Insulin-Sensitive Obesity

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PUBLICATIONS ARISING FROM THIS THESIS

Chen DL, Liess C, Poljak A, Xu A, Zhang J, Thoma C, Trenell M, Milner B, Jenkins AB, Chisholm DJ, Samocha-Bonet D and Greenfield JR. Phenotypic characterization of insulin-resistant and insulin-sensitive obesity. Journal of Clinical Endocrinology and Metabolism. 2015;100:4082-91.[1]

Chen DL, Brown R, Liess C, Poljak A, Xu A, Zhang J, Trenell M, Jenkins AB, Chisholm DJ, Samocha-Bonet D, Macefield VG and Greenfield JR. Muscle sympathetic nerve activity is linked to liver insulin sensitivity in non-diabetic men with obesity. Clinical Autonomic Research. In the process of submitting (2015).

RELATED PUBLICATION

Williams RS, Heilbronn LK, **Chen DL**, Coster ACF, Greenfield JR and Samocha-Bonet D. Dietary acid load, metabolic acidosis and insulin resistance - lessons from cross-sectional and overfeeding studies in humans. Clinical Nutrition. In press (2015).

ORAL/POSTER PRESENTATIONS

Chen DL, Liess C, Poljak A, Xu A, Zhang J, Trenell M, Jenkins AB, Chisholm DJ, Brown R, Macefield V, Samocha-Bonet D and Greenfield JR. Liver insulin sensitivity is linked with resting sympathetic nervous activity in non-diabetic obese men. Australian and New Zealand Obesity Society, Melbourne, Oral presentation (2015).

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Chen DL, Samocha-Bonet D, Liess C, Poljak A, Xu A, Zhang J, Thoma C, Trenell M, Milner B, Jenkins A, Chisholm DJ, Greenfield JR. Dual insulin resistance in muscle and liver, but not a single site, is associated with visceral and liver lipid accumulation in obesity. US ENDO, Chicago. Poster presentation (2014).

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ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adult treatment panel
BGL	Blood glucose level
BMI	Body mass index
BMSF	Bioanalytical mass spectrometry facility
BP	Blood pressure
CRF	Clinical Research Facility
CT	Computed tomography
CV	Coefficient of variation
DAG	Diacylglycerols
DBP	Diastolic blood pressure
DXA	Dual energy X-ray absorptiometry
EDTA	Ethylene-diaminetetraacetic acid
EGP	Endogenous glucose production
ELISA	Enzyme-linked immunosorbent assays
FABP4	Fatty acid-binding protein 4
FFM	Fat-free mass
FGF-19	Fibroblast growth factor 19
FGF-21	Fibroblast growth factor 21
GC	Gas chromatography
GCMS	Gas chromatography-mass spectrometry
GDR	Glucose disposal rate

GINF	Glucose infusate
GLUT4	Glucose transporter type 4
GIR	Glucose infusion rate
GIR_{HI}	Glucose infusion rate at high insulin dose
HDL	High-density lipoprotein
HIRI	Hepatic insulin sensitivity index
HOMA-IR	Homeostasis model assessment insulin resistance
Hs-CRP	High sensitivity C-reactive protein
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHTG	Intra-hepatic triglycerides content
IL6	Interleukin 6
IR	Insulin resistance
IVGTT	Intravenous glucose tolerance test
LBM	Lean body mass
LDL	Low-density lipoprotein
$Liver_{sen}$	Liver insulin-sensitive
$Liver_{res}$	Liver insulin-resistant
MET	Metabolic Equivalent of Task
MHO	Metabolically healthy obesity
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MSNA	Muscle sympathetic nervous activity
$Muscle_{sen}$	Muscle insulin-sensitive
$Muscle_{res}$	Muscle insulin-resistant

NEFA	Non-esterified fatty acids
NA	Not available
NR	Not reported
NS	Non significant
Ob_{sen}	Obese insulin-sensitive
Ob_{res}	Obese insulin-resistant
OGTT	Oral glucose tolerance test
PA	Physical activity
PAI-I	Plasminogen activator inhibitor-1
PPAR-γ	Peroxisome-proliferation activated receptor-γ
PW	Postmenopausal women
RBP4	Retinol-binding protein 4
RIA	Radioimmunoassay
ROI	Region of interest
RQ	Respiratory quotient
SAA	Serum amyloid A
SBP	Systolic blood pressure
SC	Subcutaneous
SNS	Sympathetic nervous system
SST	Serum separating tubes
SREBP 1c	sterol regulatory element-binding protein 1c
TG	Triglycerides
TM4SF1	Transmembrane 4 L six family member 1
TNF-α	Tumour necrosis factor- alpha
U/S	Ultrasound

VCAM-1	Vascular cell adhesion molecule-1
VOI	Volume of interest

CHAPTER 1
INTRODUCTION

1.1 OBESITY

Obesity is one of the major public health problems facing most developed and many developing countries. Australia has one of the highest rates of overweight and obesity among developed countries,[2] with 60% of adult Australians reported in the overweight or obese categories.[3] Obesity is associated with metabolic complications, including insulin resistance, hypertension and dyslipidaemia, which may increase the risk of cardiovascular disease and diabetes mellitus. Obesity is estimated to contribute to 23% of the ischaemic heart disease burden and 44% of the diabetes burden.[4] Apart from obesity-related metabolic complications, obesity is also associated with other co-morbidities, such as osteoarthritis, obstructive sleep apnoea, and breast and colon cancer. With the rising incidence of obesity, there is a significant impact on health costs. The total direct and indirect costs of obesity in Australia were estimated to be $8.3 billion and $2.0 billion respectively.[5]

The increasing prevalence of obesity parallels the prevalence of type 2 diabetes mellitus. It is estimated that 150 million people in the world have diabetes;[6] most of these cases are type 2 diabetes. The prevalence of diabetes increases with higher body mass index (BMI) and obese individuals have 4 times the risk of developing diabetes compared to normal-weight individuals.[7]

Obesity refers to a BMI over 30 kg/m^2 (defined as weight in kilograms / height in metres squared) in Caucasians although a lower cut off may be appropriate for some ethnic groups e.g. Asians. However, there are other ways to assess obesity, including body fat mass and/or distribution via dual energy X-ray absorptiometry (DXA) and waist circumference.

DXA is superior to anthropometric techniques in assessing whole-body fat composition. Body fat percentage is a strong predictor of insulin resistance as measured by hyperinsulinaemic-euglycaemic clamp in lean men.[8] Using DXA to define obesity by specific age- and gender-adjusted values would give rise to a different cohort of individuals compared with BMI. For example, one study defined obesity using DXA and BMI on the same cohort of individuals and identified half of obese people as metabolically healthy when using DXA compared to 34% being metabolically healthy when using BMI.[9] Although the use of DXA scan is associated with a small dose of radiation and may not be feasible due to cost.

Abdominal adiposity, often assessed as waist circumference (defined as circumferential measurement midway between the lower rib margin and iliac crest or at the umbilicus) particularly in large cohort studies, is a stronger predictor of many metabolic and cardiovascular outcomes compared with BMI.[10] However, cut-off points for waist circumference in different ethnic groups are lacking. Nevertheless, waist circumference is a good indicator of visceral adiposity[11,12] and insulin resistance/diabetes mellitus.[13] Waist-to-hip ratio and waist-to-height ratio have been advocated as better than waist alone in predicting cardio-metabolic risk in different ethnic populations, but there is no consensus on the cut-off values.[14,15]

1.2 METABOLIC HEALTHY OBESITY

The increasing prevalence in obesity globally creates a significant public health burden. However, studies have identified a group of obese individuals who appeared to be protected from developing insulin resistance and/or metabolic syndrome. The term "metabolically healthy obese (MHO)" is used to describe obese individuals with absence of some or all features of the metabolic syndrome. Several studies have shown that MHO may have lower risks of cardiovascular events or all-cause mortality.[16,17] It is important to identify MHO for a number of reasons.

First, MHO individuals may have lower risk of developing metabolic complications than other obese individuals and may not need to be monitored as closely as the metabolically-abnormal obese (MAO) individuals. This would enable efficient use of health resources and divert resources to MAO individuals, who need more active intervention to prevent development of obesity-related metabolic complications.

Second, the identification of MHO individuals would provide an opportunity to perform comprehensive metabolic analyses to identify clinical and molecular factors that protect these individuals from developing metabolic complications. This would hopefully lead to novel therapeutic interventions that allow clinicians to minimise progression of obesity-related metabolic complications and reduce health burden and cost.

1.2.1 Definition of MHO

There is no agreed definition of MHO. MHO is usually defined as obesity in the absence of all or most Adult Treatment Panel III (ATP) criteria for the metabolic syndrome. The current ATP III criteria include:[18]

- Abdominal obesity, defined as a waist circumference in men ≥ 102 cm and in women ≥ 88 cm
- Serum triglycerides ≥ 1.7 mmol/L or drug treatment for elevated triglycerides
- Serum high-density lipoprotein (HDL) cholesterol < 1 mmol/L in men and < 1.3 mmol/L in women or drug treatment for low HDL cholesterol
- Blood pressure (BP) ≥ 130/85 mmHg or drug treatment for elevated blood pressure
- Fasting plasma glucose (FPG) ≥ 5.6 mmol/L or drug treatment for elevated blood glucose

Other sets of criteria are described in Table 1.1.

MHO individuals do not need to be free of all criteria, so individuals can be defined as MHO with normal blood pressure, but have impaired fasting glucose. The lack of universal definition for MHO might result in misclassification of some individuals who actually have a low-risk phenotype as having a high-risk phenotype.[19]

There are 15-30 different definitions that have been used to identify MHO individuals, which could potentially explain conflicting results that are observed between studies.[20,21] For example, Bonora's study[22] defined MHO as an absence of metabolic syndrome plus homeostasis model assessment-insulin resistance (HOMA-IR) score < 2.8. HOMA-IR is defined as (fasting plasma insulin concentration [mU/L] x fasting

plasma glucose [mmol/L])/22.5.[23] Another study[24] devised additional criteria including white blood count and plasma fibrinogen to define MHO. The disparity in definitions used to identify MHO creates problems in comparing and utilising clinical outcomes in current obesity literature.

A newer study uses liver fat as the only criteria to define MHO. Fabbrini's study[25] used Magnetic Resonance Spectroscopy (MRS) to measure intra-hepatic triglycerides content (IHTG) to define a group of non-diabetic obese individuals into MHO (defined as IHTG < 6%) and MAO (defined as IHTG > 10%). The MAO group had higher plasma concentrations of triglycerides, HOMA-IR and lower endogenous glucose production (EGP) suppression during low-dose insulin infusion (index of hepatic insulin sensitivity) than MHO. This interesting classification highlights the potential pivotal role of liver fat in metabolic syndrome and insulin resistance, and raises the question as to whether the use of liver fat in the definition of MHO might be an objective criterion to minimize confusion and differences in the MHO literature. Nevertheless, MRS is not readily available and expensive, which limits its wide clinical use.

Without a clear set of criteria in defining MHO, our understanding on potential protective factors and mechanisms that prevent MHO individuals from developing metabolic complications is limited and unclear. Perhaps using insulin resistance as a sole criterion is more objective and relevant to cardiovascular outcomes than a constellation of poorly defined metabolic parameters.

1.3 INSULIN-SENSITIVE OBESITY

Insulin resistance is a pivotal component of the metabolic syndrome. It is an obligatory precursor to the development of type 2 diabetes and a likely contributor to cardiovascular disease.[26] Given the variability in defining and identifying MHO, whether this phenotype is predictive of lower diabetes and cardiovascular risk cannot be answered. As insulin resistance is the key unifying factor in the metabolic syndrome, a more pathophysiological definition of MHO may be one based on insulin sensitivity alone, at least for research purposes.

Insulin resistance is a multisite dysfunction of insulin action in liver, skeletal muscle and adipose tissue.[27,28] Muscle or whole body insulin sensitivity is best evaluated by gold standard hyperinsulinaemic-euglycaemic clamp. However it is labour-intensive and invasive and is only used in specialised research units. A number of less laborious and expensive methods of assessing insulin sensitivity have been proposed such as glucose/insulin ratio,[29] oral glucose tolerance test (OGTT) derived indices,[30] but HOMA-IR has been the most widely used measure. HOMA was first described in 1985[31] to estimate insulin resistance from fasting glucose and insulin concentrations. It has the advantage of only requiring a single plasma sample assayed for insulin and glucose. The relationship between glucose and insulin in the fasting state reflects mainly the balance between hepatic glucose output and insulin secretion, which is maintained by a feedback loop between the blood glucose level and beta cells in the pancreas. HOMA has become widely used in the literature, and is more frequently used for the estimation of insulin resistance than beta cell function.[23]

Studies have shown that both the prevalence and incidence of cardiovascular risk factors and/or diseases are strongly related to insulin resistance.[22,32,33] Thus, insulin sensitivity could be the key factor discriminating healthy from at-risk obese individuals, and has been used as a sole criterion in many studies to define healthy obese individuals.[34-36] These studies used the terms obese insulin-sensitive (Ob_{sen}) and obese insulin-resistant (Ob_{res}) to categorise obese subjects according to their insulin sensitivity alone.

We currently do not have a consensus on the cut-off values for insulin sensitivity that are used to define Ob_{sen}. In studies that performed hyperinsulinaemic-euglycaemic clamp, one[36] used the upper quartile of glucose infusion rate (GIR) to define Ob_{sen} while another[34] used an absolute value of 8 mg/min/kg in GIR at an insulin infusion rate of 240 $pmol \cdot m^{-2} \cdot min^{-1}$ to define Ob_{sen}. There is a need for a consensus on the definition of MHO or Ob_{sen} when examining cardio-metabolic health in obesity to solve the current conundrum in the literature.

1.4 PREVALENCE OF METABOLICALLY HEALTHY OBESITY/INSULIN-SENSITIVE OBESITY

A recent meta-analysis[21] included 27 articles/studies (14 cross sectional, 13 prospective) in their analysis and found 30 different definitions that were used to identify MHO. The prevalence of MHO was 6-75%. The wide variation in the prevalence of MHO is due to inconsistent criteria used to define MHO phenotype. This creates confusion and difficulties in comparing results between studies. The wide range in prevalence of MHO can be explained by several factors.

The baseline population is heterogeneous. Some studies used the general population (obese and non-obese) as a denominator[22,37] and this makes the prevalence of MHO lower than studies that use an obese cohort only as the background to estimate the prevalence of MHO. Therefore, the prevalence of MHO varies significantly depending on the background cohort.

There are significant variations in gender and ethnicity among the study populations. For example, Iacobellis[24] estimated the prevalence of MHO in Italian obese individuals with age between 16 and 71 years with more than 3 quarters of the study population being female, while Wildman's study[37] focussed on an American population with age above 20 years and 60% were female. Thus, it is difficult to determine the true prevalence of MHO if the study populations are widely disparate in gender and ethnicity.

The different parameters and criteria used to define MHO by different studies created immense problems in estimating true prevalence of MHO. Kuk and Ardern's study[38]

used HOMA-IR index < 2.5 to define insulin sensitivity while Wildman's[37] study used HOMA-IR < 5.1. Messier's[39] study used triglycerides < 1.7 mmol/L as a cut-off, while Bonora's[22] study used triglycerides < 2.85 mmol/L. Logically, applying different criteria to define the same cohort, there will be different prevalence rates. For example, Velho[40] used 6 different sets of criteria used in previous studies to define MHO on a group of Swiss men and women aged 35-75 years and found that the prevalence of MHO ranged between 3.3% and 32.1% in men and between 11.4% and 43.3% in women depending on the criteria used. Messier[39] examined 113 obese sedentary postmenopausal women, and used 5 different definitions to evaluate the prevalence of MHO. Sixty-seven MHO individuals were identified using the 5 different definitions and there was no one single subject that fulfilled all 5 definitions. There were also significant differences in age, triglycerides/HDL, high-sensitive C reactive protein (hs-CRP) and fasting insulin observed among MHO subjects classified by the different definitions. Their study emphasises the need to unify various parameters and criteria to delineate this entity.

The prevalence of Ob_{sen} defined solely on the basis of insulin sensitivity also had wide variation ranging between 24% and 44%.[34,36,41-44] Studies used different measurements (clamp studies *vs.* HOMA-IR) and different cut-off values to define Ob_{sen}. HOMA-IR is a better indicator of hepatic as compared to whole body insulin sensitivity;[31] therefore, this could contribute partially to the wide variations in the prevalence of Ob_{sen}. This underscores the importance of having a standardised definition of the MHO/Ob_{sen} phenotype.

1.5 LONGITUDINAL STUDIES

Though cross-sectional studies suggest that MHO/Ob_{sen} may be a benign phenotype, the long-term protective benefits of the MHO/Ob_{sen} phenotype are unclear. Several prospective studies have shown consistently that MAO individuals have higher mortality than MHO,[45,46] but MHO has an increased risk of all-cause mortality and cardiovascular events compared with metabolically healthy normal-weight individuals.[47,48]Therefore the risks of MHO developing cardiovascular disease and mortality seem to be intermediate. Due to the multiple definitions of the MHO/Ob_{sen} phenotype and the different durations of follow up, findings of meta-analyses are inconsistent.

In the Ob_{sen} literature, several longitudinal studies[49-51] have used HOMA-IR to categorise a group of obese individuals into Ob_{sen} and Ob_{res} and followed these people over 6-20 years. The Ob_{sen} group had a lower incidence of diabetes and cardiovascular disease compared with Ob_{res} group in all studies, but Ob_{sen} subjects had higher risks than normal-weight healthy controls. However, one study, reported that the Ob_{sen} group had similar all-cause and cardiovascular mortality compared to normal weight healthy controls after 17 years of follow-up.[52] By using a strict set of criteria such as Edmonton obesity staging system (to define MHO), Padwal et al were able to independently predict no increase in mortality even after adjustment for contemporary methods of classifying adiposity.[53] As far as we are aware, there is no longitudinal data on the mortality risk of Ob_{sen} *vs.* Ob_{res} individuals defined by the gold standard hyperinsulinaemic-euglycaecaemic clamp. Further studies are needed to clarify the long term morbidity and mortality in Ob_{sen} and Ob_{res} groups using the clamp methodology.

MHO may be a transient phenotype. Most longitudinal studies[38,49,54] compared the incidence of cardiovascular disease and diabetes to baseline metabolic status on the assumptions that the MHO phenotype remains stable. According to a recent study by Appleton et al,[55] one third of MHO individuals have become MAO over a period of 5-10 years, while those individuals who remained metabolically healthy had lower risk of cardiovascular events and type 2 diabetes. Maintenance of a metabolically healthy status in obesity has been linked to higher levels of physical activity and weight loss in some studies[56,57] and high level of cardiorespiratory fitness in another.[58] Therefore, despite the controversy regarding the long term outcomes of MHO, maintaining healthy lifestyle and avoiding further weight gain may be the key to sustaining metabolic health in obesity.

One recent study showed that MHO and metabolically healthy normal-weight groups with similarly low liver fat content had similar incidence of diabetes mellitus after 5 years of follow-up.[59] Inflammation may play a role in the development of diabetes; in a recent Korean study, MHO objects with low CRP levels had a similar diabetes incidence as a metabolically healthy non-obese group after 36 months of follow-up.[60] This highlights the importance and significance of central adiposity, liver fat and inflammation in insulin resistance and metabolic complications.[55] This is supported by numerous cross-sectional studies, where lower central fat is associated with a metabolically healthy obesity phenotype.[37,45] Further studies are needed to clarify the long-term outcome of MHO/Ob_{sen} phenotype, especially looking at various factors that contribute to maintenance of the MHO/Ob_{sen} phenotype with time.

In summary, current literature is inconsistent as to the potential protective benefits of MHO/Ob_{sen}. However, given the tremendous variations in the criteria used to define MHO, caution is needed when interpreting these findings. Care should be taken to recognise that MHO/Ob_{sen} might be a transient phenomenon and these individuals should be encouraged to not gain weight and adopt a healthy lifestyle to preserve their MHO status.[56] In addition, certain characteristics such as liver fat and hs-CRP could be utilised as adjunctive measures to predict long term outcome of MHO/Ob_{sen} individuals in addition to current diagnostic definitions/criteria. Future clearly-defined prospective studies are needed to explore and clarify the long term outcomes of the MHO/Ob_{sen} phenotype.

1.6 CHARACTERISTICS OF METABOLICALLY HEALTHY OBESE INDIVIDUALS

Cross sectional studies that compared MHO and MAO are detailed in Table 1.1. Studies that compared Ob_{sen} and Ob_{res} are detailed in Table 1.2. Table 1.1 highlights the complexity introduced when numerous different diagnostic criteria were used to define MHO.

1.6.1 Gender

Cross-sectional studies involving men and women showed that women tend to exhibit more favourable metabolic parameters than men across different ethnic and age groups.[42,61] This could affect findings when the distribution of men and women in studies are uneven such that the prevalence of MHO is higher in cohorts with higher proportions of women. Nevertheless, Wildman[37] studied 5440 American obese individuals over 20 years of age with 48% of cohort being men and still found more women (60%) than men being metabolically healthy. Premenopausal women who have similar BMI and age as men tend to have better whole body insulin sensitivity, most likely due to differential fat distribution. Specifically, premenopausal women tend to accumulate fat in the subcutaneous compartment, while men accumulate more intra-abdominal fat.[62,63] Therefore it is important to recognise the potential differences in fat distribution between premenopausal women and men and the potential contribution to the prevalence of MHO/Ob_{sen} phenotype.

1.6.1.1 Menstrual cycle and insulin sensitivity

There is conflicting evidence on insulin sensitivity variation at different phases in a regular menstrual cycle in premenopausal women. Insulin resistance correlated with oestradiol and progesterone in healthy normal weight premenopausal women[64] and studies that have used intravenous glucose tolerance test (IVGTT)[65,66] or HOMA-IR[64] found a lower insulin sensitivity during the luteal phase compared with the follicular phase. To the contrary, several hyperinsulinaemic-euglycaemic clamp studies conducted in normal healthy non-obese women showed no differences in insulin sensitivity (glucose disposal rate [GDR]) among various phases such as follicular, luteal and menstrual phases for single[67] and multiple insulin infusion rates.[68,69] Altogether, these differences in insulin sensitivity in different phases of the menstrual cycle are probably too small to be clinically meaningful.

It is difficult to draw conclusions from various studies due to different sample sizes (e.g., n=6[68] vs n=257[64]), and methods of measuring insulin sensitivity (HOMA-IR *vs.* clamp studies). Future larger studies using euglycaemic clamps might be warranted to delineate the role of sex hormones and insulin sensitivity in obese population.

Table 1.1 Prevalence and metabolic accompaniments of metabolically healthy obese subjects in the literature

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Aguilar-Salinas[61]	Mexican population, age 18-70 years (716)	BMI > 30 kg/m^2 • HDL > 40 mg/dl • Absence of T2DM • Absence of HTN (140/90)	24% of total cohort; 36% of obese cohort	171/299	38M/133F	NR/41	Smaller waist circumference Higher adiponectin
O'Connell[70]	Prior bariatric surgery (including DM) (29)	BMI > 48 kg/m^2 None of the below • BGL ≥ 5.6 mmol/L • BP ≥ 135/85 mmHg • TG/HDL ≥ 1.65 (male) • TG/HDL ≥ 1.32 (female)	41% of obese cohort	12/17	4M/8F	48/40	Lower preadipocyte factor-1 (omental and subcutaneous) Lower omental macrophage number and size Lower SC macrophage number
Geetha[71]	Southern Indian population, age > 20 years (2350)	BMI > 25 kg/m^2 • Absence of metabolic syndrome according to South Asian Modified National Cholesterol Education Programme	13.3% of total cohort; 19% of obese cohort	312/1335	114M/198F	27.5/36	Lower BP, HbA1c, BGL, TG
Hong[72]	Korean population age > 18 years (16190)	BMI > 25 kg/m^2 0 or 1 component of metabolic syndrome: • BP >130/85 mmHg • TG >1.7 mmol/L • HDL <1.3 mmol/L • Fasting BGL >5.6 mmol/L	14% of total cohort; 45% of obese cohort	2318/2778	56.9% (male)	27.1/42.4	Higher physical activity, lower BP, lipid, HOMA-IR, insulin, fasting blood glucose and liver enzymes

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Eglit[73]	Estonian population, age 20-74 years (495)	BMI > 30 kg/m^2 Absence of the followings: • Impaired glucose regulation • HDL < 1.3 mmol/L in women, < 1.03 mmol/L in men • TG ≥ 1.7 mmol/L • BP > 130/80 mmHg • IR- upper quartile of HOMA-IR of whole group	4% of total cohort; 12% of obese cohort	19/139	6M/13F	NR/50.4	Lower HOMA-IR and higher high molecular weight adiponectin in men
Gomez-Huelgas[74]	Spanish population age 18-80 years (2270)	BMI > 30 kg/m^2 0 or 1 of the followings: • BP > 130/85 mmHg • TG > 1.7 mmol/L • HDL-C) (< 1 mmol/L in men or < 1.3 mmol/L in women • fasting plasma glucose ≥ 5.6 mmol/L	2.2% of total population; 9.6% of obese cohort	50/470	18M/32F	33.6/42.1	Younger, higher education level and sedentary lifestyle, lower waist circumference, fatty liver index, smoking rate

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Lopez-Garcia[75]	Spanish population age > 18 years (12883)	BMI > 30 kg/m^2 0 or 1 of the following: • BP > 130/85 mmHg • TG > 1.7 mmol/L • HDL-C) (< 1 mmol/L in men or < 1.3 mmol/L in women) • fasting plasma glucose ≥ 5.6 mmol/L • HOMA-IR > 4.05 • Hs-CRP > 0.74 mg/dL	6.5% of total population; 28.9% of obese cohort	754/1857	48.5% men	48.3/32.7	Lower age, current smoking, moderate alcohol consumption (7.1-17.5 g/day), physical activity (> 33 MET-hrs/week)
Wildman[37]	USA population, age > 20 years (5440)	BMI > 30 kg/m^2 0 or 1 of the following: • BP > 130/85 mmHg • TG > 150 mg/dL • HDL-C (< 1 mmol/L in men or < 1.3 mmol/L in women) • fasting plasma glucose ≥ 5.6 mmol/L • HOMA-IR > 5.13 • Hs-CRP > 0.1 mg/L	9.7% of total population; 31.7% of obese cohort	NR	39.2% men	40.1/34.2	Younger age, non-Hispanic black race/ethnicity, Higher physical activity levels and smaller waist circumference.

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Hankinson[76]	USA population, age 40-59 years (4680)	BMI > 30 kg/m^2 Fulfils all the following criteria • Favourable blood pressure (120/80 mm Hg) and no medication or special diet for hypertension; • no physician diagnosis, medication, or special diet for other metabolic risk factors (i.e., diabetes and dyslipidaemia); • No prevalent cardiovascular disease.	3.2% of total population; 19% of obese cohort	149/626	75M/74F	47.1/34	Longer sleep duration in women
Doumatey[77]	African Americans (822)	BMI ≥ 30 kg/m^2, • fasting plasma glucose ≤ 7mmol/L, systolic blood pressure (SBP) ≤ 130 or diastolic blood pressure (DBP) ≤ 85, • HDL-C (> 1 mmol/L in men or > 1.3 mmol/L in women)	12% of total population; 28% of obese cohort	96/247	32M/64F	42.3/36	Higher adiponectin in both Men: lower insulin and HOMA-IR, Women: lower WHR and TG

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Camhi[78]	American 1.adolescents (335) 2.adults 19-44 years (635) 3.adults 45-85 years (779)	Adolescents: BMI > 30 kg/m^2 0 or 1 of the followings: • TG ≥ 1.24 mmol/L • HDL ≤ 1mmol/L • BP ≥ 90th percentile for age/gender • Fasting BGL ≥ 5.6mmol/L Adults 0 or 1 of the followings: • TG ≥1.7mmol/L • HDL ≤ 1 mmol/L in men or ≤ 1.3 mmol/L in women BP ≥ 130/85 mmHg • Fasting BGL ≥ 5.6mmol/L	Total; obese population: 1: 49%; 68% 2: 24%; 54% 3: 8%; 24%	1: 163/62 2:152/118 3:64/207	Proportion of Men 1: 67% 2:63% 3: 38%	1: 14.6/32.3 2: 30.8/34.6 3: 55.2/34.5	Adults (19-44 years)- higher vigorous physical activity and active transportation (walking/bicycle) Adults (45-85 years)- higher moderate physical activity
Pajunen[79]	Finnish population age 45-74 years (28490	Two or less of the following five components: • large waist circumference (≥ 94 cm in men and ≥ 80 cm in women), • TG ≥ 1.7 mmol/l • HDL cholesterol level < 1.0 mmol/l in men or < 1.3 mmol/l in women, BP ≥ 130/85 mmHg • Fasting BGL ≥ 5.6 mmol/l	3% of total population 28.7% of obese cohort,	94/609	28.7% men	58.5/33.2	Lower prevalence of diabetes

Study	Population (n)	Definition of MHO	Prevalence of MHO	MHO/MAO (n)	MHO Gender	MHO BMI/age	Characteristics of MHO compared with MAO
Wildman[80]	USA population Postmenopausal women age 50-79 years (1889)	Overweight and obese BMI > 25 kg/m^2 0 and 1 of the followings: • BP ≥ 130/85 mmHg • Fasting TG ≥ 1.7 mmol/L • HDL < 1.3 mmol/L • Fasting BGL ≥ 5.6 mmol/L	17.5% of total population; 27.7% of overweight/obese cohort	330/860	All women	29/67.6	Lower fibrinogen, CRP, IL-6, TNF-α, WBC, E-selectin, and PAI-1

Abbreviations: PAI = plasminogen activator inhibitor-1; VCAM-1 = vascular cell adhesion molecule-1, NR = not reported, SC = subcutaneous, PA = physical activity, DM = diabetes mellitus, BGL = blood glucose level, MHO=metabolically healthy obesity, M = male, F = female

Table 1.2 Comparison of metabolic characteristics in obese insulin-sensitive and insulin-resistant subjects in reported studies

Study*	Number Ob_{sen}/Ob_{res}	BMI (kg/m^2) Ob_{sen}/Ob_{res}	Age Ob_{sen}/Ob_{res}	Gender $Ob_{sen}F/Ob_{sen}M$; $Ob_{res}F/Ob_{res}M$	Insulin sensitivity measured by	Clamp insulin dose	Visceral fat	Liver fat	DXA	BP	Lipids	CRP (ug/ml)
Stephen[42]	31/96	33.7/34.2	46.5/45.8	19/12; 59/37	OGTT- upper quartiles and 3 lower quartiles	NA	No difference (MRI)	Ob_{sen} lower (MRS)	ND	ND	Same (FA)	Not done
Karelis[36]	22/22	32.3/34.8	56.7/59.2	PW	HEC# (upper quartile vs lower quartile)	75 $mU \cdot m^2 \cdot min^{-1}$180 min	Ob_{sen} lower (CT)	ND	Yes	No difference	Lower TG, higher HDL (same FA)	Ob_{sen} lower
Weiss[43]	14/14	37.8/37.7	13.7/13.9	7/7; 7/7	HEC # (M>8.5mg/kg-lbm.min)	80 $mU \cdot m^{-2} \cdot min^{-1}$120 min	Ob_{sen} lower (MRI)	ND	Yes	ND	Lower TG (same FA)	Not done
Kloting[81]	30/30	45.1/45.2	44.6/44.9	20/10; 20/10	HEC# (GIR >70 µmol.kg^{-1}.min^{-1}, GIR <60 µmol.kg-1.min-1)	40.3 $mU \cdot m^{-2}$ body surface $\cdot min^{-1}$ 120 min^	Ob_{sen} lower (CT or MRI)	Ob_{sen} lower (MRI)	ND	ND	Lower TG, FFA Higher HDL	Ob_{sen} lower
Brochu[34]	17/26	31.5/34.7	58/58.6	PW	HEC# (M>8 mg/min.kg lbm)	34.6 $mU \cdot m^{-2} \cdot min^{-1}$hours^^	Ob_{sen} lower (CT)	ND	Yes	No difference	Lower TG, Higher HDL	Not done

Study*	Number Ob_{sen}/Ob_{res}	BMI (kg/m^2) Ob_{sen}/Ob_{res}	Age Ob_{sen}/Ob_{res}	Gender $Ob_{sen}F/Ob_{sen}M$; $Ob_{res}F/Ob_{res}M$	Insulin sensitivity	Clamp insulin dose	Visceral fat	Liver fat	DXA	BP	Lipids	CRP (ug/ml)
Tonks[82]	16/21	29/34.1	56.2/58.4	11/5; 7/13	HOMA-IR <1.5, HOMA-IR > 3	NA	Ob_{sen} lower (CT)	Ob_{sen} lower (CT)	Yes	ND	ND	Not done
Succurro[83]	22/43	34.5/36.4	34/36	19/3; 28/15	Mffm$^{\phi}$ in upper quartile	40 $mU \cdot m^{-2} \cdot min^{-1}$	ND	ND	Yes	Lower DBP	Lower TG, FFA, Higher HDL	Not done
Tarantino[84]	21/21	39.4/41.4	19.5/19.2	NR	HOMA < 1.95	NA	No difference (U/S)	Ob_{sen} lower (U/S)	No	ND	ND	Ob_{sen} lower

$^{\phi}$Mffm = glucose disposal calculated as the mean rate of glucose infusion measured during the last 60 min of the clamp (steady state) is expressed as mg per minute per kilogram FFM

$^{\#}$ HEC = Hyperinsulinaemic-euglycaemic clamp

*All studies have excluded diabetic subjects except in Stephen's study[42]

^ Insulin infusion rate was based on Bluher's study[85] as per email correspondents with Professor Bluher, insulin units were converted from $nmol \cdot m^{-2} \cdot min^{-1}$ to $mU \cdot m^{-2} \cdot min^{-1}$

^^ Insulin units were converted from $pmol \cdot m^{-2} \cdot min^{-1}$ to $mU \cdot m^{-2} \cdot min^{-1}$

TG = triglycerides, FA = fatty acid, ND = not done, PW = postmenopausal women, U/S = ultrasound, MRI = magnetic resonance imaging, CT = computed tomography. NR = not recorded, NA = not available, LBM = lean body mass

1.6.2 Age

The MHO phenotype has been shown to correlate with a younger age and earlier age of onset of obesity. One Italian study[86] identified 13% of the obese population as metabolically healthy, defined by absence of ATP-III criteria. The study reported that MHO individuals were younger age with lower BMI compared to MAO. An American study[37] examined 5440 US citizens aged 20 years and older and identified 32% of obese adults as metabolically healthy by the definition of ATP-III plus HOMA-IR score. They found that younger age is an independent correlate of MHO among obese individuals.

These observations are supported by a recent meta-analysis which showed that the prevalence of MHO decreases with age despite differences in MHO definitions.[21] This association between younger age and MHO may be explained by the fact that in younger individuals, there is only a short period of time for obesity-related complications to develop; thus younger individuals are more metabolically healthy at that point of time. Therefore, it may be appropriate for clinicians to reinforce healthy lifestyle modification in younger MHO individuals to avoid further weight gain to potentially reduce long term metabolic and non-metabolic obesity-related complications.

1.6.3 Lifestyle

Physical activity has been shown to positively impact on energy balance and body composition[87] and an association has been noted between higher physical activity and a reduction in visceral adiposity.[88] While a plausible explanation for maintenance of metabolic health in obesity, a healthier diet and engagement in physical activity are hard

to measure and monitor in free living individuals in studies. Yet, anecdotal data suggest lower saturated fat[89] and alcohol[90] intake and higher level of physical activity[78,90] in insulin-sensitive or metabolically-healthy obese individuals.

Assessment of lifestyle factors in studies rely on self-reported physical activity and diet diary and overweight/obese subjects have been shown to substantially overestimate energy expenditure[91] and to have dietary self-report inaccuracies.[76,92] Physical activity assessed by questionnaires was not different between insulin-sensitive and insulin-resistant obese individuals in two studies.[34,93] Similarly, a healthier diet has not been shown to be associated with healthy obesity in a recent cross-sectional study involving 6964 women.[92] Another study in American men and women also did not detect any significant differences in diet composition between metabolically healthy and abnormal obesity phenotypes.[76] Further studies with comprehensive physical and diet assessment tools such as pedometers and weighed food records are needed to further clarify the role lifestyle factors play in metabolic health in obesity.

1.6.4 Visceral Fat

Visceral fat denotes the amount of fat that envelops internal organs. Visceral adipose tissue has an important association with both insulin resistance and inflammation in obesity.[94] Some studies suggested that lower visceral fat could explain the more favourable metabolic and inflammatory profile described in MHO.[34,36] These studies measured visceral fat at L4/L5 using computed tomography (CT) scan on a group of postmenopausal women. They performed clamp studies and used glucose infusion rate to identify Ob_{sen}, though using different cut-off values in GDR (Ob_{sen} was defined as GDR [M value] > 8mg/min.kg lean body mass in Brochu's study[34] and upper quartile of

GDR [M value] in Karelis's study[36]). Both studies found that Ob_{sen} women had lower visceral fat compared to Ob_{res}. Interestingly, in a stepwise regression analysis, with glucose disposal as the dependent variable, visceral adipose tissue explained 22% of variance in glucose disposal, which could suggest that visceral fat is an important contributor to insulin sensitivity.[95]

In contrast, Stefan and his group[42] did not find any difference in visceral adipose tissue between MHO and MAO. Stefan examined 314 German subjects with mean age of 45 years, who had a history of either impaired glucose tolerance/gestational diabetes and/or family history of type 2 diabetes mellitus. Stefan used the OGTT derived index (as proposed by Matsuda and DeFronzo[96]) to assess insulin sensitivity. Ob_{sen} was defined as BMI > 30 kg/m^2 and belonging to the upper quartile of insulin sensitivity, and the remainder of subjects with BMI > $30kg/m^2$ were deemed Ob_{res}. Magnetic resonance imaging (MRI) was used to measure visceral adipose tissue volume. There was no difference between Ob_{sen} and Ob_{res} in visceral adipose tissue (3.5 kg *vs.* 4 kg, $p > 0.05$). This finding was unexpected, as previous studies have demonstrated lower visceral fat in MHO. Potential explanations could be the different study populations (previous studies only examined postmenopausal women), imaging modalities (CT *vs.* MRI) and insulin sensitivity assessment (clamp studies *vs.* OGTT-derived index). Nevertheless, subsequent hyperinsulinaemic-euglycaemic clamp studies have identified lower visceral fat in Ob_{sen} compared with Ob_{res} assessed by either MRI[43] or CT.[34,36,81,82]

There are several putative mechanisms linking visceral fat to insulin resistance. Firstly the lability of lipolysis allows direct drainage of fatty acids to the liver via the portal circulation, inducing liver insulin resistance.[97] Secondly, visceral fat is suggested to

have a higher number of immune cells than other fat depots and secrete higher amounts of inflammatory molecules that may induce insulin resistance.[98] These inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin 6 (IL6) and interleukin 1-β are secreted excessively with visceral fat accumulation and this exerts a further negative effect on the production of adiponectin.[99] The exact mechanisms linking visceral fat and lower adiponectin levels are poorly understood. Adiponectin is an insulin sensitiser and therefore could potentially explain the link between visceral adiposity and insulin resistance. Despite the potential mechanism linking visceral fat to insulin resistance, there is evidence in recent literature on the pivotal role of liver fat that perhaps more closely relates to insulin sensitivity than visceral fat.[100] In summary, visceral fat has been shown in most cross-sectional studies to be strongly correlated with insulin resistance.

1.6.5 Liver Fat

Elevated lipid accumulation in the liver is accompanied by atherosclerosis and the metabolic syndrome,[101] even independent of visceral adiposity.[102] Fewer studies have directly compared the amount of liver fat between MHO and MAO. Stephen[42] used OGTT-derived index to categorise a group of obese subjects (BMI > 27kg/m^2) into Ob_{sen} and Ob_{res}. MRS was used to estimate liver fat; Ob_{sen} women had lower liver fat compared with Ob_{res} women (3.5% *vs.* 8.8%, $P < 0.001$). Liver fat was also lower in Ob_{sen} men compared with Ob_{res} men though not statistically significant (5.6% vs. 10.5%, $P = 0.054$).

A recent study from our laboratory by Tonks[82] compared liver fat between Ob_{sen} and Ob_{res}. Tonks and colleagues used HOMA-IR to categorise overweight/obese individuals

and identified 21 Ob_{res} (HOMA-IR > 3) and 16 Ob_{sen} subjects (HOMA-IR < 1.5). Peripheral insulin sensitivity was assessed by clamp studies and was similar between Ob_{sen} and lean controls, despite the fact that Ob_{sen} had double the amount of total and central fat. Liver fat was assessed by CT at T12/L1 level. Interestingly, Ob_{sen} group had similar liver fat content as lean controls, but significantly lower fat than Ob_{res} group. Tonks' findings were supported by other studies that found lower liver fat in Ob_{sen}.[42,81] These studies all support the association between liver fat and insulin resistance.

Lower liver fat could potentially protect obese individuals from developing obesity-related metabolic complications. A recent longitudinal study in a Japanese population showed that MHO individuals without fatty liver (measured by ultrasound) had a lower incidence of diabetes than MAO without fatty liver and the odds ratio was not significantly different from that for the metabolically healthy normal weight group without fatty liver.[19] Also, many other studies demonstrated an increased incidence of type 2 diabetes in individuals with high liver fat content independent of established risk factors in Asians.[103-105] Furthermore, a recent study suggested that the MHO phenotype defined by low liver fat (intra-hepatic triglycerides < 5.6%) was resistant to the adverse metabolic effect of overfeeding and weight gain with no deterioration in their muscle, hepatic and adipose tissue insulin sensitivity.[25] These observations underscore the significance of liver fat in insulin resistance and metabolic syndrome.

The relationship between liver lipid and insulin resistance is complex and bidirectional. Many studies have shown that liver adipose tissue accumulation contributes to insulin resistance.[106] Nevertheless, in the insulin-resistant hyperinsulinaemic state, a "selective insulin sensitivity" of the pathway to lipid synthesis through sterol regulatory element-

binding protein 1c (SREBP 1c) has been described,[107] which contributes to increased liver lipid production. Furthermore, increased peripheral lipolysis from insulin resistance could also contribute to increased free fatty acid supply to the liver, leading to liver fat accumulation.[108] Therefore liver fat is possibly both a contributor to and a consequence of insulin resistance, and further prospective studies could assist in establishing and clarifying the causal relationship between liver steatosis and insulin resistance in obesity.

1.6.5.1 Liver insulin resistance

Insulin resistance manifests mainly in liver, muscle and adipose tissue. The magnitude and severity of insulin resistance in different tissues might be different. Current lifestyle and therapeutic interventions target insulin resistance at different tissue sites. Metformin therapy mainly targets liver insulin resistance, physical activity predominantly targets muscle insulin resistance, while thiazolidinedione therapy and weight loss affect both liver and muscle insulin resistance.[109] Liver insulin resistance plays a vital role in regulation of glucose, lipid metabolism and systemic inflammation. It is a significant indicator of cardiovascular disease[110] and correlates with non-alcoholic fatty liver disease (NAFLD).

Hyperinsulinaemic-euglycaemic clamp is the gold standard method to measure muscle insulin resistance;[111] when combined with radiolabelled deuterated glucose tracer and using a relatively low dose insulin infusion rate, it allows measurement of both liver and muscle insulin sensitivity.[112-114] A tracer is a labelled form of a substance.[115] In glucose metabolism, either hydrogen or carbon-labelled glucose tracer are used to determine hepatic insulin sensitivity.[116] Among all the glucose tracers, 6-6 $^{2}H_2$ glucose is

considered the most accurate tracer in determining hepatic glucose output (equivalent to EGP).[117] Both tracer and tracee can be measured by mass spectrometry. EGP in the fasted state assumes the one compartment model theory under steady state,[116] while EGP measured during clamp is assessed during non-steady state, commonly using Steele's equation to assume mono-compartment with constant volume.[118]

The limitation of measuring EGP as a surrogate for liver insulin sensitivity is that the kidney can also contribute to EGP, between 5-28% in the post-absorptive state.[119,120] Nevertheless, isotope-labelled glucose tracer methodology has been used since the early 1950's,[121] and is regarded an irreplaceable tool in assessing hepatic insulin sensitivity.

Liver insulin resistance contributes to fasting and postprandial hyperglycaemia via increased hepatic glucose output. Liver insulin resistance has been shown to correlate positively with hypertriglyceridemia and inflammatory markers such as CRP,[110] which could contribute to increased cardiovascular risk. Studies have shown that liver lipid accumulation contributes to liver insulin resistance,[122,123] or is a consequence of liver insulin resistance.[106] In the state of liver insulin resistance with associated hyperinsulinemia, the lipid synthesis stimulation pathway through SREBP-1c remains insulin-sensitive,[107] in contrast to increased hepatic glucose synthesis. This causes accumulation of liver lipid which could lead to dyslipidaemia.

There are several clinical surrogates or markers for liver insulin resistance. Hepatic glucose output is regulated by liver insulin resistance, and fasting insulin level has been shown to be a surrogate of liver insulin resistance.[116] Other surrogates for liver insulin resistance include HOMA-IR and OGTT-derived index,[124] liver fat,[122] as measured by

MRS, and the liver enzyme alanine aminotransferase (ALT).[125] Further large studies are needed to specifically examine the role of liver insulin resistance and its role in glucose and lipid metabolism and devise a set of clinically available surrogate markers.

1.6.6 Adipocyte size

Adipose tissue is recognised as an important endocrine organ. Adipocyte size was shown to correlate positively with adipocyte insulin resistance four decades ago[126,127] and more recently.[81,128] Large subcutaneous abdominal adipocyte size has been shown to be an independent predictor of type 2 diabetes.[129] Adipocyte cell size decreases with weight reduction in obese individuals,[128] with associated improvement in adipocyte insulin sensitivity and both fasting plasma insulin levels,[127] and insulin levels during an oral glucose tolerance test.[126] Conversely, 28 days of overfeeding with moderate weight gain (+2.7-3.4 kg on average), did not result in a change in subcutaneous abdominal adipocyte size[130,131] in a group of non-diabetic non-obese individuals.

There is conflicting evidence as to whether subcutaneous adipocyte size associates with insulin sensitivity in humans. One study suggests that it is the omental adipocyte size that correlates with metabolic health and presence of hepatic steatosis, but not the subcutaneous adipocyte size in a severely obese population.[132] Omental adipocyte size has a stronger negative correlation with insulin sensitivity than subcutaneous adipocyte size.[81] Another study by McLaughlin and colleagues did not report any differences in adipocyte size between Ob_{sen} and Ob_{res} individuals;[133] however, the Ob_{sen} group had a higher ratio of small to large adipocyte cells and lower expression of genes encoding markers of adipose cell differentiation (peroxisome proliferator-activated receptor (PPAR)-γ1, PPAR-γ2, glucose transporter 4 (GLUT4) and adiponectin) compared to

Ob_{res} group. They postulated that impaired adipose cell differentiation may contribute to obesity-related insulin resistance rather than the size of adipocytes. Nevertheless, this study did not use hyperinsulinaemic-euglycaemic clamp to measure insulin sensitivity (HOMA-IR was used), which may affect the significance of their findings. Further studies are needed to clarify the association between adipocyte size and insulin sensitivity in obese individuals.

The exact mechanisms linking adipocyte size and insulin resistance/gluco-metabolic disorders are unknown. Adipocyte size is an important determinant of adipokine secretion, and there seems to be a differential expression of pro-inflammatory and anti-inflammatory adipokines with increasing adipocyte size. Skurk[134] has demonstrated a predominance of pro-inflammatory cytokines production (leptin, IL-6, interleukin-8, monocyte chemoattractant protein-1 (MCP1) and granulocyte colony-stimulating factor) in large subcutaneous abdominal adipocytes compared with small adipocyte cells. This pro-inflammatory status is considered to build the common soil for the development of insulin resistance, type 2 diabetes and atherosclerosis.[129,135]

Furthermore, it is thought that large adipocyte cells have a decreased response to insulin stimulation by having a reduced amount of GLUT4 in the plasma membrane; while small adipocyte cells have double the amount of GLUT4 translocation at the cell surface in response to insulin.[136] This may provide an explanation to the link between the size of the adipocyte and adipose tissue insulin resistance at the molecular level. Another mechanism could be differential gene expression between large and small adipocyte cells. Large adipocyte cells have increased serum amyloid A (SAA) and transmembrane 4 L six family member 1 (TM4SF1) genes expression by more than 19 and 22 fold,

respectively compared with small adipocyte cells in the same individuals.[137] SAA and TM4SF1 may link hypertrophic obesity to insulin resistance.[137] Further studies are necessary to clarify the association between adipocyte size and insulin resistance, preferably with use of gold-standard hyperinsulinaemic-euglycaemic clamp.

1.6.7 Adipose tissue macrophages

Macrophages in adipose tissue play an important role in low-grade chronic inflammation that could be linked with insulin resistance in obesity. Macrophages were found in white adipose tissues of obese subjects in several studies.[138-140] Macrophage infiltration in abdominal adipose tissue is increased in obese and pre-diabetic individuals relative to lean healthy individuals.[141,142] It has been shown that increased macrophage infiltration in the omental adipose tissue was associated with insulin resistance measured by HOMA-IR[143] and hyperinsulinaemic-euglycaemic clamp.[81] These cross-sectional studies have shown a significant correlation between macrophage infiltration in adipose tissue and insulin resistance, though causality cannot be proven in cross-sectional studies.

Macrophages tend to surround the mature adipocyte cytoplasm in “crowns” in obese individuals and this pattern is different to that seen in lean subjects.[144] While “crowns” have been shown to disappear following weight loss surgery (-22kg) accompanied by a significant reduction in macrophage number. Overfeeding for 28 days, which caused impairment in insulin sensitivity, has not resulted in a significant change in macrophage number, though the weight change was modest (2.7kg).[130] Interestingly, lower subcutaneous adipose tissue and visceral adipose tissue macrophage activation predicted

a greater weight loss after surgery in severely obese individuals;[145] this suggests adipose tissue immune cells might be involved in the regulation of weight loss.

The association of macrophage numbers and insulin resistance/chronic inflammation may extend to the specific phenotype of macrophages. M1 and M2 macrophages have different gene expression patterns. M1 macrophages are activated by interferon-γ or lipopolysaccharide, and are pro-inflammatory, thus contributing to a state of low-grade inflammation.[146] M2 macrophages are activated by IL-13 or IL-4 and are anti-inflammatory.[147] An increase in M1 adipose tissue macrophages and M1-to-M2 ratio was observed in obese individuals *vs.* lean control[147] and is associated with increased insulin resistance in the high fat fed mouse model.[148] Weight reduction following Roux-en-Y bypass surgery (RYBG) was associated with a reduction in M1 macrophages and M1/M2 ratio.[147] Further studies using hyperinsulinaemic-euglycaemic clamps will be helpful in clarifying the association between insulin sensitivity and adipose tissue macrophages/inflammatory cytokines.

1.6.8 Adipokines/chemokines/hepatokines

Adipocytes secrete a multitude of bioactive polypeptide hormones, known as adipokines that modulate glucose and lipid metabolism, immunity and neuroendocrine systems.[149] Adipokines/hepatokines may play a role in modulating insulin sensitivity and provide a putative mechanism that links fat depots and hepatocytes to insulin sensitivity.

1.6.8.1 Highly-sensitive C-reactive protein (hs-CRP)

CRP is secreted from the liver and is elevated in acute phase reaction or inflammation. The hs-CRP measurement is associated with insulin resistance[150] and cardiovascular morbidity in obese individuals,[151] and has been shown to be elevated in Ob_{res} compared with Ob_{sen} in studies that have measured insulin sensitivity using clamp[36,81] or HOMA-IR.[45,84] Previous studies[152,153] have suggested that CRP levels could be an important factor associated with variations in insulin sensitivity, but this relationship is abolished after controlling for visceral fat quantity, suggesting lower hs-CRP in MHO appears to be a marker of lower visceral fat.[36] The exact mechanism by which CRP may contribute to insulin resistance is unclear, perhaps relating to visceral adiposity. Further studies are needed to clarify the association between hs-CRP and insulin resistance in relation to visceral adiposity.

1.6.8.2 Leptin

Leptin secretion from adipose tissue parallels the adipose tissue mass and correlates with adipocyte size and triglyceride content.[149] In the obese population, there are increased levels of leptin, but reduced response to rising endogenous leptin suggesting leptin resistance.[154] In lean individuals, leptin stimulates fatty acid oxidation by activating AMP-activated protein kinase and inhibiting acetyl-coenzyme A carboxylase (ACC).[155] This limits accumulation of triglycerides ectopically in tissues such as muscle and liver. Serum leptin levels were similar between Ob_{sen} and Ob_{res} groups categorised by hyperinsulinaemic-euglycaemic clamp in both adolescent[43] and adults.[81] This suggests that leptin is probably more related to adiposity than insulin sensitivity.

1.6.8.3 Adiponectin

Adiponectin has an inverse relationship with body fat mass and BMI.[156] Adiponectin modulates a number of metabolic processes including glucose and fatty acid metabolism and is an insulin sensitiser in muscle and liver. Adiponectin is anti-inflammatory and has a protective effect from development of cardiovascular disease.[99] Adiponectin circulates in oligo- or multi-meric forms and the high molecular weight (polymeric) form appears to be the major mediator of its beneficial effects (insulin-sensitising).[157,158]

Studies have reported conflicting evidence regarding the association between adiponectin concentration and MHO/Ob_{sen} phenotype. Two studies have shown that adiponectin levels are higher in Ob_{sen} groups. Tonks' study[82] showed that adiponectin levels in Ob_{sen} were similar to lean controls, and were significantly higher than in Ob_{res} at both baseline and steady state hyperinsulinaemic-euglycaemic clamp. Similarly, Kloting and colleagues study[81] used a clamp study and showed Ob_{sen} subjects had higher adiponectin levels compared to an Ob_{res} group.

Conversely, Stephan's study,[42] did not find any significant differences between Ob_{sen} and Ob_{res} groups in adiponectin levels. They used HOMA-IR to measure insulin sensitivity. However this lack of significance may be due to differences in assessing insulin sensitivity (clamp *vs.* HOMA-IR) and sample size. Similarly, Fabbrini and colleagues[25] defined MHO as having liver fat content of <5.6%, and did not demonstrate a difference in adiponectin levels between MHO and MAO (defined by liver fat). Inconsistent findings between studies can also relate to BMI and body fat mass differences between groups or gender imbalance in studies that found significant differences in adiponectin compared to matched cohorts.[159]

Recently, an Estonian study showed that MHO men had higher high-molecular weight adiponectin compared to MAO men.[73] This was supported by another study involving African men and women.[77] Both studies defined MHO as absence of hypertension, dyslipidaemia and insulin resistance/hyperglycaemia. The current evidence may suggest that adiponectin is one of the determinants of the MHO/Ob_{sen} phenotype. Nevertheless, further studies with larger samples are needed to further clarify this association, particularly adjusting for visceral fat and gender.

1.6.8.4 Adipocyte fatty acid binding protein (FABP4)

FABP4 is produced in adipocytes and macrophages and is regulated by PPAR-γ agonists, insulin and fatty acids.[160] PPAR is a member of the nuclear receptor family of ligand activated transcription factors which induces expression of FABP-4 and is a key regulator of adipocyte differentiation; it plays a key role in the anti-diabetic actions of the thiazolidinediones.[161] FABP4 has been shown to play a pivotal role in the regulation and dysregulation of inflammatory and metabolic responses.[162,163] FABP4 is elevated in type 2 diabetic and metabolic syndromes[164,165] and elevated FABP4 is associated with higher cardiovascular risk.[166] FABP4 has pleiotropic roles that include the stimulation of hepatic glucose output production[167] and thereby chemical inhibition of FABP4 might show a benefit in preventing obese humans from diabetes and cardiovascular disease.[160] FABP4 has a significant role in insulin resistance and metabolic syndrome by its implication in the control of lipid homeostasis, linked to inflammatory function in macrophages and cholesterol trafficking.[162] One clamp study did not show any significant differences in FABP4 between Ob_{sen} and Ob_{res}.[82] Future studies are needed to further clarify the role of FABP4 in metabolic syndrome and obesity.

1.6.8.5 Retinol binding protein 4 (RBP4)

RBP4 is secreted from liver and adipose tissue. RBP4 has been postulated to link obesity with insulin resistance, diabetes and some features of the metabolic syndrome.[168] It is suggested that RBP4 acts on muscle and/or liver in either a retinol-dependent or retinol-independent way.[169] Several studies have suggested an association between elevated RBP4 levels and visceral adiposity,[170] and RBP4 levels decrease with weight loss and improvement in insulin sensitivity,[168] and with the insulin-sensitizing agent rosiglitazone.[171] Consistently, a cross-sectional hyperinsulinaemic-euglycaemic clamp study has reported that Ob_{res} had higher RBP4 and visceral fat compared with Ob_{sen}.[81]

There is debate on its role with insulin resistance and obesity.[172,173] Currently, the role of RBP4 in obesity and insulin resistance regulation is not certain; additional studies are needed to clarify the role of RBP4 in obesity and insulin resistance.

1.6.8.6 Fibroblast Growth Factor-19 (FGF-19)

FGF-19 is an endocrine hormone that coordinate carbohydrate and lipid metabolism in response to nutritional status. FGF-19 regulate both bile acid and glucose metabolism.[174] FGF-19 is secreted from the small intestine in response to feeding and has insulin-like action.[174] FGF-19 exerts its metabolic effect by binding to the b-klotho/ FGFR4 complex. The liver has the highest levels of b-klotho and FGFR4 and therefore liver is the main target organ of FGF-19 action.[175]

FGF-19 controls hepatic metabolism in response to nutritional status. After a meal, FGF-19 works with insulin to promote glycogen synthesis and inhibit

gluconeogenesis.[176] Interestingly, FGF-19 has been shown to suppress insulin-induced stimulation of fatty acid synthesis through suppression of SREBP-1c.[177] The ability of FGF-19 to decrease liver fat content, triglycerides, total cholesterol and plasma glucose levels and to improve insulin sensitivity makes it a potential promising therapeutic agent for management of metabolic syndrome and insulin resistance.

1.6.8.7 Fibroblast growth factor-21 (FGF-21)

FGF-21 is another, more studied, member of the FGF superfamily. It is produced in liver, white and brown adipose tissue and pancreas. It has differential roles on the liver and adipose tissue. In the fasting state, FGF-21 coordinates with glucagon in promoting gluconeogenesis, ketogenesis and fatty acid oxidation while in the fed state, FGF-21 functions like insulin to stimulate glucose uptake in white adipose tissue and decreases plasma glucose, triglycerides and insulin levels with improved insulin sensitivity.[178] FGF-21 has also been shown to potentiate thiazolidinediones effects on adipocyte differentiation and gene expression via regulation of PPAR-γ activity.[179]

FGF-21 levels are elevated in obesity and insulin resistance[180] and in type 2 diabetes.[181] This elevation appears to be compensatory due to the potential anti-diabetic effect.[106] Though, one study did not show significant differences in FGF-21 between Ob_{sen} and Ob_{res}, classified by HOMA-IR.[82] FGF-21 coordinates with PPAR-γ in promoting adipocyte glucose transport and differentiation in fed state, while works with glucagon in fasting state (or starvation) by increasing gluconeogenesis. FGF-21 has differential roles on the liver and adipose tissue; this makes FGF-21 a potential therapeutic target in management of insulin resistance in obesity.

1.6.8.8 Lipocalin-2

Lipocalin-2 is a recognized adipokine which is secreted from white adipose tissue and is involved in glucose metabolism and insulin sensitivity.[182] Lipocalin-2 is also involved in various biological functions such as apoptosis, innate immunity and tumorigenesis, and is expressed in brain, lung, liver, neutrophils, adipocytes, kidney and macrophages.[183,184] Lipocalin-2 may have a potential role in insulin resistance and glucose metabolism, as its level is elevated among diabetic patients, and reduced by the insulin-sensitising drug rosiglitazone.[185] Lipocalin-2 also correlated with the insulin resistance index and inflammatory markers.[186] One study showed a correlation between lipocalin-2 and beta cell function and insulin resistance, perhaps mediated partially through iron excess and inflammation.[182] There is evidence in animal model to suggest a potential link between visceral adiposity and elevated lipocalin-2,[186] which may explain the potential link between lipocalin-2 and insulin resistance. Though, one study did not show any differences in lipocalin-2 levels between Ob_{sen} and Ob_{res}.[82] Current evidence re the relationship between lipocalin-2 and insulin resistance is scarce and additional studies are warranted to establish its potential role in insulin resistance in obesity.

1.6.8.9 Other adipokines/cytokines/hepatokines

There are numerous other molecules secreted from adipose tissue and liver that may be involved in regulation of insulin resistance and metabolic syndrome in obesity. Chemerin is a chemoattractant protein, structurally different from any chemokine family, and has been shown to be involved in obesity and obesity related pathologies,[187] where it induces insulin resistance in adipocytes *in-vitro*.[188] Another novel chemokine is fractalkine, which is an inflammatory adipose chemokine that modulates monocyte

adhesion to adipocytes and has been reported to correlate with insulin resistance, obesity and type 2 diabetes.[189] Other markers such as fetuin A, resistin A, vifastin and omentin may have potential roles in insulin resistance in obesity.[106] Future studies are needed to delineate the roles of these adipokines/cytokines in insulin resistance in obesity.

1.6.9 Metabolic flexibility

Metabolic flexibility is a term used in the literature to denote the flexibility of skeletal muscle to switch from carbohydrate to fat oxidation during fasting and from fat to carbohydrate oxidation in response to insulin.[190] Obese insulin-resistant individuals have lower fasting lipid utilisation (in muscle) and do not switch to carbohydrate oxidation in response to insulin. Metabolic flexibility can be measured by the change in respiratory quotient (RQ) obtained from indirect calorimetry during a clamp study, i.e. the difference between baseline and insulin-stimulated RQ,[191] and has been shown to be greater in Ob_{sen} individuals compared to Ob_{res} group.[81,82] It has been postulated that metabolic flexibility is an intrinsic characteristic of skeletal muscle, at least to a certain extent, and external factors such as weight loss,[192] exercise[193] and thiazolidinedione treatment[112] may potentially influence metabolic switching. The intrinsic defect in metabolic switching of skeletal muscle may favour the accumulation of lipids and lipid intermediates, implicated in insulin resistance.[194] Further larger clinical studies are needed to ascertain the association between skeletal muscle insulin resistance and metabolic flexibility.

1.6.10 Muscle lipids

Intramuscular lipids have been shown in humans to be associated with skeletal muscle insulin resistance.[195] Skeletal muscle accounts for 80% of insulin-stimulated glucose uptake.[196] Many studies have shown that an increase in intramyocellular lipid content, measured by MRS, is associated with insulin resistance in obese individuals.[42,43] Despite this, some studies showed no difference in muscle lipid content between Ob_{sen} and Ob_{res} groups using either CT[34,36] or DXA[197] scans to measure leg fat attenuation/fat mass, which would measure both intra- and extramyocellular lipid; this could potentially explain these inconsistent findings.

Recently, there have been discussions on the roles of ceramides and diacylglycerols (DAG) in skeletal muscle insulin resistance. Chronic exercise and obesity both increase intramyocellular triglycerides, but chronically exercised humans were markedly insulin-sensitive.[198] This "athlete's paradox" suggests that there is perhaps a differential accumulation of lipid intermediates that are expressed in different amounts between chronic exerciser and obese individuals. Indeed, both ceramides[199] and DAGs[194] have been show in animal models to be associated with skeletal muscle insulin resistance.

Ceramides are members of the sphingolipid family of lipids. Ceramide is generated during the hydrolysis of plasma membrane sphingomyelin, and is a second messenger of the sphingomyelin pathways.[200] Ceramides are known to induce insulin resistance in cells[201] and have been observed to be elevated in obese individuals compared with normal-weight sedentary and exercise-trained individuals in muscle.[202] Muscle ceramides levels were higher in Ob_{res} compared to Ob_{sen} (determined by clamp studies)[200,203] and obese compared to lean groups.[194] Ceramides induce insulin

resistance in skeletal muscle via inhibition of insulin signaling, primarily through Akt.[204] Interestingly, Skovbro and colleagues did not demonstrate any differences in skeletal muscle ceramides content among type 2 diabetes, impaired glucose tolerance, healthy controls and endurance trained groups.[205] The lack of differences among 4 groups could be related to their small sample sizes. Further studies are needed to elucidate the role of ceramides in skeletal muscle insulin resistance in Ob_{sen} and Ob_{res} groups.

DAGs have conflicting evidence relating to their association with skeletal muscle insulin resistance. DAGs can be produced from TG hydrolysis, *de novo* synthesized from monoacylglycerol or from phospholipid hydrolysis.[206] Skeletal muscle DAG content was increased in obese *vs.* non-obese groups,[200,207] and diabetic *vs.* non-diabetic groups.[208] Though, other studies did not detect any differences in skeletal muscle DAG content between Ob_{sen} and Ob_{res} groups;[203,209] obese *vs.* lean groups[210] or diabetic *vs.* non-diabetic groups.[211] These inconsistent results suggest that specific DAG structures or subcellular localization (e.g. cytoplasmic membrane vs. organelle membranes *vs.* lipid droplets) could contribute to muscle insulin resistance rather than total DAG content.[212]

Our group have previously examined muscle lipids extensively in relation to insulin sensitivity[213-215] but have not repeated such measurements in this study, partly because of the known paradox of greater muscle lipid but greater insulin sensitivity in women.

1.6.11 Other factors

MHO as defined by insulin sensitivity from hyperinsulinaemic-euglycaemic clamp has been shown to be associated with lower persistent organic pollutants in plasma compared with an MHO group in a cohort of non-diabetic postmenopausal women.[216] This interesting observation may suggest a potential link between endocrine-disrupting chemicals and metabolic health in obese population

Another study has shown that an MHO group as defined by HOMA-IR had favourable heart rate variability profile compared with an MAO group in a postmenopausal cohort.[217] These two interesting studies raise other factors that may be used in predicting the MHO/Obsen phenotype.

1.6.12

Summary

Despite the conundrum in the obesity literature on the definition of MHO/Ob_{sen}, there are still some recognisable and consistent determinants of this phenotype. As summarised in Table 1.1, MHO are younger with lower waist circumference, glycaemia, HOMA-IR, and inflammatory markers and higher adiponectin and physical activity level.

On the other hand, studies that based the categorisation on insulin resistance with either HOMA-IR or hyperinsulinaemic-euglycaemic clamp have slightly more consistent results on features of Ob_{sen} phenotype (Table 1.2). These characteristics include lower liver fat, visceral fat, inflammatory markers and triglycerides.

In summary, it is necessary to standardise the definition on MHO/Ob_{sen} phenotype. This will allow clinical meaningful comparisons among studies to elucidate and identify clinical predictors of this phenotype. Furthermore, identifying potential protective mechanism in MHO/Ob_{sen} phenotype will provide potential therapeutic targets for interventions to reduce obesity-related metabolic complications.

1.7 PANCREATIC FAT

Ectopic lipid accumulation occurs when there is excessive nonesterified fatty acids (NEFA) and lipid supply that exceeds the oxidative capacity and with an inadequate or impaired lipid storage capacity of adipose tissue. Ectopic fat accumulation in liver and skeletal muscle has been associated with insulin resistance and decreased diastolic function of the heart.[218-220] There are recent emerging studies to suggest an association of pancreatic fat with glucometabolic disorders and particularly with beta cell function.[221-223]

Pancreatic fat is related to obesity[224], and increases with BMI and age.[221,222,224,225] Pancreatic fat is higher in males than females[222,224] and varies with different ethnicity,[226] where Hispanic and white subjects have higher pancreatic triglyceride levels as measured by ^{1}H MRS than African American subjects despite similar age and BMI. Pancreatic fat is positively associated with visceral fat[227,228] but the evidence with liver fat is conflicting. Many studies did not show an association between liver fat and pancreatic fat,[224,225,229,230] while some studies showed positive correlation.[227,228] This could be due to the intrinsic differences of the modality used to measure pancreatic and liver fat and the study population. In studies that reported the positive, one used CT to measure pancreatic fat[227] in a Korean population, while the other used MRI in Hispanics and an African American population[228].

So far, the literature on pancreatic fat is scarce and does not show any coherent findings in its association with glucose metabolism and beta cell function (Table 1.3). Most studies used MRS to evaluate pancreatic fat, while others used CT or MRI. This may have an impact on the finding. The study cohorts were heterogeneous and different in

age, duration and severity of dysglycaemia (i.e. normal glucose tolerance [NGT], impaired glucose tolerance [IGT]/impaired fasting glucose [IFG] or Type 2Diabetes Mellitus) and gender. These differences make direct comparison between studies difficult, for example, one only recruited obese men,[225] while the comparison groups in other studies were not matched by age.[221,224] As pancreatic fat is positively associated with age, especially in men,[222] pancreatic fat measurement between diabetic and non-diabetic groups cannot be directly compared without adjusting for the effect of age. Therefore, caution is needed when interpreting these studies.

1.7.1 Measurement of pancreatic fat

CT/PET has been used in the past to estimate pancreatic fat volume by subtracting parenchymal pancreas from total pancreas volume.[222] This estimation of pancreatic fat cannot differentiate between pancreatic fat within interlobular adipocytes and fat within acinar cells. More recent studies used MRS or MRI to quantify pancreatic fat. Nevertheless, methods used to measure pancreatic fat are not standardized. Lingvay[224] selected the body of pancreas, while others selected the distal pancreas[223,225] to measure pancreatic fat. They used 2-2.5 cm^3 of volume of interest (VOI) to estimate pancreatic fat content. MRI has been used to measure pancreatic fat, using two[231] or three[221] different regions of interest. The lack of standardising in estimating pancreatic fat and the use of different medical imaging modalities would make comparison between studies difficult.

1.7.2 Pancreatic fat and glucose metabolism

There are inconsistent findings regarding the association between pancreatic fat and glucometabolic disorders. Saisho[222] demonstrated a positive association between pancreatic fat with age and obesity, but did not find any difference in pancreatic fat volume between non-diabetic and diabetic groups. The pancreatic fat assessment was performed using a CT scan. However, several subsequent studies did show a positive correlation between pancreatic fat and impaired glucose metabolism.[223-225] All three studies evaluated pancreatic fat volume using MRS, and demonstrated that there is increased pancreatic fat volume in groups with type 2 diabetes or prediabetes (IGT/IGT) when compared with a normal glucose tolerance group. These groups were matched for age and BMI except one study.[224] A recent study showed that pancreatic fat significantly decreased in obese patients 6 months after bariatric intervention. The change in pancreatic fat was related to the improvement in insulin resistance and reversal of type 2 diabetes.[232] This suggests a potential link between pancreatic fat and glucometabolic status.

1.7.3 Pancreatic fat and beta cell function

Recent studies have suggested that pancreatic fat is one of the mechanisms leading to beta cell dysfunction (Table 1.3). However, the evidence in this area is inconsistent. Van der Zijl[223] used combined hyperinsulinaemic-euglycaemic and hyperglycaemic clamps with subsequent arginine stimulation to assess insulin sensitivity and secretion in a group of non-diabetic overweight individuals. They showed that increased pancreatic fat was associated with worsening glycaemic metabolism and a lower Disposition Index (DI, insulin sensitivity adjusted beta cell function). Tushuizen et al[225] examined the association of pancreatic fat and beta cell function in diabetic and non-

diabetic men using HOMA-B to assess beta cell function, and found a negative correlation between pancreatic fat and beta cell function. However, this association was significantly affected by the diabetic state, such that a significant association of pancreatic fat with beta cell dysfunction was only present in the non-diabetic group.

Heni's group[221] demonstrated a negative association between pancreatic fat and insulin secretion in subjects with pre-diabetes (IGT and IFG). They used OGTT with insulin and C-peptide to calculate the insulinogenic index, early insulin release and other parameters. They identified a significant negative association between pancreatic fat content and insulin secretion in a pre-diabetic cohort, but no association in a normal glucose tolerant group. One study[228] did not demonstrate any association between pancreatic fat content and beta cell function in a group of young non-diabetic Hispanic and African Americans. In this study, they used an IVGTT to calculate acute insulin response, DI and insulin sensitivity index as measurements of beta cell function. Cross-sectional studies so far have not shown much clarity in the association between pancreatic fat and beta cell function, perhaps, attributable to different background cohorts and/or different glucometabolic groups and small sample size.

Interventional studies again showed inconsistent findings on possible involvement of pancreatic fat in beta cell function. Lim et al[231] examined the effect of caloric restriction on reversal of beta cell failure and insulin resistance. They recruited type 2 diabetic patients matched with non-diabetic controls for weight, age and gender. These participants underwent 8 weeks of very-low-energy diet. MRI was used to assess pancreatic fat and beta cell secretion function was assessed by a hyperglycaemic clamp followed by arginine bolus. The study that showed a reduction in pancreatic fat was

associated with an improvement in beta cell function. Another interventional study[232] examined the relationships between pancreatic fat and beta cell function following bariatric surgery. They measured beta cell function by HOMA-B and pancreatic fat with MRS and found that, despite a reduction in pancreatic fat following bariatric surgery, there was no change in HOMA-B.

The differences in observations of reduction in pancreatic fat and beta cell function may be attributed to differences in measurement of pancreatic fat (MRI *vs.* MRS) and methods used to assess beta cell function (hyperglycaemic clamp *vs.* the surrogate HOMA-B). One theory postulated that the negative effect of pancreatic fat on beta cell function is exerted by toxic intermediaries such as ceramides and DAGs, which alter rapidly in response to acute metabolic changes, rather than by stored triacylglycerol *per se*.[233] Therefore, these changes in toxic intermediaries in pancreatic fat *per se* might not be reflected by pancreatic fat measurement assessed by MRI/MRS.

Based on the evidence, pancreatic lipid content increases with deterioration of the glucometabolic state in humans. However, there is currently no clear evidence that these lipids interfere with beta cell function. The controversy involving beta cell function and pancreatic fat might be due in part to the heterogeneity of study population mixing different ethnic, gender and dysglycaemic groups (e.g. pre-diabetic and non-diabetic groups together). Perhaps pancreatic fat is positively associated with beta cell secretion in the early stage of dysglycaemia, and as dysglycaemia progresses (pre-diabetes), pancreatic fat contributes to beta cell function decline.[232] Further studies are needed to delineate the association between pancreatic beta cell function and pancreatic fat by

using standardized pancreatic fat imaging and gold-standard assessment of beta cell function (hyperglycaemic clamp) with clearly defined metabolic/glycaemic groups.

Table 1.3 Cross sectional studies involving pancreatic fat and its association with glycaemia/beta cell function

	Population	Groups/number (n)	Gender	Mean Age (years)	BMI (kg/m^2)	Imaging	β cell function assessed by	Association with glycaemia	Association with β cell function
Saisho[222]	USA, 74% Caucasian	1886 adults (165 with diabetes)	Both	66	27.7	PET/CT	NA	PF not increased in diabetic subjects	NA
Tushuizen[225]	Netherland, Caucasian	Non-diabetics (24) Diabetics (12)	Men	55	29/31.1	MRS	OGTT	Higher in DM subjects	Negative correlation in non-diabetic group only
Lingvay[224]	USA	79 adults 1. NGT, BMI < 25 kg/m^2 (15) 2. NGT, BMI > 25 kg/m^2 (30) 3. IFG/IGT (23) 4. DM (11)	Both	1.31 2.39 3.42 4.48	1.22.2 2.32.4 3.32.4 4.32.9	MRS	NA	Increased with impaired glycaemia and BMI	NA
Heni[221]	Germany, Caucasians	51 adults NGT (28) IFG/IGT (23)	Both	43/53	29.6/30.3	MRI	OGTT	No difference	Negative correlation only in IFG/IGT group
Van der Zijl[223]	Netherland, Caucasian	64 adults 1. NGT (16) 2. IFG (29) 3. IFG/IGT (19)	Both	1.55 2.57 3.56	1.27.5 2.28.7 3.28.2	MRS	Hyperglycaemic clamp with arginine stimulation	Increased with impaired glucose metabolism	No association
Szczepaniak[226]	USA, Hispanic, White, Black	100 non-diabetic adults	Both	39	30	MRS	IVGTT	NA	Positive in White and black, negative in Hispanic
Le[228]	USA, Hispanic and African American	138 non-diabetic adolescents	Both	17	35	MRI	IVGTT	NA	No association

IVGTT= intravenous glucose tolerance test, NGT=normal glucose tolerance, IFG=impaired fasting glucose, IGT= impaired glucose tolerance, T2DM=Type 2 diabetes mellitus, NA=not available, PF=pancreatic fat

1.8 SYMPATHETIC NERVOUS ACTIVITY AND INSULIN RESISTANCE IN OBESITY

The sympathetic nervous system (SNS) plays an important role in metabolic and circulatory control. The degree of sympathetic activation seems to influence the development of obesity-related complications including hypertension, insulin resistance, diastolic cardiac function and renal impairment.[234] Acute sympathetic activation may increase gluconeogenesis in the liver, inhibit insulin release by the pancreas and stimulate fatty acid oxidation in muscle.[234] SNS activation may be a hallmark of the metabolic syndrome and relate to insulin resistance via positive-feedback mechanisms.[235,236] This has been shown previously where SNS as expressed in muscle sympathetic nervous activity (MSNA) was elevated in subjects with metabolic syndrome even in the absence of hypertension.[236,237]

It remains unknown whether changes in SNS tone are primary and contribute to the development of obesity and insulin resistance or whether they develop secondary to the obese state.[238] Elevated SNS activity has been shown to predict insulin resistance in prospective studies,[239,240] perhaps due to vasoconstriction and increased adipose tissue lipolysis which may lead to reduced glucose utilisation and muscle insulin resistance.[241,242] Conversely, insulin has been known to stimulate the SNS in humans directly in the forebrain area [243] or indirectly via a reflex response due to vasodilation.[244] This has been supported by studies that have shown that obese individuals have higher MSNA than lean individuals.[245,246] Furthermore, in an obese cohort, Ob_{res} has higher resting MSNA compared with Ob_{sen}.[89,247]

Contrary to the resting state, the MSNA response in the postprandial state is blunted in obese individuals. Tenolouris[248] examined the effect of meal-induced hyperinsulinaemia on sympathovagal balance in lean and obese subjects using high and low-carbohydrate meals. They found that in obese subjects, the insulin-induced increase in SNS tone following the meal was blunted despite having higher postprandial insulin levels. Within the obese cohort, Ob_{res} have a blunted SNS response to oral glucose compared with Ob_{sen} individuals when stratified by OGTT-derived HOMA-IR.[89] A potential explanation for these observations could be that chronic sympathetic stimulation may cause adrenoreceptor down-regulation leading to a blunted responsiveness to sympathetic stimuli such as insulin.[248,249] The other explanation could be that Ob_{res} individuals already have maximally stimulated MSNA from resting hyperinsulinaemia so that further increase with increase in insulin levels during a meal is not possible.[250] Blunted SNS responsiveness may lead to reduced postprandial thermogenesis and fat oxidation, and consequently to development of increased body fat. Additional studies are necessary to clarify and delineate the role of MNSA in obesity, particular its association with insulin resistance.

1.8.1 Measurement of sympathetic nervous activity

Various techniques have been used to measure autonomic nervous system activity in humans.[251] Catecholamines in 24 hour urine, plasma or platelet are commonly used to assess whole-body sympathetic nervous activity. Other measures include microneurography and noradrenaline turnover. Microneurography measures MSNA which is a direct indicator of central sympathetic outflow to the vasculature. Noradrenaline turnover measures noradrenaline spill over and clearance following

infusion of radioactive noradrenaline. Heart rate variability can also be measured by spectral analysis to assess both sympathetic and parasympathetic nervous activity.[252]

1.9 STATEMENT OF AIMS

MHO is an interesting identity that is vitally important and intriguing. Given the increasing trend in the prevalence of obesity and diabetes mellitus, MHO provides great opportunities to allow clinicians to investigate potential clinical and molecular factors that protect these individuals from developing metabolic complications associated with obesity. Given that obesity is a massive public health burden, any novel findings in MHO could improve quality of care to obese individuals and minimize or prevent obesity-related cardiovascular and metabolic complications.

We propose by using insulin sensitivity as a sole measurement to identify non-diabetic obese individuals who are insulin-sensitive (similar to lean controls) using a two-step hyperinsulinaemic-euglycaemic clamp. The aims of the thesis are:

1. To compare subjects who are muscle insulin-sensitive with those who are muscle insulin-resistant ($Muscle_{sen}$ and $Muscle_{res}$, respectively) and identify predictors that are associated with muscle insulin sensitivity in obesity
2. To compare liver insulin-sensitive ($Liver_{sen}$) and liver insulin-resistant ($Liver_{res}$) groups and identify predictors that are associated with liver insulin sensitivity
3. To stratify our cohort into four groups according to muscle and liver insulin sensitivity, to determine whether being insulin-sensitive at either site predicts a favourable metabolic profile
4. To compare sympathetic activation in $Muscle_{sen}$ and $Muscle_{res}$ to determine whether sympathetic activation is independently linked to insulin resistance independent of obesity.
5. To assess the role of pancreatic fat and its relationship with insulin resistance and beta cell function.

CHAPTER 2
METHODS

2.1 SUBJECT RECRUITMENT

Subjects (n=184) were recruited via advertisements placed around the hospital campus, the Metabolism and Obesity Service at Royal Prince Alfred hospital and in a state-wide newspaper, the Daily Telegraph, and in health column articles published in the Sydney Morning Herald. The inclusion criteria were individuals aged 18-70 years, with a BMI over 30 kg/m^2. BMI was calculated as weight in kilograms divided by height in metres square.

Exclusion criteria were as follows:

- Excessive alcohol intake (> 20g/day in women; > 40g/day in men)
- Pregnancy
- Severe known renal, cardiac, liver disease and cancer
- Treatment with medications known to affect carbohydrate metabolism (e.g. steroid, anti-psychotics, anti-diabetic medications)
- Weight loss or gain > 5% of body weight in the previous 3 months
- Participation in an organised exercise program (> 1hr, 5 times per week)
- Type 2 diabetes as defined by either HbA1c > 6.5% or OGTT (fasting plasma glucose ≥ 7mmol/L and/or 2 hour post glucose ≥ 11.1 mmol/L) and
- Claustrophobia.

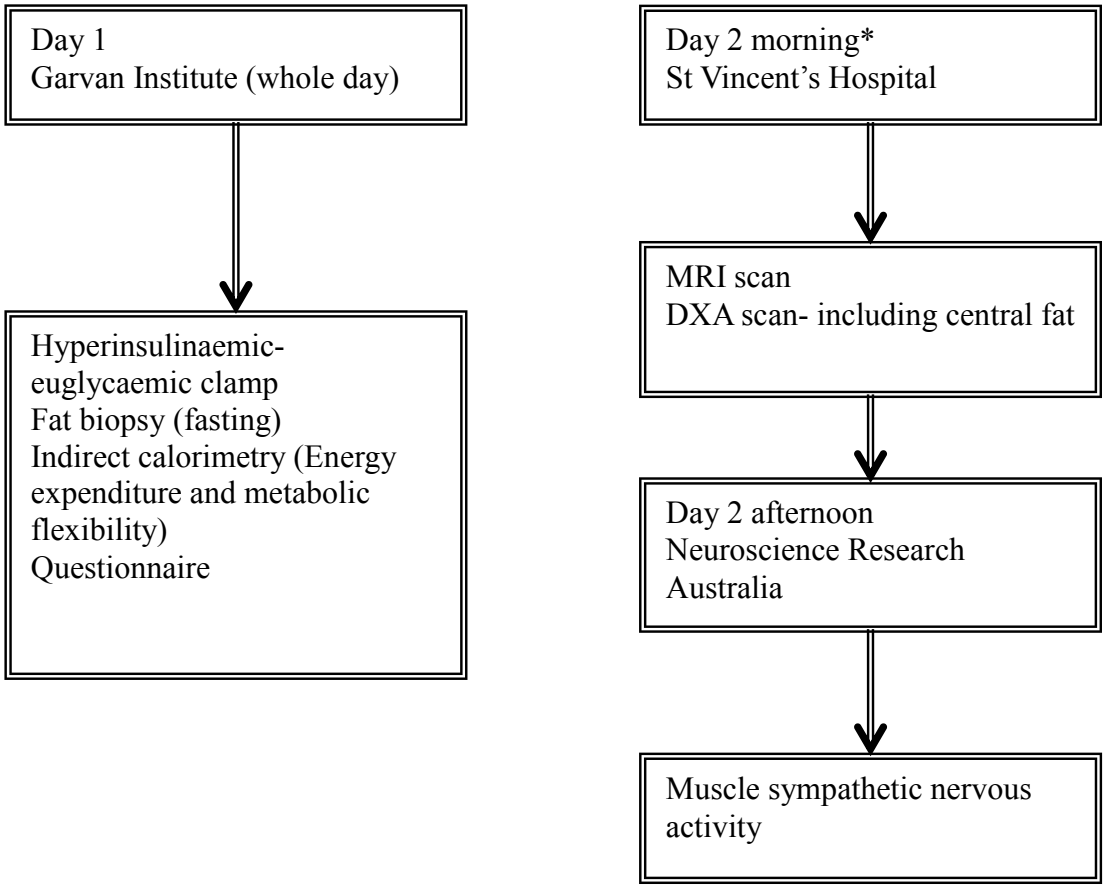
There were 104 subjects excluded from screening due to the above exclusion criteria.

Eighty subjects were invited to Clinical Research Facility (CRF) at the Garvan Institute of Medical Research, Sydney for an initial screening visit, which included an OGTT. Two subjects who were diagnosed with diabetes from OGTT or HbA1c were excluded from the study. Fourteen subjects did not proceed with clamp study due to various

reasons including loss of interest (n = 4), systemic illness (n = 4), and difficult venous access (n= 6). Hence, 64 subjects proceeded to metabolic assessment including clamp and body composition studies. Fourteen subjects out of 64 subjects had pre-diabetes (2 had both IFG and IGT, one had IFG alone; 11 had IGT alone). Women were defined postmenopausal if they had stopped menstruating for more than 1 year, n=22 out of 35. None of postmenopausal women were on hormone replacement therapy. Nine of 13 premenopausal women had their clamp studies performed during follicular phase . Two men and 1 woman were smokers. All participants were Caucasian.

Both OGTT and hyperinsulinaemic-euglycaemic clamps were performed in the CRF. MRI scans were performed at medical imaging department at St Vincent's hospital. DXA scans were performed at in the Nuclear Medicine department at St Vincent's Clinic. Muscle sympathetic nervous activity was performed by our collaborators at Neuroscience Institute Australia. The studies were approved by the Research and Ethics Committee at St Vincent's Hospital. Subjects were provided with informed written consent prior to commencement of the study, which was conducted under the guidelines of the Declaration of Helsinki. The outline of metabolic assessment is displayed below (Figure 2.1)

Figure 2.1 Outline of metabolic assessment



*About one-third of subjects had their MSNA assessed in the morning due to time availability, followed by MRI and DXA.

2.2 ORAL GLUCOSE TOLERANCE TEST

An OGTT was performed in the CRF at the Garvan institute of Medical Research. Dietary advice was given for 3 days before the OGTT to ensure high carbohydrate intake (> 120 grams/day). Blood glucose and insulin were taken immediately before and 30, 60, 90 and 120 minutes after the 75 grams glucose challenge. Basal samples were also analyzed for lipid and C-peptide levels. We measured blood glucose levels via the YSI 2300 Stat Plus Glucose and Lactate analyzer (Yellow Springs, Ohio 45387, USA). Diabetes and pre-diabetes were defined according to the American Diabetes Association criteria.[253] We sent blood samples to SydPath, St Vincent's Hospital, Sydney, Australia if the blood glucose readings were close to the cut-off point (i.e. fasting blood glucose level (BGL) ≥ 6 mmol/L, 2 hours BGL ≥ 10 mmol/L) for formal validation. Three subjects were living outside the local area and had their OGTT performed at local pathology collection centers under standard OGTT instructions.

2.3 DEUTERATED GLUCOSE PREPARATION

Deuterated glucose (6,6-D2, 99%) is an isotope labeled glucose, which is used as a tracer in metabolic studies to assess hepatic glucose output. This is used to measure hepatic insulin sensitivity during clamp study. The use of deuterated glucose had been documented in literature.[254-257] Deuterated glucose (DLM-349-MG, Cambridge Isotopes Laboratories; Andover, Massachusetts, USA) were ordered from USA and sterilised with gamma knife irradiation (Stenlake compound pharmacy, Bondi Junction, Sydney). Isotope labelled glucose to glucose ratio before and after gamma-knife irradiation (analysed in Bioanalytical Mass Spectrometry Facility [BMSF], UNSW, Australia) was similar. The relative deuterated-to-normal glucose ratio was 0.043 and 0.036 before and after gamma knife irradiation respectively.

Preparation and packaging of sterilised deuterated glucose were processed by Stenlake compound pharmacy (Bondi Junction, Australia). Deuterated glucose powder was packaged into 8 grams and 1 gram plastic vials. Baxter Pharmacy (Darlinghurst, Australia) prepared both deuterated glucose syringe driver and hot glucose infusate (hot GINF) (25% dextrose bag enriched with deuterated glucose) for all subjects. The deuterated glucose syringe was used for deuterated glucose infusion during the basal and low insulin stage. Hot GINF was used during low and high insulin stage at various infusion rates to maintain a plasma glucose level of 5 mmol/L.

2.3.1 Subjects who weighed below 125 kg (n = 56/64)

Eight thousand milligrams of deuterated glucose was mixed with 40 ml of normal saline (200mg/mL). The exact procedure involved mixing deuterated glucose powder with

40mL of normal saline via a transfer set via a 60mL syringe. The vial was shaken until all deuterated glucose powder was dissolved. The syringes infusion was sterilised prior to dispensing by passing through a 0.22 um filter. The procedure was carried out in a laminar flow hood contained in a sterile room using aseptic techniques. 9mL (of 40mL) of deuterated glucose was transferred into a 60mL Terumo Luer lock syringe via filter and 31mL of normal saline was added subsequently to make up 40mL of volume in the syringe driver (1800mg of deuterated glucose in 40mL of normal saline = 45mg/mL). The remainder deuterated glucose (31/40 mL) was mixed with 19mL of normal saline to give a total volume of 50mL (6200mg of deuterated glucose in 50mL of normal saline). Fifty millilitres of 1 Litre 25% dextrose solution was removed and discarded using sterile technique and was replaced with 50mLs of deuterated glucose (6200mg deuterated glucose) to make up the hot GINF.

2.3.2 Subjects who weighed above 125 kg (n = 8/64)

The preparation process was identical except different volume and quantity of deuterated glucose powder. In brief, 9000mg deuterated glucose was added in 45ml of normal saline (200mg/mL). 14mL of (45mL) of deuterated glucose was mixed with 26mL of normal saline to make up 40mL of deuterated glucose solution in the syringe driver (2800mg of deuterated glucose in 40mL NS = 70mg/mL). For hot GINF preparation, the remainder deuterated glucose solution (31mL/45mL) was mixed with 19 mL of normal saline to give a total volume of 50mL (6200mg of deuterated glucose in 50mL of normal saline). The rest of procedure was the same as the preparation for lighter subjects.

Both heavier and lighter weight subjects had the same GINF concentrations (6200mg of deuterated glucose in 50mL of normal saline in the 1L 25% dextrose bag). The main difference between heavier and lighter weight subjects was the concentration of the syringe driver.

2.4 METABOLIC ASSESSMENT DAY 1

Subjects attended the CRF after an overnight fast. All premenopausal women had a urine pregnancy test (beta-hCG) to exclude pregnancy at the time of metabolic assessment Subjects were instructed not to perform any vigorous exercise two days and abstain from alcohol for 3 days prior to the study.

2.4.1 Anthropometric indices

Weight and height were measured in a hospital gown. BMI was calculated by weight in kilograms divided by height in metres squared. Waist circumference was measured as the widest circumference between the lower end of the ribs and the anterior superior iliac spines. Hip circumference was defined as the widest circumference between and greater trochanters and anterior superior iliac spines. Brachial blood pressure and heart rate were measured supine after a 5-minute rest on the right arm by an automated sphygmomanometer (OMRON 1A1B Intelligence HEM-70000-C1L, OMRON Corp., Tokyo, Japan).

2.4.2 Physical activity and dietary assessment

Stanford 7-day activity questionnaires were used to assess physical activity in the preceding 7 days as described previously.[258] Subjects were asked if they participated in regular physical activity and if so, how many minutes/week they engaged in light, moderate, hard and very hard activities. The duration of physical activity needed to exceed 10 minutes during one day for it to be counted. The amount of time was recorded and rounded to 15 minutes.

- 10 and 20 min are rounded to 15 min = .25 h

- 25 and 35 min are rounded to 30 min = .50 h
- 40 and 50 min are rounded to 45 min = .75 h
- 55 and 65 min are rounded to 60 min = 1.0 h

Light activity was defined as activities such as leisure walking or bowling; moderate activity was defined as activities such as brisk walking, cycling or swimming, hard activity was defined as activities such as tennis and moderate effort swimming, very hard activity was defined as activities such as jogging and circuit training. Physical activity was calculated and assessed according to physical activity calculator and was expressed as Metabolic Equivalent of Task hours per week (MET-hr/week), as described previously.[259,260]

Subjects were asked to record their diet in the preceding two days and dietary intake was evaluated using Foodworks version 7 with the use of Australian food-composition database (Xyris software, Highgate Hill, Queensland, Australia) which has been used in a previous study.[131] Average daily macronutrient intake was expressed as % energy intake.

2.4.3 Hyperinsulinaemic-euglycaemic clamp

Two intravenous cannulas were inserted into each antecubital fossa in both arms. One cannula was used for intravenous infusion connected to an access port. The other cannula on the other side of the arm was used for blood sampling. In the basal stage, deuterated glucose ([6,6-$^{2}H_2$] glucose) boluses were given (5mg/kg) after baseline blood tests were taken (t = 0 min). This was followed by a 2 hour continuous (3mg/kg/hour) infusion of [6,6-$^{2}H_2$] glucose to determine basal hepatic glucose output. Steady state

was defined as 90-120 minutes after [6,6-$^2H_2$] glucose bolus. Adipose tissue biopsy was performed in the first 90 minutes of the basal stage.

This was followed by a two-step hyperinsulinaemic-euglycaemic clamp using low-dose (15 $mU \cdot m^{-2} \cdot min^{-1}$) and high-dose (80 $mU \cdot m^{-2} \cdot min^{-1}$) insulin infusion (Actrapid; Novo Nordisk Pharmaceuticals, New South Wales, Australia) each step being 2 hours (Figure 2.2). Deuterated glucose ([6,6-$^2H_2$] glucose) infusion rate was halved during the low dose infusion at 1.5mg/kg/hour and ceased at the end of the low dose stage. Prior to the commencement of high-dose infusion, an insulin bolus was administered at 320 $mU \cdot m^{-2} \cdot min^{-1}$ for 2 minutes Steady state was defined as the last 30 minutes of each stage of the clamp.

Our insulin infusion rates at low dose were calculated as (ml/hr):

= BSA x insulin infusion dose per minute x 0.06 / concentration in bag

= BSA x 15 x 0.06 / 0.1

= BSA x 9

Our insulin infusion rates at high dose (ml/hr) were calculated as follows:

= BSA x insulin infusion dose per minute x 0.06 / concentration in bag

= BSA x 80 x 0.06 / 0.1

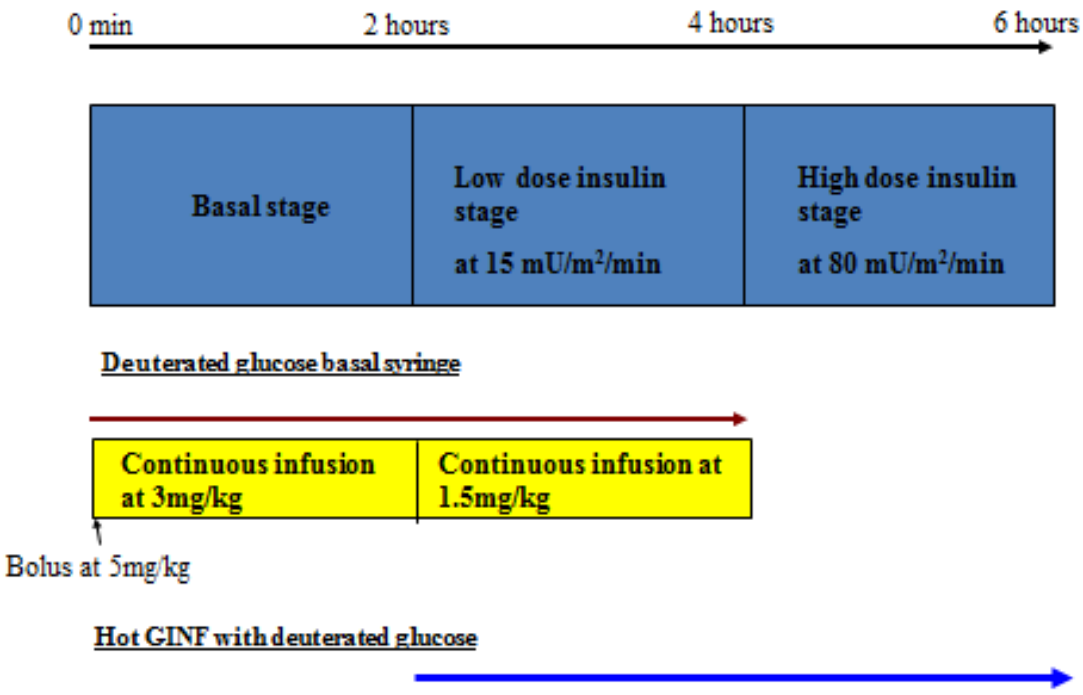
= BSA x 48

Where Body Surface Area (BSA) = weight (kg) ^0.425 x height (cm) ^0.725 x 0.007184 i.e. using the Dubois formula[261]

During the low and high insulin infusion, the plasma glucose level was maintained at 5 mmol/L with a variable-rate infusion of 25% dextrose (25g /100 mL enriched to

approximately 2.5% with deuterated glucose, Baxter Healthcare, Darlinghurst), adjusted according to 10-minute results during non-steady state and 5-minute results during steady state. The steady state glucose infusion rate represented net whole-body glucose disposal and was calculated as a mean at 5-minute intervals during the last 30 minutes of low- and high-dose insulin clamp.

Figure 2.2 Time Line for the hyperinsulinaemic-euglycaemic clamp



2.4.4 Serum samples

In the basal stage, blood samples were collected at time 0 and 120 minutes into the ethylene-diaminetetraacetic acid (EDTA) plasma tubes (1.8mL), while blood samples were collected at 0,30,60,90 minutes during non-steady state and every 10 minutes during steady state into serum separating tubes (SST). During low and high insulin infusion, EDTA plasma and SST (1mL) were collected at every 30 and 10 minutes in non-steady state and steady state respectively.

All tubes were centrifuged at 3500 relative centrifugal force for 7 minutes at temperature of 4 degree with a Heraeus centrifuge machine (Megafuge 1.0R DJB Labcare, Buckinghamshire, UK). The plasma in EDTA tubes was aliquoted in 2 vials. Serum samples were aliquoted in 3 vials for 5mL SST tubes and 4 vials in 8mL SST tubes respectively. SST tubes serum were stored to measure deuterated glucose, insulin, inflammatory markers and non-esterified fatty acid (NEFA) (using the 8mL SST tube). These vials were transported in dry ice and stored in -80ºC freezer.

As described previously, blood samples were collected every 10 minutes during non-steady state and every 5 minutes during steady state into fluoride-oxalate (grey top) tubes to measure plasma glucose levels during the low and high dose insulin infusion

2.4.5 Calculations of various clamp measurement

EGP was calculated using a template based on the Steele's one-compartment fixed-volume model (assuming volume of distribution of 20% of body weight and pool-fraction of 0.65[118]), as modified by Finegood.[262] The template used information including deuterated glucose enrichment in serum samples/GINF, steady state glucose

infusion rate, blood glucose levels and weight to calculate hepatic glucose output. Systemic glucose appearance and disappearance (Ra and Rd) were estimated using non-steady state calculations.[118,262] Hepatic insulin resistance index (HIRI) was calculated as fasting serum insulin*EGP.[96] HOMA2-beta % was calculated as previously described,[263] the details are in the next section. As EGP was fully suppressed during the high-dose insulin infusion, glucose infusion rate at high dose (GIR_{HI}) reflects peripheral (mainly muscle) insulin sensitivity.

2.4.6 HOMA2-β

HOMA estimates steady state beta cell function and insulin sensitivity, as percentages of normal reference population. These measures estimate insulin sensitivity and beta cell function, and correspond well to gold standard measurements including hyperinsulinaemic-euglycaemic clamp, intravenous glucose tolerance test and hyperglycaemic clamp. HOMA has been updated in 1998 (HOMA2), which incorporated variations in peripheral and hepatic glucose resistance, contribution of proinsulin and increases in insulin secretion curve for plasma glucose level above 10 mmol/L.[263] HOMA2 has been shown to be more accurate than the original HOMA.[23] The output of the model is calibrated to give normal beta cell function of 100%. HOMA2 is a computer model with non-linear solutions.[23] The computer model allows estimate of HOMA2-beta with paired fasting glucose and radioimmunoassay derived insulin, specific insulin or c-peptide concentrations. We calculated our HOMA2-beta% by using the HOMA2 calculator, downloaded from University of Oxford (Oxford, UK).

2.4.7 Definition of insulin sensitivity in muscle and liver

Hepatic insulin sensitivity was defined as the percentage of EGP suppression during the low dose stage. The low- and high-glucose infusion rates were calculated at last 30 minutes of each clamp stage adjusted for fat free mass (FFM). Muscle insulin sensitivity was defined as GIR_{HI} derived from steady state during the high dose clamp adjusted for FFM.

Study participants were assigned to the obese insulin-sensitive ($Muscle_{sen}$) group if GIR_{HI} was in the upper tertile of the cohort and to the insulin-resistant ($Muscle_{res}$) group if GIR_{HI} fell in the lower two tertiles. We will define Ob_{sen} as $Muscle_{sen}$ and Ob_{res} as $Muscle_{res}$ for rest of the thesis, as our GIR_{HI} at high dose insulin infusion (80 $mU \cdot m^{-2} \cdot min^{-1}$) is a measure of predominantly muscle insulin sensitivity.

In separate analyses, subjects were reclassified by the degree of EGP suppression during low-dose insulin. $Liver_{sen}$ were in the upper tertile of EGP suppression and $Liver_{res}$ were in the lower two tertiles. GIR_{HI} and EGP suppression correlated ($R^2 = 0.14$, $P = 0.003$). Muscle and liver stratification to groups was performed in men and women separately.

2.4.8 Fat biopsy

Periumbilical subcutaneous fat biopsy was performed during the basal stage of the clamp study, under sterile condition as described previously.[131,264] In detail, the abdominal area was cleaned with povidone iodine solution under sterile conditions. A sterile fenestrated drape with adhesive aperture was place overlying the abdomen with the target area exposed to create a sterile field. Five to ten mL of 2% lignocaine was administered into the skin before an incision (5 mm) was made with a surgical scalpel.

Further lignocaine was administered at various angles into the subcutaneous tissue. Prior to the procedure, the Bergstrom needle set was assembled by inserting the cutting cannula into the trocar and checked to ensure appropriate sliding action and position. A 5 mm trocar (UCH skeletal muscle biopsy, Micrins, Illinois, USA) was inserted into subcutaneous periumbilical abdominal tissue. The biopsy technique was described by Bergostrom.[265] Once the trocar was placed in the subcutaneous abdominal tissue, the cutting cannula was withdrawn a few centimetres and the needle angled at 15-20º to allow adipose tissue to enter the cutting chamber. One research assistant applied suction via a syringe applied to the cutting cannula to draw adipose tissue into the cutting chamber before fully advancing the cannula to guillotine a section of adipose tissue. This process was repeated 2-3 times each time with rotating the instrument by 90º to obtain an optimal yield (200-300 mg). This procedure has been reported previously.[131,264]

Fat samples were fixed in Bouin's fluid (Sigma), dehydrated, paraffin-embedded and then sectioned (4 μm). Sections were stained with haematoxylin and eosin. Processed images were acquired by a microscope camera system (Leica DMR, core LAS 4.2). There were 53 subjects with measurable histological adipose tissue. Fat cell size (diameter) was measured by Image J software 1.46r (NIH, Bethesda, Maryland, USA). Adipocyte diameter was measured blindly by two independent observers (Daniel Chen and Sebastian Tattam) and in at least two fields of view. The mean diameter was calculated from an average of 100 cells per sample.

2.4.9 Indirect calorimetry

Indirect calorimetry is commonly used to measure energy production and nutrient oxidation in humans. It involves a ventilated hood system with high airflow to measure gaseous exchange including O_2 consumption and CO_2 production to assess energy expenditure and fat and carbohydrate oxidation. This is based on the principle that oxidation to water and carbon dioxide is the common final pathway for lipid and carbohydrate.

Indirect calorimetry (Parvo Medics True One, Utah, USA) with ventilator hood technique (Deltatrack Metabolic Monitor, Helsinki, Finland) was conducted to measure whole-body energy expenditure and glucose and fat oxidation during the last 30 minutes of each stage (basal, low insulin and high insulin). Oxidative glucose disposal was calculated from the final 20 minutes of indirect calorimetry recordings in the steady state as described previously.[266,267] A transparent plastic ventilated hood was placed over the participants' head and made airtight around their head and chest. Gentle negative pressure was maintained in the hood to avoid loss of expired air. Air flow, O_2 and CO_2 concentrations in both inspired and expired air were measured by a continuous open-circuit system. Air flowing out of the hood was sampled every 30 seconds.

Indirect calorimetry measured gas exchange involving whole-body O_2 uptake (VO_2) and CO_2 release (VCO_2)[268]. The RQ was defined as the ratio between VCO_2 and VO_2 (VCO_2/VO_2) and reflected substrate use.[269] Daily average energy expenditure in kilocalories was usually calculated using the modified Weir equation with substitution of the measured VO_2 and VCO_2 values.[270]

Energy expenditure (kcal/day) = $VO2_{(ml/min)}$ x 5.675 + $VCO_{2\,(ml/min)}$ x 1.593- uN_2 x 2.17

Urinary nitrogen component (uN_2) is often excluded when calculating energy expenditure as it only contributes to a small error of 1-2% in the calculation of final energy expenditure in outpatients.[268] The formula for resting energy expenditure without urinary nitrogen (assuming it negligible) is as below as per ParvoMedics manual.[271]

Energy expenditure (kcal/day) = $VO2_{(ml/min)}$ x 5.616 + $VCO_{2\,(ml/min)}$ x 1.584

The reproducibility of indirect calorimetry has been reported previously, with the day to day coefficient of variation (CV) for repeated measure of energy expenditure and RQ at 4.3% and 3% respectively.[272]

2.4.10 Measurement of metabolites and hormones

Serum insulin concentrations were measured by radioimmunoassay (RIA) (Millipore, St Charles, Missouri, USA) performed by Daniel Chen and Dorit Samocha-Bonet at the Garvan Institute of Medical Research. Serum samples were thawed overnight in -20º fridge. Standards and quality controls were prepared according to the RIA manual. Serum samples were transferred to standard tubes via pipet with addition of assay buffer, I-125 human insulin tracer and human insulin antibody. All tubes were vortexed and covered with aluminium wrap and left at room temperature overnight (22-25ºC). On day two, precipitating reagents were added to all tubes. All tubes were vortexed and subsequently incubated at 4ºC for 40 minutes. These tubes were centrifuged at 4ºC at

3000 revolutions per minute for 40 minutes and decanted. All tubes were placed in a gamma counter to measure insulin levels. C-peptide concentrations were measured by RIA kit (Millipore, St Charles, USA) and prepared similar to the insulin assays by Daniel Chen.

Plasma lipid profiles including total cholesterol, HDL, low-density lipoprotein (LDL) and serum triglycerides were determined by conventional automated analyzer, (Roche Modular-P platform, Mannheim, Germany) performed at SydPath (Darlinghurst, St Vincent's Hospital). Serum NEFA was analyzed by an enzymatic calorimetric assay (Wako, Osaka, Japan).

Serum hs-CRP, FGF-19,[273] FGF-21, total adiponectin, FABP4, lipocalin 2[274] and RBP4 were analyzed by sandwich enzyme-linked immunosorbent assays (ELISA) by Professor Aimin Xu (Antibody and Immunoassay service, The University of Hong Kong) as previously described.[275-277] The intra- and inter- assay CVs for hs-CRP, FGF-19, FGF-21, total adiponectin, FABP4, lipocalin 2 and RBP4 were 4.3% and 5.9%; 4.5% and 5.6%; 4.4% and 9.2%; 5.1% and 6.2%; 4.8% and 5.7%; 3.8% and 5.2%; 4.1% and 7.2%, respectively.

2.4.11 Measurement of deuterated glucose tracer

Isotope glucose assays were performed in BMSF at the University of New South Wales (Sydney, Australia) by Gas Chromatography-mass spectrometry (GCMS). The protocol for glucose derivatization and assay was adapted from previously published work (Mcintosh[278], Magni[279] and Petersons[114]).

Serum samples were initially transferred from -80ºC to -20ºC for storage prior to analysis. On the day of samples preparation, serum samples were transferred from -20ºC to room temperature to thaw. Ice cold acetone (1mL) was added to 1.7 mL polypropylene centrifuge tubes followed by serum (50 µL) in a fume hood. Tubes were capped, vortexed and stored at -20ºC for one hour to precipitate proteins. Tubes were then spun in a microfuge for 10 minutes at ambient temperature (14,926g) to remove the protein pellet. The supernatant was transferred to the glass culture tube using Pasteur pipettes and dried under vacuum and centrifugation (Savant Speedvac, ThermoFischer).

Derivatization steps are required for GCMS analysis[280,281] of compounds which are not intrinsically volatile, such as glucose. Dried serum samples were derivatized using a two-step method which has been described previously.[279,282,283] The glass tubes were screw capped using phenolic caps containing Teflon inserts during the derivatization steps. The first derivatization step involved adding hydroxylamine.HCL (0.2M in neat pyridine, 150uL) to each sample in a fume hood. Tubes were capped, briefly vortexed and left standing at ambient temperature overnight (*ca* 18hrs). The second derivatization step involved adding neat acetic anhydride to each tube (200uL, *ca* 18hrs, ambient temp) creating the aldononitrile acetate derivative. Finally the glass tubes were placed in the Savant speedvac system for 1-2 hours to dry. Toluene (1mL) was added to each tube, vortexed briefly and the contents transferred to 1.5 mL glass crimp cap gas chromatography (GC) vials for analysis.

The GCMS analysis was performed using an Agilent GC/MSD 5973 system (Sydney, Australia) using positive chemical ionisation and methane reagent gas (Gas A, gas flow setting is 20%). Samples (1uL) were injected splitless-into the GC inlet in automated

mode (7683 series autosampler and injector, Agilent Technologies, Sydney, Australia). A Restek Rxi-5Sil 30 meter capillary column was used (0.25mm id, 0.25um df, 5%diphenyl/95% dimethyl polysiloxane stationary phase). Inlet and interface temperatures were 230^{o}C and 280^{o}C respectively, and helium carrier gas was used with a pressure of 7.39 psi. Using a temperature ramp of 70^{o}C to 250^{o}C at a rate of 35^{o}C/min, and a final time of 2min at 250^{o}C, the retention time of glucose was *ca* 6.08 min. Single ion monitoring was used to maximise sensitivity, with acquisition of ions *m/z* 328 (glucose) and *m/z* 330 (6,6-$^{2}H_2$ glucose), using a 50ms dwell time. Peak areas were used for all calculations, and integration achieved using the ChemStation RTE integrator. To maintain Gaussian peak shape, peak areas were maintained at < 500,000 counts. Baseline 6,6-$^{2}H_2$ glucose samples were run in duplicate with a mean CV of 0.5%.

The output was calculated as the relative peak area ratio of deuterated glucose tracer to endogenous plasma glucose. Dextrose solution (25% glucose enriched with deuterated glucose) that was used during hyperinsulinaemic-euglycaemic clamp was also sampled for each individual subject using a procedure similar to that described above. Mathematical calculations/formulae were devised to account for the natural abundance of deuterated glucose (without exogenous D_2 tracers), which was approximately 0.03. The between-run and within-run CV% for unenriched 25% glucose was 0.8% and 0.4% respectively as reported previously.[114]

2.5 DUAL-ENERGY X-RAY ABSORPTIOMETRY

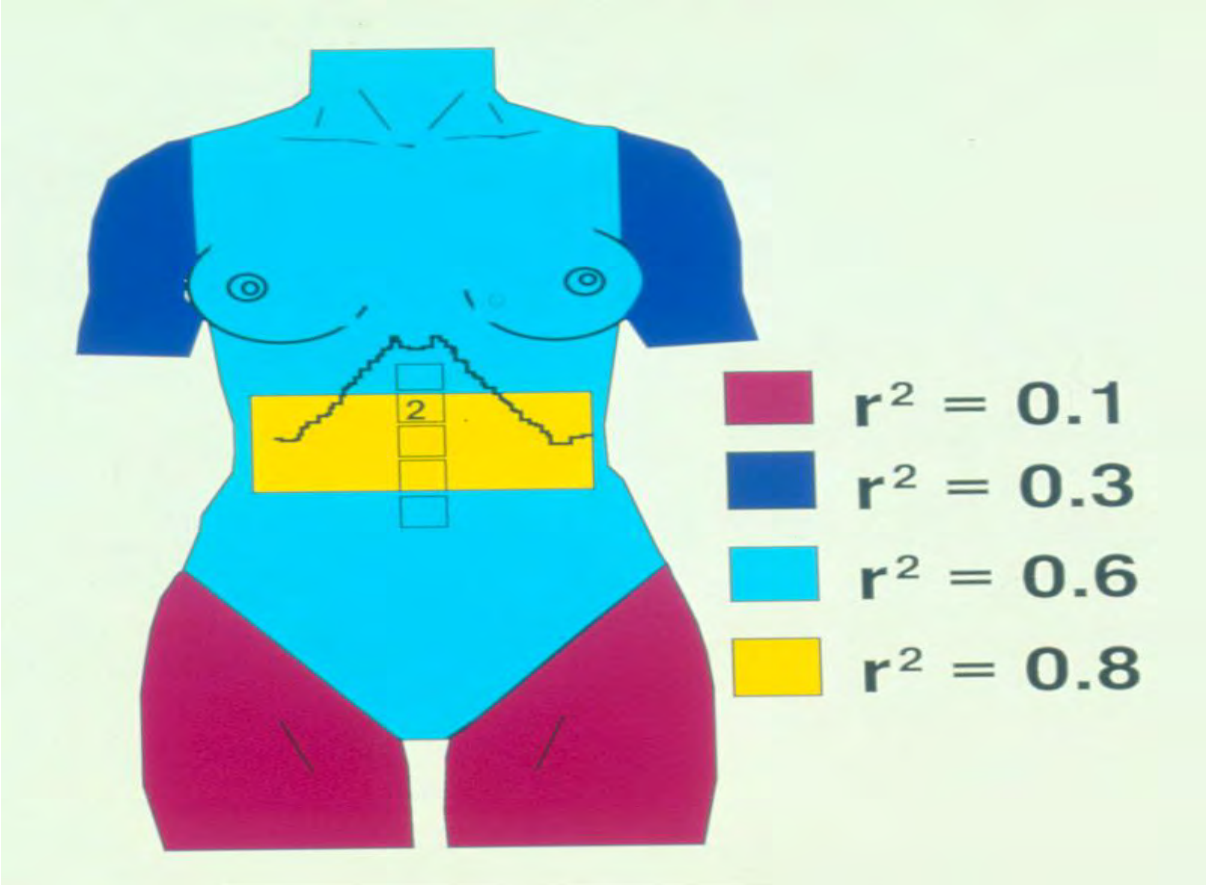
All subjects underwent anthropometric and DXA measurements of body fat. DXA was used to measure body composition, comprising fat mass, lean tissue and bone mineral content. Fat-free mass (defined as lean mass without bone mineral content) was used to adjust for glucose infusion rate during hyperinsulinaemic-euglycaemic clamp. This was undertaken at St Vincent's Clinic, using the Lunar Prodigy GE-Lunar DXA scanner (Madison, Winsconsin, USA) using software version 12.20. Three subjects could not undergo DXA scan due to their weight (bed limit of 136 kg), and had bio-impedance analysis instead. Bio-impedance analysis cannot provide information on central abdominal fat distribution, however can provide estimated lean mass for glucose infusion rate during hyperinsulinaemic-euglycaemic clamp. Bio-impedance determines FFM based on the differences in resistance when an electrical current is conducted through different components of body tissue. It has been used widely in determining body composition, but it lacks specificity and accuracy.[284]

Due to the large size of our obese cohort, most of them could not fit in the measurement field of DXA scan. We have aligned these subjects on one side, and copied the body composition data of one arm to the other arm. Four subjects did not have both arms inside the measurement field; however, the omitted part of the arm was minimal to account for any significant differences in lean mass evaluation.

Central abdominal fat was defined by a set window, which was bordered by the upper margin of the second and the lower margin of the fourth lumbar vertebral bodies and the outer margins of the ribs (Figure 2.3). Central abdominal fat is expressed in absolute weight (kg) and percentage of the soft tissue content and has a strong association with

insulin sensitivity.[285] Despite total abdominal fat measured by DXA and CT was similar, DXA is inferior in assessing intra-abdominal fat compared with CT.[286]

Figure 2.3 Central abdominal fat measured by DXA scan.[285]



This is a diagram of DXA regional fat measurement and their correlation with insulin sensitivity expressed in r^2. Legs, arms, trunk and central abdominal regions are coloured in order of increasing strength of correlation.

2.6 MAGENETIC RESONANCE IMAGING

Each subject has undergone MRI of the abdomen to assess liver, visceral and subcutaneous fat within 1 month after the clamp study. Three subjects were excluded from MRI assessment due to their sizes (MRI scanner maximal diameter was 60 cm). We used 3.0 T MRI machine (3.0 T Philips Achieva machine) with modified Dixon techniques (mDIXON software, Philips, Eindhoven, Netherland) to acquire our images. Modified Dixon techniques used flexible two-point method to achieve fat suppression images. These images were acquired with "in-phase" and "out-phase" images with the liberty of choosing echo times freely and therefore allowing shorter scan times. The Modified Dixon technique allows efficient water-fat imaging that is less impacted by lower signal-to-noise ratio (SNR) and has a higher tolerance to field inhomogeneities.[287] Dual-echo (two point) Dixon imaging has been shown to allow accurate quantification of liver fat content in a surgical population, even outperforming traditional histopathologic analysis[288].

One significant restriction with dual-echo (two point) Dixon technique is that the sampled two echoes must not provide redundant information.[287] This technique is similar and compatible with previous Reeder's method.[289] Nevertheless, two-point Dixon technique cannot account for the confounding T2 effect of iron content in the liver, affecting accuracy of liver fat measurement in patients with increased liver iron stores.[290] Dixon technique imaging can also lead to artifacts. One of the potential source of artifacts is flow, as this can leads to phase errors that may mislead the separation, resulting in artifacts.[287]

The setting for the MRI of the abdomen (to cover from top of liver down to sacrum) was established with a field of view of 450 x 402 x 200mm, with 100 slices each with thickness of 2mm. Breath holds were 15.7 seconds for liver and pancreas and 10.7 seconds for L4/L5 segments. Modified Dixon technique involved water/in phase/out of phase/fat echoes.

Image J software 1.46r (NIH, USA) was used to measure subcutaneous, visceral, liver and pancreatic fat. Subcutaneous and visceral fat was measured in 5 horizontal MRI imaging slices counting upwards from the L4/L5 intervertebral disc level. Visceral fat was calculated as the difference between total fat and subcutaneous fat in each slice. Visceral and subcutaneous fat were expressed as the average of 5 slices. Liver fat was evaluated from 3 liver regions of interest (ROI) (15mm x 15mm; one on left lobe of the liver, one above and one below the portal vessels). Pancreatic fat was assessed from two pancreatic regions of interest (10 mm x 10 mm; at the head and the tail of pancreas). These intraorgan fat was defined and averaged in a blinded fashion by one observer (Daniel Chen) with assistance from a radiologist (Brad Milner) locating the pancreas. Liver and pancreatic fat data was obtained in a fashion to avoid contamination from blood vessels. Liver and pancreatic fat were expressed in percentages, and were determined by the average of individual ROIs.

2.7 MUSCLE SYMPATHETIC NERVOUS ACTIVITY

MSNA assessments were performed at Neuroscience Research Australia, Sydney by Rachael Brown, supervised by Vaughan Macefield. MSNA was recorded in a resting state in room temperature at 22ºC with two-thirds of subjects studied in the afternoon. Subjects refrained caffeine in the four hours prior to recording. Subjects voided before commencement. Subjects lay in a chair with their backs at 45º and their legs supported horizontally. Spontaneous resting MSNA was recorded by inserting tungsten microelectrode (Frederick Haer and co, Bowdoinham, Maine, USA) percutaneously at the level of the fibular head into the left common peroneal nerve muscle fascicles supplying the toe extensor and foot everter muscles and the ankle. The tungsten microelectrode was advanced toward a muscle fascicle of the nerve while delivering electrical impulses (0.01-1 mA, 0.2ms, 1Hz). An uninsulated reference electrode was inserted subdermally 2-3cm from the recording electrode and a surface Ag-AgCl electrode applied to the leg served as the ground electrode.

A muscle fascicle was defined as such if intraneural stimulation evoked muscle twitches of the ankle, toe dorsiflexors or foot everter muscles with no radiating paraesthesia. Neural activity was amplified (band-pass 0.3-5 kHz, gain 2 x 10^4) using an isolated amplifier and headstage (NeuroAmpEx, AD Instruments, Sydney, New South Wales, Australia) once a muscle fascicle had been entered. The position of the electrode was then adjusted manually until spontaneous bursts of MSNA were identified. Once an acceptable nerve-recording site was obtained with both acoustic and visual identification of spontaneous sympathetic bursts, resting measurements were recorded and stored on computer (10 kHz sampling) using a computer based data acquisition and analysis system (PowerLab 16SP hardware and LabChart 7 software; AD Instruments).

Radial artery tonometry (CBM-7000, Colin Corp. Komaki city, Japan) was used to record continuous no-invasive blood pressure. The electrocardiogram (0.3 Hz- 1 kHz) was recorded with surface electrodes on the chest and sampled at 2 kHz and respiration (DC-100Hz).

MSNA burst amplitudes were measured from the RMS-processed signal (200ms moving average) using the Peak Parameters feature of LabChart7 (AD Instruments, Sydney, Australia). The entire process has been described previously.[291,292] MSNA was manually analyzed and expressed as burst frequency (bursts per minute) and burst incidence (bursts per 100 heartbeats), averaged over 15 minutes. Forty-five subjects (22 male, 23 female) had successful MSNA recordings; none of them were on beta-blockers.

2.8 STATISTICAL ANALYSIS

Abnormally distributed data were logarithmically transformed prior to statistical analysis. Student's t-test and one-way analysis of variance (ANOVA) were used to detect significant differences between phenotypes, according to the number of groups compared. Correlations were performed using Pearson's correlation coefficient. Repeated measures-ANOVA assessed differences in EGP and NEFA suppression from basal to hyperinsulinemia between phenotypes. In 4-groups comparisons, one-way ANOVA determined statistical significance and Tukey post-hoc analyses detected differences between groups

Stepwise regression was used to assess the contributions of continuous clinical and metabolic variables to peripheral insulin sensitivity (defined as GIR_{HI}) and liver insulin sensitivity (defined as EGP suppression during low insulin dose). Variance inflation factors were calculated to exclude potential co-linearity. AUC for glucose, insulin and C-peptide response to the OGTT were calculated using the trapezoidal model. Data were expressed as means ± SD unless otherwise specified. Statistical analysis was carried out using SPSS version 21 (Chicago, Illinois, USA).

CHAPTER 3
COMPARISON OF METABOLIC CHARACTERISTICS BETWEEN MUSCLE INSULIN-SENSITIVE AND MUSCLE INSULIN-RESISTANT SUBJECTS

3.1 INTRODUCTION

The prevalence of obesity is rapidly increasing, with 600 million adults affected worldwide.[293] The associated increase in diabetes, cardiovascular disease and cancer carries a significant financial and health burden.[294] However, not all obese subjects are similarly affected, and some obese individuals are observed to have normal blood pressure, insulin sensitivity and lipid profile. To target intervention most effectively, it is critical to identify obese individuals carrying the highest metabolic risks.

MHO is a term used to describe obese individuals, who, despite excessive adiposity, remain free from metabolic complications and have relatively normal insulin sensitivity. The prevalence of MHO ranges between 6% to 43% depending on the definitions used (>30 definitions).[40] MHO is defined as absence of some or all metabolic syndrome criteria in most studies, while in other studies is solely or partly based on insulin sensitivity.[48]

Insulin resistance is an integral component of the metabolic syndrome. It is a likely contributor to cardiovascular metabolic disease and an obligatory precursor to the development of type 2 diabetes.[26,295] Given the variability in defining and identifying MHO, whether this phenotype is predictive of lower cardio-metabolic risk and diabetes cannot be answered. As insulin resistance is the key unifying factor in the metabolic syndrome, a more pathophysiological definition of MHO may be one based on insulin sensitivity alone.

3.2 AIMS

In non-diabetic obese individuals, using a two-step hyperinsulinaemic-euglycaemic clamp with deuterated glucose tracers. The aims of this study are:

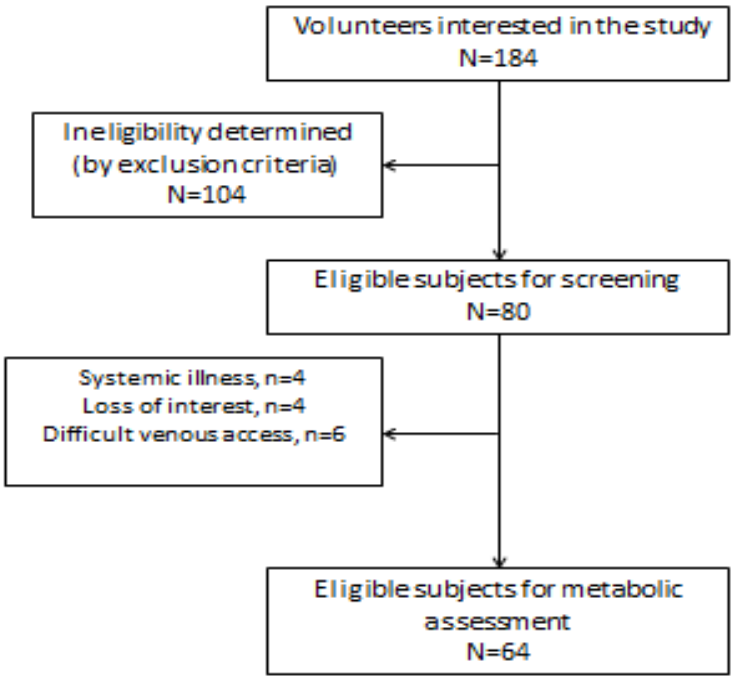
1. To compare insulin-sensitive and insulin-resistant subjects stratified by muscle insulin sensitivity
2. To perform linear regression analyses on pancreatic fat, adipose cell size and muscle insulin sensitivity with other clinical and metabolic variables
3. To identify potential metabolic and clinical parameters that explain variability of muscle insulin sensitivity by using a multiple linear regression analysis

3.3 METHODS

3.3.1 Subjects

Details of subjects recruitment is shown below (Figure 3.1). Inclusion criteria were age 18-70 years and BMI >30 kg/m^2. Exclusion criteria were known diabetes, treatment with medications that affect glucose metabolism (e.g. glucocorticoids, anti-psychotics), alcohol intake >20 g/day in women and >40 g/day in men, weight change >5% in preceding 3 months, known renal, cardiac, liver disease and current cancer. We used the ADA criteria to exclude screened subjects with undiagnosed diabetes.[296] Fourteen subjects did not proceed with clamp study due to various reasons including loss of interest (n = 4), systemic illness (n = 4), and difficult venous access (n= 6). There were no significant differences in age (P = 0.63), BMI (P = 0.61), systolic blood pressure (SBP) (P = 0.07), diastolic blood pressure (DBP) (P = 0.13), waist circumference (P = 0.26), smoking (P = 0.09) and alcohol (P = 0.78) status between our final cohort and these 14 subjects. Hence, 64 subjects underwent clamp and body composition studies. All studies were conducted at the CRF at the Garvan Institute of Medical Research, Sydney (with the exception of 3 subjects who had their OGTT at local pathology centres). Fourteen subjects (out of 64 subjects) had pre-diabetes (2 had both IFG and IGT, one had IFG alone; 11 had IGT alone). Women were defined postmenopausal if they had stopped menstruating for more than 1 year, n=22 out of 35.

Figure 3.1 Subject recruitment flowchart



3.3.2 Dietary and physical activity

Dietary intake and physical activity was evaluated using the Australian-based food composition software FoodWorks 7 (Xyris, Highgate Hill, Australia) and Stanford 7-day activity questionnaires.[258]

3.3.3 Hyperinsulinaemic-euglycaemic clamp studies

Details of the clamp are included in Chapter 2. In short, subjects underwent a 6-hour two-step hyperinsulinaemic-euglycaemic clamp with deuterated glucose tracers (6,6-$^{2}H_{2}$, Cambridge Isotope Laboratories, Andover). Indirect calorimetry (Parvo Medics True One, Utah) measured whole-body energy expenditure and RQ at baseline and

during the last 30 minutes of each stage of the clamp. Subjects were assigned to the obese insulin-sensitive ($Muscle_{sen}$) group if GIR_{HI} was in the upper tertile of the cohort and to the insulin-resistant ($Muscle_{res}$) group for the remaining bottom two tertiles.

3.3.4 Body composition and MRI measurements

DXA was used to measure body composition and fat free mass. Three subjects were too large to undergo DXA scanning; instead, bioimpedance analysis (Tanita Body Composition Analyzer) was used to estimate fat and lean mass. Bio-impedance and DXA derived measures of fat mass have previously showed strong correlations (r=0.92).[297] MRI was performed to measure subcutaneous, liver, visceral and pancreatic fat.

3.3.5 Adipose Tissue Cell size

Periumbilical subcutaneous fat biopsy was performed in 53 subjects during the basal clamp stage under sterile conditions using a trocar, as previously described.[131] Fat cell size (diameter) was measured using Image J software 1.46r (National Institute of Health, USA) by two blinded independent observers (Daniel Chen and Sebastian Tattam). The mean diameter was calculated from an average of approximately 100 cells per sample.

3.3.6 Measurement of metabolites and hormones

Whole blood glucose was measured using YSI 2300 STAT analyzer (Yellow Springs Ohio). Insulin and C-peptide were measured by radioimmunoassay (Millipore, St Charles, USA) by Daniel Chen and Dorit Samocha-Bonet; lipid profiles by an

automated analyzer (Roche, IN, USA); non-esterified fatty acids (NEFA) by an enzymatic colorimetric assay (Wako, Osaka, Japan hs-CRP, FGF-19, FGF-21, total adiponectin, FABP4, lipocalin 2 and RBP4 by ELISA (Antibody and Immunoassay service, HK).[275-277]

3.3.7 Statistical analysis

Data were expressed as mean ± SD unless otherwise specified. Abnormally distributed data were logarithmically transformed prior to statistical analysis. $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using SPSS version 21 (Chicago, IL, USA).

3.4 RESULTS

3.4.1 Metabolic characteristics of $Muscle_{sen}$ and $Muscle_{res}$ individuals

Characteristics of the cohort are presented in Table 3.1, characterized by GIR_{HI} ($Muscle_{sen}$ vs $Muscle_{res}$). By design, $Muscle_{sen}$ had higher GIR_{HI} than $Muscle_{res}$ (120 ± 25 *vs.* 76 ± 21 µmol/min/FFM kg). Importantly, GIR_{HI} in $Muscle_{sen}$ was similar to a group of lean healthy individuals previously assessed by our group using the same protocol (GIR 92 ± 23 µmol/min/FFM kg).[298] Age and BMI were not different between groups. Waist circumference and waist-to-hip ratio tended to be lower in $Muscle_{sen}$ subjects. Despite similar total body fat content, $Muscle_{sen}$ had lower central abdominal fat than $Muscle_{res}$ (Table 3.1). Mean abdominal subcutaneous adipocyte size was not different between $Muscle_{sen}$ and $Muscle_{res}$.

After exclusion of subjects treated with anti-hypertensive medications, $Muscle_{sen}$ had lower systolic and diastolic BP than $Muscle_{res}$ (Table 3.1). After exclusion of subjects treated with lipid-lowering medications, $Muscle_{sen}$ had lower fasting plasma concentrations of triglycerides compared with $Muscle_{res}$; both groups had similar total cholesterol and HDL cholesterol (Table 3.1).

Fasting blood glucose was not significantly different between groups, but 1hr blood glucose following a 75 g glucose load, $AUC_{glucose}$, $AUC_{insulin}$ and $AUC_{C\text{-}peptide}$ during the OGTT were significantly lower in $Muscle_{sen}$ compared to their insulin-resistant counterpart (Table 3.1). Fasting insulin was significantly lower in $Muscle_{sen}$ compared to $Muscle_{res}$ (Table 3.1). Circulating insulin concentrations during the low- and high-dose

insulin clamps were similar between insulin-sensitive and insulin-resistant groups. While fasting serum NEFA levels were not different between insulin-sensitive and insulin-resistant groups, NEFA concentrations at low-dose insulin infusion were significantly lower in $Muscle_{sen}$ compared to $Muscle_{res,}$ suggesting increased adipose tissue insulin sensitivity (Table 3.1).

Basal RQ was not significantly different between groups, but ΔRQ (RQ during the high-dose insulin infusion minus baseline RQ) was significantly higher in $Muscle_{sen}$ compared to $Muscle_{res,}$ reflecting increased metabolic flexibility (Table 3.1).

FGF-19 and total adiponectin tended to be higher in $Muscle_{sen}$ vs $Muscle_{res}$; other circulating inflammatory markers were not different (Table 3.1)

3.4.2 Endogenous glucose production in $Muscle_{sen}$ and $Muscle_{res}$ individuals

Baseline EGP was not different between groups (96 ± 12 *vs.* 104 ± 24 $mg.m^{-2}.min^{-1}$) [Figure 3.1A]. However, HIRI was significantly lower in $Muscle_{sen}$ (Figure 3.1B). $Muscle_{sen}$ had lower EGP during low-dose insulin (Figure 3.1C) and EGP suppression was significantly blunted in $Muscle_{res}$ compared to $Muscle_{sen}$ (Figure 3.1D). EGP was not significantly different from zero during high-dose insulin infusion (P = 0.22).

Table 3.1 Anthropometric, clinical and metabolic characteristics of obese individuals stratified based on muscle insulin sensitivity (glucose infusion rate during high dose clamp)

Characteristics	$Muscle_{sen}$ (9M, 12F)	$Muscle_{res}$ (20M, 23F)	P value
Age	50 ± 12.6	50 ± 11.0	0.97
Anthropometry, body composition and abdominal fat distribution			
BMI (kg/m^2)	35.3 ± 4.2	37.1 ± 4.8	0.16
Waist circumference (cm)	107 ± 12	113 ± 14	0.08
Waist/hip ratio	0.89 ± 0.09	0.93 ± 0.1	0.14
Whole body fat (kg)‡	45 ± 11	46 ± 10	0.62
Whole body fat (%)‡	46 ± 9	46 ± 7	0.90
Fat-free mass (kg)‡	53 ± 12	55 ± 11	0.55
Fat-free mass (%)‡	54 ± 9	54 ± 7	0.92
Central abdominal fat (kg)‡	3.1 ± 0.6	3.6 ± 0.7	**0.01**
Mean adipocyte diameter (µm)π	71 ± 9	75 ± 9	0.12
Median adipocyte diameter (µm) π	70 ± 9	75 ± 10	0.11
Blood pressure and lipid profile			
Systolic BP (mmHg)**	118 ± 8	127 ± 13	**0.01**
Diastolic BP (mmHg)**	78 ± 8	84 ± 10	**0.03**
Total cholesterol (mmol/L)^	5.0 ± 0.8	5.0 ± 0.8	0.79
LDL cholesterol (mmol/L)^	3.1 ± 0.6	3.1 ± 0.7	0.96
HDL cholesterol (mmol/L)^	1.3 ± 0.3	1.3 ± 0.3	0.27
Triglycerides (mmol/L)^Φ	0.8 (0.6-1.2)	1.1 (0.8-1.4)	**0.02**
Oral glucose tolerance test, glycaemia and hyperinsulinaemic clamp measures			
Fasting blood glucose (mmol/L)	4.7 ± 0.4	4.8 ± 0.5	0.12
OGTT 1h blood glucose (mmol/L)	6.6 ± 1.4	8.3 ± 2.1	**0.001**
OGTT 2h blood glucose (mmol/L)	5.7 ± 1.5	6.6 ± 1.6	**0.04**
OGTT $AUC_{Glucose}$ (mmol/L·120min)	754 ± 121	887 ± 176	**0.003**
OGTT $AUC_{Insulin}$ (mU/L·120min)Φ	7600 (6323-9854)	11410 (8287-17157)	**0.002**
OGTT $AUC_{C\text{-}peptide}$ (µg/L·120min)Φ	513 (385-666)	750 (588-1027)	**<0.001**
HbA1c (%)	5.2 ± 0.2	5.6 ± 0.3	**<0.001**
Fasting insulin (mU/L)Φ	12 (9-16)	18 (13-26)	**0.001**
$Insulin_{LO}$ (mU/L)Φ	38 (32-47)	41 (36-47)	0.35
$Insulin_{HI}$ (mU/L)Φ	215 (171-238)	205 (168-240)	0.72
Fasting NEFA (mmol/L)Φ	0.36 (0.29-0.45)	0.37 (0.26-0.46)	0.97
$NEFA_{LO}$ (mmol/L)Φ	0.04 (0.02-0.05)	0.05 (0.04-0.06)	**0.02**

Characteristics	$Muscle_{sen}$ (9M, 12F)	$Muscle_{res}$ (20M, 23F)	P value
$NEFA_{HIGH}$ (mmol/L)$^{\Phi}$	0.02 (0.01-0.03)	0.05 (0.02-0.03)	0.14
Basal RQ	0.79 ± 0.03	0.80 ± 0.03	0.47
Δ RQ ($RQ_{HI} - RQ_{baseline}$)	0.19 ± 0.04	0.14 ± 0.05	**0.002**
Circulating cytokines			
Hs-CRP (mg/L)$^{\Phi}$	2.3 (1.5-4.2)	3.9 (1.8-5.6)	0.16
FGF-19 (ng/L)$^{\Phi}$	128 (69-232)	94 (59-142)	0.09
FGF-21(ng/L)$^{\Phi}$	72 (20-109)	83 (44-140)	0.32
FABP4 (µg/L)	60 ± 27	64 ± 27	0.63
Lipocalin 2 (µg/L)	40 ± 14	41 ± 14	0.67
RBP 4 (mg/L)	11 ± 2	11 ± 3	0.78
Adiponectin (mg/L)	17 ± 9	14 ± 7	0.08

*Data are means ± SD. Φ Data are median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Muscle_{sen}$ n = 17 and $Muscle_{res}$ n = 34).

^ Subjects treated with lipid medications excluded from the analysis (included: $Muscle_{sen}$ n = 18 and $Muscle_{res}$ n = 37).

‡ DXA, n=61

πAdipocyte data, n=53 (included: $Muscle_{sen}$ n = 17 , $Muscle_{res}$ n = 36)

3.4.3 Adiposity variables in $Muscle_{sen}$ and $Muscle_{res}$ individuals

$Muscle_{sen}$ had significantly less abdominal visceral (213 ± 50 cm^2 *vs.* 289 ± 82 cm^2, $P < 0.001$) and liver fat (5 ± 5% *vs.* 17 ± 2%, $P < 0.001$) than $Muscle_{res}$ (Figure 3.2A-B). Subcutaneous and pancreatic fat were not different between groups ($P = 0.99$, Figure 3.2C; $P = 0.66$, Figure 3.2D respectively).

3.4.4 Diet and physical activity

Reported dietary energy and macronutrient intake did not differ between groups, although sugar and saturated fat intake were lower in $Muscle_{sen}$ (Table 3.2). Physical activity level was not different between groups (34 ± 1 and 34 ± 2 MET-hrs/day in $Muscle_{sen}$ and $Muscle_{res}$, $P = 0.7$).

Figure 3.1

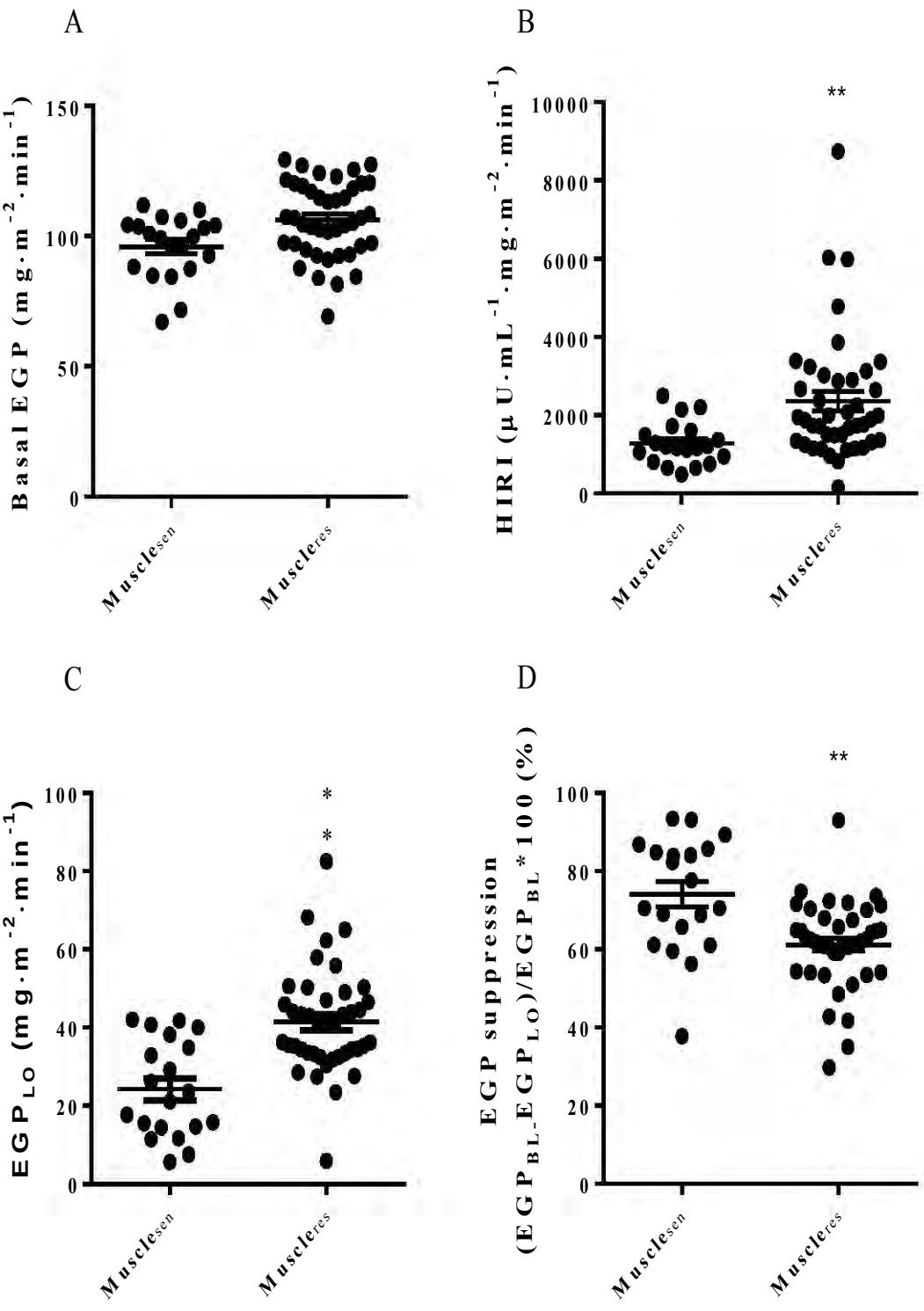


Figure 3.1: Basal endogenous glucose production (EGP, A), hepatic insulin resistance index (HIRI, B), EGP during the low dose insulin clamp (EGP_{LO}, C) and EGP suppression during the low dose clamp (D) in obese individuals stratified based on muscle insulin sensitivity. Differences by Student's t-test are noted, $^{**}P < 0.01$.

Figure 3.2

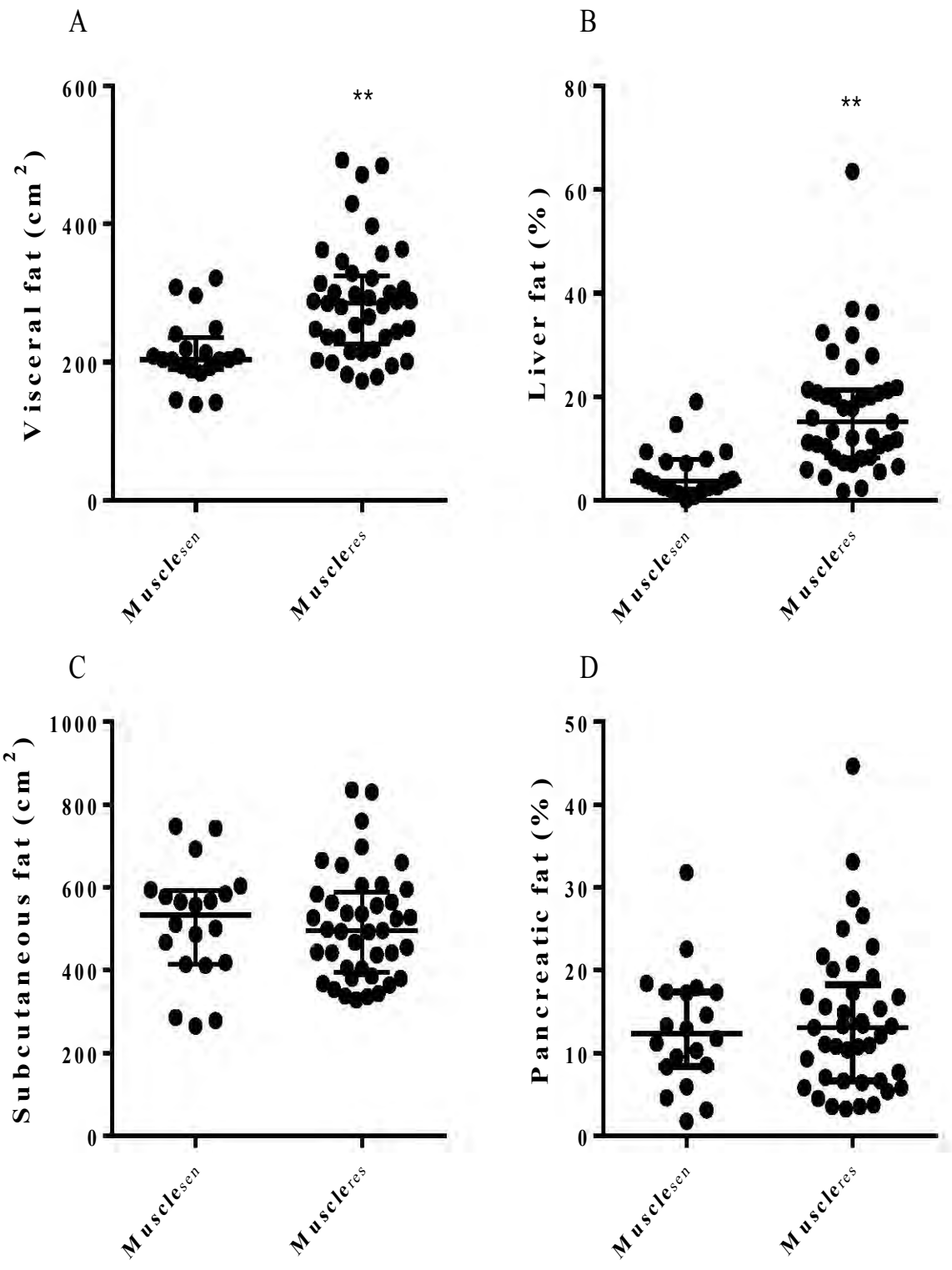


Figure 3.2 Abdominal visceral (A), liver (B), subcutaneous (C) and pancreatic (D) fat in individuals stratified based on muscle insulin sensitivity. $^{**}P < 0.001$ between $Muscle_{sen}$ and $Muscle_{res}$.

Table 3.2 Dietary intake of obese individuals stratified based by muscle insulin sensitivity (glucose infusion rate during the high-dose clamp) according to diet diary

Daily intake*	$Muscle_{sen}$ (M9:F12)	$Muscle_{res}$ (M20:F23)	P value
Energy (kcal)	1830 ± 556	2129 ± 870	0.16
Protein (g)	105 ± 47	104 ± 39	0.92
Protein (% of energy)	22.9 ± 6.2	20.7 ± 5.6	0.16
Total fat (g)	72 ± 33	86 ± 44	0.20
Fat (% of energy)	34 ± 8	35 ± 6	0.56
Saturated fat (g)	26 ± 12	35 ± 18	**0.05**
Saturated fat (% of energy)	12± 4	14 ± 3	0.06
Polysaturated fat (g)	13 ± 11	13 ± 7	0.84
Monosaturated fat (g)	28 ± 13	33 ± 19	0.28
Carbohydrate (g)	178 ± 58	223 ± 110	0.08
Carbohydrate (% of energy)	39 ± 9	41 ± 8	0.45
Sugar (g)	73 ± 29	112 ± 82	**0.007**
Alcohol (g)	1.25 ± 3.15	0.94 ± 3.89	0.75
Dietary fibre (g)	20.5 ± 6.2	22.4 ± 9.4	0.39

*Data are means ± SD

3.4.5 $Muscle_{sen}$ and $Muscle_{res}$ in men and women

We compared clinical factors between $Muscle_{sen}$ and $Muscle_{res}$ in men and women separately (Table 3.3). In men, $Muscle_{sen}$ subjects had lower central abdominal fat, basal insulin and higher metabolic flexibility compared with $Muscle_{res}$ men. Visceral and liver fat were lower in $Muscle_{sen}$ men and women compared with their insulin-resistant counterparts. $Muscle_{sen}$ men had lower diastolic blood pressure compared with $Muscle_{res}$ men while $Muscle_{sen}$ women had lower systolic blood pressure compared with $Muscle_{res}$ women. There were no differences in lipid profile in $Muscle_{sen}$ and $Muscle_{res}$ men and $Muscle_{sen}$ and $Muscle_{res}$ women respectively. Both $Muscle_{sen}$ men and women had lower

OGTT 1h BGL and HbA1c. There were no differences in adipokines between $Muscle_{sen}$ and $Muscle_{res}$ groups when stratified by gender (men $P > 0.14$, women $P > 0.08$).

Table 3.3 Clinical and metabolic characteristics of obese individuals stratified based on muscle insulin sensitivity (GIR_{HI}) in men and women

	$Muscle_{sen}$ Men (n = 9)	$Muscle_{res}$ Men (n = 20)	P value	$Muscle_{sen}$ Women (n = 12)	$Muscle_{res}$ Women (n = 23)	P value
Age	46 ± 15	49 ± 9	0.57	53 ± 10	51 ± 13	0.63
Anthropometry and abdominal fat distribution						
BMI (kg/m^2)	34.6 ± 5.2	36.7 ± 4.5	0.27	35.9 ± 3.2	37.0 ± 5.4	0.46
Central abdominal fat (kg) ‡	3.3 ± 0.5	4.0 ± 0.6	**0.01**	3.0 ± 0.6	3.3 ± 0.6	0.23
Mean adipocyte size (µm) π	72 ± 10	76 ± 9	0.28	70 ± 8	74 ± 10	0.27
Median adipocyte size (µm) π	72 ± 11	76 ± 10	0.36	69 ± 7	74 ± 10	0.21
Visceral fat Φ	208 (193-285)	298 (249-363)	**0.01**	203 (185-218)	262 (202-308)	**0.01**
Liver fat (%) Φ	2.9 (2.0-6.9)	20 (12-26)	**<0.01**	4.3 (2.2-8.9)	10.7 (6.9-18.3)	**0.01**
Clinical measurements						
SBP (mmHg)**	123 ± 8	130 ± 16	0.28	114 ± 6	125 ± 10	**0.004**
DBP (mmHg)**	76 ± 10	85 ± 9	**0.04**	79 ± 7	83 ± 11	0.30
Total cholesterol (mmol/L) ^	4.7 ± 0.7	5.2 ± 0.8	0.19	5.2 ± 0.7	4.8 ± 0.7	0.11
LDL (mmol/L) ^	3.0 ± 0.7	3.6 ± 0.7	0.13	3.2 ± 0.6	2.8 ± 0.6	0.15
HDL (mmol/L) ^	1.1 ± 0.2	1.1 ± 0.2	0.94	1.5 ± 0.3	1.4 ± 0.2	0.28
Triglycerides (mmol/L) ^ Φ	1.0 (0.6-1.2)	1.2 (0.9-1.5)	0.23	0.8 (0.6-1.2)	1.0 (0.7-1.2)	0.06
Oral glucose tolerance test, glycaemia and hyperinsulinaemic clamp measures						
OGTT1 h BGL (mmol/L)	6.6 ±1.3	8.6 ± 2.1	**0.02**	6.6 ±0.6	8.1 ± 2.1	**0.04**
HbA1c (%)	5.3 ± 0.2	5.6 ± 0.3	**0.01**	5.2 ±0.3	5.6 ± 0.3	**0.003**
Basal insulin (mU/L) Φ	12 (10- 19)	25 (16-39)	**0.004**	12 (8-15)	16 (11-21)	0.06
Metabolically flexibility	0.19 ±0.04	0.10 ± 0.03	**<0.01**	0.18 ±0.05	0.18 ± 0.04	0.68
Circulating cytokines						
Hs-CRP (mg/L) Φ	1.9 (1.3-2.9)	4.1 (1.2-5.4)	0.14	3.6 (1.5-4.4)	3.1 (1.9-5.7)	0.53
FGF-19 (ng/L) Φ	128 (42-198)	76 (45-104)	0.94	126 (76-334)	118 (66-166)	0.35
FGF-21 (ng/L) Φ	21 (16-132)	65 (27-151)	0.35	93 (63-110)	90 (56-140)	0.56
FABP4 (µg/L)	43 ± 26	46 ± 16	0.75	73 ± 19	79 ± 24	0.45
Lipocalin 2 (µg/L)	44 ± 16	44 ± 12	0.99	36 ± 13	38 ± 14	0.62
RBP 4 (mg/L)	12 ± 2	11 ± 2	0.65	11 ± 2	11 ± 3	0.51
Total adiponectin (mg/L)	11 ± 5	9 ± 3	0.23	22 ± 8	17 ± 7	0.08

*Data are means ± SD. Φ Data are median (interquartile range) for non-normally distributed data
**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Muscle_{sen}$ men n = 7, $Muscle_{res}$ men n = 15; $Muscle_{sen}$ women n = 10 and $Muscle_{res}$ women n = 19).
^ Subjects treated with lipid medications excluded from the analysis (included: $Muscle_{sen}$ men n = 6, $Muscle_{res}$ men n = 15; $Muscle_{sen}$ women n = 12 and $Muscle_{res}$ women n = 22).
‡ DXA, n=61
πAdipocyte data, n = 53 (included: $Muscle_{sen}$ men n = 8, $Muscle_{res}$ men n = 17; $Muscle_{sen}$ women n = 9 and $Muscle_{res}$ women n = 19)

3.4.6 Linear regression analyses

In the whole cohort, GIR_{HI} correlated inversely with waist circumference, waist/hip ratio, central abdominal fat, systolic BP, serum triglycerides, fasting glucose, glucose concentrations during OGTT, $AUC_{glucose}$, $AUC_{insulin}$ and $AUC_{C\text{-}peptide}$ during OGTT, HbA1c and $NEFA_{LO}$ (Table 3.4). Positive associations were noted with serum HDL, ΔRQ, serum FGF-19 and total adiponectin (Table 3.4). All associations remained significant upon adjustment for total body fat (Table 3.4).

GIR_{HI} correlated inversely with liver, visceral and pancreatic fat (Figure 3.3A-C), but not with abdominal subcutaneous fat (Figure 3.3D). These correlations remained significant after adjusting for total body fat ($P < 0.01$).

Pancreatic fat correlated positively with liver ($r = 0.29$, $P = 0.02$) and visceral ($r = 0.40$, $P=0.001$) fat, but not subcutaneous fat ($P = 0.25$). Pancreatic fat correlated positively with OGTT-derived measures of pancreatic function ($AUC_{C\text{-}peptide}$ [$r = 0.33$, $P = 0.01$] and $AUC_{insulin}$ [$r = 0.33$, $P = 0.01$]) and HOMA2-β ($r = 0.29$, $P = 0.02$).

Positive significant associations were noted between adipocyte diameter and fasting serum triglycerides ($r = 0.47$), waist circumference ($r = 0.31$), visceral fat ($r = 0.30$), liver fat ($r = 0.29$) and HbA1c ($r = 0.36$) (Figure 3.4 C-G, respectively). GIR_{HI} ($r = -0.29$), metabolic flexibility ($r = -0.37$) and HDL cholesterol ($r = -0.42$) correlated negatively with adipocyte diameter (Figure 3.4 A-B, H respectively).

Table 3.4: Correlations between glucose infusion rate at high dose clamp (GIR_{HI}) and clinical and metabolic variables

Variable	GIR_{HI}, Pearson's coefficient (*P*)
Age	NS
Waist circumference (cm)	-0.47 (<0.001)[¥]
Waist/hip ratio	-0.58 (<0.001)[¥]
Central abdominal fat[‡] (kg)	-0.51 (<0.001)[¥]
SBP [*] (mmHg)	-0.46 (0.001)[¥]
DBP [*] (mmHg)	NS
Serum cholesterol[^] (mmol/L)	NS
Serum LDL cholesterol[^] (mmol/L)	NS
Serum HDL cholesterol[^] (mmol/L)	0.53 (<0.001)[¥]
Serum triglycerides[^] (mmol/L)	-0.39 (0.004)[¥]
Fasting blood glucose (mmol/L)	-0.28 (0.03)[¥]
OGTT 1h blood glucose (mmol/L)	-0.51 (<0.001)[¥]
OGTT 2h blood glucose (mmol/L)	-0.36 (0.004)[¥]
OGTT $AUC_{glucose}$ (mmol/L·120min)	-0.49 (<0.001)[¥]
OGTT $AUC_{insulin}$ (mU/L·120min)	-0.67 (<0.001)[¥]
OGTT $AUC_{C\text{-}peptide}$ (µg/L·120min)	-0.53 (<0.001)[¥]
HbA1c (%)	-0.44 (<0.001)[¥]
Fasting insulin (mU/L)	-0.59 (<0.001)[¥]
Fasting NEFA (mmol/L)	NS
$NEFA_{LO}$ (mmol/L)	-0.5 (<0.001)[¥]
Δ RQ ($RQ_{HI} - RQ_{baseline}$)	0.64 (<0.001)[¥]
Hs-CRP (mg/L)	NS
FGF-19 (ng/L)	0.30 (0.02)[¥]
FGF-21(ng/L)	NS
FABP4 (µg/L)	NS
Lipocalin 2 (µg/L)	NS
RBP 4 (mg/L)	NS
Serum adiponectin (mg/L)	0.54 (<0.001)[¥]

[‡] DXA, n=61
[*]Subjects treated with anti-hypertensive medications excluded from the analysis (included: n = 51)
[^]Subjects treated with lipid medications excluded from the analysis (included: n = 55).
[¥] P<0.05 after adjusting for total body fat, NS, non-significant *P* > 0.05

Figure 3.3 Correlations with GIR_{HI}

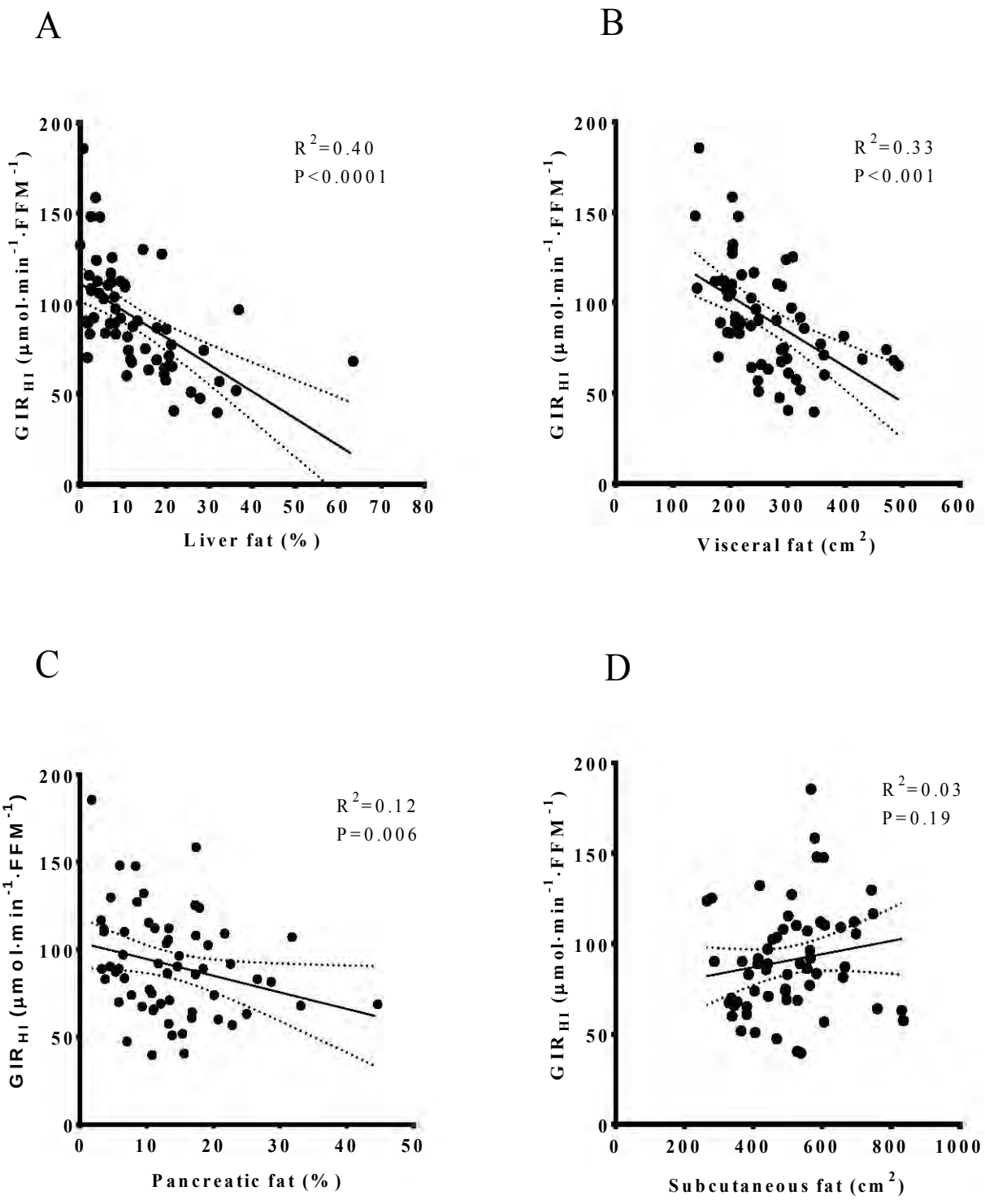


Figure 3.3 linear regressions between liver, visceral, pancreatic and subcutaneous fat with muscle insulin sensitivity (glucose infusion rate during the high dose insulin clamp, GIR_{HI}, A-D). Depicted are the line of fit and the 95% confidence curves that were obtained from linear regression. MRI-derived measures were logarithmically transformed prior to statistical analysis

Figure 3.4 Correlations with adipocyte size

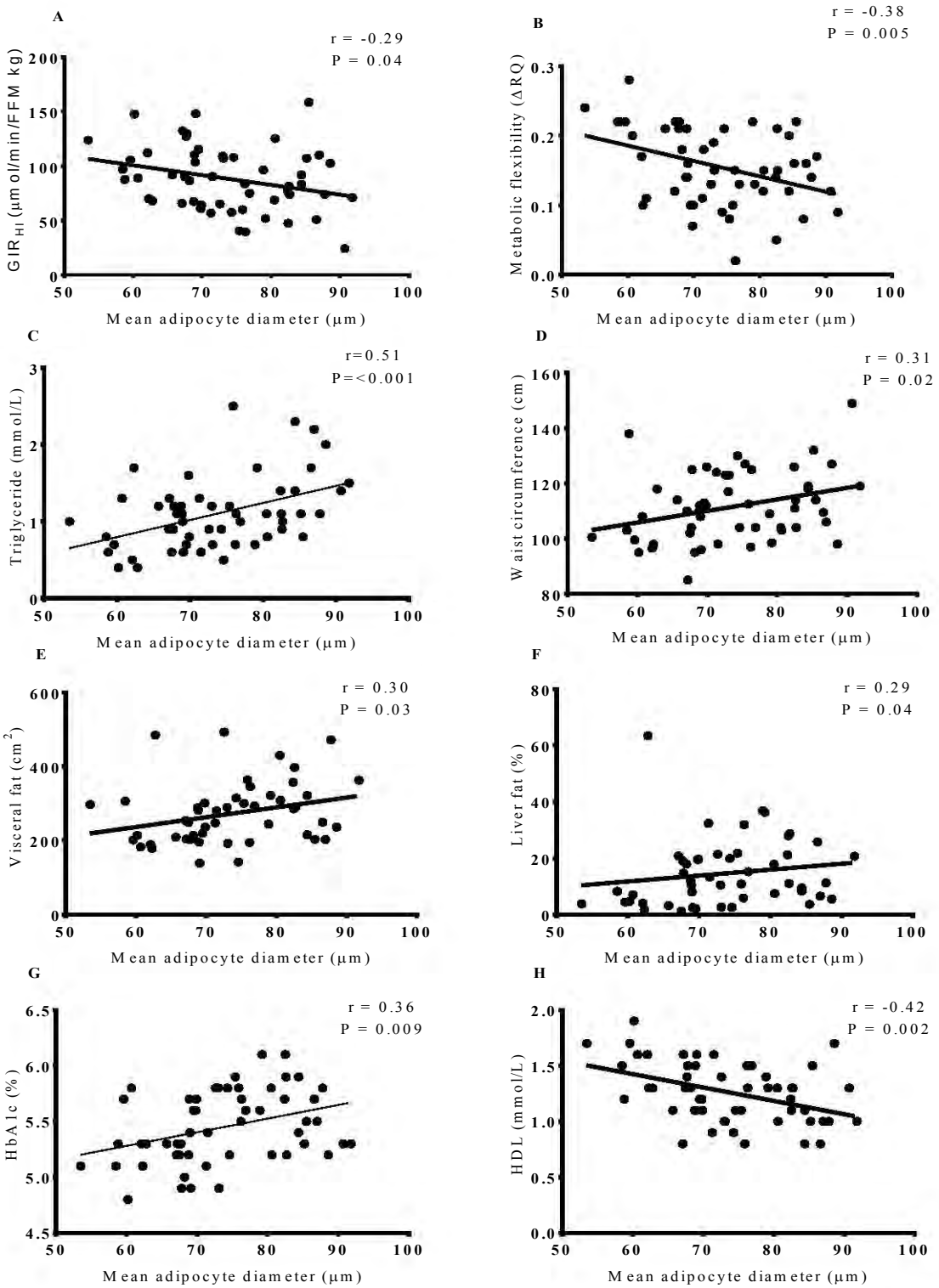


Figure 3.4 Correlation between adipocyte size and various factors including GIR_{HI}, metabolic flexibility, triglycerides, waist circumference, visceral fat, liver fat, HbA1c and. Depicted are the line of fit and the 95% confidence curves that were obtained from Pearson's correlation analysis. MRI-derived measures (liver and visceral fat) and triglycerides were logarithmically transformed prior to statistical analysis.

3.4.7 Multiple linear regression analyses in total cohort

Sixty-four percent of GIR_{HI} variability was explained by ΔRQ, liver fat, HDL cholesterol, and SBP (Table 3.5). Since ΔRQ is, to an extent, an alternative measure of insulin sensitivity, a second multiple linear regression model was carried out including the same variables, but without ΔRQ. Liver fat, HDL cholesterol, SBP and HbA1c explained 63% of GIR_{HI} variability. In a third model including clinically-relevant markers (HDL cholesterol, OGTT 1h blood glucose, SBP, triglycerides, hs-CRP, HbA1c, and waist circumference), HDL, OGTT 1h blood glucose, SBP and serum triglycerides explained 53.7% of GIR_{HI} variability (Table 3.5).

3.4.8 Multiple linear regression analyses by gender

When we analysed clinical model in men and postmenopausal women separately using the same clinical markers, we found different but interesting results (Table 3.6). In men, HbA1c and waist circumference explained about a quarter of the GIR_{HI} variability while in postmenopausal women (n=22 out of 35 women), OGTT 1h BGL predicted 67% of variability in muscle insulin sensitivity. Postmenopausal women were analysed due to their larger sample size and absence of oestrogen influence which lessens the disparity between men and women in regional adiposity.

Table 3.5. Linear regression models to explain the variability in muscle insulin sensitivity (GIR_{HI})

		Model	Adjusted R^2	Standardized coefficients β	P value
GIR_{HI}	***Model 1.*** *Clinical, metabolic and abdominal adipose tissue imaging variables*[*]	ΔRQ	0.39	0.63	<0.001
		ΔRQ		0.45	
		Liver fat	0.55	-0.44	<0.001
		ΔRQ		0.32	
		Liver fat		-0.40	
		HDL cholesterol	0.61	0.28	0.008
		ΔRQ		0.29	
		Liver fat		-0.34	
		HDL cholesterol		0.30	
		SBP	0.64	-0.20	0.0028
	Model 2. *Clinical, metabolic and abdominal adipose tissue imaging variables*[**] *excluding ΔRQ*	Liver fat	0.38	-0.62	<0.001
		Liver fat		-0.48	
		HDL cholesterol	0.54	0.42	<0.001
		Liver fat		-0.40	
		HDL cholesterol		-0.43	
		SBP	0.59	-0.23	0.02
		Liver fat		-0.32	
		HDL		0.41	
		SBP		-0.22	
		HbA1c	0.63	-0.23	0.02

		Model	Adjusted R^2	Standardized coefficients β	P value
GIR_{HI}	***Model 3.*** *Clinical variables only*[***]	HDL cholesterol	0.288	0.548	<0.001
		HDL cholesterol		0.462	
		OGTT 1h BGL	0.443	-0.411	<0.001
		HDL cholesterol		0.442	
		OGTT 1h BGL		-0.349	
		Systolic blood pressure	0.487	-0.236	0.02
		HDL cholesterol		0.334	
		OGTT 1h BGL		-0.364	
		Systolic blood pressure		-0.271	
		Triglycerides	0.537	-0.258	0.009

R^2 = explained variance; β = beta-estimate of linear regression model

[*] HbA1c (P = 0.1), adiponectin (P = 0.2), $NEFA_{LO}$ (P = 0.2), hs-CRP (P = 0.6), triglycerides (P = 0.2), visceral fat (P = 0.2), age (p=0.2), OGTT 1hour BGL (P=0.2) and total body fat (P = 0.5) were not retained in the regression model.

[**]OGTT 1h BGL (P = 0.3), age (P = 0.5), adiponectin (P = 0.1), hs-CRP (P = 0.2), $NEFA_{LO}$ (P = 0.1), triglycerides (P=0.2), visceral fat (P = 0.5), and total body fat (P = 0.2) were not retained in the regression model.

[***]HbA1c (P = 0.5), age (P = 0.07), hs-CRP (P = 0.8), and waist circumference (P = 0.2) were not retained in the regression model

Table 3.6 Linear regression models to explain the variability in muscle insulin sensitivity (GIR_{HI}) in men and postmenopausal women

		Model	Adjusted R^2	Standardized coefficients β	P value
Men (n=29)					
GIR_{HI}					
	Clinical variables^	HbA1c	0.15	-0.42	0.03
		HbA1c		-0.38	
		Waist circumference	0.26	-0.37	0.04
Postmenopausal women (n=22)					
GIR_{HI}	*Clinical variables*^^	OGTT 1h BGL	0.67	-0.83	<0.001

R^2 = explained variance; β = beta-estimate of linear regression model
^hs-CRP (P = 0.8), age (P = 0.7), HDL (P = 0.8), OGTT-1 hour BGL (P = 0.5), triglycerides (P = 0.2), and systolic blood pressure (P = 0.7) and were not retained in the regression model
^^hs-CRP (P = 0.7), age (P = 0.6), HDL (P = 0.7), HbA1c (P = 0.1), triglycerides (P = 0.4), waist circumference (P = 0.8) and systolic blood pressure (P = 0.1) and were not retained in the regression model

3.5 DISCUSSION

The main findings in this chapter that:

1. $Muscle_{sen}$ subjects had:
    a. Lower blood pressure, plasma concentrations of triglycerides, glycaemia, compared with $Muscle_{res}$.
    b. Greater metabolic flexibility than $Muscle_{res.}$
    c. Lower visceral and liver fat compared with $Muscle_{res}$ despite similar subcutaneous fat.
    d. Lower sugar and saturated fat intake compared with $Muscle_{res}$ despite similar calorie intake and physical activity.
2. Pancreatic fat correlated positively with pancreatic β-cell function via OGTT-derived measures of pancreatic function.
3. HDL cholesterol, OGTT 1h blood glucose, SBP and serum triglycerides explained >50% of GIR_{HI} in total cohort, where OGTT 1h blood glucose explained two thirds of GIR_{HI} in postmenopausal women.
4. Subcutaneous abdominal adipocyte diameter was correlated with muscle insulin sensitivity (GIR_{HI}) and metabolic flexibility.

Similar to previous clamp studies, we have confirmed that $Muscle_{sen}$ individuals have lower plasma concentrations of triglycerides, blood pressure and glycaemia.[34,36,81] Higher levels of plasma concentrations of triglycerides have been shown to relate to insulin resistance[299] and cardiovascular disease.[300] These findings suggest a close link between insulin resistance and dyslipidaemia, perhaps associated with higher visceral and liver fat. Our study also demonstrated that by using insulin sensitivity as a unifying

key criterion, we were able to identify a group of obese individuals with features of metabolic syndrome including higher plasma concentrations of triglycerides, blood pressure and glycaemia.

Lower visceral and liver fat were key features of insulin-sensitive obesity. This has been supported by many hyperinsulinaemic-euglycaemic clamp studies looking at obese postmenopausal women,[34,36] adolescents,[43] and others.[81,82] Most previous studies used CT to measure visceral and liver adiposity. We used MRI to quantify adiposity, which involves no radiation and its accuracy and precision has been validated compared with weighing of adipose tissue in human cadavers.[301] Visceral fat is an important correlate with insulin resistance and metabolic disease[302] and potential mechanisms include increased lipolysis, direct flow of fatty acids into the portal circulation and greater accumulation of inflammatory cells.[106]

Earlier clamp studies did not assess the relationship between liver fat and insulin resistance, but recent studies have shown lower liver fat content in obese-insulin sensitive groups.[81,82] Both liver and visceral fat may contribute to components of metabolic syndrome,[303,304] but some suggest that only liver fat contributes to multi-organ insulin resistance and metabolic syndrome independent of visceral fat.[305] Our findings supported the idea that liver fat (but not visceral fat) is an independent predictor of muscle insulin sensitivity. Indeed it explained 38% of variability of GIR_{HI}. We also showed a stronger correlation between GIR_{HI} and liver fat compared with visceral fat. Interestingly, in insulin-resistant states, de-novo lipogenesis remains "insulin-sensitive" and hyperinsulinaemia stimulates hepatic lipid accumulation via the

SREBP-1c pathway.[107] This may explain the fact that our $Muscle_{res}$ individuals had higher liver fat compared to $Muscle_{sen}$ possibly due to higher fasting insulin levels. Our study has shown that the determinants of insulin resistance in obese individuals are not attributed to the amount of excess adipose tissue, but by the distribution and ectopic fat deposition, especially in the liver.

Dietary intake, macronutrient composition and physical activity habits are difficult to measure with accuracy in free living individuals. Yet, anecdotal data suggest lower saturated fat[89] and alcohol[90] intake and greater physical activity[90] in insulin-sensitive or metabolically-healthy obese individuals. In our study, $Muscle_{sen}$ consumed lower sugar and saturated fat than $Muscle_{res}$, but no significant differences were detected in intake of other macronutrients, energy intake or physical activity. This could be due to the inaccuracy associated with self-reporting. Other similar studies also showed $Muscle_{sen}$ group did not have a higher physical activity score[41,42] when assessed by self-reporting, in contrast to a recent study which showed higher physical activity in MHO group when assessed by an accelerometer.[306] Future studies with detailed physical and diet assessment tools such as pedometers/accelerometer and weighed food records are warranted to further evaluate lifestyle factors in insulin-sensitive obesity.

Evidence relating pancreatic fat to glucose homeostasis is conflicting.[221,223-225] Here, pancreatic fat content correlated weakly with muscle insulin sensitivity and no differences were observed between $Muscle_{sen}$ and $Muscle_{res}$. Pancreatic fat has been reported to inversely correlate with beta cell function in prediabetic[221] and diabetic patients.[225] However, we reported a positive correlation between pancreatic fat and C-

peptide and insulin responses to OGTT and HOMA2-β. As we only included normoglycaemic or pre-diabetic patients in our study, it is not surprising that we had shown a positive correlation. Consistent with a previous study,[307] pancreatic fat correlated positively with liver and visceral, but not subcutaneous, fat. This may support the metabolic importance of ectopic fat deposition in internal organs.

We have shown that larger subcutaneous adipocyte size correlated with an unfavourable metabolic profile including higher fasting triglycerides and HbA1c, lower HDL and metabolic flexibility and insulin resistance (GIR_{HI}). Other clamp studies have shown similar results between adipocyte diameter and triglycerides, HDL and insulin resistance in obese patients undergoing Roux-en-Y[308] and non-diabetic individuals.[309] This interesting association between adipocyte size and unhealthy metabolic profile could be explained by an increase in proinflammatory cytokines observed in larger adipocytes,[310,311] though not shown in our study, perhaps due to small sample size. At the cellular level, large adipocyte size is associated with blunted translocation of GLUT4 to the plasma membrane in response to insulin, which may support a causal correlation between large adipocytes and insulin resistance.[136] Further larger studies are needed to dissect the relationships between adipocyte size and metabolic profiles.

In the multiple linear regression model, ΔRQ strongly predicted muscle insulin sensitivity (GIR_{HI}), in agreement with a previous study in obese adolescents.[43] The switch from fat to carbohydrate oxidation during hyperinsulinemia is termed metabolic flexibility[312] and is impaired in insulin-resistant individuals.[190] Since ΔRQ is, to an extent, an alternative measure of insulin sensitivity, ΔRQ was excluded in an alternative

model demonstrating that liver fat and HDL explained more than half (54%) the variability in insulin sensitivity, with further, relatively small, contributions from SBP (5%), and HbA1c (4%).

A regression model including clinically-available markers only, revealed that HDL cholesterol, OGTT 1h blood glucose, SBP and serum triglycerides explained half the variability in insulin sensitivity. These findings should encourage use of these relatively simple tests in identifying obese individuals at greater metabolic risk. Interestingly, in postmenopausal obese women, OGTT 1h blood glucose predicted two thirds of variability in insulin sensitivity. Our finding was different to other clamp studies involving postmenopausal women; CRP, triglycerides and lean body mass predicted one third of variability in glucose disposal value in one study[36] while visceral adipose tissue and earlier age-related onset of obesity were independent predictors of muscle insulin sensitivity in another study.[34] Both studies did not incorporate OGTT results in their analyses. Our findings suggest that predictors of muscle insulin sensitivity may be different depending on the gender and menopausal status of obese individuals. Further studies are needed to looking at both obese men and women to further consolidate our findings in identification of metabolically abnormal obese individuals.

The main strengths of this study are:

1. The careful phenotyping of participants by performing a detailed metabolic assessment on various clinical, radiological and metabolic factors on a relatively large sample size (n=64). We have used the gold standard hyperinsulinaemic-euglycaemic clamp developed by De Fronzo, where we used 80 $mU \cdot m^{-2} \cdot min^{-1}$ of

high dose insulin to assess muscle insulin sensitivity.[111] We also used a lower insulin infusion rate with deuterated glucose tracer to assess endogenous glucose output to assess liver insulin sensitivity using Steele's equation,[118] as modified by Finegood.[262]

2. The use of MRI to measure pancreatic fat, and correlated pancreatic fat with muscle and liver insulin sensitivity using gold standard measures. This has not been reported previously.

3.6 SUMMARY AND CONCLUSIONS

In conclusion, $Muscle_{sen}$ subjects are characterised by lower blood pressure, lower visceral and liver fat. While the debate regarding the long-term protective value of insulin sensitivity in obesity persists,[4] insulin resistance in muscle is associated with the poor cardiometabolic profile and is characterized by visceral and liver fat accumulation. Identification of obese individuals at high risk of metabolic disease is vital for early and effective interventions to minimize disease and health costs. Factors readily measured in clinical practice may serve as early detection tools, guiding targeted intervention, perhaps, with different factors tailored according to gender and/or menopausal status

CHAPTER 4
COMPARISON OF METABOLIC CHARACTERISTICS BETWEEN LIVER INSULIN-SENSITIVE AND LIVER INSULIN-RESISTANT SUBJECTS

4.1 INTRODUCTION

Insulin resistance may manifest at different magnitudes and severity in various insulin-targeted sites (liver, muscle and adipose tissue), and different interventions may target at different sites of insulin resistance. For examples, metformin therapy predominantly improves liver insulin resistance, while exercise improves muscle insulin resistance.[109] Therefore, it is important to dissect insulin resistance at different sites. Liver insulin resistance is important clinically as it contributes to fasting hyperglycaemia and, consequently, to impaired fasting glucose/pre-diabetes.[109] The best way to assess liver insulin resistance is to assess hepatic glucose output, most commonly undertaken by isotope dilution technique using a tracer.[116] Hyperinsulinaemic-euglycaemic clamps with glucose tracers have been used to determine liver and muscle insulin sensitivity.[112] We have used a similar clamp study protocol with glucose tracers to identify and characterise liver insulin sensitive obese individuals.

4.2 AIMS

1. To compare insulin-sensitive and insulin-resistant subjects stratified by liver insulin sensitivity.
2. To determine whether being insulin-sensitive at one site (muscle or liver) is sufficient in predicting a favourable metabolic phenotype.

4.3 METHODS

A detailed description of the methodology used in these studies is outlined in Chapter 2 and 3. In separate analyses, subjects were reclassified by the degree of EGP suppression during low-dose insulin infusion. $Liver_{sen}$ individuals were in the upper tertile of EGP suppression and $Liver_{res}$ individuals were in the lower two tertiles. Muscle and liver stratification to groups was performed in men and women separately. We divided subjects into 4 groups based on the site of insulin resistance (muscle, liver or both) in our secondary analyses.

4.4 RESULTS

4.4.1 Metabolic characteristics of $Liver_{sen}$ and $Liver_{res}$ individuals

Subjects were reclassified as $Liver_{sen}$ and $Liver_{res}$ based on the degree of EGP suppression (80 ± 9 *vs.* 58 ± 9 % respectively) during low dose insulin infusion. GIR_{HI} and EGP suppression correlated (R^2 = 0.14, P = 0.003). The characteristics of $Liver_{sen}$ and $Liver_{res}$ subjects are outlined in table 4.1. Despite similar age, BMI and total body fat (P > 0.19), $Liver_{sen}$ had significantly lower waist circumference compared with $Liver_{res}$. There was a trend of lower central abdominal fat in $Liver_{sen}$ (P = 0.09). Both mean and median adipocyte diameters were significantly lower in $Liver_{sen}$ compared with $Liver_{res}$. This was different to the $Muscle_{sen}$ and $Muscle_{res}$ groups where there were no differences in adipocyte size.

$Liver_{sen}$ and $Liver_{res}$ had similar systolic and diastolic blood pressure. After exclusion of subjects treated with lipid-lowering medications, $Liver_{sen}$ had lower fasting triglycerides compared with $Liver_{res}$; the groups had similar total cholesterol, LDL and HDL cholesterol. $Liver_{sen}$ group had lower OGTT 1h-blood glucose, $AUC_{glucose}$, $AUC_{insulin}$, $AUC_{C\text{-}peptide}$, during OGTT, HbA1c and fasting insulin concentrations compared with $Liver_{res}$ (Table 4.1). NEFA suppression during low-dose insulin infusion was blunted in $Liver_{res}$ subjects suggesting reduced adipose tissue insulin sensitivity (Table 4.1).

Basal RQ was not significantly different between groups, but ΔRQ (RQ during the high-dose insulin infusion minus baseline RQ) was significantly higher in $Liver_{sen}$ compared

to $Liver_{res}$ respectively, reflecting increased metabolic flexibility (Table 4.1). Hs-CRP (P = 0.02) was lower and FGF-21 (P = 0.09) trended lower in $Liver_{sen}$.

Similar to differences observed between $Muscle_{sen}$ and $Muscle_{res}$, $Liver_{sen}$ had lower visceral (227 ± 51 cm^2 *vs.* 288 ± 86 cm^2, P = 0.003) and liver (8 ± 8% *vs.* 16 ± 12%, P < 0.001) fat (Figure 4.1A-B), but no differences between groups were noted for subcutaneous abdominal fat and pancreatic fat (Figure 4.1C-D).

4.4.2 Dietary intake and physical activity

In the $liver_{sen}$ and $liver_{res}$ groups, there were no differences between dietary energy, macronutrient (Table 4.2) and physical activity (34 ± 1 and 34 ± 2 MET-hrs/day in $Liver_{sen}$ and $Liver_{res}$, P = 0.6). Sugar intake trended lower in $Liver_{sen}$ compared to $Liver_{res}$ group (P = 0.07).

4.4.3 $Liver_{sen}$ and $Liver_{res}$ in men and women

We compared various clinical factors between $Liver_{sen}$ and $Liver_{res}$ in men and women separately, as shown in table 4.3. In men, $Liver_{sen}$ had lower central abdominal fat, mean and median adipocyte size, visceral and liver fat compared with $Liver_{res}$. There were no differences in blood pressure and lipid profile in men. $Liver_{sen}$ men had lower OGTT 1h BGL, HbA1c, fasting insulin and greater metabolic flexibility than $Liver_{res}$ men. $Liver_{sen}$ men had lower hs-CRP and higher FGF-19 compared with $Liver_{res}$ men. In $Liver_{sen}$ women, the significant findings included lower liver fat, triglycerides, HbA1c and basal insulin levels compared with $Liver_{res}$ women. There was a trend of lower FGF-21 in $Liver_{sen}$ women.

Table 4.1 Anthropometric, clinical and metabolic characteristics of obese individuals stratified based on liver insulin sensitivity (EGP suppression).

Characteristics	$Liver_{sen}$ (M9:F12)	$Liver_{res}$ (M18:F23)	P value
Age	49 ± 12	51 ± 11	0.52
Anthropometry, body composition and abdominal fat distribution			
BMI (kg/m^2)	35.6 ± 3.9	36.1 ± 4.1	0.65
Waist circumference (cm)	106 ± 11	114 ± 14	**0.046**
Waist/hip ratio	33.5 ± 1.3	33.7 ± 1.6	0.59
Whole body fat (kg) ‡	45 ± 10	46 ± 10	0.64
Whole body fat (%)‡	45 ± 8	47 ± 7	0.54
Fat-free mass (kg) ‡	54 ± 11	53 ± 11	0.67
Fat-free mass (%)‡	55 ± 8	53 ± 7	0.53
Central abdominal fat (kg) ‡	3.2 ± 0.7	3.5 ± 0.7	0.09
Mean adipocyte diameter (µm)$^{\pi}$	69 ± 7	76 ± 10	**0.01**
Median adipocyte diameter (µm) $^{\pi}$	69 ± 8	76 ± 10	**0.01**
Clinical measurements			
SBP (mmHg)**	121 ± 9	126 ± 14	0.16
DBP (mmHg)**	80 ± 12	83 ± 9	0.23
Total cholesterol (mmol/L)^	5.1 ± 0.9	4.9 ± 0.7	0.45
LDL cholesterol (mmol/L)^	3.2 ± 0.8	3.1 ± 0.7	0.53
HDL cholesterol (mmol/L)^	1.3 ± 0.4	1.3 ± 0.3	0.54
Triglycerides (mmol/L)^ $^{\Phi}$	0.9 (0.6-1.2)	1.1 (0.9-1.4)	**0.03**
Oral glucose tolerance test, glycaemia and hyperinsulinaemic clamp measures			
Fasting blood glucose (mmol/L)	4.7 ± 0.5	4.9 ± 0.4	0.11
OGTT 1h blood glucose (mmol/L)	6.8 ± 1.8	8.2 ± 2.0	**0.01**
OGTT 2h BGL (mmol/L)	5.8 ± 1.6	6.6 ± 1.6	0.07
OGTT $AUC_{glucose}$ (mmol/L·120min)	763 ± 154	887 ± 168	**0.01**
OGTT $AUC_{insulin}$ (mU/L·120min)$^{\Phi}$	7497(6106-11353)	11978 (7941-17114)	**0.004**
OGTT $AUC_{C\text{-}peptide}$ (µg/L·120min)$^{\Phi}$	425 (371-612)	746 (586-992)	**<0.001**
HbA1c (%)	5.2 ± 0.3	5.6 ± 0.3	**<0.001**
Fasting insulin (mU/L) $^{\Phi}$	12 (10-16)	18 (13-27)	**<0.001**
$Insulin_{LO}$ (mU/L) $^{\Phi}$	38 (32-46)	41 (37-51)	0.09

Characteristics	$Liver_{sen}$ (M9:F12)	$Liver_{res}$ (M18:F23)	P value
$Insulin_{HI}$ (mU/L) $^{\Phi}$	200 (169-226)	217 (180-254)	0.11
Fasting NEFA (mmol/L) $^{\Phi}$	0.35 (0.25-0.45)	0.37 (0.28-0.46)	0.97
$NEFA_{LO}$ (mmol/L) $^{\Phi}$	0.03 (0.02-0.05)	0.05 (0.04-0.06)	**0.02**
$NEFA_{HIGH}$(mmol/L) $^{\Phi}$	0.02 (0.01-0.02)	0.02 (0.02-0.03)	0.20
Basal RQ	0.79 ± 0.02	0.80 ± 0.03	0.1
Δ RQ ($RQ_{HI} - RQ_{baseline}$)	0.18 ± 0.04	0.15 ± 0.05	**0.048**
Circulating cytokines			
Hs-CRP (mg/L) $^{\Phi}$	2.1 (1.1-3.7)	4.2 (2.0-5.7)	**0.02**
FGF-19 (ng/L) $^{\Phi}$	120 (69-187)	96 (56-152)	0.12
FGF-21 (ng/L) $^{\Phi}$	68 (23-105)	91 (46-159)	0.09
FABP 4 (μg/L)	61 ± 28	65 ± 26	0.56
Lipocalin 2 (μg/L)	44 ± 14	40 ± 14	0.29
RBP 4 (mg/L)	12 ± 2	11 ± 3	0.36
Total adiponectin (mg/L)	16 ± 8	14 ± 8	0.34

*Data are means ± SD. $^{\Phi}$ Data are median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Liver_{sen}$ n = 19 and $Liver_{res}$ n = 30).

$^{\wedge}$ Subjects treated with lipid medications excluded from the analysis (included: $Liver_{sen}$ n = 20 and $Liver_{res}$ n = 34).

‡ DXA, n = 59

$^{\pi}$Adipocyte data, n=51 (included: $Liver_{sen}$ n = 17 , $Liver_{res}$ n = 34)

Figure 4.1

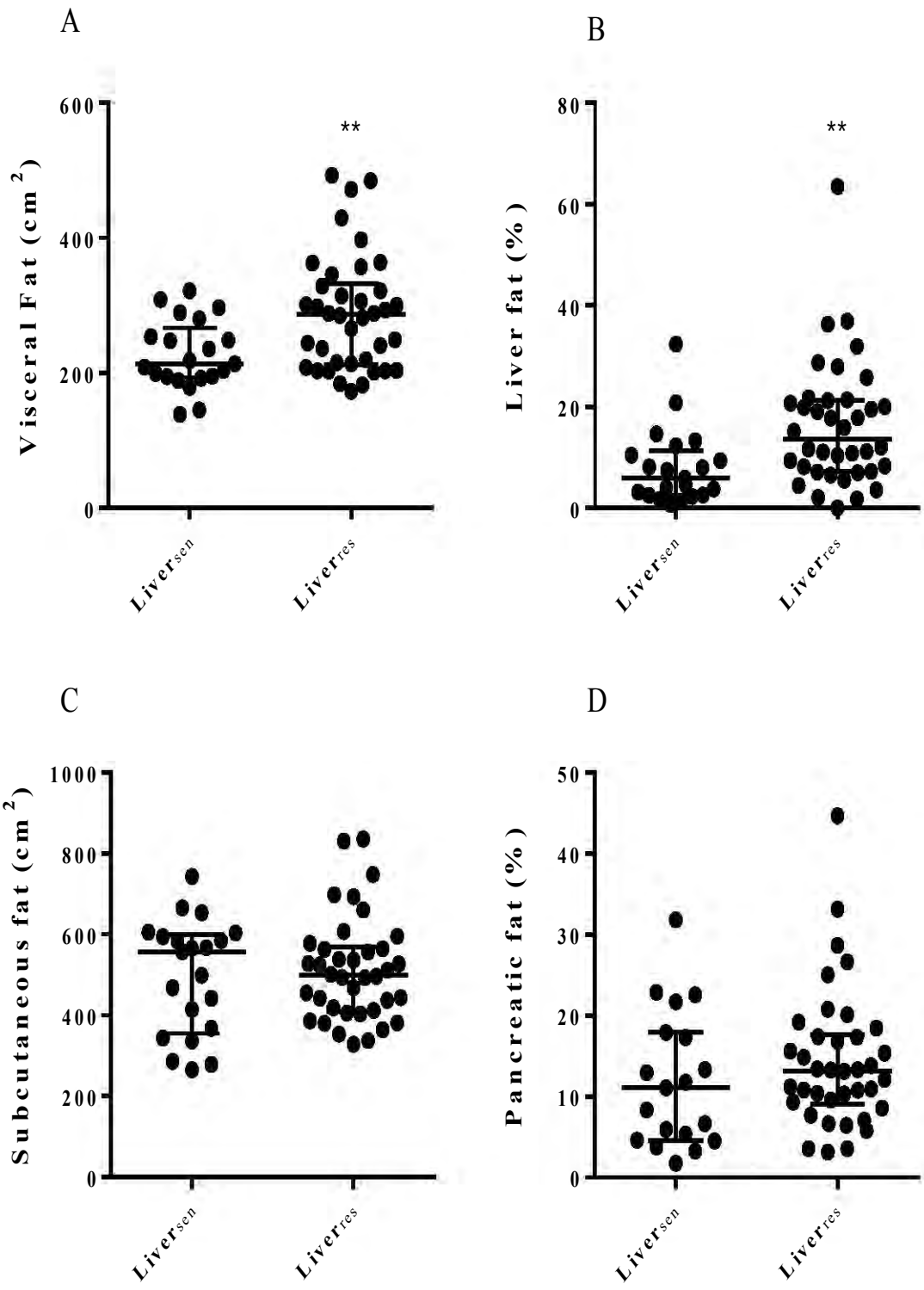


Figure 4.1 Abdominal visceral (A), liver (B), subcutaneous (C) and pancreatic (D) fat in individuals stratified based on hepatic insulin sensitivity. $^{**}P < 0.01$ between $Liver_{sen}$ and $Liver_{res}$.

Table 4.2 Dietary intake of obese individuals stratified based on liver insulin sensitivity (glucose infusion rate during the high dose clamp) according to diet diary

Daily intake*	$Liver_{sen}$ (M9:F12)	$Liver_{res}$ (M18:F23)	P value
Energy (kcal)	2020 ± 630	2043 ± 874	0.92
Protein (g)	105 ± 45	101 ± 37	0.74
Protein (% of energy)	21 ± 5	21 ± 6	0.83
Total fat (g)	82 ± 39	81 ± 43	0.98
Fat (% of energy)	35 ± 8	34 ± 6	0.94
Saturated fat (g)	31 ± 15	32 ± 18	0.80
Saturated fat (% of energy)	13 ± 3	13 ± 3	0.75
Polyunsaturated fat (g)	13 ± 10	13 ± 6	0.93
Monounsaturated fat (g)	32 ± 15	31 ± 19	0.89
Carbohydrate (g)	204 ± 52	216 ± 115	0.66
Carbohydrate (% of energy)	41 ± 7	41 ± 9	0.94
Sugar (g)	83 ± 25	110 ± 85	0.07
Alcohol (g)	1.2 ± 3.1	1.0 ± 4.0	0.82
Dietary fibre (g)	23 ± 8	21 ± 9	0.59

*Data are means ± SD

4.4.4 Linear regression analyses

There was a significant negative correlation between EGP suppression at low dose insulin infusion with liver fat (Figure 4.2A), a trend to inverse correlation with visceral fat (Figure 4.2B) and no significant correlations with pancreatic and subcutaneous fat (Figure 4.2C and 4.2D, respectively). EGP suppression had significant inverse associations with $AUC_{insulin}$, $AUC_{C\text{-}peptide}$, HbA1c, fasting insulin, $NEFA_{LO}$, and hs-CRP (Table 4.3).

There was a significant inverse correlation between mean adipocyte diameter and HIRI index (r = 0.27, P = 0.03) and a borderline relationship with EGP suppression (r = -0.26,

P = 0.06). In men, mean adipocyte size correlated negatively with HIRI index (r = -0.43, P = 0.04) and EGP suppression (r = -0.46, P = 0.03), but not in women (P = 0.40 and P = 0.64 respectively).

4.4.5 Multiple linear regression analyses

When HbA1c, hs-CRP, triglycerides, SBP and liver fat were entered into a multiple linear regression model using EGP suppression, HbA1c and liver fat explained 22% of the variability and all other variables were not retained (Table 4.4).

Table 4.4 Linear regression models to explain the variability in liver insulin sensitivity (EGP suppression)

		Model	Adjusted R^2	Standardized coefficients β	P value
EGP suppression	*Clinical and abdominal imaging variables*^	HbA1c	0.160	-0.42	0.001
		HbA1c		-0.30	
		Liver fat	0.22	-0.29	0.03

R^2 = explained variance; β = beta-estimate of linear regression model
^hs-CRP (P = 0.5), triglycerides (P = 0.4), and systolic blood pressure (P = 0.7) and were not retained in the regression model

Table 4.3 Clinical and metabolic characteristics of obese individuals stratified based by liver insulin sensitivity (endogenous glucose production suppression) in men and women

	$Liver_{sen}$ Men (n = 9)	$Liver_{res}$ Men (n = 18)	P value	$Liver_{sen}$ Women (n = 12)	$Liver_{res}$ Women (n = 23)	P value
Age	45 ± 14	51 ± 9	0.18	53 ± 9	52 ± 13	0.81
Anthropometry and abdominal fat distribution						
BMI (kg/m^2)	33.9 ± 2.5	37.0 ± 4.9	0.08	36.3 ± 4.6	36.8 ± 4.9	0.79
Central abdominal fat (kg) ‡	3.4 ± 0.5	4.0 ± 0.6	**0.03**	3.7 ± 0.1	3.2 ± 0.2	0.5
Mean adipocyte diameter (µm) π	68 ± 8	79 ± 9	**0.01**	70 ± 7	74 ± 10	0.33
Median adipocyte diameter (µm) π	68 ± 9	79 ± 10	**0.01**	70 ± 7	74 ± 11	0.38
Visceral fat Φ	248 (194-275)	301 (271-364)	**0.01**	209 (190-269)	238 (203-308)	0.08
Liver fat (%) Φ	3.8 (2.2-15.1)	20 (11-25)	**0.01**	6.7 (2.9-11.8)	9.9 (6.2-19.2)	**0.03**
Clinical measurements						
SBP (mmHg) **	123 ± 7	132 ± 18	0.14	119 ± 11	122 ± 10	0.48
DBP (mmHg) **	79 ± 10	85 ± 10	0.21	81 ± 13	83 ± 8	0.69
Total cholesterol (mmol/L) ^	5.1 ± 1.0	5.1 ± 0.7	0.91	5.1 ± 0.8	4.9 ± 0.6	0.40
LDL (mmol/L) ^	3.5 ± 0.8	3.4 ± 0.7	0.89	3.1 ± 0.7	2.9 ± 0.6	0.55
HDL (mmol/L) ^	1.0 ± 0.2	1.1 ± 0.2	0.67	1.5 ± 0.3	1.4 ± 0.2	0.13
Triglycerides (mmol/L) ^ Φ	1.1 (0.8-1.4)	1.1 (0.9-1.4)	0.73	0.7 (0.6-1.1)	1.1 (0.8-1.3)	**0.004**
Oral glucose tolerance test, glycaemia and hyperinsulinaemic clamp measures						
OGTT1 h BGL (mmol/L)	6.4 ± 1.7	8.8 ± 1.8	**0.003**	7.1 ± 2.0	7.8 ± 2.0	0.31
HbA1c (%)	5.2 ± 0.2	5.6 ± 0.3	**<0.001**	5.3 ± 0.4	5.5 ± 0.3	**0.04**
Basal insulin (mU/L) Φ	14 (11- 21)	26 (17-41)	**0.01**	11 (8-15)	17 (13-22)	**0.01**
Metabolically flexibility	0.17 ± 0.05	0.11 ± 0.04	**0.01**	0.18 ± 0.05	0.14 ± 0.05	0.70
Circulating cytokines						
Hs-CRP (mg/L) Φ	1.7 (1.1-2.8)	4.3 (2.2-5.6)	**0.03**	3 (1.4-4)	4.2 (1.9-5.9)	0.24
FGF-19 (ng/L) Φ	124 (79-198)	58 (34-99)	**0.03**	106 (67-193)	120 (90-182)	0.73
FGF-21 (ng/L) Φ	45 (15-132)	77 (24-162)	0.44	78 (30-105)	93 (62-162)	0.09
FABP 4 (µg/L)	38 ± 13	50 ± 21	0.11	78 ± 23	76 ± 23	0.82
Lipocalin 2 (µg/L)	43 ± 14	46 ± 13	0.64	44 ± 14	34 ± 13	0.06

	Liver$_{sen}$ Men (n = 9)	**Liver$_{res}$ Men (n = 18)**	**P value**	**Liver$_{sen}$ Women (n = 12)**	**Liver$_{res}$ Women (n = 23)**	**P value**
RBP 4 (mg/L)	12 ± 2	11 ± 2	0.27	11 ± 1	11 ± 3	0.88
Total adiponectin (mg/L)	10 ± 5	9 ± 3	0.33	21 ± 8	18 ± 7	0.41

[*]Data are means ± SD. [Φ] Data are median (interquartile range) for non-normally distributed data

[**]Subjects treated with anti-hypertensive medications excluded from the analysis (included: Liver$_{sen}$ men n = 8 and Liver$_{res}$ men n = 12, Liver$_{sen}$ women n = 11, Liver$_{res}$ women n = 18;).

[^] Subjects treated with lipid medications excluded from the analysis (included: Liver$_{sen}$ men n = 8 and Liver$_{res}$ men n =12, Liver$_{sen}$ women n = 12, Liver$_{res}$ women n = 22).

[‡] DXA, n = 59

[π] 51 subjects were included in the analysis of adipocyte size (included: Liver$_{sen}$ men n = 9, Liver$_{res}$ men n = 14; Liver$_{sen}$ women n = 8 and Liver$_{res}$ women n = 20)

Figure 4.2

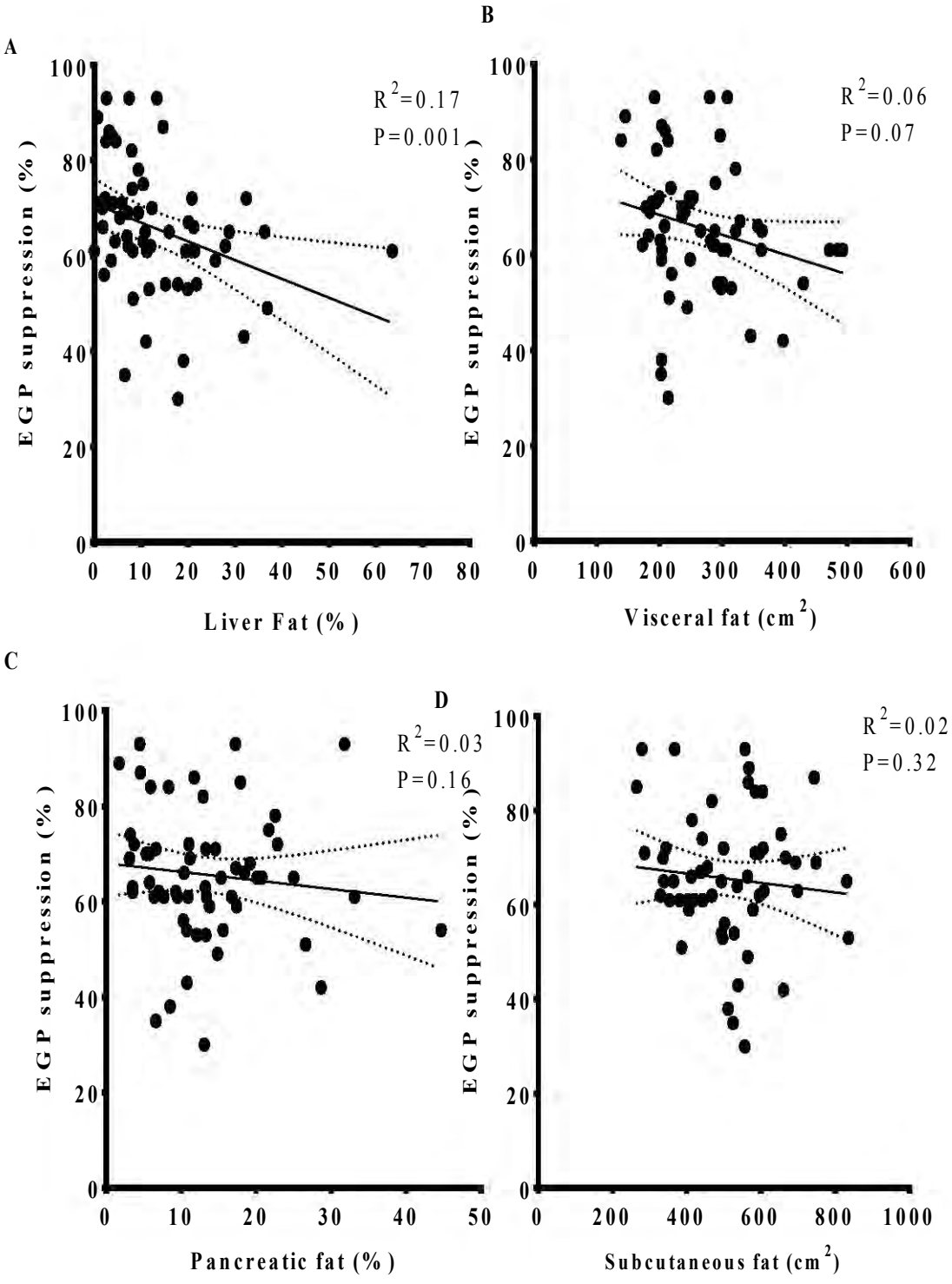


Figure 4.2 Linear regression between liver, visceral, pancreatic and subcutaneous fat with liver insulin sensitivity (EGP suppression during the low dose insulin clamp, 4.2A-D). Depicted are the line of fit and the 95% confidence curves that were obtained from linear regression. MRI-derived measures were logarithmically transformed prior to statistical analysis.

Table 4.3: Correlations between endogenous glucose production (EGP) suppression at low insulin dose with clinical and metabolic variables

Variable	EGP suppression Pearson's coefficient (*P*)
Age	NS
Waist circumference (cm)	NS
Waist/hip ratio	NS
Central abdominal fat (kg)‡	NS
SBP (mmHg) *	NS
DBP (mmHg) *	NS
Total cholesterol (mmol/L)^	NS
LDL cholesterol (mmol/L)^	NS
HDL cholesterol (mmol/L)^	NS
Triglycerides (mmol/L)^	NS
Fasting blood glucose (mmol/L)	NS
OGTT 1h blood glucose (mmol/L)	NS
OGTT 2h blood glucose (mmol/L)	NS
OGTT $AUC_{Glucose}$ (mmol/L·120min)	NS
OGTT $AUC_{Insulin}$ (mU/L·120min)	-0.31 (0.02) ¥
OGTT $AUC_{C\text{-}peptide}$ (µg/L·120min)	-0.44 (0.001) ¥
HbA1c (%)	-0.41 (0.001) ¥
Fasting insulin (mU/L)	-0.43 (0.001) ¥
Fasting NEFA (mmol/L)	NS
Serum $NEFA_{LO}$ (mmol/L)	-0.35 (0.01) ¥
Δ RQ ($RQ_{HI} - RQ_{baseline}$)	NS
Hs-CRP (mg/L)	-0.26 (0.04)
FGF-19 (ng/L)	NS
FGF-21(ng/L)	NS
FABP4 (µg/L)	NS
Lipocalin 2 (µg/L)	NS
RBP 4 (mg/L)	NS
Total adiponectin (mg/L)	NS

*Subjects treated with anti-hypertensive medications excluded from the analysis (included: n = 49).
^Subjects treated with lipid medications excluded from the analysis (included: n = 54).
¥ $P < 0.05$ after adjusting for total body fat, NS, non-significant $P > 0.05$

4.4.6 Multiple linear regression analyses by gender

When we analysed the above model in men and postmenopausal women separately, we found that HbA1c explained 37% and 22% of the EGP suppression variability in men and postmenopausal women (22 out of 35 women studied), respectively (table 4.5).

Table 4.5 Multiple linear regression models to explain the variability in liver insulin sensitivity (EGP suppression)

		Model	Adjusted R^2	Standardized coefficients β	P value
Men (n=29)					
GIR_{HI}	*Clinical variables*^				
		HbA1c	0.37	-0.62	0.001
Postmenopausal women (n=22)					
GIR_{HI}	*Clinical variables*^^	HbA1c	0.22	-0.51	0.02

R^2 = explained variance; β = beta-estimate of linear regression model
^hs-CRP (P = 0.1), liver fat (P = 0.07), triglycerides (P = 0.6), and systolic blood pressure (P = 0.2) and were not retained in the regression model
^^hs-CRP (P = 0.4), liver fat (P = 0.8), triglycerides (P = 0.9) and systolic blood pressure (P = 0.4) and were not retained in the regression mode

4.4.7 Characterisation based on both muscle and liver insulin sensitivity

An additional classification of the cohort was carried out based on both GIR_{HI} ($Muscle_{sen}$ and $Muscle_{res}$) and EGP suppression ($Liver_{sen}$ and $Liver_{res}$). As expected, the most significant differences were noted between the two extreme groups (Table 4.6). Specifically, $Muscle_{sen}Liver_{sen}$ had significantly lower glycaemia (i.e. glucose AUC during OGTT and HbA1c), insulin and C-peptide AUC during OGTT and fasting insulin compared with $Muscle_{res}Liver_{res}$. There were no differences in circulatory cytokines between the four groups. $Muscle_{res}Liver_{res}$ had significantly greater visceral adiposity than all other groups and greater liver fat when compared with $Muscle_{sen}Liver_{sen}$ and $Muscle_{sen}Liver_{res}$ (Figure 4.3 A, B). Subcutaneous fat and pancreatic fat content were not different between the four groups (Figure 4.3 C, D).

Table 4.6 Features of $Muscle_{sen}Liver_{sen}$, $Muscle_{sen}Liver_{res}$, $Muscle_{res}Liver_{sen}$ and $Muscle_{res}Liver_{res}$ obese individuals

Characteristics	$Muscle_{sen}$ $Liver_{sen}$ (n=12)	$Muscle_{sen}$ $Liver_{res}$ (n=8)	$Muscle_{res}$ $Liver_{sen}$ (n=9)	$Muscle_{res}$ $Liver_{res}$ (n=33)	P ANOVA
Age	49 ± 4	54 ± 4	50 ± 4	50 ± 2	0.76
Anthropometry, body composition and abdominal fat distribution					
BMI (kg/m^2)	35.4 ± 3.5	34.1 ± 2.9	35.9 ± 4.7	36.6 ± 4.3	0.52
Waist circumference (cm)	106 ± 12	109 ± 14	107 ± 10	115 ± 14	0.16
Waist/hip ratio	0.90 ± 0.11	0.87 ± 0.07	0.89 ± 0.08	0.94 ± 0.10	0.19
Whole body fat (kg)	46 ± 11	45 ± 11	44 ± 9	47 ± 10	0.90
Whole body fat (%)	45 ± 9	50 ± 8	46 ± 8	46 ± 7	0.60
Fat-free mass (kg)	56 ± 11	46 ± 11	52 ± 12	55 ± 10	0.20
Fat-free mass (%)	55 ± 9	50 ± 8	54 ± 8	54 ± 7	0.60
Central abdominal fat (kg)	3.2 ± 0.7	3.0 ± 0.5	3.3 ± 0.7	3.6 ± 0.7	**0.04**
Mean adipocyte diameter (μm) $^{\pi}$	68 ± 9	75 ± 9	70 ± 5	76 ± 10	0.09
Median adipocyte diameter (μm)$^{\pi}$	68 ± 9	74 ± 10	70 ± 6	76 ± 11	0.13
Clinical measurements					
Systolic BP (mmHg)**	120 ± 8	112 ± 4	122 ± 11	130 ± 14¶	**0.006**

Characteristics	$Muscle_{sen}$ $Liver_{sen}$ (n=12)	$Muscle_{sen}$ $Liver_{res}$ (n=8)	$Muscle_{res}$ $Liver_{sen}$ (n=9)	$Muscle_{res}$ $Liver_{res}$ (n=33)	P ANOVA
Diastolic BP (mmHg)**	78 ± 10	78 ± 7	82 ± 13	85 ± 9	0.206
Total cholesterol (mmol/L)^	5.2 ± 0.8	5.0 ± 0.5	5.0 ± 0.9	4.9 ± 0.7	0.87
LDL cholesterol (mmol/L)^	3.2 ± 0.7	3.1 ± 0.4	3.2 ± 0.9	3.1 ± 0.7	0.93
HDL cholesterol (mmol/L)^	1.4 ± 0.4	1.4 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	0.65
Triglycerides (mmol/L)^Φ	0.8 (0.6-1.2)	1.0 (0.3-1.2)	0.9 (0.7-1.3)	1.1 (0.9-1.4) ± 0.5	0.19
Oral glucose tolerance test, glycaemia and hyperinsulinaemic clamp measures					
Fasting BGL (mmol/L)	4.5 ± 0.5	4.9 ± 0.2	4.8 ± 0.4	4.8 ± 0.5	0.19
OGTT 1h BGL (mmol/L)	6.3 ± 1.5	7.1 ± 1.3	7.4 ± 2.2	8.5 ± 2.0‡	**0.008**
OGTT 2h BGL (mmol/L)	5.5 ± 1.7	6.2 ± 1.4	6.3 ± 1.3	6.8 ± 1.7	0.14
OGTT $AUC_{Glucose}$ (mmol/L·120min)	728 ± 124	806 ± 117	815 ± 187	905 ± 174‡	**0.02**
OGTT $AUC_{Insulin}$ (mU/L·120min)Φ	6827 (5352-8660	7888 (7341-13916)	10076 (6905-11499)	13504 (8466-117188)‡	**0.007**
OGTT $AUC_{C\text{-}peptide}$ (µg/L·120min)Φ	422 (378-557)	533 (460-768)	546 (363-1027)	785 (598-1032)‡	**<0.001**
HbA1c (%)	5.1 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	5.6 ± 0.1‡	**<0.001**
Fasting insulin (mU/L) Φ	11 (8-13)	16 (13-23)	15 (11-20)	19 (13-31) ‡	**<0.001**
$Insulin_{LO}$ (mU/L) Φ	37 (32-47)	41 (38-47)	42 (34-45)	41 (36-53)	0.41

Characteristics	$Muscle_{sen}$ $Liver_{sen}$ (n=12)	$Muscle_{sen}$ $Liver_{res}$ (n=8)	$Muscle_{res}$ $Liver_{sen}$ (n=9)	$Muscle_{res}$ $Liver_{res}$ (n=33)	P ANOVA
$Insulin_{HI}$ (mU/L) $^{\Phi}$	216 (176-231)	223 (150-266)	173 (156-202)	217 (180-254)	0.20
Fasting NEFA (mmol/L) $^{\Phi}$	0.32 (0.26-0.45)	0.38 (0.32-0.46)	0.37 (0.25-0.46)	0.36 (0.27-0.46)	0.87
$NEFA_{LO}$ (mmol/L) $^{\Phi}$	0.03 (0.02-0.05)	0.05 (0.04-0.06)	0.04 (0.02-0.05)	0.05 (0.04-0.06) ‡	**0.04**
$NEFA_{HIGH}$ (mmol/L) $^{\Phi}$	0.02 (0.01-0.03)	0.02 (0.02-0.04)	0.02(0.01-0.02)	0.02 (0.02-0.03)	0.17
Basal RQ	0.78 ± 0.02	0.80 ± 0.03	0.79 ± 0.01	0.80 ± 0.04	0.36
Δ RQ ($RQ_{HI} - Ba_{seline}$)	0.19 ± 0.04	0.17 ± 0.04	0.15 ± 0.04	0.14 ± 0.06‡	**0.03**
Circulating cytokines					
Hs-CRP (mg/L) $^{\Phi}$	2.0 (1.2-3.3)	4,3 (2.6-5.7)	3.1 (1.1-4.7)	4.2 (1.9-5.7)	0.08
FGF-19 (ng/L) $^{\Phi}$	130 (76-228	109 (24- 338)	90 (66-147)	94 (56-147)	0.31
FGF-21(ng/L) $^{\Phi}$	80 (29-110)	80 (15-159)	45 (17-94)	91 (51-159)	0.14
FABP 4 (µg/L)	56 ± 26	72 ± 24	68 ± 31	63 ± 26	0.57
Lipocalin 2 (µg/L)	40 ± 10	40 ± 21	49 ± 16	39 ± 12	0.32
RBP 4 (mg/L)	12 ± 2	10 ± 2	11 ± 2	11± 3	0.44
Total adiponectin (mg/L)	17 ± 10	19 ± 8	16 ± 7	12 ± 7	0.17

Data are means ±SD or Φ median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Muscle_{sen}Liver_{sen}$ n=10, $Muscle_{sen}Liver_{res}$ n=6, $Muscle_{res}Liver_{sen}$ n=9 and $Muscle_{res}Liver_{res}$ n=24)

$^{\wedge}$Subjects treated with lipid medications excluded from the analysis (included: $Muscle_{sen}Liver_{sen}$ n=11, $Muscle_{sen}Liver_{res}$ n=6, $Muscle_{res}Liver_{sen}$ n=9 and $Muscle_{res}Liver_{res}$ n=28)

[π] 51 subjects were included in the analysis of adipocyte size (included: $Muscle_{sen}Liver_{sen}$ n=11 , $Muscle_{sen}Liver_{res}$ n=5, $Muscle_{res}Liver_{sen}$ n=6 and $Muscle_{res}Liver_{res}$ n=29) Significance was tested by one-way ANOVA with Tukey posthoc. [‡]P<0.05 between $Muscle_{res}Liver_{res}$ and $Muscle_{sen}Liver_{sen}$, [¶]P<0.01 between $Muscle_{res}Liver_{res}$ and $Muscle_{sen}Liver_{res}$

Figure 4.3

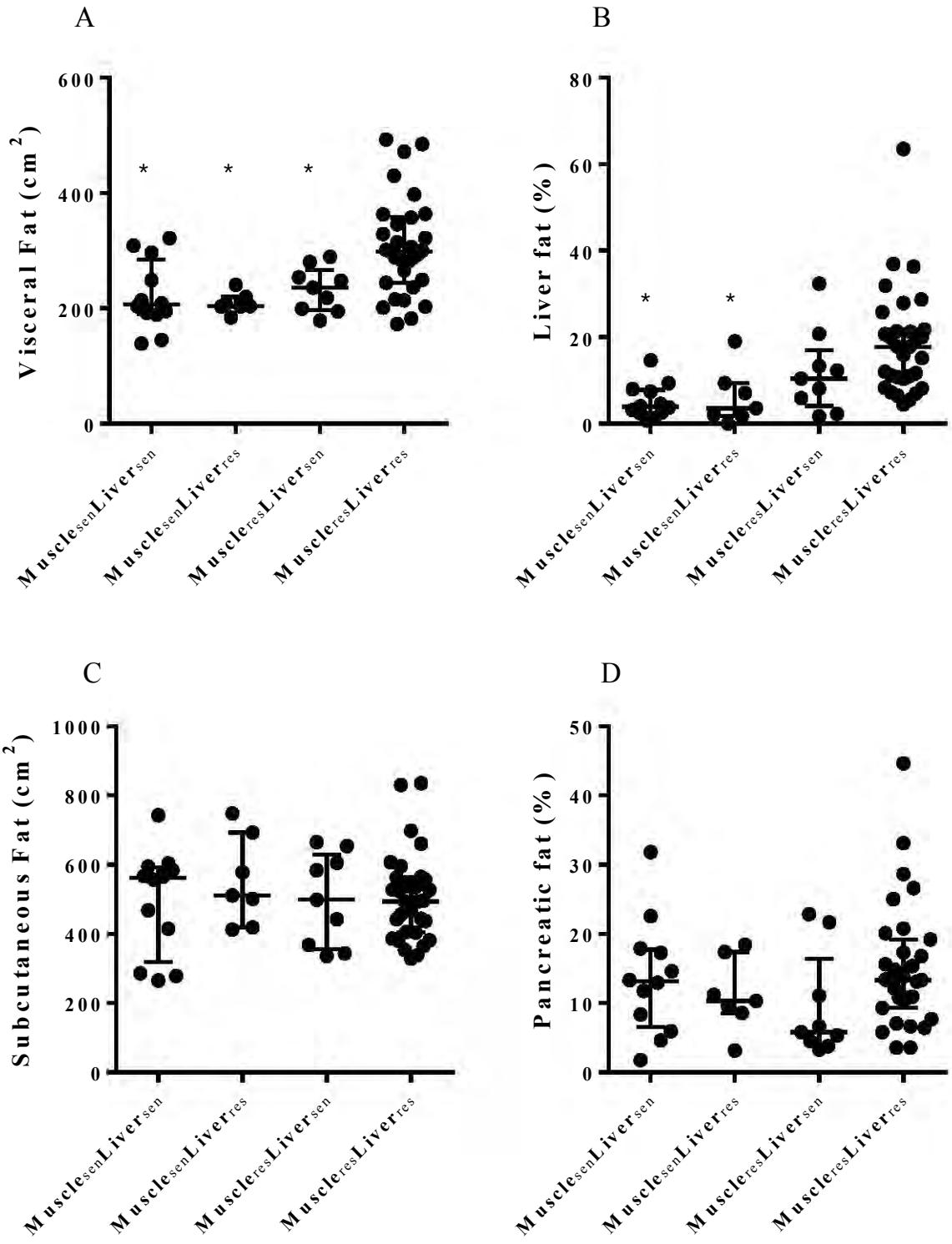


Figure 4.3: Abdominal visceral (A), liver (B), subcutaneous (C) and pancreatic (D) fat in obese individuals stratified based on muscle and liver insulin sensitivity. Differences by one-way ANOVA with Tukey post-hoc from $Muscle_{res}Liver_{res}$ as the reference group are depicted, $^{*}P < 0.05$. MRI-derived measures were logarithmically transformed prior to statistical analysis.

4.5 DISCUSSION

The main findings in this Chapter that:

1. $Liver_{sen}$ subjects had:
    a. Lower visceral and liver fat than $Liver_{res}$
    b. Lower waist circumference, OGTT 1h-blood glucose, OGTT-derived $AUC_{insulin}$/$AUCc_{\text{-peptide}}$/$AUC_{glucose}$, HbA1c and fasting insulin concentrations compared with $Liver_{res}$
    c. Lower hs-CRP (P = 0.02), with a significant inverse correlation with liver insulin sensitivity (EGP suppression at low insulin dose)
2. There were gender-specific differences such that in:

    Men:

    a. $Liver_{sen}$ men had lower mean and median adipocyte diameter compared with $Liver_{res}$ men,
    b. $Liver_{sen}$ men had lower hs-CRP and higher FGF-19

    Women:

    a. $Liver_{sen}$ women had lower fasting plasma concentrations of triglycerides than $Liver_{res}$ women
3. HbA1c and liver fat explained 22% of variability in liver insulin sensitivity (EGP suppression) in total cohort.
    a. HbA1c is the major determinant of liver insulin sensitivity in men (explained 37% of variability in EGP suppression) and postmenopausal women (22% of variability in EGP suppression)
4. $Muscle_{res}Liver_{res}$ had significantly greater visceral adiposity than all other groups and greater liver fat when compared with $Muscle_{sen}Liver_{sen}$ and $Muscle_{sen}Liver_{res}$

The $Liver_{sen}$ group had lower glycaemia and waist circumference compared with $Liver_{res}$ group. Interestingly, there was no difference in blood pressure between $Liver_{sen}$ and $Liver_{res}$ group. This is in contrast with the previous chapter where $Muscle_{sen}$ group had lower systolic and diastolic blood pressure. Correlation analyses (Table 3.3 and 4.3) also suggested a significant correlation between systolic blood pressure and muscle insulin resistance, but not liver insulin resistance. This has been supported by a previous study that divided a group of obese adolescents into high and low liver fat content groups and found no difference in blood pressure between two groups despite significant differences in liver insulin sensitivity (as defined by EGP suppression during low insulin dose).[313] Furthermore, in healthy non-obese hypertensive individuals, insulin resistance was demonstrated in skeletal muscle, but not in liver[314] assessed by hyperinsulinaemic-euglycaemic study. This suggests that hypertension is associated with insulin resistance in the muscle, but not liver.

CRP is a pro-inflammatory hepatokine which is elevated in obese insulin-resistant humans; and low concentrations have been reported in metabolically-healthy obese individuals, defined by either absence of metabolic syndrome features[80,315] or hyperinsulinaemic-euglycaemic clamps.[36,81] CRP has been shown to correlate more strongly with liver insulin resistance than peripheral insulin resistance[110] and may therefore be considered as a hepatic insulin resistance surrogate. $Liver_{sen}$ men had higher FGF-19 than $Liver_{res}$ men. FGF-19 is an endocrine hormone which in response to nutritional status inhibits glucose synthesis and stimulates glycogen and protein synthesis.[174] FGF-19 has the ability to lower liver fat content, triglycerides and plasma

glucose levels and improve insulin sensitivity.[176] FGF-19 has been shown to be lower in insulin-resistant obese adolescents,[316] supporting our finding. It is unknown why the observation was not evident in women; further larger studies are needed to delineate the role of FGF-19 and insulin resistance in different genders.

Consistent with muscle insulin sensitivity, $Liver_{sen}$ had lower liver and visceral fat. Liver insulin resistance correlated strongly with liver fat, but not visceral fat. It is known that hepatic steatosis (liver fat) inversely predicts liver insulin sensitivity,[305] and that liver fat is also a marker of liver insulin resistance.[317] It is not surprising to see a strong association between liver insulin resistance with liver fat in our cohort, supported by other similar studies using two-step hyperinsulinaemic-euglycaemic clamps.[313,318] Future prospective studies are needed to examine potential causal relationship between liver insulin resistance and liver fat.

HbA1c was one of the main clinical factors that explained some of the variability in EGP suppression. HbA1c is a glycosylated haemoglobin, and is a reflection of overall glycaemia over the life span of the red cell (around 3 months).[319] HbA1c at levels below the diabetic range seems to be a predictor of both liver and muscle insulin sensitivity in men from our findings. This may suggest a potential utility of HbA1c in screening non-diabetic obese men to identify insulin resistant individuals.

$Liver_{sen}$ men had lower adipocyte diameter compared with $Liver_{res}$ men. Adipocyte size correlated inversely with liver insulin sensitivity (expressed as EGP suppression and HIRI) in men. The role of liver insulin sensitivity and adipocyte physiology has not

been examined previously. Several cross-sectional hyperinsulinaemic-euglycaemic studies have shown that larger adipocyte size/volume was associated with peripheral insulin resistance,[308,309] but there have been no studies examining the association between liver insulin sensitivity and adipocyte size. It is interesting to note that a significant association was only observed in obese men, but not women. This is similar to a previous study where a significant association existed between fat cell size and insulin sensitivity in middle aged men, but not women.[320] The absence of a significant association in women may be due to small sample size compounded by menopausal status (about 1/3 women were premenopausal). This observation raises the "adipocyte overflow hypothesis"[106] where perhaps, larger adipocyte size in obese men leads to preferential ectopic fat deposition in liver causing liver insulin resistance. Further studies are needed to clarify the potential association between liver insulin sensitivity and adipocyte size in different genders.

Muscle and liver are major insulin target tissues and key players in glucose homeostasis. Visceral fat deposition is associated with metabolic disease and a strong correlation between visceral fat and peripheral insulin resistance is maintained even when BMI is >30 kg/m^2.[321] Strikingly, when our cohort was bi-dimensionally stratified based on both muscle and liver insulin sensitivity, individuals who were insulin-resistant at either muscle or liver were not different in abdominal visceral fat from those who were insulin-sensitive in both tissues. Those who were insulin-resistant in *both* muscle and liver had significantly greater visceral adiposity than the group sensitive at both sites.

Liver lipid accumulation is common in obesity and is associated not only with hepatic insulin resistance, but also with muscle insulin resistance.[25,122] Our study showed that in obese individuals, liver fat was lower in obesity, irrespective of liver insulin resistance, if muscle remained insulin-sensitive, suggesting that liver fat aligns more closely with muscle than liver insulin sensitivity. This is consistent with other studies in which obese cohorts matched for visceral adiposity showed liver fat predicted not only hepatic, but also skeletal muscle insulin resistance.[25,305] In support, liver fat correlated with both GIR_{HI} and EGP suppression in our study and predicted both muscle and liver insulin sensitivity.

4.6 CONCLUSION

$Liver_{sen}$ group had lower liver and visceral fat, glycaemia and waist circumference. $Liver_{sen}$ men had lower CRP, adipocyte size and higher FGF-19. Liver insulin resistance has been shown to correlate with cardiovascular disease risk factors in men.[110] Our findings may highlight the pivotal importance of liver insulin resistance and its contribution to obesity-related metabolic complications via inflammation, ectopic fat deposition and glycaemia especially in men. Longitudinal studies will be helpful in determining potential causation between liver insulin resistance and liver fat/metabolic diseases in different genders.

Our four group analysis demonstrated that individuals with dual-site insulin resistance carry the worse metabolic profile and are characterised with the highest amount of visceral fat. Liver fat is associated with both muscle and liver insulin resistance. Visceral fat could be used as an indicator to identify obese individuals with dual-site insulin resistance, while HbA1c can be used to predict liver insulin resistance. These factors may assist clinicians to identify obese individuals at high risk of metabolic disease so that appropriate monitoring and intervention can be implemented to minimise obesity-related metabolic complications and health costs.

CHAPTER 5
COMPARISON OF MUSCLE SYMPATHETIC NERVOUS ACTIVITY IN INSULIN-SENSITIVE AND INSULIN-RESISTANT SUBJECTS

5.1 INTRODUCTION

The sympathetic nervous system plays an important role in body metabolism, affecting resting metabolic rate, energy expenditure, and glucose and lipid metabolism.[322,323] Resting sympathetic nervous activity, as reflected by MSNA, has been shown to be increased in obese individuals[245,324] and MSNA decreases with weight loss.[325]

Obesity is associated with insulin resistance and multiple metabolic complications possibly contributed to by increased sympathetic nervous activity. Hyperinsulinaemia is observed commonly in obesity, is closely linked to peripheral and hepatic insulin resistance and has been shown to stimulate SNS activity in man.[238,243] Higher resting MSNA and a blunted response to oral glucose is a characteristic of obese insulin-resistant individuals when defined by oral glucose tolerance test indices.[89] However, the associations between SNS activity and peripheral or hepatic insulin resistance remain unclear.

MSNA measured by microneurography is a reliable and reproducible assessment of sympathetic nervous activity.[326] Despite MSNA only accounting for about 20% of whole body release of norepinephrine to plasma, it has been shown to closely associate with cerebral, cardiac and renal noradrenaline spillover.[327-330]

5.2 AIMS

1. To determine whether resting MSNA correlates with muscle and/or liver insulin sensitivity in an obese cohort

2. To assess whether MSNA is higher in liver and/or muscle insulin-resistant subjects compared to their insulin-sensitive counterparts.

3. To elucidate potential metabolic/biochemical factors that correlate with MSNA in non-diabetic obese individuals

5.3 METHODS

5.3.1 Subjects

As described in detail in Chapter 2, 64 subjects underwent metabolic assessment. MSNA measurement was performed at Neuroscience Research Australia, Randwick and MRI was performed at St Vincent's Hospital, Sydney. Only 45 subjects were willing to undergo studies and had successful MSNA recordings and are included in our analysis.

5.3.2 Experimental protocol

The study protocols on hyperinsulinaemic-euglycaemic clamp, MRI/body composition are summarised in detail in Chapter 2, and Figure 2.2. Definitions of liver insulin-sensitive and liver insulin-resistant and muscle insulin-sensitive and muscle insulin-resistant groups are outlined in Chapter 2.3. The classification was re-categorised based on our sub-cohort of 45 subjects in men and women.

Laboratory analyses of various parameters are detailed in Chapter 2.4. Statistical analysis was carried out using SPSS version 21 (Chicago, IL, USA), detailed in Chapter 2.5. Additional statistical analyses included the followings:

Correlations were performed using Pearson's correlation coefficient adjusted for total body fat. Chi-square test was used to detect differences in timing of MSNA measurement in $Liver_{sen}$ and $Liver_{res}$ groups while Fisher's exact test was used in men and women (expected number <5). P value < 0.05 was considered statistically significant.

5.3.3 MSNA measurement

MSNA measurement protocol is summarised in Chapter 2.4. In brief, subjects voided before the commencement of MSNA measurement. MSNA was performed in resting state with two thirds of subjects studied in the afternoon due to attendance at the Garvan in the morning. There were no significant differences in MSNA burst incidence/frequency between subjects who had MSNA recorded in the morning and afternoon in the total cohort (MSNA burst frequency 32 ± 12 vs. 37 ± 13 burst per minutes; P = 0.25, MSNA burst incidence 54 ± 18 vs. 58 ± 18 burst per 100 HB; P = 0.46) or when men (MSNA burst frequency 33 ± 12 vs. 35 ± 8 burst per minutes; P = 0.70, MSNA burst incidence 54 ± 18 vs. 60 ± 13 burst per 100 HB; P = 0.47) and women (MSNA burst frequency 28 ± 6 vs. 37 ± 14 burst per minutes; P = 0.37, MSNA burst incidence 52 ± 17 vs. 57 ± 20 burst per 100 HB; P = 0.72) were analyzed separately (independent T test- all P > 0.25)We performed Chi square/Fisher's exact test to evaluate whether the proportion of subjects who underwent MSNA measurement (in the morning *vs.* afternoon) differed between $Liver_{sen}$ and $Liver_{res}$. There were no differences in the total cohort (P = 0.88), men (P = 0.59) and women (P = 0.65).

Spontaneous resting MSNA was recorded by inserting tungsten microelectrodes (Frederick Haer and co, Bowdoinham, ME, USA) percutaneously at the level of the fibular head into the left common peroneal nerve muscle fascicles. A large uninsulated tip was inserted nearby as the reference electrode subdermally (within 2-3cm). The position of the electrode was adjusted manually until spontaneous cardiac-locked MSNA was encountered. Once an acceptable nerve-recording site was obtained with both visual and acoustic identification of spontaneous sympathetic bursts, resting measurements were recorded.

MSNA burst amplitudes were measured from the RMS-processed signal (200ms moving average) using the Peak Parameters feature of LabChart7 (AD Instruments, Sydney, Australia). The entire process has been described previously.[291,292] MSNA was manually analyzed and expressed as burst frequency (bursts per minute) and burst incidence (bursts per 100 heartbeats), averaged over 15 minutes. Both measurements quantify MSNA bursts, but burst incidence accounts for differences in heart rate when comparing between individuals. Forty-five subjects (22 male) had successful MSNA recording; none of them were on β-blockers.

5.4 RESULTS

5.4.1 Baseline characteristics

Baseline characteristics of men, women and total cohort are included in table 5.1. Both men and women had similar age, BMI and HbA1c. Systolic (P = 0.26) and diastolic blood pressure (P = 0.56) were similar in men and women. MSNA burst frequency, incidence and heart rate were similar between men and women (P > 0.13). As expected, women had higher total body fat (P=0.004), but lower fat free mass (P < 0.001) and central abdominal fat (P = 0.03) than men (Table 5.1).

MSNA-derived measures were all similar between men and women (P > 0.13, Table 5.1). Total adiponectin and FABP4 levels were significantly lower in men compared with women (Table 5.1).

Table 5.1 Baseline clinical characteristics of men, women and total cohort

Characteristics	Men (22)	Women (23)	P value	Total cohort (45)
Age	48 ± 12	54 ± 10	0.07	51 ± 11
Adiposity				
BMI (kg/m^2)	34.7 ± 3.3	36.9 ± 5.3	0.11	35.8 ± 4.6
Waist circumference (cm)	114 ± 9	108 ± 13	0.13	111 ± 12
Whole body fat (kg)	40 ± 7	49 ± 12	**0.004**	45 ± 11
Fat-free mass (kg)	64 ± 6	46 ± 8	**<0.001**	55 ± 12
Central abdominal fat (kg)	3.7 ± 0.6	3.2 ± 0.6	**0.026**	3.4 ± 0.7
Blood pressure				
SBP (mmHg)**	127 ± 14	122 ± 11	0.26	125 ± 13
DBP (mmHg)**	83 ± 11	81 ± 8	0.56	82 ± 9
MSNA-derived measures				
Burst incidence (bursts/100 beats)	56 ± 17	57 ± 19	0.86	56 ± 18
Burst frequency (bursts/minute)	34 ± 11	37 ± 14	0.41	35 ± 13
Heart rate (BPM)	66 ± 7	70 ± 11	0.09	68 ± 9
Lipid profile				
Total cholesterol (mmol/L)^	5.0 ± 0.9	4.9 ± 0.7	0.84	4.9 ± 0.8
LDL cholesterol (mmol/L)^	3.3 ± 0.8	3.0 ± 0.6	0.12	3.1 ± 0.7
HDL cholesterol (mmol/L)^	1.1 ± 0.2	1.4 ± 0.3	**<0.001**	1.3 ± 0.3
Triglycerides (mmol/L)^ Φ	1.1 (0.8-1.5)	0.9 (0.7-1.2)	0.32	1.0 (0.7-1.3)
Fasting NEFA (mmol/L) Φ	0.27(0.25-0.32)	0.44 (0.37-0.51)	**<0.001**	0.36 (0.27-0.45)
Glycaemia				
HbA1c (%)	5.5 ± 0.3	5.5 ± 0.3	0.95	5.5 ± 0.3
Fasting insulin (mU/L) Φ	18 (12-26)	13 (11-18)	**0.02**	15 (11-23)
Circulatory cytokines				
Hs-CRP (mg/L) Φ	2.2 (1.4-4.9)	3.1 (1.9-5.7)	0.22	2.9 (1.7-5.0)
FGF-19 (ng/L) Φ	97 (56-152)	118 (66-166)	0.06	98 (64-152)
FGF-21(ng/L) Φ	49 (16-159)	93 (62-161)	0.07	84 (32-159)
FABP 4 (µg/L)	42 ± 17	78 ± 21	**<0.001**	61 ± 26
Lipocalin 2 (µg/L)	44 ± 14	37 ± 11	0.09	40 ± 13
RBP 4 (mg/L)	12 ± 2	12 ± 3	0.96	12 ± 2
Total adiponectin (mg/L)	10 ± 4	18 ± 7	**<0.001**	14 ± 7

Data are means ± SEM. $^{\Phi}$ Data are median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: Men=18 and women n=18).

$^{\wedge}$ Subjects treated with lipid medications excluded from the analysis (included: Men n=17 and women n=22).

5.4.2 Simple linear regression analyses

5.4.2.1 Linear regression analyses in total cohort

MSNA burst incidence was positively associated with age (r = 0.48; P = 0.001), SBP (r = 0.32, P = 0.03) and visceral fat (r = 0.30, P = 0.05). However, DBP (P = 0.32), subcutaneous fat (P = 0.58), liver fat (P = 0.84), triglycerides (P = 0.27) and HbA1c (P = 0.36) were not related. MSNA burst incidence was not associated with muscle (P = 0.74) or liver (P = 0.86) insulin sensitivity. The findings remained unchanged when adjusted for total body fat.

MSNA burst incidence was not associated with adipokines/hepatokines including FGF-19 (P = 0.44), FGF-21 (P = 0.83), hs-CRP (P = 0.21), total adiponectin (P = 0.13), Lipocalin 2 (P = 0.77), FABP4 (P = 0.36) and RBP4 (P = 0.62). MSNA burst frequency had no significant correlations with any variables.

5.4.2.2 Linear regression analyses in men

When male subjects were assessed independently, liver insulin sensitivity (expressed as EGP suppression) was inversely correlated with MSNA burst frequency (r = -0.53, P = 0.02) and remained significant after adjusting for age (r = -0.54, P = 0.02). There was a trend to an inverse correlation with MSNA burst incidence (r = -0.36, P = 0.12). Muscle insulin sensitivity was not related to MSNA burst frequency (P = 0.09) or burst incidence (P = 0.40).

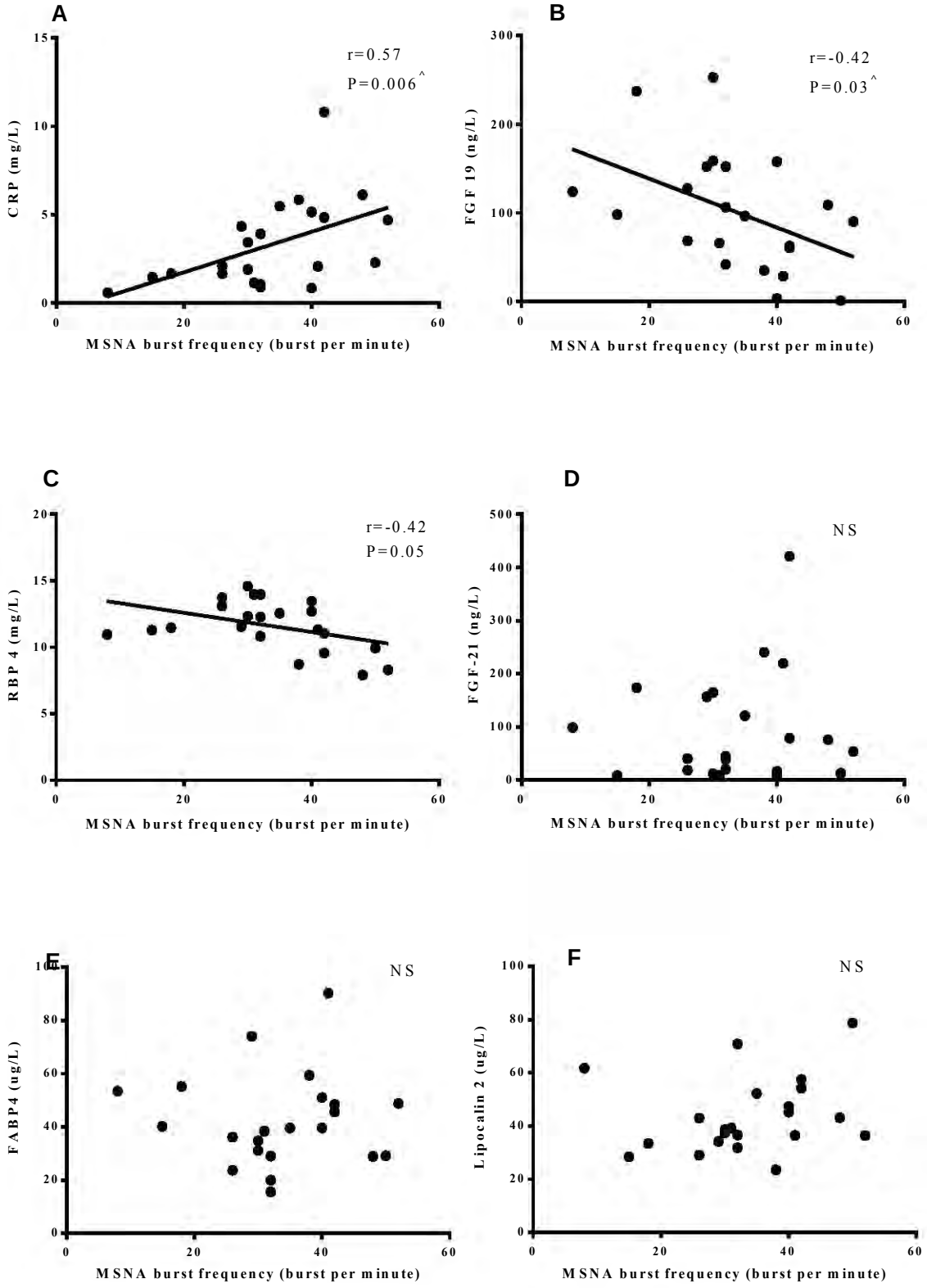
Age correlated positively with MSNA burst incidence (r = 0.61, P = 0.002), with a trend to positive correlation with MSNA burst frequency (r = 0.37, P = 0.09). However, SBP, DBP, subcutaneous and liver fat, triglycerides and HbA1c were not related to MSNA burst incidence and frequency (P > 0.09).

Highly sensitive-CRP correlated positively with MSNA burst frequency and incidence (Figure 4.1A and r = 0.45, P = 0.04 respectively). FGF-19 correlated negatively with MSNA frequency (Figure 4.1B) and had a trend to inverse correlation with MSNA incidence (r = -0.37, P = 0.09). Other significant correlations with MSNA burst frequency include basal insulin (r = 0.42, p = 0.05) and RBP4 (Figure 4.1 C). Factors unrelated to MSNA burst frequency include FGF-21 (Figure 4.1D), FABP4 (Figure 4.1E), Lipocalin 2 (Figure 4.1F) and total adiponectin (P > 0.16). The association between MSNA burst frequency and hs-CRP, FGF-19 and RBP4 remained significant after adjusting for age. MSNA burst incidence had no significant correlations with other inflammatory cytokines besides hs-CRP.

5.4.2.3 Linear regression analyses in women

There were no significant correlations in women. Clinical variables (P > 0.08) including age, SBP, triglycerides and HbA1c, muscle (P > 0.97) and liver (P > 0.41) insulin sensitivity were not related to MSNA burst incidence or frequency. There was a positive correlation between DBP and MSNA burst incidence (r = 0.47, P = 0.03). FABP4 had a weak inverse correlation with MSNA burst frequency (r = -0.43, P = 0.04), all other hepatokines/adipokines were unrelated to MSNA.

Figure 5.1: Correlations between MSNA burst frequency and circulating hepatokines in men.



^ P<0.05 after adjusting for total body fat

5.4.3 Multiple linear regression in men

In men, 60% of the variability in MSNA burst frequency was contributed to by CRP and FGF-19 (Table 5.2). Variables excluded in the model were EGP suppression, basal insulin and RBP4. Age explained 35% of variability in MSNA burst incidence in men.

Table 5.2 Multiple linear regression models to explain the variability in MSNA burst frequency and incidence in men

	Model	Adjusted R^2	Standardized coefficients β	P value
MSNA burst frequency*	Hs-CRP	0.28	0.57	0.01
	Hs-CRP		0.65	
	FGF-19	0.59	-0.57	<0.001
MSNA burst incidence^	Age	0.35	0.61	0.02

R^2 = explained variance; β = beta-estimate of linear regression model
* EGP suppression (P = 0.84), RBP4 (P = 0.34), and basal insulin (P = 94) were not retained in the regression model.
^ hs-CRP (P=0.1) was not retained in the regression model.

As there was no more than 1 clinical factors that correlated with MSNA burst frequency or incidence in women, multiple linear regression model was not performed in women.

5.4.4 Analysis stratified by liver insulin sensitivity

The total MSNA-assessed cohort was stratified based on EGP suppression level to $Liver_{sen}$ and $Liver_{res}$ (top tertile vs. lower 2 tertiles, 72% EGP suppression in men and 70% in women respectively). $Liver_{sen}$ subjects had lower HbA1c and fasting insulin than $Liver_{res}$ subjects, but similar age, BMI and waist circumference (Table 5.3).

Heart rate was similar between $Liver_{sen}$ and $Liver_{res}$ subjects (P = 0.1, Table 5.3), with a trend of lower MSNA burst frequency in $Liver_{sen}$ subjects (P = 0.09).

$Liver_{sen}$ men had lower HbA1c, fasting insulin and CRP than $Liver_{res}$ men (Table 5.4). $Liver_{sen}$ men had lower HDL than $Liver_{res}$ men. MSNA burst frequency was significantly lower in $Liver_{sen}$ men compared with $Liver_{res}$ men (Figure 5.2A). There was a trend towards lower MSNA incidence (P = 0.06) in $Liver_{sen}$ men (Figure 5.2A), but no difference in either MSNA-derived variables in women (Figure 5.2B, P > 0.6).

5.4.4 Analysis stratified by muscle insulin sensitivity

The total cohort was stratified according GIR_{HI}, which is roughly equivalent to muscle insulin sensitivity. Resting MSNA tended to be lower in $Muscle_{sen}$ group (Table 5.3). $Muscle_{sen}$ group had lower HbA1c, fasting insulin and systolic and diastolic blood pressure than $Muscle_{res}$ group (Table 5.3) despite similar age and BMI.

The details of $Muscle_{sen}$ and $Muscle_{res}$ men groups are shown in Table 5.4. $Muscle_{sen}$ men also had lower heart rate at the time of microneurography compared with $Muscle_{res}$ men (Table 5.4). The groups were matched for age, BMI, total body fat, waist circumference and FFM. $Muscle_{sen}$ men had lower MSNA frequency than $Muscle_{res}$ men and a trend to lower MSNA incidence (P = 0.1). $Muscle_{sen}$ men had lower HbA1c, fasting insulin, hs-CRP and FGF-19 than $Muscle_{res}$ men (Table 5.4). There was no difference in MSNA-derived variables in women (P > 0.6)

Table 5.3 – Clinical and metabolic characteristics of obese individuals stratified by muscle (GIR_{HI}) and liver insulin sensitivity (EGP suppression) in total cohort

Characteristics	$Liver_{sen}$ (15)	$Liver_{res}$ (28)	P value	$Muscle_{sen}$ (15)	$Muscle_{res}$ (30)	P value
Age	49 ± 12	53 ± 10	0.21	50 ± 14	51 ± 10	0.75
Adiposity measures						
BMI (kg/m^2)	35.7 ± 4.3	35.8 ± 4.5	0.94	34.6 ± 3.3	36.5 ± 5.0	0.20
Waist circumference (cm)	109 ± 11	112 ± 12	0.43	108 ± 12	112 ± 12	0.20
Whole body fat (kg)	45 ± 10	45 ± 11	0.89	44 ± 10	46 ± 11	0.56
Fat-free mass (kg)	55 ± 12	53 ± 12	0.62	53 ± 13	55 ± 12	0.56
Central abdominal fat (kg)	3.3 ± 0.6	3.5 ± 0.7	0.52	3.2 ± 0.6	3.6 ± 0.7	0.06
Blood pressure						
SBP (mmHg)**	121 ± 8	127 ± 15	0.13	118 ± 9	128 ± 13	**0.02**
DBP (mmHg)**	81 ± 9	82 ± 10	0.83	77 ± 8	84 ± 9	**0.02**
MSNA derived measures						
MSNA burst incidence (burst per 100 beats)	52 ± 21	59 ± 16	0.21	51 ± 16	59 ± 19	0.18
MSNA burst frequency (burst per minute)	31 ± 13	38 ± 12	0.09	31 ± 11	37 ± 13	0.08
MSNA Heart rate (BPM)	65 ± 9	70 ± 9	0.1	65 ± 8	70 ± 10	0.13

Characteristics	$Liver_{sen}$ (15)	$Liver_{res}$ (28)	P value	$Muscle_{sen}$ (15)	$Muscle_{res}$ (30)	P value
Lipid profile						
Total cholesterol (mmol/L)^	4.9 ± 0.7	5.0 ± 0.8	0.71	4.9 ± 0.7	5.0 ± 0.9	0.80
LDL cholesterol (mmol/L)^	3.1 ± 0.6	3.2 ± 0.7	0.70	3.1 ± 0.5	3.2 ± 0.8	0.72
HDL cholesterol (mmol/L)^	1.3 ± 0.2	1.3 ± 0.4	0.73	1.3 ± 0.3	1.3 ± 0.3	0.81
Triglycerides (mmol/L)^ Φ	0.9 (0.6-1.2)	1.1 (0.9-1.4)	0.13	0.8 (0.6-1.2)	1.1 (0.9-1.4)	0.21
Fasting NEFA (mmol/L) Φ	0.37 (0.25-0.45)	0.36 (0.27-0.45)	0.96	0.36 (0.26-0.44)	0.36 (0.27-0.46)	0.60
Glycaemia						
HbA1c (%)	5.3 ± 0.3	5.6 ± 0.3	**0.01**	5.2 ± 0.2	5.6 ± 0.3	**<0.001**
Fasting serum insulin (mU/L) Φ	11 (10-15)	18 (13-28)	**0.002**	11 (8-14)	18 (14-27)	**<0.001**
Circulatory cytokines						
Hs-CRP (mg/L) Φ	2.5 (1.7-3.9)	4.1 (2.0-5.8)	0.13	2.1 (1.3-4.1)	3.1 (1.9-5.5)	0.13
FGF-19 (ng/L) Φ	106 (70-237)	97 (53-152)	0.17	128 (21-237)	95 (60-130)	0.06
FGF-21(ng/L) Φ	84 (41-106)	92 (47-173)	0.72	72 (21-106)	87 (44-163)	0.34
FABP 4 (µg/L)	62 ± 30	62 ± 24	0.96	57 ± 26	63 ± 27	0.53
Lipocalin 2 (µg/L)	43 ± 12	40 ± 13	0.47	37 ± 10	42 ± 14	0.22
RBP 4 (mg/L)	12 ± 2	12 ± 3	0.86	12 ± 2	11 ± 3	0.52
Total adiponectin (mg/L)	15 ± 7	13 ± 8	0.54	16 ± 8	13 ± 7	0.28

Data are means ± SD. $^{\Phi}$ Data are median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Muscle_{sen}$ n = 12 and $Muscle_{res}$ n = 24; $Liver_{sen}$ n = 13; $Liver_{res}$ n = 21).

$^{\wedge}$ Subjects treated with lipid medications excluded from the analysis (included: $Muscle_{sen}$ n = 14 and $Muscle_{res}$ n = 25; $Liver_{sen}$ n = 14; $Liver_{res}$ n = 24).

Table 5.4 Clinical characteristics of obese men stratified by liver insulin sensitivity (endogenous glucose production suppression) and muscle insulin sensitivity

Characteristics	$Liver_{sen}$ men (7)	$Liver_{res}$ men (13)	P value	$Muscle_{sen}$ men (7)	$Muscle_{res}$ men (15)	P value
Age	43 ± 15	51 ± 9	0.14	43 ± 16	50 ± 8	0.33
Adiposity measures						
BMI (kg/m^2)	34.6 ± 2.3	34.3 ± 2.5	0.79	33.1 ± 2.5	35.5 ± 3.5	0.12
Waist circumference (cm)	114 ± 9	113 ± 9	0.81	110 ± 9	115 ± 9	0.23
Whole body fat (kg)	41 ± 7	39 ± 7	0.65	38 ± 5	41 ± 8	0.40
Fat-free mass (kg)	66 ± 6	62 ± 7	0.31	65 ± 3	64 ± 8	0.74
Central abdominal fat (kg)	3.6 ± 0.4	3.7 ± 0.7	0.58	3.3 ± 0.4	3.8 ± 0.7	0.10
Blood pressure						
SBP (mmHg)**	121 ± 6	131 ± 17	0.10	125 ± 8	128 ± 17	0.6
DBP (mmHg)**	81 ± 8	84 ± 13	0.66	77 ± 10	86 ± 10	0.1
MSNA derived measures						
MSNA burst incidence (burst per 100 beats)	48 ± 21	61 ± 12	0.06	47 ± 20	60 ± 14	0.10
MSNA burst frequency (burst per minute)	27 ± 14	38 ± 7	**0.03**	24 ± 9	38 ± 9	**0.004**
MSNA Heart rate (BPM)	65 ± 9	67 ± 6	0.48	60 ± 5	68 ± 6	**0.008**
Lipid profile						
Total cholesterol (mmol/L)^	4.9 ± 0.6	5.2 ±1.0	0.55	4.7 ± 0.7	5.2 ±0.9	0.28

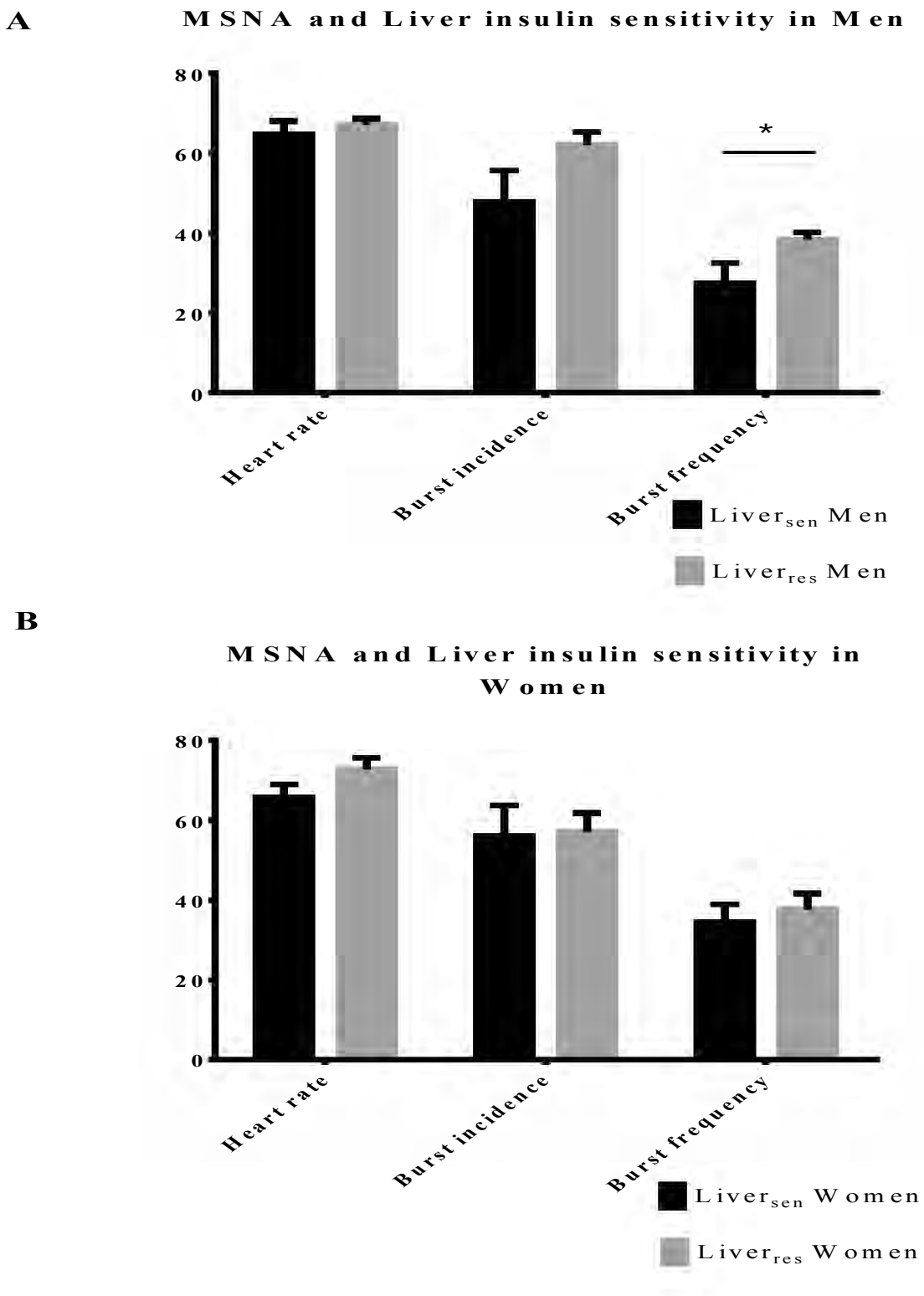
Characteristics	$Liver_{sen}$ men (7)	$Liver_{res}$ men (13)	P value	$Muscle_{sen}$ men (7)	$Muscle_{res}$ men (15)	P value
LDL cholesterol (mmol/L)^	3.4 ± 0.5	3.4 ± 0.9	0.86	3.0 ± 0.7	3.5 ± 0.8	0.24
HDL cholesterol (mmol/L)^	1.0 ± 0.1	1.2 ± 0.2	**0.03**	1.1 ± 0.2	1.1 ± 0.2	0.87
Triglycerides (mmol/L)^ Φ	1.1 (0.9-1.6)	1.2 (0.8-1.6)	0.78	0.9 (0.6-1.5)	1.2 (0.9-1.6)	0.38
Fasting NEFA (mmol/L) Φ	0.29 (0.25-0.37)	0.27 (0.24-0.31)	0.43	0.29 (0.25-0.30)	0.27 (0.23-0.35)	0.68
Glycaemia						
HbA1c (%)	5.2 ± 0.2	5.6 ± 0.3	**0.01**	5.2 ± 0.2	5.6 ± 0.3	**0.003**
Fasting insulin (mU/L) Φ	14 (11-22)	25 (17-41)	**0.03**	12 (10-14)	23 (17-40)	**0.004**
Circulatory cytokines						
Hs-CRP (mg/L) Φ	1.7 (1.0-2.1)	4.3 (2.2-5.7)	**0.03**	1.7 (0.9-2.1)	4.3(1.4-5.5)	**0.04**
FGF-19 (ng/L) Φ	124 (90-237)	62 (32-188)	0.08	152 (124-237)	66 (35-106)	**0.05**
FGF-21(ng/L) Φ	54 (41-165)	76 (13-188)	0.64	41 (19-165)	54 (13-157)	0.91
FABP 4 (μg/L)	38 ± 15	47 ± 18	0.28	36 ± 14	45 ± 19	0.28
Lipocalin 2 (μg/L)	45 ± 16	45 ± 14	0.97	40± 11	45 ± 15	0.42
RBP 4 (mg/L)	12 ± 2	11 ± 2	0.69	13 ± 1	11 ± 2	0.08
Total adiponectin (mg/L)	11 ± 5	8 ± 4	0.17	10 ± 5	9 ± 4	0.53

Data are means ± SD. Φ Data are median (interquartile range) for non-normally distributed data

**Subjects treated with anti-hypertensive medications excluded from the analysis (included: $Liver_{sen}$ men n = 6 and $Liver_{res}$ men n = 10, $Muscle_{sen}$ men n = 6 and $Muscle_{res}$ men n = 12).

^ Subjects treated with lipid medications excluded from the analysis (included: $Liver_{sen}$ men n = 6 and $Liver_{res}$ men n = 10, $Muscle_{sen}$ men n = 6 and $Muscle_{res}$ men n = 11).

Figure 5.2: Heart rate, burst incidence and burst frequency measured by MSNA in obese individuals stratified by liver in men (A) and women (B) respectively.



Differences between groups were analysed by Student T-test, $^{*}P < 0.05$

5.5 DISCUSSION

The main findings of the study described in this Chapter that:

1. In men, liver insulin sensitivity correlated negatively with muscle sympathetic nervous activity.
2. In men, hepatokines including CRP and FGF-19 explain 60% of variability in MSNA burst frequency, supported by their significant correlations with MSNA.
3. There were no significant correlations in muscle insulin sensitivity and MSNA in men, and there were no significant correlations between MSNA and any clinical variables in women
4. MSNA burst frequency is lower in insulin-sensitive men compared with insulin-resistant men.

Basal SNS activity, as measured by MSNA was closely associated with liver insulin sensitivity in obese men in the present study. The metabolic profile of the obese insulin-sensitive men in the present study was consistent with previous reports, including improved glycaemia and lower fasting insulin and CRP (as reviewed[331]). Interestingly, however, MSNA findings in this cohort were limited to men. Our study highlighted significant importance of inflammatory markers CRP and FGF-19 explaining 60% of MSNA burst frequency variability in obese men. The significant correlation between MSNA burst frequency and liver insulin sensitivity (EGP suppression) suggests a potential hepato-endocrine-sympathetic axis modulated by inflammatory markers.

Our results showed gender-specific findings on the correlates with MSNA mostly in men. MSNA was correlated with age, hs-CRP, FGF-19 and RBP4 in men and with DBP

and FAB4 in women. Gender has a role in regulation of sympathetic nervous activity. MSNA has been shown to be regulated differently in men and women. Premenopausal women are protected against hypertension because of a reduced activation of SNS.[308] The exact mechanisms for the role of female sex hormones protecting women from cardiovascular diseases are unknown but there is evidence to suggest that SNS activation is attenuated or sympatho-adrenal inhibition is augmented in women.[246] MSNA has been shown to correlate with BMI and waist hip ratio[332] in men, but only with blood pressure in women[309], again these findings support a gender difference in the regulation of MSNA. Our finding also showed a significant positive correlation between DBP and MSNA in women only.

We have observed higher levels of total adiponectin and FABP4 in women compared with men despite similar age and BMI. These observations were similar to previous studies examining normal weight and overweight individuals.[165,333] FABP4 was thought to relate to fat percentage and women tend to have higher fat percentage than men, thus result in gender differences in FABP4 levels.[275] It is known that there is a sexual dimorphism in adiponectin levels with women having higher adiponectin levels than men.[334] It is thought to relate to the different distribution in adipose tissue depot where adiponectin is preferentially secreted by subcutaneous tissue[335] and women generally have more subcutaneous tissue than men.[275] Interestingly, one study showed that testosterone might contribute to the gender dimorphism in adiponectin levels by inhibiting high molecular weight adiponectin.[336] Further studies are needed to clarify gender differences in inflammatory cytokines and MSNA.

The relationships between insulin resistance and MSNA have been shown to be different in men and women, thought to be due to different adipose tissue distribution.[337] We have demonstrated a significant correlation between liver insulin sensitivity and MSNA in obese men, but not in women. Previous studies that have performed both hyperinsulinaemic-euglycaemic clamp and microneurography[247,338] did not highlight any gender-specific differences in the relationship between MSNA and peripheral insulin sensitivity. Nevertheless, younger (18-35 years) non-obese (BMI <28 kg/m2) individuals [338] or pre-diabetic and diabetic individuals[247] were included, making direct comparisons with our study difficult. Interestingly, one clamp study with deuterated glucose showed significant association between MSNA and muscle and liver insulin sensitivity in women only.[338] Nevertheless, this association applied to lean young women. Other factors such as age and BMI might confound the gender effect in MSNA. Future larger studies are needed to dissect the relationship between MSNA and insulin resistance controlling for gender, age and BMI.

The lack of association between MSNA and other clinical variables in our female cohort was surprising. Nevertheless, one third of female cohort was pre-menopausal. Menstrual cycle can have effects on MSNA[339] and insulin sensitivity[193]. Our female sample size was too small to divide into premenopausal and postmenopausal. This could potentially account for the lack of significant findings in MSNA in women.

This is the first study to our knowledge examining the relationship between liver insulin sensitivity using a two-step hyperinsulinaemic-euglycaemic clamp with deuterated glucose tracers and MSNA in obese non-diabetic individuals. Previous studies have

reported that resting MSNA was higher in obese insulin-resistant subjects compared to obese insulin-sensitive subjects stratified by an OGTT-derived index.[89] The OGTT-derived Matsuda and Defronzo index of insulin sensitivity measures a composite of hepatic and muscle insulin action which has been shown to correlate strongly (r=0.73) with hyperinsulinaemic-euglycaemic clamp with glucose tracers.[96] Our findings suggest that the relationship between insulin sensitivity and resting MSNA reported in previous studies is probably contributed more by the liver.

In non-diabetic obese men, FGF-19 correlated inversely with resting MSNA and insulin-sensitive men had higher FGF-19 levels than insulin-resistant men. FGF-19 has a role to coordinate bile acid and glucose metabolism.[340] Liver is the main target organ of FGF-19 action, where FGF-19 binds to the FGF receptor 4/β-klotho complex to reduce gluconeogenesis and triglycerides in the liver.[176] FGF-19 works in a coordinated temporal fashion with insulin to inhibit gluconeogenesis and promote glycogen synthesis after a meal.[176] MSNA correlated inversely with FGF-19 but positively with basal insulin, this suggests that FGF-19 and insulin might have differential actions in regulation of SNS. We have also shown a positive correlation between hs-CRP and resting MSNA. Both hs-CRP and FGF-19 are hepatokines with pivotal roles and actions in the liver and they explained more than half of MSNA variability in our study. Our findings raise the possibility of a hepato-endocrine-autonomic axis, where inflammatory hepatokines may play a role in regulating sympathetic nervous activity or vice versa. Further studies are warranted to delineate the role of hepatokines, hepatic insulin sensitivity and SNS in no-diabetic obese men.

We found a positive correlation between resting MSNA burst incidence and visceral fat in the whole cohort. This is congruent with previous studies that examined the correlation between visceral fat and MSNA in overweight[341] and lean men[324]. Increased visceral fat has been hypothesized to be the consequence of a neuroendocrine disorder associated with sympathetic nervous system activation and hypothalamic pituitary axis dysregulation.[342] On the other hand, visceral fat is associated with insulin resistance and consequent hyperinsulinaemia is thought to stimulate sympathetic outflow directly and indirectly via baroreceptor-reflex mediated sympathetic flow activation due to insulin-induced peripheral vasodilatation.[89] However, the mechanisms linking visceral adiposity and MSNA remain unclear.

Our study has some limitations. First, resting MSNA was not uniformly measured in the fasting state and feeding has been shown to stimulate sympathetic nervous activity.[343] Nevertheless, testing for differences between measurements performed fasting or non-fasting confirmed that our findings were not affected by timing of measurement. Second, the small sample size may account for the lack of association between insulin sensitivity and MSNA in obese women. Third, MSNA measured in the peroneal nerve might not represent hepatic sympathetic nervous activity. Dissociation between regional and systemic sympathetic nervous activity has been described previously and regional noradrenaline spillover measurement might more precisely assess regional sympathetic nervous activity.[326]

5.6 SUMMARY AND CONCLUSIONS

In summary, basal sympathetic outflow to the muscle vascular bed is lower in insulin-sensitive compared to insulin-resistant men. Resting sympathetic nervous activity is inversely associated with liver insulin sensitivity and with hepatokines, including hs-CRP (directly) and FGF-19 (inversely). These interesting findings raise the potential for a link between liver insulin sensitivity and autonomic nervous activity in non-diabetic obese men, but further studies are needed to examine a putative hepato-endocrine-autonomic axis.

CHAPTER 6
SUMMARY AND CONCLUSIONS

6.1 THE OBESE INSULIN-SENSITIVE PHENOTYPE

The obesity epidemic has an increasing trend globally with an estimated prevalence of 1 billion people by 2030.[344] Obesity is associated with increasing risks of cardiovascular disease, hypertension and diabetes and increased mortality shown in many cross-sectional and prospective studies.[345-347] Nevertheless, obesity is heterogeneous and not all obese individuals carry the same risks of developing metabolic complications. The term MHO has been used since 1980 to denote a group of individuals who, despite having excessive amount of adipose tissue, remain protected from developing metabolic derangement.[348] The current difficulty and confusion in the identity of MHO lies with the conundrum of various definitions.

We have successfully demonstrated that by using insulin resistance as the sole unifying factor, we could potentially identify those obese individuals with fewer metabolic syndrome features and/or lower metabolic complications. We have shown that obese insulin-sensitive individuals had similar insulin sensitivity as lean normal control as studied previously.[82] The term Ob_{sen} phenotype might be a more appropriate definition than MHO where we could use one set of criteria (insulin sensitivity) to define healthy obese individuals with lower metabolic complications. This also highlights the pivotal role of insulin resistance in association with and/or contribution to metabolic disease such as hypertension, dyslipidaemia and diabetes in obesity. We acknowledge that there are still potential issues using insulin sensitivity as a sole definition for Ob_{sen} phenotype using the hyperinsulinaemic-euglycaemic clamp. First, the clamp study is time-consuming and could only be applied to an academic research setting. Second, there is no standardisation in the cut-off value in GIR_{HI} used to define $Ob_{sen,}$ as various clamp

studies used different values or tertiles/quartiles.[36,43,81] Third, gender and menopausal status may play a role in insulin sensitivity,[62,63] and therefore different cut-off values may apply to men, premenopausal women and postmenopausal women.. Lastly, there is lack of standardisation of insulin assay; therefore a specific insulin sensitivity cut-off value in one study may not be extrapolated to other studies that used a different insulin radioimmunoassay. Nevertheless, by using a gold-standard clamp study, we can confidently identify Ob_{sen} and Ob_{res} phenotypes and determine various clinical factors that are associated with and may contribute to insulin resistance. This will assist in clarifying the current confusion regarding the definition of "metabolically health obesity".

6.2 INSULIN RESISTANCE

Insulin resistance involves multi-organs including muscle, liver and adipose tissue. Obesity and diabetes are associated with skeletal muscle and liver insulin resistance. Different therapeutic interventions may target different sites of insulin resistance. For example, metformin predominantly improves hepatic insulin resistance while physical activity decreases muscle insulin resistance and thiazolidinediones and weight reduction ameliorate both.[109] Therefore, it is important to evaluate the magnitude of insulin resistance at various sites to enable directed therapeutic intervention in obese individuals. We have performed a two-step hyperinsulinaemic-euglycaemic clamp with deuterated glucose tracers to differentially measure muscle and liver insulin sensitivity and correlate various metabolic and clinical factors with the specific site of insulin resistance.

6.3 DETERMINANTS OF THE METABOLICALLY HEALTHY OBESITY/OBESE INSULIN-SENSITIVE PHENOTYPE

Cross-sectional studies have highlighted common determinants of MHO/Ob_{sen} phenotypes. These include lower blood pressure, waist circumference, lipid profile, inflammatory markers including CRP and glycaemia.[61,71,73,80] Other features include lower visceral and liver fat shown from various clamp studies.[42,43,81,82] There is conflicting evidence on the association of pancreatic fat[222-225] and adipocyte size[81,128,129] with insulin sensitivity in MHO/Ob_{sen}. These determinants may vary depending on the background population and criteria/definitions used to identify MHO/Ob_{sen}, but some could hopefully be utilised to identify and prioritize high-risk obese individuals to allow efficient use of public health resources to minimise potential obesity-related complications.

Longitudinal studies have shown different long term outcomes on the potential protection of MHO/Ob_{sen} from cardiovascular disease and diabetes. Studies have shown that MHO might not be a stable condition and MHO individuals may develop overt cardiovascular risk factors such as hypertension[349] and about one third may convert to metabolically unhealthy phenotype.[51,55] Potential factors that may preserve MHO phenotype with time include physical activity and weight loss.[56] Thus it is important for clinicians not to assume MHO as a benign phenotype but to encourage lifestyle intervention in MHO individuals to reduce potential metabolic complications and/or preserve MHO status.

6.4 RESULTS

6.4.1 The obese insulin-sensitive phenotype

In chapter 3, we have performed 64 clamp studies on a group of non-diabetic obese individuals (BMI > 30 kg/m^2) including both men and women. We have demonstrated similar insulin sensitivity in our Ob_{sen} (=$Muscle_{sen}$) cohort compared to lean control in another similar study.[82] Similar to previous studies,[34,43,82] we have shown that Ob_{sen} phenotype was associated with lower blood pressure, glycaemia and triglycerides and greater metabolic flexibility. Again, we have shown that non-obese individuals have different fat distribution and liver and visceral fat correlated closely with muscle insulin resistance. Consistent with other studies,[305,350,351] liver fat appeared to be a stronger correlate with muscle insulin resistance relative to visceral fat and explained 38% of variability in GIR_{HI}.

We did not show significant differences in dietary intake and physical activity between $Muscle_{sen}$ and $Muscle_{res}$, perhaps due to potential inaccuracy in self-reporting. Nevertheless, our finding was similar to other studies.[76,93] Adipocyte size correlated positively with muscle insulin resistance in our study. This raises an interesting concept on adipose tissue physiology where MHO/Ob_{sen} phenotype is associated with preserving healthy adipose tissue with the ability to further expand and engage in adipogenesis to preserve elevated adiponectin.[4]

Our multiple linear regression identified clinically available factors that predict muscle insulin resistance, these factors include HDL, OGTT 1h BGL, SBP and TG. These factors will assist clinicians to identify obese insulin-resistant individuals who require

more frequent medical follow-up. Though $Muscle_{sen}$ group might have lower risks of metabolic complications compared with $Muscle_{res}$ group, this might be a transient phenomenon[55] and lifestyle modification and weight reduction should still be implemented to maintain and preserve their metabolic status.[56] Furthermore, lifestyle measures and weight reduction could reduce other obesity-related complications such as osteoarthritis, sleep apnoea, colonic polyps and depression. Further longitudinal studies are needed to assess the long term outcome of Ob_{sen} phenotype.

6.4.2 The Liver insulin-sensitivity phenotype

Liver insulin sensitivity plays an important role in obesity with significant association with inflammation and dyslipidaemia and may contribute to cardiovascular disease.[110] In chapter 4, we have stratified $Liver_{sen}$ and $Liver_{res}$ using EGP suppression during low dose insulin infusion. Similar to $Muscle_{sen}$ phenotype, $Liver_{sen}$ group had lower waist circumference, glycaemia (OGTT-derived $AUC_{insulin}$/$AUC_{c\text{-}peptide}$/$AUC_{glucose,}$ HbA1c), fasting insulin compared with $Liver_{res}$. Again, $Liver_{sen}$ group is characterised by lower liver and visceral fat. In contrast to $Muscle_{sen}$ group, $Liver_{sen}$ group had lower adipocyte size and highlighted the significant associations between liver insulin sensitivity and inflammatory markers including hs-CRP and FGF-19, especially in men. These findings support the concept of healthy adipocyte physiology where insulin sensitive obese individuals exhibit a healthier adipocyte size with less inflammation. It also shows that adipocyte physiology and adipokines have a closer association with liver insulin sensitivity than muscle.

Our sub-analysis by dividing the total cohort into four groups highlighted the importance of visceral and liver fat. Obese individuals who were insulin resistant in dual sites carried the worst metabolic profile and were characterized by a significantly greater amount of visceral fat. Interestingly, liver fat was lower in obesity, irrespective of liver insulin resistance, if muscle remained insulin-sensitive, suggesting that liver fat aligns more closely with muscle than liver insulin sensitivity. Liver fat is also an independent predictor of liver and muscle insulin sensitivity. Congruent with other studies, liver fat may be the most significant determinant of Ob_{sen} phenotype contributing to insulin resistance and metabolic disease[352] and diabetes.[353] Therefore, detection of liver fat in obese individuals is a crucial component in MHO/Ob_{sen}. Further larger studies are needed to devise a gender-specific diagnostic cut-off value for liver fat content using simple non-invasive ultrasound. This could allow wider clinical application and enable clinicians to identify those obese individuals with greatest need for medical attention.

Pancreatic fat is a novel measurement in recent literature, due to advances in MRI/MRS technology. There is conflicting evidence on the association between pancreatic fat and beta cell function.[221,225,226] We have shown a positive correlation between pancreatic fat and OGTT-derived beta cell function in non-diabetic obese individuals. Perhaps pancreatic fat is associated positively with pancreatic beta cell function in normoglycaemic individuals, but as glycaemia deteriorates, this association becomes a negative one.[232] The heterogeneity in the direction of association between pancreatic fat and beta cell function could be contributed by various ethnicity, gender and glucometabolic disorders in the study populations. Further studies are needed to dissect

and clarify the role of pancreatic fat and glycaemia/beta cell function looking at different gender, ethnicity and glycaemic state.

6.4.4 Sympathetic nervous activity in insulin-sensitive obesity

Sympathetic nervous activity is an integral component of metabolic disease in obesity, linking insulin resistance with other components of metabolic syndrome. Studies have shown an increase in MSNA in resting state and a blunted MSNA response post an oral glucose tolerance test in obese insulin-resistant individuals.[89,354] There is ample evidence to suggest a bidirectional relationship between insulin resistance and sympathetic activity. Hyperinsulinaemia is known to stimulate central sympathetic activity directly in the hypothalamus[355] and via baroreflex activity.[236] On the other hand, increased sympathetic activity has been shown to precede insulin resistance and diabetes in prospective studies,[239,240] perhaps via neural vasoconstriction and increased adipose lipolysis which reduce skeletal muscle insulin sensitivity.[242,247] These findings suggest sophisticated complex interactions between sympathetic activity and insulin resistance in obesity and metabolic disease.

In Chapter 5, we have shown an inverse correlation between MSNA and liver insulin sensitivity (EGP suppression during low dose insulin infusion) in non-diabetic men, but not in women. This raises an interesting concept of a differential regulation of sympathetic nerve activity in obesity that is gender-specific. Studies have shown different correlations with MSNA in men and women and the differences are thought to be related to oestrogen status, adipose tissue distribution or sympathetic-adrenal axis.[246,337] Our finding suggests a link between liver insulin sensitivity and MSNA in men, perhaps contributed by inflammatory cytokines CRP and FGF-19. Both hs-CRP

and FGF-19 explained 35% of variability in MSNA burst frequency in obese men. A hepato-endocrine-autonomic axis is suggested to explain the potential link that hepatokines may direct/indirectly interact with sympathetic nerve activity. Future larger studies are needed to delineate potential roles of hepatokines, liver insulin sensitivity and sympathetic nerve activity in different genders.

6.5 CONCLUSIONS

In conclusion, insulin resistance plays an integral part in obesity and is a precursor to development of diabetes. Differential magnitude in insulin resistance in skeletal muscle and liver may play important roles in specifying different metabolic and clinical characteristics and site-specific therapeutic interventions in non-diabetic obese individuals. The criteria used to define MHO needs to be clarified to reduce the current conundrum in the literature. The use of clinical markers such as HDL, TG, OGTT 1h BGL and SBP should help clinicians to identify obese insulin-resistant individuals who can be prioritized with current health resources to allow frequent monitoring and interventions to reduce their metabolic complications.

The complex relationship between sympathetic nerve activity, liver insulin resistance, liver fat and circulating molecules released from insulin-sensitive tissues, including hepatokines and adipokines in non-diabetic obese humans will need to be clarified with larger gender-specific studies. The importance of liver fat in insulin sensitivity in obesity is highlighted in our study and provides a clearer picture on its association and/or contribution to insulin resistance perhaps via inflammatory cytokines. Lastly, the concept of an Ob_{sen}/MHO phenotype allows clinicians to identify obese individuals at lower or higher risks of developing metabolic disease. Nevertheless, it is important to understand that Obsen/MHO individuals may develop potential metabolic complications in the future and weight loss and lifestyle changes should be encouraged and other non-metabolic obesity-related complications such as depression, osteoarthritis and sleep apnoea need to be addressed to reduce potential co-morbidities.

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