother will not be asked to attend for any visits or blood tests at hospital.

All data that I will obtain throughout this research project will be anonymised, but should I encounter any adverse results both on examination or on blood testing a particular person I will contact you personally to make the necessary arrangements for further tests or treatment as required via the government health service either through their family doctor or in one of my afternoon clinics at Mater Dei Hospital (according to your preference).

I would like to participate in this research study and I confirm that:

The purpose and details of the study have been explained to me by Dr. Rachel Agius (principle investigator) and any difficulties which I raised have been adequately clarified.

I give my consent to the Principal Investigator (Dr. Rachel Agius) and her supervisor (Prof. S. Fava) to either make the appropriate observations/tests or both and to take the necessary blood samples. I am also aware that blood samples may be taken and stored for future genetic studies. I am aware of the inconveniences which this will cause.

I understand that the results of this study may be used for medical or scientific purposes and that the results achieved from this study in which I am participating may be reported or published: however, I shall not be personally identified in any way, either individually or collectively, without my express written permission.
I am under no obligation to participate in this study and am doing so voluntarily.

I may withdraw from the study at any time, without giving any reason. This will not influence in any way the care and attention and treatment normally given to me.

I am not receiving any remuneration for participating in this study.

In case of queries during the study I may contact Dr. Rachel Agius (principle investigator) on Tel No **79847509**

Signature of participant: _____

Name of participant (in block letters) _____

Id. No.: _____

Signature of principle investigator: _____
Name of principle investigator: Dr Rachel Agius MD MRCP MSc
Id. No.: 276781M

Appendix 1C: Copy of Study Proforma

A) Index case anthropometric data

Name Surname

Identification No. Age DOB

Gender Race/ethnicity

Area of current residence north □ central □ south □

Town of current residence

Level of education: primary □ secondary □ tertiary □

Occupation (ISCO-08) 1-Managers
2-Professionals
3-Technicians and associate professionals
4-Clerical support workers
5-Service and sales workers
6-Skilled agricultural, forestry and fishery workers
7-Craft and related trades workers
8-Plant and machine operators, and assemblers
9-Elementary occupations)
0-Armed forces occupations

Smoking status: lifelong non-smoker: (<100 cigarettes in their lifetime & not currently smoking □

current smoker: (>100 cigarettes in their lifetime & current smokers) □
no. per day □
ex-smoker: (>100 cigarettes in their lifetime but not presently smoking) □

Alcohol intake: non-drinker (<12 alcoholic beverages in their lifetime) □
< 1 drink/day □ 1-2 drinks/day □ >2 drinks/day□

Physical activity: type of activity

No. of times performed per day week month

Duration of each activity (minutes)

Known medical illness: T2DM □ HT □ hypercholesterolaemia □ CVD □

Others:

Past surgical history:

Drug history antihypertensives

Anti diabetic agents

Lipid lowering agents

Others

Ht Wt neck circumference

BMI normal weight □ overweight □ obese □

WC waist index hip circumference waist/hip ratio

R arm circumference L arm circumference

R thigh circumference L thigh circumference

B) Measurement of cardiometabolic components and body size phenotype

LFT TC LDL HDL TG

FBG Hba1c Insulin

Uric acid CRP ferritin Renal profile Vitamin D TFTs

BP

Body phenotype category: normal weight □ 0-1 abnormalities □ ≥2 abnormalities □

Obese □ 0-1 abnormalities □ ≥2 abnormalities

Appendix 2: Definitions of the Metabolic Syndrome as proposed by different organisations.

Clinical measure	World Health Organization [WHO] (Alberti *et al.*, 1998)	European Group for the Study of Insulin Resistance [EGIR] (Balkau *et al.*, 1999)	National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) (2005 revision)	American Association of Clinical Endocrinologists (Einhorn *et al.*, 2003)	International Diabetes Federation (Alberti *et al.*, 2005)	Joint Interim Statement (JIS)* (Alberti *et al.*, 2009)
Required criteria	Insulin resistance (including IGT, IFG, T2DM, or lowered insulin sensitivity)[a] **Plus two of the following:**	Hyperinsulinemia (plasma insulin >75th percentile) **Plus two of the following:**	**Three or more of the following:**	IFG or IGT **Plus any of the following based on clinical judgement:**	Increased waist circumference (ethnic specific) **Plus two of the following:**	**Three or more of the following:**
Measures of obesity assessment	Waist/hip ratio: >0.90 (M), >0.85 (F); and/or BMI >30 kg/m^2	Waist circumference: ≥94 cm (M), ≥80cm (F)	Waist circumference: >102cm (M), >88 cm (F)	BMI ≥25kg/m^2	Waist circumference: ethnicity specific (already required)	Waist circumference: population- and country- specific definitions[b]
Blood pressure	≥140/90 mmHg	≥140/90 mmHg or Rx	≥130/≥85 mmHg or Rx	≥130/85 mmHg	≥130/≥85 mmHg or Rx	≥130/≥85 mmHg or Rx
Lipid profile	Triglycerides: ≥ 1.7 mmol/l *and/or* HDL-C: <0.9 mmol/l (M), <1.0 mmol/l (F)	Triglycerides: >2.0mmol/l or Rx *and/or* HDL-C: <1.0 mmol/l or Rx	Triglycerides: ≥1.7 mmol/l or Rx HDL-C: <1.03 mmol/l (M), <1.29 mmol/l (F) or Rx	Triglycerides: >1.7 mmol/l HDL-C: <1.03 mmol/l (M), <1.29 mmol/l (F)	Triglycerides: ≥1.7 mmol/l or Rx HDL-C: <1.03 mmol/l (M), <1.29 mmol/l (F) or Rx	Triglycerides: ≥1.7 mmol/l or Rx HDL-C: <1.0 mmol/l (M), <1.3 mmol/l (F) or Rx
Glucose	IGT, IFG, or T2DM	Fasting plasma glucose ≥6.1 mmol/l but no diabetes	Fasting plasma glucose ≥5.6 mmol/l or Rx	IFG or IGT but not diabetes	Fasting plasma glucose ≥5.6 mmol/l or diagnosis of T2 DM	Fasting plasma glucose ≥5.6 mmol/l or Rx
Other criteria	Albumin:creatinine ratio: ≥30 mg/g or Urinary albumin excretion rate ≥20 µg/min			Other risk factors: family history of T2 DM, PCOS, sedentary lifestyle, advancing age, ethnic groups at high risk for T2 DM		

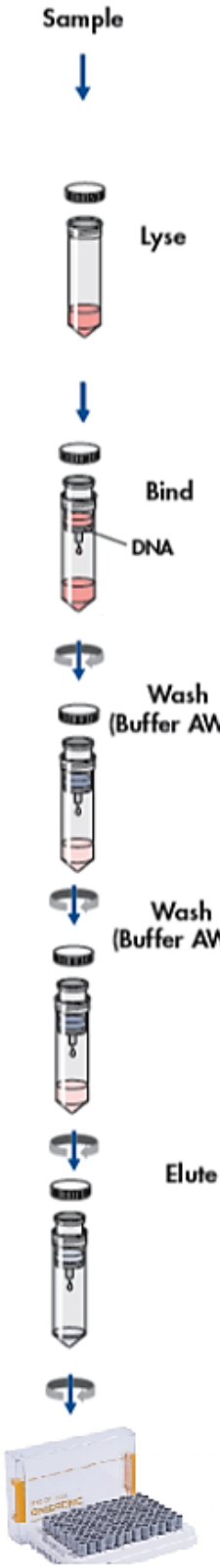
*A joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the study of Obesity.

[a]Insulin sensitivity as measured under hyperinsulinemic euglycemic clamp conditions.

[b]IDF cut points are recommended to be used for non-Europeans and either the IDF or AHA/NHLBI cut points for people of European origin until more data are available.

Abbreviations: IGT, impaired glucose tolerance; IFG, impaired fasting glucose; T2 DM, type 2 diabetes; M, males; F, females; Rx, drug treatment; HDL-C, high density lipoprotein cholesterol

Appendix 3: An overview of the DNA extraction process.



Sample

1mL of thawed blood at room temperature, mixed on a rotator

Protein digestion and cell lysis

Add 200µL of protease, 1.2mL buffer AL to 1mL of blood in 15mL tube, homogenised and mixed by inversion, incubated at 70°C for 10 minutes using a water bath

Binding

Add 1mL of 96% ethanol, mix by inversion, transfer to QIAmp Midi spin column in 15mL tube, centrigure 3000 rpm x 3 minutes. Discard the filtrate.

Washing step 1

Add 2mL of buffer AW1 to spin column and centrifuge at 5000rpm for 1 minute, do not discard flow-through.

Washing step 2

Add 2mL of buffer AW2 to the spin columand centrifuge at 5000rpm for 15 minutes.

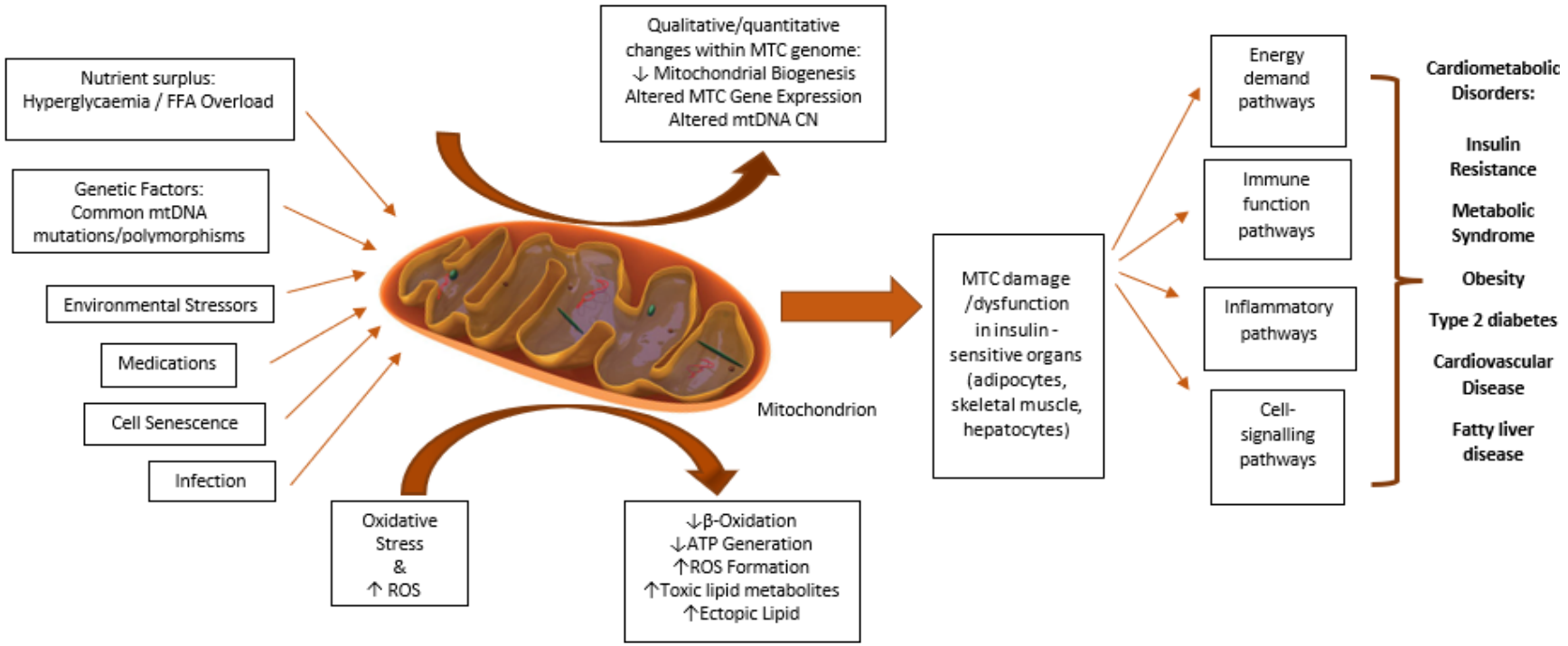
Elution

Transfer spin column to a new 15mL tube. Add 200µL of Buffer AE, incubate at room temperature for 5 minutes, and centrifuge at 5000rpm for 2 minutes. Repeat elution using a fresh tube to ensure maximal DNA yield.

Storage

Transfer to 0.5mL screw capped tube in 96 well-storage rack format till further analysis. Store at 4°C

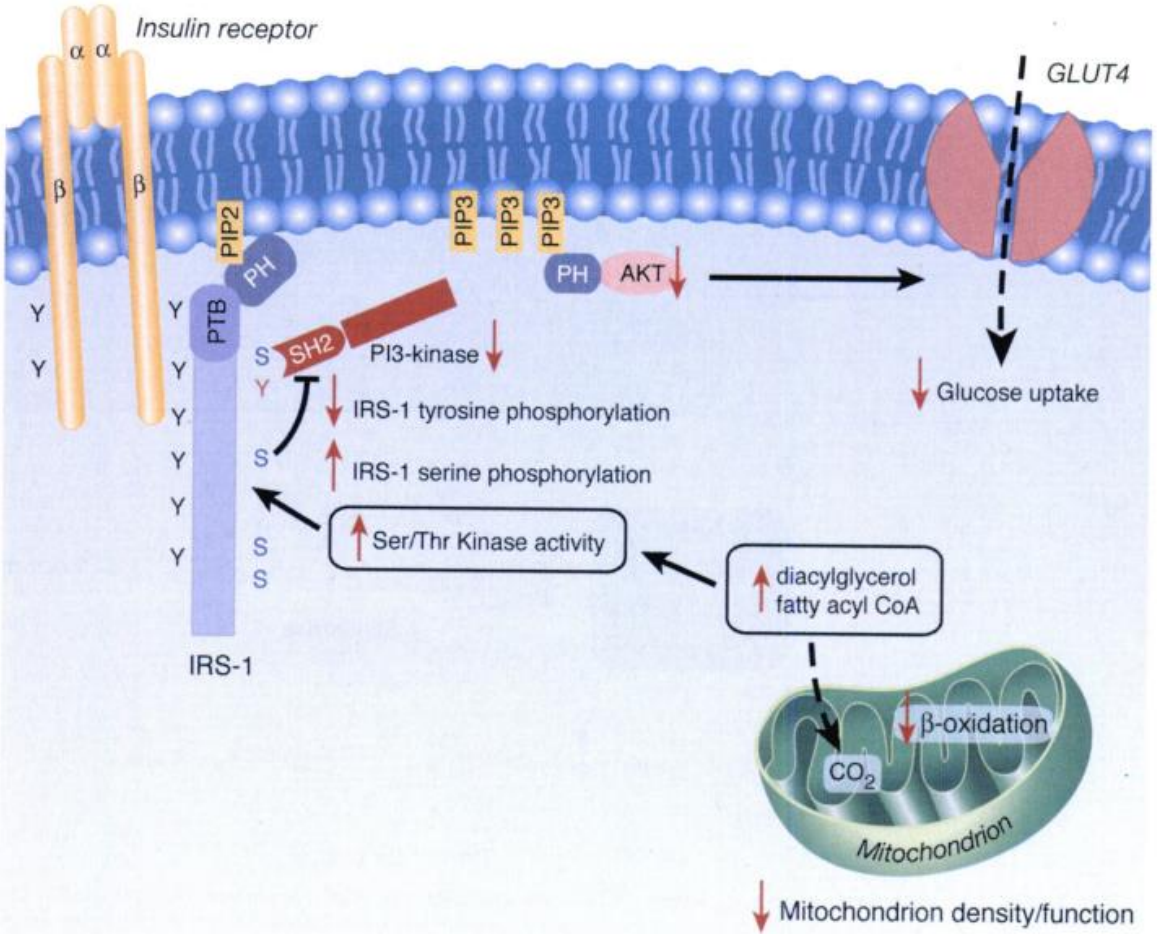
Appendix 4: Summary of proposed pathways linking AT mitochondrial dysfunction and cardiometabolic disease.



Mitochondrial dysfunction may be mediated through poor nutrition (including a high fat or glucose diet), inactivity or other environmental stressors such as infection, certain medications, and cell senescence or through common mitochondrial DNA mutations/polymorphisms. This causes increased ROS formation and impairment of mitochondrial oxidative function (oxidative stress) leading to decreased β-oxidation of fatty acids and ATP generation, further ROS production and intracellular lipotoxic lipid accumulation which concomitantly bring about a reduction in mitochondrial biogenesis and gene expression. These processes eventually lead to the downstream activation of various pathways resulting in the onset of insulin resistance and several chronic cardiometabolic diseases

AT- adipose tissue, ROS-reactive oxygen species, ATP-adenosine triphosphate, MTC- mitochondria, FFA – free fatty acid

Appendix 5: Mitochondrial dysfunction. lipotoxicity and insulin metabolic signalling.



The cellular mechanisms linking mitochondrial dysfunction, lipotoxicity and onset of insulin resistance in classical insulin target tissues such as skeletal myocytes. In this model mitochondrial dysfunction or a reduction of mitochondrial content leads to a decrease in fatty acid beta-oxidation. This is associated with intracellular accumulation of toxic metabolites such as fatty acyl CoA and diacylglycerol. The presence of these molecules causes activation of novel protein kinase C which in turn activates a serine kinase cascade (which possibly involves various stress pathways including NF-kB and c-Jun N-terminal kinase (JNK) leading to increased phosphorylation of serine residue on IRS-1 (pS). Increased serine phosphorylation blocks IRS-1 tyrosine phosphorylation (Y) by the insulin receptor which subsequently inhibits activity of PI 3-kinase. This culminates in the suppression of insulin-mediated glucose uptake and the onset of insulin resistance.

IRS-1, insulin receptor substrate-1; PIP3, phosphatidylinositol 3,4,5-trusphosphate; PI3-kinase, phosphatidylinositol 3-kinase; PTB, phosphotyrosine binding domain; PH, pleckstrin homology domain; SH2, src homology domain

(Source: Lowell and Shulman, 2005)

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