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Energy metabolism in obesity: Role of vitamin D status, cold exposure and leucine supplementation

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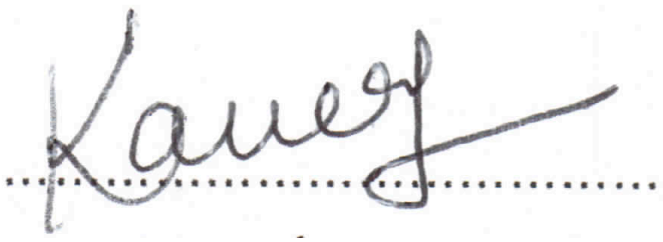
Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics: The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Numbers: HR103/2012, HR 108/2013 and SPH-46-2014.



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Publications and Awards

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2. Pathak K, Calton EK, Soares MJ, Zhao Y, James AP, Keane K and Newsholme P. Forearm to finger-tip skin temperature gradients in the thermoneutral zone maybe causally related to resting energy metabolism: potential implications for nutrition research. **European Journal of Clinical Nutrition. 2017**. 71: 1074-1079; advance online publication, April 5, 2017; doi:10.1038/ejcn.2017.30. Journal impact factor- 3.057.

3. Pathak K, Soares MJ, Zhao Yun, James AP, Sherriff JS & Newsholme P. Postprandial changes in glucose oxidation and insulin sensitivity in the metabolic syndrome: influence of fibroblast growth factor 21 and vitamin D status**. Nutrition**. **2016**. 37, 37-42. doi: http://dx.doi.org/10.1016/j.nut.2016.12.007. Journal impact factor- 3.42

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Publications related to but not part of this thesis

1. Calton EK, Pathak K*, Soares MJ, Alfonso H, Keane KN, Newsholme P, Cummings NK, Chan She Ping‐Delfos W, Hamidi A. Vitamin D status and insulin sensitivity are novel predictors of resting metabolic rate: a cross‐sectional analysis in Australian adults. European Journal of Nutrition. 2016. 55. 2075-2080. doi: 10.1007/s00394-015-1021-z. Journal impact factor – 3.239. Cited by 6.

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3. Soares MJ, Pathak K, Calton EK. Calcium and Vitamin D in the Regulation of Energy Balance: Where Do We Stand? International Journal of Molecular Science. 2014, 15, 4938-4945. Journal impact factor – 3.226. Cited by 20.

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Abstract

The overarching aim of this thesis was to expand our understanding of energy metabolism in obesity and in those at risk of and with the metabolic syndrome (MetS). I have studied adaptive and postprandial thermogenesis through studies that focussed on three aspects: cold-induced thermogenesis, vitamin D status and through supplementation with the branched chain amino acid leucine, during weight loss.

First, a randomized controlled trial (RCT) was conducted with twenty individuals with (MetS+; n =9) and without MetS (MetS-; n =11) where a 3.5 hour exposure to mild cold of 20^{o}C was contrasted in a within-subject design, with two temperatures in the classical thermoneutral range (25^{o}C and 27^{o}C). Mild cold exposure stimulated resting thermogenesis only in those with MetS+ and was accompanied by significant vasoconstriction and a drop in in-the- ear temperature (IET). Specifically, this increased thermogenesis was only evident as a significant difference between MetS groups at 25°C and 20°C, but not at 27°C. There were no differences in postprandial energy metabolism that was monitored following glucose, across the three temperatures and between MetS groups. Circulating irisin and fibroblast growth factor 21 (FGF21) did not differ between temperatures but their postprandial change associated with glucose oxidation (Chapter 4). In these studies, a large variation in forearm to fingertip skin temperature gradient (FFG) was noticed at 25°C; a temperature at the midpoint of classical thermoneutral zone (TNZ). FFG reflects skin blood flow and a collation of cross-sectional and longitudinal data indicated that variations in FFG contributed to both resting metabolic rate (RMR) and respiratory quotient (RQ). Such data could signal a re-assessment of criteria for determining thermoneutrality, which is an essential prerequisite for the proper measurements of resting energy metabolism in the clinical and research settings (Chapter 5).

Secondly, a systematic review (n=18 studies) and meta-analysis (n=12 studies) of quality RCTs indicated that vitamin D supplementation did not impact on weight or fat lost in the absence of caloric restriction (included in Chapter 2). A follow up cross-sectional trial (n=48) with overweight and obese older adults showed that higher 25(OH)D was associated with greater metabolic flexibility and postprandial insulin sensitivity. Though FGF21 increased

significantly post-glucose challenge, the change did not account for these effects observed (Chapter 6).

Lastly, a double-blind placebo controlled weight loss RCT was conducted over 8 weeks examining the effects of l-leucine supplementation (n=18) versus placebo (lactose; n=19). We did not uncover any additional effect of leucine on body composition, insulin sensitivity, lipid profile or liver function. However incidental improvements in vitamin D status (as measured by circulating 25(OH)D) during weight loss resulted in less body fat and more skeletal muscle compared to those who had a fall in 25(OH)D during the course of the trial. Postprandial thermogenesis was not different due to leucine or to change in 25(OH)D. However postprandial RQ was significantly higher following leucine, suggestive of an improved metabolic flexibility (Chapter 7).

Collectively, through a series of diverse approaches, my studies have highlighted some novel aspects of energy metabolism in obesity and the metabolic syndrome. Future trials are needed to build on these outcomes and arrive at tangible approaches for prevention and management of this global malady.

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List of Abbreviations

AcoA	acetyl-CoA
AEE	activity induced energy expenditure
AM	appendicular mass
ATP	adenosine triphosphate
AUC	area under the curve
BAT	brown adipose tissue
BCAA	branched chain amino acid
BD	twice a day
BMI	body mass index
CIT	cold-induced thermogenesis
CKD	chronic kidney disease
DIT	diet induced thermogenesis
EE	energy expenditure
EER	estimated energy requirement
ER	energy restriction
ETC	electron transport chain
FFG	forearm-fingertip gradient
FGF21	fibroblast factor 21
FM	fat mass
FNDC5	fibronectin type 3 containing 5

FP	functional power
Gp	group
HDL	high density lipoprotein
HFD	high fat diet
HOMA-IR	homeostasis model assessment - insulin resistance
IER	impaired energy regulation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IIT	insulin induced thermogenesis
IL-6	intraleukine factor 6
IMTG	intramyocellular triacylglycerol
IR	insulin resistance
IS	insulin sensitivity
ISI	insulin sensitivity index
ITT	intention to treat
IU	international unit
LB	lower body
LBM	lean body mass
LDL	low density lipoprotein
MA	meta-analysis
MetS	metabolic syndrome
MLR	multiple linear regression

MM	muscle mass
MRI	magnetic resonance imaging
NEFA	non-esterified fatty acid
NST	non-shivering thermogenesis
NO	non-obese
OGTT	oral glucose tolerance test
OW/OB	overweight/obese
PA	physical activity
PCOS	polycystic ovarian syndrome
PET- scan	positron emission tomography scan
(PGC-1a)	gamma coactivator-1a
PPAR	peroxisome proliferator activator receptor
PTH	pituitary thyroid hormone
QUICKI	quantitative insulin sensitivity check index
RCT	randomized controlled trial; DB, double blind
RMR	resting metabolic rate
RQ	respiratory quotient
SBP	systolic blood pressure
SNS	sympathetic nervous system
SR	systematic review
ST	shivering thermogenesis
TEE	total energy expenditure

TEF	thermic effect of food
TAG	triglycerides
T2DM	type 2 diabetes
TNZ	thermoneutral zone
UB	upper body
UCP	uncoupling protein
VLDL	very low-density lipoprotein
WAT	white adipose tissue
Wks	weeks
Wt	weight
Y	years

Chapter 1 Introduction and Thesis Outline

1.1 Background - Concepts and Definitions

Obesity

Obesity is a pandemic of major public health concern due to its intimate consequences for insulin resistance, metabolic syndrome, type 2 diabetes, cardiovascular disease and even some cancers. The latest Australian Health Survey (Australian Bureau of Statistics, 2015) reported the percentage prevalence of major health conditions of 2014-15 which included: overweight or obesity - 11.2 million people (adults) (63.4%), high cholesterol - 1.6 million people (7.1%), diabetes - 1.2 million people (5.1%), heart disease - 1.2 million people (5.2%), hypertension - 2.6 million people (11.3%). The data firmly places Australia in the top 3 countries worldwide with adverse health profiles (World Health Organisation, 2007).

Obesity can be described as "abnormal or excessive fat accumulation that presents a risk to health" (WHO, 2017). One of the measures of obesity is the body mass index (BMI), which is the ratio of weight (in kilograms) to the square of a person's height (in metres). A person with a BMI of 30 or more is considered obese and BMI equal to or more than 25 is overweight. Diet, medication and exercise are the main avenues to address obesity whether the intent of the treatment is curative or preventative. In essence, creating a negative energy balance by increasing energy expenditure and reducing energy intake is usually accomplished by therapies that utilize caloric restriction/dietary modification or specific nutrient supplementation and drugs that target energy expenditure or energy intake. Sustainability of the ensuing weight loss then becomes an important consideration for translation of newer lifestyle modifications to health policy.

The prevalence of obesity is still on the rise suggesting that current strategies of reducing energy intake or increasing energy expenditure via physical activity are not having the desired outcomes. A recent focus is on increasing energy expenditure through

involvement of adaptive thermogenesis by maximising activity of uncoupling protein 1 (UCP-1) (Bonet, Mercader & Palou, 2017). Adaptive thermogenesis can be modulated by modifying dietary composition, treatment with hormone mimetics, as well as by cold exposure. In humans, a large part of the adaptive thermogenic response is controlled by the capacity and activity of brown adipose tissue (BAT), and by the skeletal muscle mass via the process of mitochondrial uncoupling (Cannon & Nedergaard, 2004).

Total Energy Expenditure

Total energy expenditure (TEE) is divided into three components: basal metabolic rate (BMR), thermogenesis and physical activity. Resting metabolic rate (RMR) is closely related to BMR. It is often used interchangeably with BMR, though it is ~5% higher as the prerequisite for its measurement permits the volunteer to come to the place of measurement from home (Soares, Piers, Kraai & Shetty, 1989). In this thesis, we have measured RMR. Resting metabolic rate (RMR) is the minimum energy required to perform vital body functions such as respiration, digestion, brain activity, heart rate, etc. in the resting state (Clapham, 2011). About 60% of one's daily energy expenditure can be attributed to RMR, 10% from DIT and 30% to physical activity. The prerequisites for a RMR measurement include a 10-12 hour post-absorptive state, a restful sleep and abstinence from any vigorous physical activity at least 36 hours prior to the measurement (Calton, Soares, James & Woodman, 2016; Nsatimba, Pathak & Soares, 2016; Henry, 2006; Piers, Soares, Makan & Shetty, 1992; Soares et al., 1989). RMR is best measured before 9 AM since there is a circadian rhythm to metabolic rate (Li, Yu, Wang & D, 2013; Kräuchi & Wirz-Justice, 1994; . A properly measured RMR is used as the basis for judging energy requirements of individuals and this assists the nutritionist or dietitian in prescribing the number of calories needed for weight gain or weight loss, depending on the situation. When measured on instrumentation that also has a carbon dioxide analyser, an estimate of the respiratory quotient (RQ) given by the ratio of carbon dioxide production to oxygen consumption is also obtained. The RQ then provides information as to the type of fuel being burnt at rest, where values from 0.71 to 1.0 indicate the range of fat to carbohydrate oxidation respectively (Widmaier, Raff & Strang, 2016).

The most common method to measure metabolic rate is calorimetry, which as the name denotes is the measurement of heat production. This can be done through direct methods where all the heat losses (radiative, convective and evaporative) from the body are measured (direct calorimetry). An alternative approach is indirect calorimetry whereby measurements of oxygen consumption, carbon dioxide production and urinary nitrogen loss are related to energy expenditure through standard equations (Ferrannini, 1988). A key principle in these measures is that they be conducted in a thermoneutral environment. This thermoneutral zone (TNZ) is a range of ambient temperatures where heat is neither gained nor lost from the body. It is only in the TNZ that heat production from indirect calorimetry is equivalent to energy expenditure. Non-calorimetric methods include isotope-dilution by doubly-labelled water and heart rate monitoring (Levine, 2005). Indirect calorimetry provides comparable data to direct calorimetry, it is simpler to measure and delivers high quality and repeatable data. It has become the norm for clinical investigators (Ferrannini, 1988).

Thermogenesis

Thermogenesis in simple terms means heat production. This may occur during chemical reactions in response to food intake, as in diet-induced thermogenesis, or in response to mild cold exposure where it is termed non-shivering thermogenesis. Classical non-shivering thermogenesis (NST) is adaptive (i.e. it can be turned on and off within minutes), but also has a long term component as it can be switched on by either overfeeding or exposure to cold and may take weeks to develop to its full capacity (Van Marken Lichtenbelt & Daanen, 2003). It may contribute up to 20% of the total energy expenditure. In reanalysis of data from different studies from 1965-1999 (Stock, 1999), the author suggested that out of total increase in body weight, 60% is due to increase in fat mass and rest may be because of shift in either fat free mass or total body water. The author proposed that overfeeding in at least 40% of these subjects displayed an increase in DIT, to varying extents (Stock, 1999).

Mitochondria are the powerhouse of the cell. Mitochondria are intimately involved in ATP generation through the consumption of oxygen. This occurs in the electron transport chain (ETC) within mitochondria; the final common pathway that links intermediates of

carbohydrate, fat and protein metabolism to energy generation. Mitochondria are not fully efficient since not all oxygen consumption is coupled to mitochondrial ATP production, and thus some of the available free energy is lost as heat. Proton leakage through the inner mitochondrial membrane is a significant source of such uncoupling and has been estimated to account for 20–25% of *in vivo* basal metabolic rate (BMR) (Rolfe & Brand, 1996). Similarly, cold exposure is suggested to increase energy expenditure through recruitment of BAT (Cannon & Nedergaard, 2004) using uncoupling protein 1(UCP-1) in skeletal muscle. BAT is rich in mitochondria which gives BAT their brown colour. Cold exposure increases volume as well as activity of BAT; however, this change is more observable in lean individuals (van Marken Lichtenbelt, Vanhommerig & Smulders, 2009). Sympathetic nervous system (SNS) plays a major role in the central control of thermogenesis (Clapham, 2011) and augmented SNS activity resulting from exposure to low temperatures leads to BAT activation and hence increased energy expenditure.

Nutrient Utilization & Energy Balance

Carbohydrates (starches, sugars), fats (triglycerides) and proteins are the primary sources of dietary energy. Glucose and fatty acids are the simplest forms in which energy is supplied to the body. These nutrients provide fuel to produce adenosine triphosphate (ATP) in various metabolic activities. Any excess of glucose in the cell after glucose oxidation is complete is stored as glycogen. This glycogen is released in a catabolic reaction during times of need. Overall, carbohydrate and protein balances are brought about by increases/decreases in carbohydrate and protein oxidation. Fat oxidation in the short term is determined by the difference between energy produced (from available carbohydrates/ proteins) and the energy spent by the body, and not by the fat content in the diet. In effect, one can rewrite the energy balance equation as a nutrient balance equation that indicates fat intake minus fat oxidation equals fat/energy balance (Flatt, 1995). Thus, expert consultations and national bodies concerned with guidelines for weight control always emphasize restrictions on fat intake, particularly saturated fat, with increases in physical activity to promote fat oxidation.

Metabolic Flexibility

In the fasted state, stored fat is used as the main source of fuel. After a mixed meal, the system switches to use carbohydrate as a primary source. This capacity to switch fuel usage is termed 'metabolic flexibility'. The inability to switch fuel usage is called 'metabolic inflexibility' (Kelley, He, Menshikova & Ritov, 2002)). In obese and diabetic individuals this inflexibility manifests in a range of metabolic pathways and tissues including: failure of cephalic-phase insulin secretion (impaired early-phase prandial insulin secretion concomitant with a failure to suppress hepatic glucose production and NEFA efflux from adipose tissue), failure of skeletal muscle to appropriately move between use of lipid in the fasting state and use of carbohydrate in the insulin stimulated prandial state; and impaired transition from fatty acid efflux to storage in response to a meal. In short, metabolic inflexibility can be defined as a reduction in mitochondrial content and/or density leading to reduction in oxidative capacity of skeletal muscle. This metabolic inflexibility is a main feature of 'Metabolic Syndrome' (Storlien, Oakes & Kelley, 2004), impaired fasting glycaemia (IFG) and impaired glucose tolerance (IGT) (Faerch & Vaag, 2011). Metabolic flexibility can be measured *in vivo* as a change in RQ after a high carbohydrate diet (Weyer, Vozarova, Ravussin & Tataranni, 2001), oral glucose tolerance test (Wu & Yu, 2004), or high fat diet (Kardinaal et al., 2015; Stumvoll et al., 2000). *In vitro* measurements performed in myotubes tested for suppressibility (glucose suppression of fat oxidation) and adaptability (an increase in fat oxidation in the presence of high palmitate concentration) by treating incubated labelled skeletal muscle cells (1 **μ**Ci/mL; NEN Life Science Products) and 20 **μ**M cold palmitate with 0, 0.5, 1.5, and 5 mM glucose. The occurrence of fatty acid oxidation in presence of glucose was described as the metabolic switch (Ukropcova et al., 2005). In the same study, metabolic flexibility was positively correlated with insulin sensitivity and maximal oxygen uptake but inversely with fat percentage. Whole body metabolic flexibility is best measured using the gold standard method to measure insulin sensitivity, namely the euglyacemic hyper-insulinemic clamp technique (Faerch & Vaag, 2011). However, this technique is only available in dedicated laboratories that have the medical cover due to its invasive nature and risk, since it entails insulin infusions high enough to 'clamp' the normal functions of the pancreas, while concomitantly infusing glucose to maintain euglycemia. Post-prandial metabolism may be compromised in most chronic diseases such as obesity (Blaak et al., 2006), type 2 diabetes (Wu & Yu, 2004) and MetS (Van Oostrom, Alipour, Plokker, Sniderman & Cabezas, 2007) putting them more at risk. Modifications in lifestyle including composition of meals, physical

activity and weight reduction have been used to examine improvements in metabolic inflexibility (Corpeleijn, Saris & Blaak, 2009).

1.2 Research Question

Obesity continues to be one of the leading health issues in Australia. There is very little data on resting and post-prandial energy metabolism in the Australian population, while the literature is surfeit with studies from the US and Europe. The overarching aim of this thesis was to better understand energy expenditure in obesity and those at risk of metabolic syndrome. Specifically, resting and post-glucose energy metabolism and associated pathophysiological markers were studied to appreciate the 'metabolic switch' in fuel utilization under various experimental conditions.

1.3 Rationale for the Study

The studies in this thesis explore ways to modulate RMR and glucose induced thermogenesis (GIT) through the use of mild cold temperature and the role of nutrients (vitamin D and leucine) among individuals with obesity/MetS and whether the outcomes can be used to treat obesity and related disorders. I also examined the role of vitamin D status in post-prandial metabolism. This thesis hence presents a novel three-pronged approach to understanding thermogenesis (resting energy expenditure and GIT) and their association with ambient temperature, vitamin D status and leucine-induced weight loss among Australian adults with obesity/MetS.

The studies presented in this thesis explored the mechanisms that underscore the changes in energy metabolism due to obesity/MetS. These are novel and emerging avenues that may be of benefit in combating the obesity crisis by focusing on ways to increase energy expenditure and alter whole-body fuel utilization. Post-prandial metabolism is altered in many chronic diseases and could be an area targeted to reduce the occurrence of these diseases. Animal studies strongly implicate a role for vitamin D in energy metabolism and body composition (Wong et al., 2011; Wong et al., 2009). We are starting to understand more

about the role of vitamin D in human energy metabolism (Calton, Pathak et al., 2016; Soares, Murhadi, Kurpad, Chan She Ping-Delfos & Piers, 2012) but further work is required. Perth has a Mediterranean climate with an average annual temperature of 25°C and differs to the conditions reported in many previous studies. Demonstration of cold-induced thermogenesis requires different temperature ranges when compared to the studies in Europe where the ambient temperatures are much lower. We have used exposure to mild cold as a way to stimulate adaptive thermogenesis and potentially change fuel utilization and insulin sensitivity. Lastly, in a weight loss clinical trial, the role of leucine, a branched chain amino acid, in changes to body composition and insulin sensitivity was investigated where it was observed to have improvements over and above those of placebo.

This thesis consists of three sub-sections each exploring a potential pathway to increase energy metabolism and improve metabolic flexibility:

1. Cold exposure and energy metabolism

The most common predictors of RMR are age, gender, ethnicity, physical activity (PA), fat mass (FM) and fat-free mass (FFM). Recently our group has added to this list the consideration of vitamin D status and insulin sensitivity (Calton, Pathak et al., 2016), and as part of this thesis, forearm fingertip gradient (FFG) (Pathak, Calton et al., 2017). In response to low temperatures, the human body increases energy expenditure and vasoconstriction to maintain homeothermy (Landsberg, 1984 #706) (Calton, Soares et al., 2016). Energy balance and substrate utilisation are largely influenced by factors such as gender (Blaak, 2001) and prevalence of obesity and related disorders such as metabolic syndrome (Soares, Cummings & Chan She Ping-Delfos, 2011). Modern housing assures in-house temperatures remain constant despite wide variations in external temperatures throughout the year. This may potentially limit our capacity for thermoregulatory thermogenesis. Exposure to temperatures below our thermal comfort zone (TCZ) may be one of the non-nutritional approaches to increase energy expenditure (Celi et al., 2010; van Marken Lichtenbelt & Schrauwen, 2011). There have been studies in the past comparing the effect of cold on lean and obese subjects (Wijers, Wim & Van Marken Lichtenbelt, 2010) with either acute (Cannon & Nedergaard, 2010) or long term exposure (Lee, Linderman et al., 2014). Cold exposure increases brown adipose tissue (BAT) activity or non-shivering thermogenesis (NST). It is now confirmed that

adult humans have BAT; a body compositional feature that was previously thought to be restricted to newborns and hibernating animals. Moreover, two recently identified hormones, irisin and FGF21, may play a role in both shivering thermogenesis (ST) and NST respectively (Lee, Linderman et al., 2014) and both have the capability of converting white adipose tissue (WAT) to BAT (Choi et al., 2014; Fisher et al., 2012). Both hormones are raised in the presence of metabolic syndrome (Lee, Linderman et al., 2014) where the former is increased in ST and the latter in NST. We have tried to investigate the beneficial effects of cold exposure on metabolic rate and fuel utilisation among obese adults with or without metabolic syndrome. We were interested in understanding non-shivering thermogenesis and whether irisin and FGF21 mediated thermoregulatory changes to acute mild cold exposure.

2. Vitamin D and Energy Metabolism

Poor vitamin D status has been associated with many chronic diseases (Holick & Chen, 2008; Peterlik, Boonen, Cross & Lamberg-Allardt, 2009) including factors related to obesity, including an increase in body fat stores (Jafri, Naureen, Moatter & Khan, 2016) and decrease in insulin sensitivity (Sukumar, Shapses & Schneider, 2015). The main effects of vitamin D in the human body can be derived from vitamin D receptors (VDR), members of the nuclear steroid hormone receptor family, and the ligand-inducible transcription factor that functions to control gene expression. Activation of VDR stimulates the production of the active form of hormone 1,25 dihydroxy vitamin D_3 which is present in pancreatic beta cells, immune cells, skin and bone (Bouillon et al., 2008). VDR-null mice when compared to wild-type mice exhibited increased fat oxidation, up-regulation of UCP-1 and a lean phenotype (Narvaez et al., 2013; Wong et al., 2009). Two recent studies by our group support the potential role of vitamin D status in RMR (Calton, 2016d) and modulating the post-prandial state and fuel oxidation (Pathak, Soares et al., 2017). However, whether vitamin D status regulates adiposity is not clear (Soares, Chan She Ping-Delfos & Ghanbari, 2011). A recent bi-directional Mendelian analysis suggested that increased adiposity is associated with low vitamin D levels, but that low vitamin D status is less likely to cause obesity (Vimaleswaran et al., 2013). A systematic review by Pannu and co-workers (2016) forwarded the idea that much of the vitamin D is sequestered or interconverted to other metabolites since, due to the weight loss, the rise in 25OHD is much less than it should have been considering the enormous stores of

25OHD available in adipose tissue. This is important to studies examining the effects of vitamin D supplementation, since the active form 1,25 OHD has a close relationship to 25OHD levels, and unless an optimal circulating level of the latter is achieved, there may be no effect on the active hormone (Heaney, 2008). This is particularly true for tissues that have the metabolic machinery to convert 25OHD to active form. Thus, maintaining optimal vitamin D levels could be an approach to treating obesity.

In this thesis, the role of prevailing vitamin D status on post-prandial glucose metabolism was investigated in subjects with metabolic syndrome; a clinical condition directly linked to obesity. FGF21, as it is also a metabolic regulator, was studied with vitamin D status in one of the chapters in this thesis as a cross-sectional study. I examined their potential beneficial role in post-prandial metabolism and insulin sensitivity.

3. Weight Loss and Energy Metabolism: Role of Leucine Supplementation and Vitamin D Status

Any change in weight status brings about an alteration to metabolic rate. In fact, energy restriction will reduce RMR well before a change in weight is observed. Usually during weight loss, energy expenditure is further decreased due to a reduction in fat mass and fat free mass (Soares, Cummings et al., 2011). As aforementioned, the relationship between vitamin D status and obesity is not straight-forward (Pannu, Zhao & Soares, 2016; Vimaleswaran et al., 2013). Leucine is abundantly found in whey protein, which is strongly suspected to increase energy expenditure and reduce energy intake by its contribution to increasing satiety levels (Veldhorst et al., 2008). Leucine is therefore suggested to promote weight loss, increase protein anabolism and metabolism (Anthony, Anthony, Kimball & L.S, 2001; Lynch, Hutson, Patson, Vaval & Vary, 2002). We supplemented leucine, a branched-chain amino acid (BCAA), within a calorie restricted meal plan in an eight-week weight loss clinical trial and compared the outcomes with a control group receiving only a calorie-restricted meal plan to note any changes in energy expenditure, substrate utilisation, and body composition. One recent study looked at the combined effect of leucine and vitamin D on people with sarcopenia and interestingly found that this combination actually increased appendicular lean mass resulting

in improved muscle strength (Bauer et al., 2015). Hence, to take our analysis a step further, we also investigated whether vitamin D status could determine the extent of weight loss with leucine supplementation in obese individuals.

1.4 Objectives of the Study

1. To examine whether an acute cold exposure could stimulate adaptive thermogenesis, alter basal metabolism and glucose-induced thermogenesis (GIT) (Chapter 4).

2. To explore if metabolic hormones such as irisin and FGF21 play a role in human energy metabolism and GIT (Chapter 4).

3. To investigate if variations in forearm fingertip gradient (FFG) contribute to RMR and RQ measured in a thermoneutral zone (Chapter 5).

4. To understand the influence of prevailing vitamin D status on basal and post-prandial energy metabolism in overweight and obese adults (Chapter 6).

5. To ascertain whether leucine supplementation increases weight loss and modifies body composition through changes in energy metabolism (Chapter 7).

6. To examine the modifying effect of prevailing vitamin D status on the energy metabolism and GIT in subjects with obesity/MetS during leucine supplementation (Chapter 7).

Thesis Structure Addressing Objectives of Thesis

This thesis is comprised of chapters that provide a critical review of the recent literature and a number of studies addressing the objectives of the thesis. Many of these chapters have now been published as peer-reviewed manuscripts in international journals.

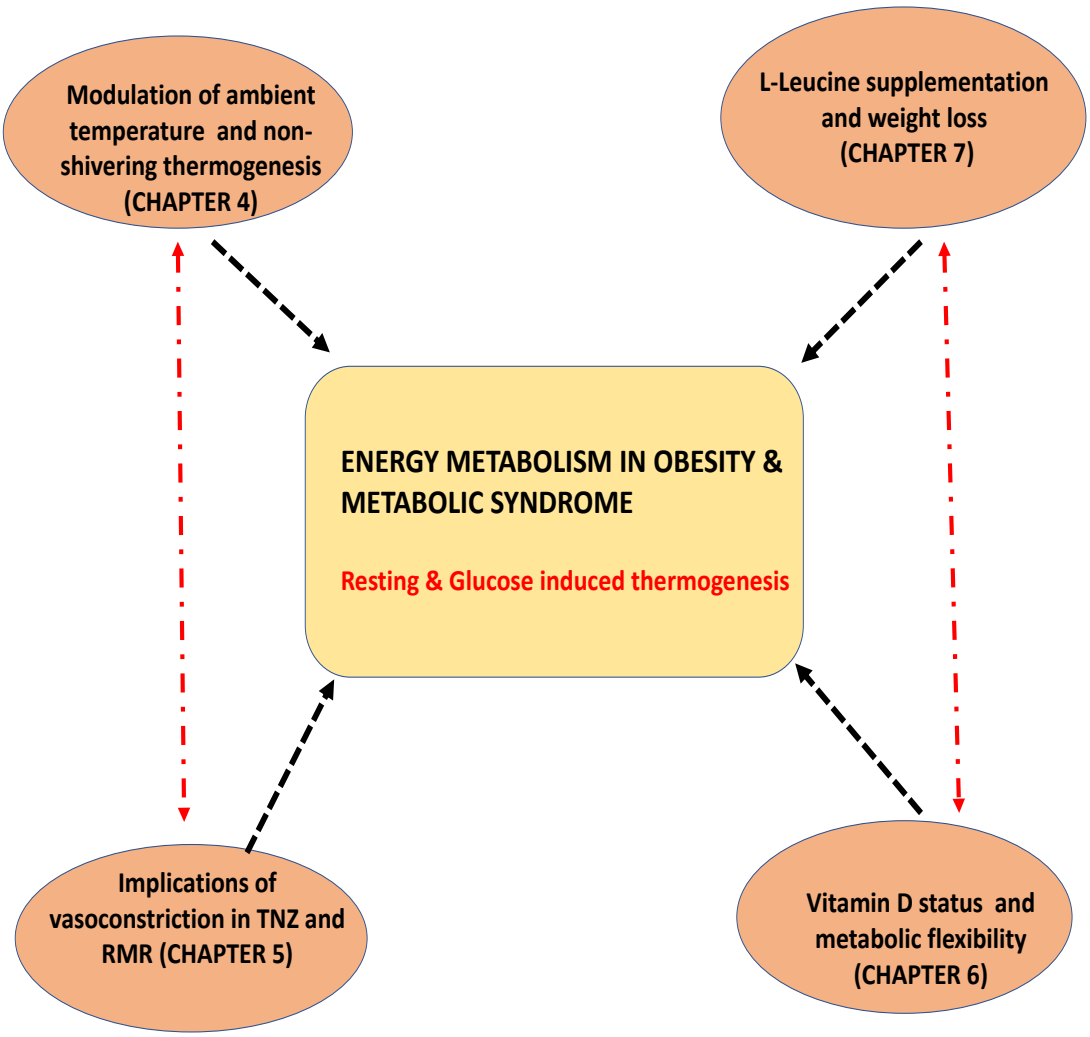

Figure 1.1 Schematic diagram of thesis.

CHAPTER 1: Introduction

This chapter provides the background, objectives and rationale for the topics covered in the thesis.

CHAPTER 2: Literature Review

This chapter provides evidence from research conducted in the relevant fields covering the thesis and concludes with knowledge gaps that were explored in this thesis.

A systematic review and meta-analysis are included as part of this chapter on the effect of vitamin D supplementation in the absence of weight loss and the potential effects of vitamin D on weight loss and changes in body composition. This has been published as: Pathak K, Soares MJ, Calton EK, Zhao Y, Hallett J. Vitamin D supplementation and body weight status: A systematic review and meta-analysis of randomized controlled trials. *Obesity Reviews.* 2014, 5(6):528-37 doi:10.1111/obr.12162.

CHAPTER 3: Methods

This chapter details study design, data collection, equipment used and clinical chemistry measured regarding energy expenditure, glucose-induced thermogenesis and body composition. The statistical analyses used varied with each study and hence are described within each chapter.

Section A: AMBIENT TEMPERATURES AND ENERGY METABOLISM

CHAPTER 4: Fasting and glucose-induced thermogenesis in response to three ambient temperatures: A randomized crossover trial in the metabolic syndrome.

OBJECTIVES:

1. To examine whether an acute cold exposure could stimulate adaptive thermogenesis and alter basal metabolism and glucose-induced thermogenesis.
2. To explore whether metabolic hormones such as irisin and FGF21 played a role in glucose oxidation.

HYPOTHESIS: We hypothesized that mild cold exposure will increase energy expenditure, improve metabolic flexibility and insulin sensitivity in MetS.

In this chapter the activation of adaptive thermogenesis in cold conditions is discussed as well as how this is altered due to body fat and the presence of metabolic syndrome. A summarised version of the chapter is published in the *European Journal of Clinical Nutrition*, 2017. Article in press.

CHAPTER 5: Potential influence of forearm to fingertip skin temperature gradients in the thermoneutral zone on resting energy metabolism.

OBJECTIVE:

1. To investigate through cross-sectional and longitudinal observations whether variations in FFG contribute to RMR and RQ measured in TNZ.

This chapter has been published as: Pathak K, Calton EK, Soares MJ, Zhao Y, James AP, Keane K and Newsholme P. Forearm to fingertip skin temperature gradients in the thermoneutral zone were significantly related to resting metabolic rate: potential implications for nutrition research. European Journal of Clinical Nutrition. 2017 71: 1074-1079; advance online publication. DO1: 10.1038/ejcn.2017.30.

Section B: VITAMIN D STATUS AND ENERGY METABOLISM

CHAPTER 6: Post-prandial changes in glucose oxidation and insulin sensitivity in the metabolic syndrome: influence of fibroblast growth factor 21 and vitamin D status.

OBJECTIVE:

1. To understand influence of prevailing vitamin D status on basal and post-prandial energy metabolism in overweight and obese adults.

HYPOTHESIS: Vitamin D status and fibroblast growth factor 21 could increase resting and post-prandial glucose oxidation and insulin sensitivity in metabolic syndrome.

This chapter has been published as: Pathak K, Soares MJ, Zhao Yun, James AP, Sherriff JS & Newsholme P. Postprandial changes in glucose oxidation and insulin sensitivity in the metabolic syndrome: influence of fibroblast growth factor 21 and vitamin D status. Nutrition. 2016. 37, 37-42. DOI: http://dx.doi.org/10.1016/j.nut.2016.12.007.

Section C: WEIGHT LOSS AND ENERGY METABOLISM

CHAPTER 7: Alterations in body composition and energy metabolism following 8 weeks of L-Leucine supplementation during weight loss: Unexpected effect of vitamin D status.

OBJECTIVES:

1. To ascertain whether leucine supplementation increases weight loss and modifies body composition through changes in energy metabolism.
2. To examine the added effect of vitamin D status on the above parameters.

HYPOTHESIS: Leucine, a branched-chain amino acid may increase weight loss during caloric restriction, preserve fat-free mass and improve insulin sensitivity. Prevailing vitamin D status may have permissive effects on these changes.

CHAPTER 8: FINAL CONCLUSIONS

This chapter summarizes the key findings based on thesis objectives and provides some insights for future research.

References are included at the end of this thesis and are inclusive of all chapters. There are appendices, describing information about the study tools utilized and some additional data not included in published papers/chapters. The author contribution declarations and permissions for each published paper are also provided.

Chapter 2 Literature Review

2.1 Introduction to Concepts and Definitions

Metabolic Syndrome

Metabolic syndrome (MetS) is a global health challenge. MetS is not a condition or disease but a cluster of risk markers for central obesity, including cardiovascular risk factors such as decreased high density lipoprotein-cholesterol (HDL-C) concentration, higher levels of triglycerides, elevated blood pressure and elevated fasting plasma glucose concentration (Alberti, Zimmet & Shaw, 2006) (figure 2.1). Presence of three or more of the above-mentioned criteria means a person is considered MetS positive (+) and although possessing two or less of these risk factors indicates a negative result, it can indicate that the individual may be at risk of becoming MetS+ in the future. It is more common in populations with a sedentary lifestyle, old age or in those with a higher waist circumference (Mankowski et al., 2015). However, the adoption of a healthy lifestyle and behaviour modification that aims to promote weight loss might reduce the risk of MetS (Corpeleijn et al., 2009; Soares, Cummings et al., 2011).

Determination of Metabolic Syndrome (MetS)

In this thesis, the presence of Metabolic Syndrome was judged by criteria proposed by Alberti et al. (2006) as the occurrence of any three out of five characteristics. These five factors are:

- Waist circumference (wherein all values are country/ethnic specific)
- TG level: ≥ 1.7 mmol/l (150 mg/ dl)
- HDL-cholesterol: < 1.03 mmol/l (40 mg/dl) in males and < 1.29 mmol/ l (50 mg/ dl) in females (or specific treatment for these lipid abnormalities)

- blood pressure (systolic BP≥130 mmHg or diastolic BP≥85 mmHg) (or treatment of previously diagnosed hypertension)
- fasting plasma glucose [FPG≥5.6 mmol/ l (100 mg/dl)] (or previously diagnosed type 2 diabetes).

Country/ethnic group	**Waist circumference† (as measure of central obesity)**
Europids*†	Male ≥ 94 cm Female ≥ 80 cm
South Asians‡	Male ≥ 90 cm Female ≥ 80 cm
Chinese	Male ≥ 90 cm Female ≥ 80 cm
Japanese§	Male ≥ 85 cm Female ≥ 90 cm
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available
Sub-Saharan Africans	Use European data until more specific data are available
Eastern Mediterranean and Middle East	Use European data until more specific data are available

Ethnicity should be the basis for classification, not the country of residence.
*In the USA the Adult Treatment Panel III values (102 cm male; 88 cm female) are likely to continue to be used for clinical purposes.
†In future epidemiological studies of populations of Europid origin, prevalence should be given using both European and North American cut-points to allow better comparisons.
‡Based on a Chinese, Malay and Asian-Indian population.
§Subsequent data analyses suggest that that Asian values (male 90 cm; female 80 cm) should be used for Japanese populations until more data are available.

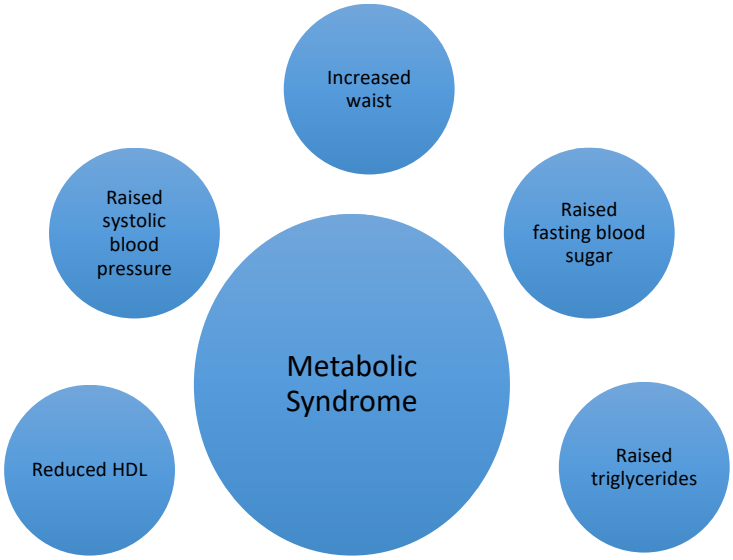

Figure 2.1 Diagnostic features of metabolic syndrome (MetS).

Insulin Resistance

Insulin resistance (IR) is a condition in which the human body produces insulin but is refractory to the action of insulin. Accordingly, normal glucose levels are attained through increased insulin secretion to overcome this resistance. However, with ongoing excessive insulin secretion there is increasing beta cell failure resulting in excessive hyperglycemia. Eventually, β cell exhaustion and IR together leads to type 2 diabetes (T2DM). Insulin resistance hence predisposes one to the development of T2DM (Defronzo, 2009; Kahn, 2003). The prevalence of diabetes is rapidly rising all over the world at an alarming rate (Huizinga & Rothman, 2006; Wild, Roglic, Green, Sicree & King, 2004). According to the Australian Health Survey (AHS) in 2011-13, 5.1% of people (i.e. 1.2 million) in Australia live with diabetes. The occurrence was greater in males than in females (5.7 % vs 4.6%) and increased with age. One in five people over the age of 75 years (i.e. 18.4%) had some type of diabetes in 2014-15 (type 1 and type 2) (Australian Bureau of Statistics, 2011-12). It is estimated that by 2031, 3.3 million Australians will have T2DM (Wild et al., 2004).

A lower respiratory quotient (RQ~ 0.70) reflects a higher fat oxidation. Upon mixed-meal ingestion, an insulin-sensitive person will increase their RQ through greater carbohydrate oxidation and reduced fat oxidation. Altered energy expenditure and substrate oxidation are key features of insulin resistance and T2DM. In the presence of insulin resistance, there may be either impaired fasting fat oxidation or impaired switch from fat to carbohydrate oxidation under insulin-stimulated conditions (Corpeleijn et al., 2009; Galgani, 2008). This state is termed *metabolic inflexibility*. This inflexibility resides in the mitochondria and, to an extent, skeletal muscle function. Further, in response to a cold environment, individuals who exhibit IR and T2DM do not mount an appropriate increase in thermogenesis. Instead, the defence of core temperature occurs through vasoconstriction and insulation (Maeda, Fukushima, Ishibashi & Higuchi, 2007).

Calculations of Insulin Sensitivity Indexes

Calculations of insulin sensitivity indexes (ISI) used further in these thesis chapters (Lorenzo, Haffner, Stancˇa´ kova´ & Laakso, 2010):

Table 2.1 Calculating ISI based on fasting measurements

Index	Formula
ISI_{basal}	$10^4/(I_0 \times G_0)$
HOMA IR	$(I_0 \times G_0)/22.5$
IGR	I_0/G_0
QUICKI	$1/[\log I_0 + \log G_0]$
McAuley	e^x, where $x = 2.63 - 0.28 \ln(I_0) - 0.31 \ln(TG_0)$

Table 2.2 Calculating ISI based on post-prandial measurements using oral glucose tolerance tests (OGTT)

Index	Formula
ISI_{2h}	$10^4/(I_{120} \text{ X } G_{120})$
IGR2h	I_{120}/G_{120}
$Stumvoll_{(0,120)}$	$0.156 - 0.0000459 \times I_{120} - 0.000321 \times I_0 - 0.00541 \text{ X } G_{120}$
Stumvoll with demographics	$0.222 - 0.00333 \times BMI - 0.0000779 \text{ X } I_{120} - 0.000422 \times age$
IGI	$I_{30} - I_0 / G_{30} - G_0$

Where: G_0, fasting glucose; I_0, fasting insulin; G_{120}, 2 hour glucose; I_{120}, 2hour insulin; Tg_0, fasting triglycerides; I_{30}, insulin at 30 min; ; G_{30}, glucose at 30 min; ISI, insulin sensitivity index; HOMA-IR, homeostatic model of assessment- insulin resistance; IGR, insulin glucose ratio; QUICKI, quantitative insulin sensitivity check index; IGI, Insulinogenic index.

Energy Metabolism

Thermogenesis: Definition

Thermogenesis, as the name suggests, is the 'production of heat' to maintain homeothermy. It can be of two types. The first is obligatory thermogenesis, which maintains body temperature at rest, performing the body's vital functions such as digestion, absorption, excretion, respiration, and the functions of the heart, muscle and liver which are metabolically-active organs and tissues. The second is adaptive thermogenesis, which functions to maintain body temperature at ambient temperatures and is switched on in situations of increased need, such as overfeeding or upon exposure to cold.

There are three prominent environmental factors that affect thermogenesis: environmental temperature (e.g. cold-induced thermogenesis), food quantity and quality (e.g. diet-induced thermogenesis), and systemic inflammation in response to infection or tissue damage (e.g. fever) (Stock, 1999) (Rothwell, 1994). Thermogenesis is chiefly controlled by the sympathetic nervous system (SNS) (Clapham, 2011). Noradrenaline combines with the β-adrenergic receptor to induce an intracellular signal cascade that activates adenylate

cyclase at the mitochondrial level. More than half (60%) of our total energy expenditure (TEE) results from basal metabolic rate (BMR) with the remainder composed of physical activity (30-40%) and diet, or cold-induced (adaptive) thermogenesis (5-10%).

Role of Mitochondria in ATP and Heat Generation

ATP (Adenosine triphosphate) is the primary energy currency of the cell. It was explained earlier that 'hooking and unhooking that last phosphate [on ATP] is what keeps the whole world operating' (Trefil, 1992). Mitochondria are intimately involved in ATP generation through the consumption of oxygen. This occurs in the electron transport chain (ETC) within mitochondria; the final common pathway that links intermediates of carbohydrate, fat and protein metabolism to energy generation. Mitochondria are not fully efficient since not all oxygen consumption is coupled to mitochondrial ATP production, and thus some of the available free energy is lost as heat (Figure 2.2 a and b). Proton leakage through the inner mitochondrial membrane is a significant source of such uncoupling and has been estimated to account for 20–25% of the *in vivo* BMR (Rolfe & Brand, 1996).

Figure 2.2a **Figure 2.2b**

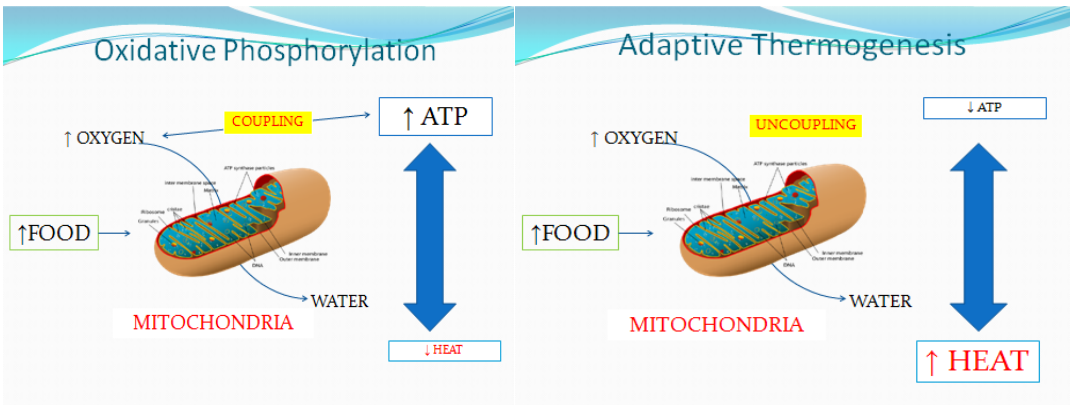

Figure 2.2 Working hypothesis of mitochondrial function in adaptive thermogenesis.

2.2a: Oxidative Phosphorylation – oxygen molecule gets coupled with ATP production and very little heat is produced.

2.2b: Adaptive thermogenesis – some of the oxygen and ATP in mitochondria is uncoupled, leading to increased heat production.

Direct calorimetry measurements are most suitable to detect the effect of uncoupling of oxidative phosphorylation on energy expenditure production. However, in this study we have used indirect calorimetry which measures oxygen use instead of heat production, and this may be a limitation to the study.

Metabolic Flexibility and Substrate Oxidation

The ability to maintain a balance between the supply and demand for energy requires the capacity to utilize lipid and carbohydrate fuels, and to transition between them. Such capacities characterizes the healthy state and are termed 'metabolic flexibility'(MF) (Kelley et al., 2002). In obese and diabetic individuals there is an inflexibility which is manifested in a range of metabolic pathways and tissues including the failure of cephalic-phase insulin secretion; failure of skeletal muscle to appropriately move between use of lipid in the fasting state and use of carbohydrate in the insulin-stimulated prandial state, and impaired transition from fatty acid efflux to storage in response to a meal (Storlien et al., 2004). When humans with impaired glucose tolerance (IGT) were challenged with insulin, they displayed reduced insulin insensitivity including reduced glucose oxidation (Kelley & Mandarino, 2000). Metabolic flexibility is impaired in obesity, insulin resistance and T2DM but whether it exists in the pre-diabetic state is still unclear. It has been reported that those with impaired fasting glucose (IFG) also exhibit reduced glucose oxidation and a slightly elevated lipid oxidation rate during insulin infusion, despite having normal total peripheral glucose disposal (Faerch & Vaag, 2011). However, more studies are needed to support this statement. Metabolic flexibility can be estimated by calculating the difference between the respiratory quotient (RQ) in the basal state, and the quotient during insulin stimulation (Kelley & Mandarino, 2000). In healthy, lean individuals, the RQ equals ~0.8 in the fasting state and rises to ~1.0 in the post-prandial or insulin-stimulated state, reflecting the switch from a predominance of lipid oxidation in the fasting state to glucose oxidation in a fed state. This ‘phenotype flexibility’ represents the absence of any inflammation or metabolic disorder predisposition (van Ommen, van der Greef, Ordovas & Daniel, 2014). Metabolic inflexibility can be tested after giving a simple single meal as glucose in an OGTT (Shaham et al., 2008), triglycerides in a fat meal (Krug et al., 2012), a protein meal (Fernandes & van de Kamer, 1968), different

combinations of glucose, triglycerides and proteins (Pellis et al., 2011; Wopereis et al., 2013), or as mixed meal challenges (Phillips et al., 2010; Van Dijk et al., 2012).

This ability to regulate fat oxidation is impaired during fasting and after a meal in a person with insulin resistance, but it could be partly reversed by weight loss (Corpeleijn et al., 2009). Weight loss improves insulin sensitivity, reduces fasting glucose, insulin and the levels of plasma free fatty acids (FFA) among obese insulin-resistant individuals, thus improving metabolic flexibility. The sustainability of such changes is doubtful and leaves scope for future research. In a study conducted in Europe, individuals with T2DM were observed to have a lower basal metabolic rate, reduced rates of hepatic glucose production and reduced lipid oxidation (Franssilla-Kallunk & Groop, 1992). It has been proposed that in the early stages of the development of T2DM, increased BMR and decreased insulin-induced thermogenesis could be seen, a possible indication of the metabolic abnormalities that are common during the development of the disease (Weyer, Bogardus & Pratley, 1999). Mitochondrial uncoupling, the process whereby substrate oxidation is uncoupled from adenosine triphosphate (ATP) production, generating heat, and resulting in increased energy expenditure. This is mainly due to high oxidative capacity of skeletal muscle (Conley, Jubrias, Amara & Marcinek, 2007), although newer evidence indicates a potential role of brown adipose tissue (BAT) in adult humans as well. BAT uncouples the respiratory chain from the production of ATP and this results in an increase in heat released (Vosselman, Vijgen, Kingma, Brans & van Marken Lichtenbelt, 2014). BAT contributes to metabolism in two ways: firstly via triglyceride clearance and secondly via activation of glucose disposal in BAT abundant organs (Bartelt et al., 2011; Nedergaard, Bengtsson & Cannon, 2011). Thus, metabolic inflexibility may be an early marker of metabolic derangement even after adjustment for body composition and insulin sensitivity.

Metabolic flexibility is dependent on metabolism in both skeletal muscle and adipose tissue. Metabolic flexibility in skeletal muscle may be affected by changes in either mitochondrial content or function (Ritov et al., 2005; Ukropcova et al., 2005). Fat cells may increase in size or number, and both may eventually lead to reduced metabolic flexibility. Thus, metabolic flexibility might be reduced with increased body fat, larger adipocytes, reduced adiponectin, increased chemokines and macrophage infiltration and impaired

suppression of lipolysis by insulin, leading to an increased supply of non-esterified free fatty acids (NEFA) (Sparksa et al., 2009).

Metabolic inflexibility is the reduced capacity of oxidative tissues and organs to maintain balance between lipid oxidation and availability, and this defect in the system results in an accumulation of lipids as triglycerides (Morino, Peterson & Shulman, 2006). Whenever there is an oversupply of any fuel in tissues and organs, anabolic pathways get activated, whereas in a shortage of fuel, hydrolytic and lipolytic pathways come into action. Mostly, genetics determine the individual variation in the balance between the storage of nutrients and oxidative capacity.

Energy balance refers to the situation when macronutrient intake balances macronutrient oxidation. This means that not only must the 24-hour energy expenditure equal the 24-hour energy intake, but the 24-hour RQ must also equal 24-hour food quotient (FQ). Several studies suggest that fasting RQ is elevated in skeletal muscle in the obese insulin-resistant state (Kelley, Goodpaster, Wing & Simoneau, 1999). Loss of muscle-specific PGC1α produces impaired mitochondrial function and a lower RQ (Handschin et al., 2007) which might not always be the case in mitochondrial dysfunction (Ukropcova et al., 2007). However, Galgani (2008) proposed that it is better to measure MF during exercise than in the resting condition, when oxidation of both fuels is maximal at the skeletal muscle level, as compared to the resting state when lipid oxidation is minimal. Skeletal muscle accounts for 55% of total body mass and is responsible for up to 80% of whole body insulin-stimulated glucose uptake in a healthy state, but is reduced to 40% in a normo-weight, insulin resistant state (DeFronzo, Gunnarsson, Björkman, Olsson & Wahren, 1985). Thus, RQ measured during exercise combined with muscle lipid and glycogen content determination may reflect the role of mitochondrial density/function on metabolic flexibility to lipids (Galgani, 2008).

Mitochondrial Dysfunction in Obesity/Insulin Resistance

Energy metabolism is compromised in obesity at the whole-body level as well as the cellular level. This can be seen through either decreased fat oxidation, increased lipid accumulation in skeletal muscle or low basal ATP concentrations. It may not always be possible to correct this defective fat oxidation after weight loss either by exercise or caloric restriction (Rogge, 2009). Mitochondrial function is impaired in insulin resistance as well as T2DM which could be due to either changes in the number and density of mitochondria (Abdul-Ghani & DeFronzo, 2008) or reduced oxidative capacity (Ritov et al., 2005; Mogensen et al., 2007) of individual mitochondria. A reduction in mitochondrial content has been observed during insulin resistance (Chomentowski, 2011; Schrauwen-Hinderling et al., 2007). It is not always seen in the fasting state but can be quite evident in the insulin stimulated state (insulin clamp or diet induced). This is because those with insulin resistance fail to increase their mitochondrial activity or oxidative phosphorylation capacity during normoglycaemic and hyperinsulaemic clamps. This leads to low levels of glucose 6 phosphate and glucose transport (Szendroedi, Phielix & Roden, 2012). On the molecular level, mitochondrial uncoupling is the result of proton leakage. The major protein involved in the process of skeletal muscle mitochondrial uncoupling is UCP-1, which is present in abundance in BAT (Ribeiro et al., 2010). Mitochondrial oxidative capacity in skeletal muscle is significantly decreased in diabetic patients (Phielix & Mensink, 2008).

Mitochondrial dysfunction causes a reduction in the capacity to transport fatty acids into the mitochondrial matrix which further reduces fat oxidation and there is an abundance of fatty acid availability in the body. Sometimes these transporters continue to supply fatty acids from triglyceride droplets in the cells even when the demand is low, leading to an accumulation of lipid, thus promoting insulin resistance. After a carbohydrate meal, there should be increased carbohydrate oxidation and reduced fat oxidation whereas consumption of a high fat meal leads to a reduced rate of both fatty acid and glucose oxidation. In a weight loss study of obese adults following a sedentary life, a decrease in weight and improved insulin sensitivity and reduced intramyocellular triacylglycerol (IMTG) accumulation were observed, but the addition of exercise training also improved aerobic capacity, enhanced mitochondrial content and activated oxidative phosphorylation activity (Toledo et al., 2008).

Exercise can decrease malonyl-coenzyme A (von Hurst, #254) production via reduced acetyl-CoA carboxylase activity in skeletal muscle and upregulate fat oxidation in leg muscle mitochondria (Roepstorff et al., 2006). Furthermore, fatty acid partitioning may vary among fatty acids (Gaster, Rustan & Beck-Nielse, 2005). Intake of unsaturated fatty acids favours less lipid accumulation in the muscle, thus improving metabolic flexibility.

To summarize, increasing mitochondrial uncoupling in skeletal muscle to stimulate adaptive thermogenesis can be targeted for weight loss and to improve insulin sensitivity. This has been proposed in earlier studies in individuals with type 2 diabetes (Van-Den-Berg, Berga, van Marken Lichtenbelt, van Dijka K.W. & Schrauwen, 2011). Therefore, lifestyle changes such as a lowering of dietary fat intake, an increase in physical activity and weight loss may partly improve metabolic flexibility in skeletal muscle, and contribute to the prevention of type-2 diabetes (Corpeleijn et al., 2009).

2.2 Energy Metabolism and Temperature

2.2.1 Thermoregulation

The human body will maintain homeothermy for its survival. This ability to regulate body temperature allows people to reside in both polar regions and warmer climates. Usually, thermoregulation is maintained by either loss of heat through 'sweating' or conservation of heat via 'vasoconstriction'. However, in recent times the extent to which we are exposed to large fluctuations in environmental temperature has decreased due to widespread use of climate control devices in our work and home environments.

The mechanisms by which we modify the core body temperature are mediated by alterations in energy expenditure, skin blood flow or by sweat and heat loss. Generally, our body tries to maintain its temperature at around 37 degrees centigrade with slight individual variation (Silva, 2006). Ideally, heat balance is maintained with appropriate energy balance.

Energy balance refers to the state where energy intake equals energy output. Prolonged periods when energy intake is greater than energy output can result in obesity and many other related metabolic conditions.

2.2.2 Resting Metabolic Rate: Definition and Measurement

Resting Metabolic Rate (RMR) is the minimum amount of energy required to maintain vital functions without any physical movement, measured while awake and in a thermoneutral environment. RMR is the largest component (almost 60%) of total daily energy expenditure. It is a repeatable and valid measure of energy production and closely parallels the measures of mitochondrial function (Lars en et al., 2011). Indirect calorimetry is one of the best methods to measure energy expenditure and is done by measuring the amount of oxygen consumed and carbon dioxide expired (Ferrannini, 1988). Metabolic rate is largely determined by the metabolic requirement of the organ tissue mass, including the liver, brain, heart, etc. (Soares & Shetty, 1992). Metabolic rate is dependent on mitochondrial function as well as the involvement of the thyroid and sympathetic nervous systems (Clapham, 2011). Other factors influencing BMR include age (Geisler, 2011; Van Pelt, Dinneno & Seals, 2001), gender (Blaak, 2001), physical activity (Speakman & Selman, 2003), fat mass (FM) and fat free mass (FFM) (Muller, Bosy-Westphal, Later, Haas & Heller, 2009). There are few novel predictors of RMR like vitamin D status (Calton, 2016 #130) and temperature gradient (Pathak, Calton et al., 2017) that respond to the metabolic health of individuals.

2.2.3 Thermoneutral Zone

The thermoeutral zone (TNZ) is a range of ambient temperatures where the core body temperature can be maintained through modulation of non-evaporative heat loss and without compensatory changes in RMR (Claessens-van Ooijen, 2008). In the thermoneutral zone the transfer of heat from the core to the skin balances the heat transfer from skin to the environment (Kingma, Frijns, Schellen & van Marken Lichtenbelt, 2014). When temperatures fall below the lower limit of the TNZ, energy expenditure increases and

together with increased peripheral vasoconstriction will counter-balance the changes to core body temperature. Such changes are seen in the non-shivering response on exposure to a mild cold temperature (Calton, Soares et al., 2016; Landsberg, 1984; Roth & Sheard, 1948). Similarly, at higher temperatures above the TNZ, RMR will also rise and be accompanied by evaporative heat loss (Kingma et al., 2014; Roth & Sheard, 1948). Determining whether a participant is in the TNZ is practically very difficult, and the literature rarely provides information on compliance to TNZ conditions (Nsatimba et al., 2016). People who live in the tropics or in warmer climates have lower BMR as compared to their temperate counterparts (Hayter & Henry, 1993). The term adaptation refers to pheno-typic or genotypic changes that reduce the physiologic stress produced by cold exposure (Adolph, 1956, 1964). Thus, it can be said that TNZ can be different depending on place of residence (Hayter & Henry, 1993), age, gender (Kingma, Frijns & van Marken Lichtenbelt, 2012) and obesity (Pathak, Calton et al., 2017).

2.2.4 Core Body Temperature and Thermoregulation

Our body temperature varies during the day-night cycle following our circadian rhythm (Li et al., 2013; Van Someren, Raymann, Scherder, Daanen & Swaab, 2002), in a fasting or fed state, and related hormonal changes (MacDonald, Bennett, Gale & Hilary Green, 1982). In an early study, some authors postulated that for each degree increase in temperature during fever, the energy expenditure is raised by 13% (Jéquier & Schutz, 1981). Obese subjects have predominantly lower body temperatures and thus reduced metabolic rates. During weight loss, energy intake is reduced which causes a decrease in core body temperature and consequently BMR (Landsberg, 2012). Upon cold exposure, body temperature is reduced, then adaptive thermogenesis is turned on and the energy expenditure is increased to maintain homeothermy. Conversely, adaptation to cold might also be determined by RMR as suggested by Maeda et al. (2007), in which individuals with higher RMR were able to reduce heat loss upon cold exposure (adiabatic-type adaptation) much better than those with lower RMRs (calorigenic-type adaptation) which used peripheral vasoconstriction.

2.2.5 Other Factors Influencing Metabolic Rate

There are many possible explanations for these variations in metabolic rates including the role of the sympathetic nervous system (Clapham, 2011) or thyroid gland (Landsberg, 1984). Obese individuals have increased energy expenditure due to increased heat generation requirements given their larger surface area. It has been suggested that low SNS activity can be a risk factor for the development of obesity (Speakman & Selman, 2003) whereas other studies have shown that SNS activity is raised in obesity (Lambert, Straznicky, Lambert, Dixon & Schlaich, 2010). More recently, it has been suggested that energy metabolism may be influenced by the presence of metabolic syndrome in different ethnic groups as concluded in a large cohort of ethnically diverse US and Taiwanese population groups. In their anatomical-physiological model of impaired energy regulation (IER), measures of waist circumference, fasting glucose and triglyceride concentrations formed a homogenous cluster across all ethnic groups and determined the greatest variance in MetS (Wahlqvist et al., 2010).

2.2.6 Adaptive Thermogenesis

As discussed previously, thermogenesis is of two types. Obligatory thermogenesis is the energetic cost of digestion, absorption and transportation of food, while adaptive thermogenesis is switched on in situations of increased energy demand when the body needs to conserve heat such as following cold exposure or to dissipate heat following overfeeding. Adaptive thermogenesis accounts for about 10% of total energy expenditure. Heat is generated by chemical reactions that address a physiological need, most notably cold exposure (non-shivering thermogenesis, NST) or dietary intake (diet-induced thermogenesis, DIT). The processes that generate heat in these situations are regulated by the SNS. Existence during long term cold exposure is maintained via elevated sympathetic activity (Landsberg, Saville & Young, 1984; Leduc, 1961), with some deterioration in the degree of SNS activity with time (Leduc, 1961). This increase in sympathetic activity during cold exposure is evident predominantly in the heart, pancreas, lung, spleen, skeletal muscle, skin and brown adipose tissue (BAT), while either no change or even suppression is observed in the submaxillary gland, liver, intestine and kidneys (Walther, Iriki & Pflugers, 1970; Young & Landsberg, 1979a;

Young, Saville, Rothwell, Stock & Landsberg, 1982). Thyroid hormones play a permissive role in BAT cell membranes and may potentiate thermogenic response to noradrenaline (Landsberg et al., 1984). Adaptive thermogenesis is mainly mediated by uncoupling protein-1 (UCP-1) that activates BAT and increases energy expenditure. But not all adult humans have sufficient BAT to upregulate their metabolic rates. This is the reason why the same weight-loss regime may be effective on one person but not on another.

Non-shivering Thermogenesis

Non-shivering thermogenesis (NST) is a mechanism whereby heat is produced using chemical energy and SNS without the involvement of muscle. Thus, basal energy metabolism in the post absorptive state and at thermoneutral conditions is mostly due to NST. This obligatory NST acts to maintain the vital functions in the body to maintain homeothermy. At cold temperatures, the excess heat production is termed regulatory NST where it may involve diet induced thermogenesis (DIT) which is also termed as the thermic effect of food (Payne, Steck, George & Steffens). Figure 2.3 below describes the involvement of obligatory and regulatory NST in thermogenesis on cold exposure.

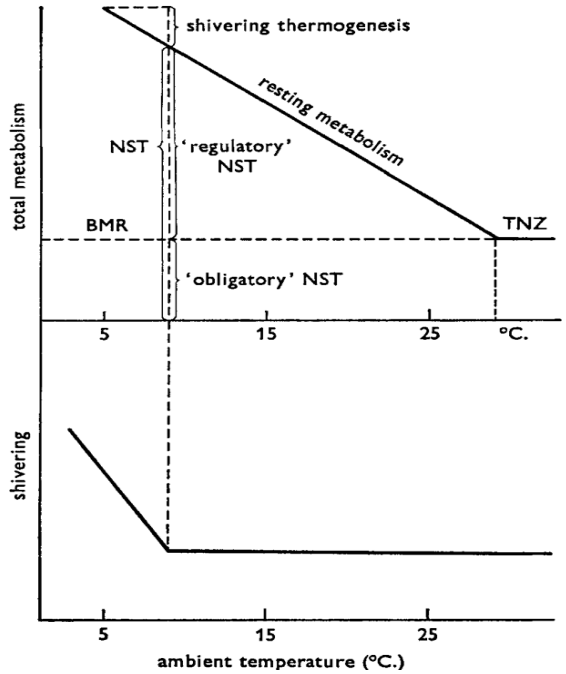

Figure 2.3 Obligatory and regulatory NST during cold exposure.

Figure obtained from (JANSKý, 1973), with permission (Appendix C).

Diet-induced Thermogenesis (DIT)

Metabolic rates can rise by as much as 15-30% post-meal depending on the meal composition (Garrow, Blaza, Warwick & Ashwell, 1980). Consumption of food raises the body temperature and hence increases the energy expenditure. DIT may also vary with seasons. A study in Japan found that DIT is reduced from autumn to winter when the mean outdoor temperatures changed from 22°C in autumn to 16°C in winter (Tobe, 2004). RMR is higher at mild cold temperatures along with increased heat production. In contrast, there is partial contribution of cold-induced and diet-induced thermogenesis following post-meal (Dauncey, 1981). Both NST and DIT are mediated by the sympathetic nervous system and they produce heat. While during DIT heat is produced and this energy is distributed by the body to prevent fat accumulation. On the other hand, NST conserves heat to maintain the core body temperature (van Marken Lichtenbelt & Schrauwen, 2011).

2.2.7 Cold Exposure and Adaptive Thermogenesis: Role of BAT

Mild cold exposure has re-emerged as a therapeutic target for the prevention and treatment of obesity and related metabolic disorders. This has stemmed from several recent observations that BAT is present and measurable in adult humans (Celi et al., 2010; Cannon & Nedergaard, 2004). Using innovative positron emission tomography-computed tomography (PET/CT) scans, biopsies, magnetic resonance imaging (MRI) (Branca et al., 2013; Chondronikola et al., 2016; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009; Vosselman et al., 2014) and other techniques, investigators have shown that BAT most commonly resides in the supra-clavicular, visceral and subcutaneous sites (Sacks & Symonds, 2013).

Brown adipose tissue (BAT) is present in many animal species and is sometimes referred to as the thermogenic organ. BAT has large numbers of mitochondria and it is because of this that it appears brown in colour. For a long time it was believed that BAT was only present in newborns and infants, and not in adult humans, hence its role in thermogenesis was debated.

The key aspect of BAT is that it has high amounts of mitochondria with more uncoupling proteins and a rich sympathetic nerve innervation (Cannon & Nedergaard, 2004) leading to an uncoupling of oxidative phosphorylation and ATP production during cold exposure. Current PET-CT scans estimate the BAT mass in humans to total approximately 13 g (0.02% of body weight for a person weighing 70 kg) (Cypess et al., 2009) whereas the mouse BAT mass is approximately 400 mg for 40 g body weight (1%) (Geisler, 2011). While BAT can account for 25% of fat mass in human infants (Lean, 1989), it was thought to contribute little (i.e. only 0.2%) to thermogenesis in adults (Jung, Leslie, Nicholls, Cunningham & Isles, 1988).

Cold exposure increases SNS activity that leads to BAT activation. In turn, there is greater thermogenesis and thus increased energy expenditure (Celi et al., 2010). This can be evidenced in lean, obese, insulin-sensitive and insulin-resistant individuals. Although, in some of the obese and insulin-resistant individuals the effects might be blunted (Wijers et al., 2010). The thermal comfort range for a naked man is 25-27°C (Juhani-Leppaluoto, 2009). It must be remembered that for most people who live an indoor life in temperature-controlled rooms (i.e. work in offices, sleep and rest at home), the indicated temperatures are only slightly below and above the usual comfortable set points (Kingma et al., 2014).

Canon & Nethergard (2010) suggest that acute cold exposure does not necessarily demonstrate non-shivering thermogenesis (NST) capacity, rather cold tolerance only. It validates the contribution of BAT to total thermogenesis by increasing carbohydrate and lipid utilisation through uncoupling protein-1 (UCP-1) in skeletal muscle. Elevation in RMR may sometimes be triggered to enable survival at lower temperatures and to defend the body temperature even in the absence of BAT. Thus, metabolic rate may be increased due to BAT thermogenesis (Frank, Raja, Bulcao & Goldstein, 2000). Frank et al. (2000) propose that the thermal threshold to cold, perception of thermal comfort and response intensity to vasoconstriction varies with ageing. At the same comfort level, the cold threshold capacity and vasoconstriction may vary between young and older adults.

Data generated in rodents and humans suggest that increasing adaptive thermogenesis by increasing skeletal muscle mitochondrial uncoupling indeed elevates the total energy expenditure (Szendroedi et al., 2012).

There are many studies on cold-induced thermogenesis with variable use of temperature range including varying methods for its determination. In general, studies that have used a cold temperature to stimulate thermogenesis have employed temperatures of 18°C and 20°C with a comparison temperature of about 5-6 degrees higher on the same day within a few hours (Blondin et al., 2015; Muzik, Mangner & Granneman, 2012; Vosselman et al., 2014), longer hours (Wijers et al., 2010) or 2 to 3 separate days (Calton, Soares et al., 2016; Celi et al., 2010; Westerterp-Plantenga, van Marken Lichtenbelt, Strobbe & Schrauwen, 2002) (Table 2.3). Other studies have experimented with a much longer exposure of up to one month (Lee, Smith et al., 2014) for acclimation. A few studies have also used cold water or the placement of subjects' feet in ice (Lee, Linderman et al., 2014; Yoneshiro & Saito, 2015), thermal suits (Haman, Pe'rronet & Kenny, 2002) or thermal blankets (Lee, Linderman et al., 2014) (Table 2.3), whereas others have used a whole body calorimeter or a temperature-controlled chamber (Calton, Soares et al., 2016; Celi et al., 2010; Maeda et al., 2007). Some studies have further examined the effect of cooling and rewarming on energy expenditure (EE) and BAT activity (van Marken Lichtenbelt et al., 2009; Van Ooijena, van Marken Lichtenbelt, van Steenhovenb & K.R, 2004). Most of these have used minimal clothing to avoid any thermal effect (Table 2.3).

In a long-term exposure study, healthy subjects were exposed to a mildly cold environment (15 – 16 °C) for up to 6 hours a day, for 10 consecutive days. The group showed a significant increase in upper body BAT activity with 2.4 ± 0.7 standard uptake value (SUV) as the mean before cold acclimation versus 2.8 ± 0.5 SUV mean after cold acclimation, ($P < 0.01$) in parallel with increased energy expenditure (10.8% ± 7.5% before cold acclimation versus 17.8% ± 11.1% after cold acclimation, ($P < 0.05$)). Furthermore, cold tolerance seemed to increase in these subjects during the cold acclimation period, as subjects tended to report more comfort and less shivering towards the end of the cold acclimation period (van der Lans et al., 2013). In a second similar study, healthy adults were subjected to a daily 2-hour cold exposure at 17°C for 6 weeks. This cold acclimation protocol resulted in an increase in BAT activity from 2.46 ± 0.40 to 3.89 ± 0.64 SUV ($P < 0.01$) accompanied with a significant increase in EE (Yoneshiro & Saito, 2015). Thus, the longer the duration of exposure, the greater the energy expenditure and BAT activity. Recruitment of BAT did not differ in acute cold exposures (Lee, Linderman et al., 2014; Vosselman, Brans & van der Lans, 2013) and the energy expenditure was increased (Celi et al., 2010; Muzik et al., 2012) with involvement of NEFA (Blondin et al., 2015) and hormone such as Irisin (Lee, Linderman et al., 2014). Cold exposure also altered

the fuel utilization (Lee, Linderman et al., 2014; Calton, Soares et al., 2016; van Marken Lichtenbelt et al., 2009). Post-meal cold-induced thermogenesis (CIT) had the additional effect of DIT (Westerterp-Plantenga et al., 2002).

While exposure to mild cold elevated energy expenditure with BAT volume and activity in all studies, lean participants demonstrated a larger increase in BAT activity (van Marken Lichtenbelt et al., 2009) as compared to obese participants whose response was mostly blunted (Wijers et al., 2010). Body fat that accumulates in response to increasing age diminishes the amount of BAT and therefore CIT (Yoneshiro & Saito, 2015). Activation of BAT is possible via various endogenous pathways such as SNS, thyroid hormones, fibronectin type 3 containing 5 (FNDC5)/Irisin and Fibroblast growth factors 21 (FGF21) as well as insulin, cardiac natriuretic peptides (ANP/BNP), bile acids, vascular endothelial growth factor and retinoic acids (Broeders, Bouvy & van Marken Lichtenbelt, 2015). Thermogenesis is driven by stimulations caused by shivering, heat production, thyroid hormones release or cardiovascular responses to heat (Clapham, 2011).

A recent study on mice (Carrière et al., 2014) identified 'lactate' as an intermediate in the conversion of white adipose tissue (WAT) to BAT. Lactate is produced in high amounts during intense exercise levels. Similarly, many other hormones such as irisin and FgF21 also have the capacity to convert WAT to BAT. Short-term exposure to mild cold does not necessarily involve increased BAT activity. There has to be long term exposure to mild cold if we aim to achieve improved BAT activity in individuals. This statement could be explained by a study done on Iceman (who has had frequent exposures to acute mild cold) and his monozygotic twin (no previous exposure to mild cold). A shift from 31°C (45 min) to 12°C (2.5 hours) demonstrated similar changes to increased BMR, peripheral vasoconstriction, CIT and BAT activity, thus explaining why short term exposures do not recruit BAT. Higher thermal comfort and a smaller decrease in core body temperature in Iceman indicated his better capacity to maintain body temperature at mild cold conditions (Vosselman et al., 2014).

It has also been opined that those who have lower BMR respond to cold temperature to a greater extent than those with already raised BMR to maintain core body temperature (Maeda et al., 2007). Since they do not have sufficient BAT, they might choose to increase

vasoconstriction over increasing energy expenditure. This switch may also depend on seasons (Table 2.3) (Van Ooijena et al., 2004).

The following Table (2.1) includes RCTs which serve to demonstrate the effect of acute and long term cold exposure on human energy expenditure and substrate utilisation using different techniques for exposure to cold.

Table 2.3 Relevant Studies on Cold-induced Thermogenesis In Humans

Author, Year	Study Location	Subjects	Clothing	Methods of Cold-induced Thermogenesis	Outcomes
(Calton, Soares et al., 2016)	Australia	22 overweight adults	Clothing worn by participants in the chamber was standardized to within 0.5 clo. All participants were provided with a gown (0.46 clo), females wore their own bra and underwear (0.04 clo) and males wore their own briefs (0.04 clo)*	90 minutes of exposure to 20 °C and 25 °C on two separate days	3.1% increase in RMR at 20 °C, significant vasoconstriction at 20 °C and reduce in ear temperature. Fold change in irisin related to change in RQ after adjustments
(Blondin et al., 2015)	Canada	12 healthy males	Shorts only	120 min baseline period at ambient temperature (25°C) followed by 180 min of exposure to a mild cold, elicited using a liquid-conditioned suit perfused with water at 18°C using a temperature- and flow-controlled circulation bath	Cold-induced increase in NEFA and BAT volume activity were related. BAT glucose uptake only in supraclavicular area but net BAT glucose uptake was not related with BAT oxidative mechanism. Skeletal muscle involved in glucose uptake during cold exposure and whole-body EE
(Vosselman et al., 2014)	Netherlands	Monozygotic twins, A with frequent cold exposures, B with no previous cold exposure	Shorts only (clo 0.06)	Baseline 45 min at TN conditions (31 °C) followed by 2.5 h of mild cold (13 °C) exposure in a temperature controlled chamber	No BAT activity in both subjects at TN condition, presence of BAT greater in Subject B with less previous cold exposure. Both reduced skin and core temperature and displayed vasoconstriction at cold, EE increased in both

Lee et al., 2014	USA	10 healthy adults	Hospital scrubs	Room temperature was maintained at 24 °C. Water infused thermal blankets used to modulate temperature rapidly. 30 min resting RR measured at 27 °C followed by 18 °C and then then dropped after each 2 °C each 3 min until it reached 12 °C when it was measured for 5 min	EE increased with decreasing temperature. RQ highest at 18 °C and lowered at low temperature as well high temperature (27 °C). FGF21 dropped at cold directing shivering. Irisin higher in those who shivered
(Lee, Linderman et al., 2014)	USA	5 healthy men	Standardized hospital clothing with a combined thermal insulation value of 0.4 (clo)	4 consecutive blocks of 1 month each [24°C (month 1) > 19°C (month 2) > 24°C (month 3)> 27°C (month 4)	BAT active at cold temperature, no change in CIT, DIT improved at 19 °C, IS improved in cold
(van der Lans et al., 2013)	Netherlands	Female	Shorts and T-shirt	15-16 °C for 6 h for 10 days	BAT activity an EE increase more after cold acclimation
Muzik, 2013	Michigan	25 healthy adults		30 min rest at 25 °C followed by cold stress at 15.5 °C with 2 fans for low level air flow in a room	BAT recruited, EE increased and more in high BAT Gp
(Yoneshiro & Saito, 2015)	Japan	162 healthy adults	T-shirt with underwear	Room set at 19 °C. Feet applied on ice block for 4 min every 5 min. 1 h later PET scan performed at 24 °C for 30 min	Body fat associated with age was related to BAT amount. CIT induced BAT reduced with age

(Celi et al., 2010)	USA	18-60 year-old healthy adults, BMI> 27kg/m^2	Normal hospital scrub	Whole room indirect calorimeter at 24 °C and 19 °C for 12 hours each on 2 different days with a recovery period of 36 h	Increase EE
(Wijers et al., 2010)	Netherlands	10 lean and 10 obese	Standardized (0.8 clo), subjects received a standard set consisting of a pair of socks (0.02 clo), a shirt (0.09 clo), sweatpants (0.28 clo) and a sweater (0.37 clo). Subjects wore their own underwear (about 0.04 clo), At night, subjects slept under a duvet (7.0 clo).	36 h at 22 ººC followed by 16 °C for next 12 h in a respiration chamber	EE increase in lean and blunted on obese, lean more insulin sensitive
(van Marken Lichtenbelt et al., 2009)	Netherlands	24 healthy men	Standardized clothing (0.49 clo)	Thermoneutral conditions (22 °C) for 1 hour and were then exposed to mild cold (16 °C) for 2 hours. In a climate chamber	BAT activity increase in cold for all but more in non-obese men, EE increased significantly in cold, increased fat oxidation, reduced skin temperature and vasoconstriction at cold temperature with no differences in lean and obese except vasoconstriction higher in obese
(Wijers, Schrauwen, Saris & van Marken Lichtenbelt, 2008)	Netherlands	11 lean male	Standardized (0.8 clo): subjects received a standard set consisting of a pair of socks (0.02 clo), a shirt (0.09 clo), sweatpants (0.28 clo) and a sweater (0.37 clo). Subjects wore their own underwear (about 0.04 clo). At night, subjects slept under a duvet (7.0 clo).	34 h at 22 ° C, 82 h at 16 ° C on 2 separate occasions in respiration chamber	Increased EE via mitochondrial uncoupling

(Maeda et al., 2007)	Japan	10 healthy males	Short-sleeved cotton T-shirt and cotton short pants (0.3 clo)	28° C for 60 min and 10 ° C for following 90 min	Individuals with low BMR respond to cold exposure more than with high BMR as they vasoconstricted
(Claessens-van Ooijen et al., 2006)	Netherlands	10 obese and 10 lean men	0.71 clo (Icl = 0.109 m2. °C/W), consisting of sweatpants (0.28 clo), a sweater (0.37 clo), socks that covered only the feet (0.02 clo), and briefs (0.04 clo).	After 1 h of TNZ (under duvet, 375 g/m^2) exposed to cold air (15 °C) for 1 h followed by 1 h of rewarming under duvet	EE increase was larger among lean than obese in the cold
(Van Ooijena et al., 2004)	Netherlands	10 women and 10 men	Standard clothing with an insulative value of 0.71 clo (Icl = 0.109 m2_j C/W), consisting of sweatpants (0.28 clo), a sweater (0.37 clo), socks that cover only the feet (0.02 clo) and panties and a bra for women and briefs for men (0.04 clo).	22 °C for 1 h and 16 ° C for following 3 hrs in climatic chamber, repeated in summer and winter	Three hours of cold exposure revealed an increase in MR of 7.0% in summer and 11.5% in winter. Greater increase in EE and reduced skin temperature in winter
(Westerterp-Plantenga et al., 2002)	Netherlands	9 healthy male	T-shirt, cotton shirt, one jogging-shirt (70% cotton, 30% polyester), one pair of jogging trousers (50% cotton, 50% polyester) and a pair of sport shoes during the day (insulation 1.2 clo (ISO 9920, 1995). At night, one T-shirt and boxer-shorts, used a cotton sheet and a duvet (375 g/m^2)	60 h at 22 °C once and twice at 16 °C in respiration chamber	Increase in sleeping metabolic rate (SMR), DIT. Proximal and distal temperature decrease, no change in RQ

(Westerterp-Plantenga et al., 2002)	Netherlands		Subjects were required to wear the same outfit three times: underwear, bermuda shorts, two T-shirts and a pair of sports shoes during the day (insulation 0.6 clo [35]). At night, subjects wore one T-shirt and they slept under a cotton sheet and a light duvet (375 g/m2)	Once at 22 °C and twice at 27 °C for 48 h each in climate chamber	EE at 27 ºC was less due to reduced DIT & AEE (activity-induced energy expenditure), RQ and skin temperature increased
(Haman et al., 2002)	Canada	6 heathy and trained men	Liquid-conditioned suit	Suit temperature at 10 °C for 2 h	Increased heat production by 2.6-fold
(Dauncey, 1981)	Cambridge	9 females	Thin cotton trouser-suit	30 h at 22 ºº C and 28 °C on two occasions in whole body calorimeter	EE increased at lower temperature. No difference post-meal. DIT partially replaced CIT

RMR, resting metabolic rate; RQ, respiratory quotient; NEFA, non-esterified fatty acid; BAT, brown adipose tissue; EE, energy expenditure; TN, thermoneutral; FGF21, fibroblast factor 21; CIT, cold-induced thermogenesis; Gp, group, Y, years; DIT, diet induced thermogenesis; AEE, activity induced energy expenditure.

*1 Clo = 0.155 m^2K/W, Clo = 0 corresponds to a naked person.

2.2.8 Obesity and Thermogenesis: Effect of Mild Cold

Vital organs like the brain, heart, liver and kidneys comprise only 10% or less of our total body weight but contribute over 60% of BMR where skeletal muscle representing 35-40% of body weight contribute to only 20-25% (Dulloo, Jacquet, Montani & Schutz, 2012). This concept explains why a certain phenotype is more susceptible to obesity as is obvious in ethnic studies and even a small change in organ mass can create large variations in BMR. Additionally, a large inter-individual variation (around 26%) in fat free mass is also a causative factor affecting RMR (Johnstone, Murison, Duncan, Rance & Speakman, 2005).

Obese individuals might have increased energy expenditure over their counterparts due to their large surface area. But they might have impaired, normal or activated sympathetic nervous response (Young & Macdonald, 1992). It is possible that in a same situation, different tissues and organs in a body may be dissimilar in their response to sympathetic drive (Clapham, 2011).

The results from two studies comparing heat regulation systems in lean vs obese participants showed that upon cold exposure, heat is dissipated via peripheral extremities (fingertips, in this case) and conserved in the abdominal region via insulative layers in obese individuals. This helps to maintain normal body temperature but a contrast is observed among lean individuals who had high temperatures along abdominal regions as compared to fingertips. These temperatures were measured using infrared thermography (Chudecka & Lubkowska, 2016; Savastano et al., 2009). Although metabolic heat production is higher in obese individuals, their skin temperature is low due to the presence of abdominal adiposity and larger FFM which serve to reduce their capacity to respond to thermoregulation and increased vasoconstriction on mild cold exposure. CIT is blunted in overweight/obese individuals and eventually produces differences in insulative cold response when compared with the lean group (Claessens-van Ooijen et al., 2006).

Obesity is the main feature of metabolic syndrome. In a study examining obese participants with and without MetS, the obese group with MetS had significantly lower resting metabolic rate than those without (6472 ± 197 vs. 7205 ± 193 kJ/d; P<0.01). Moreover, inclusion of T2DM subjects with the obese MetS + group did not make a difference (Buschemi, 2007). Interestingly, after weight loss, those who recover from MetS have been reported to have approximately 250 kJ/d lower RMR than those who never had MetS (Soares, Cummings et al., 2011). Recruitment of BAT may not only prevent obesity but may also improve MetS due to its triglyceride clearance capacity (Bartelt et al., 2011).

2.2.9 Cold Exposure and Insulin Resistance

It has been well-documented that metabolic rates increase with insulin resistance and presence of T2DM (Bogardus, 1996; Nair et al., 2008). However, these individuals have low thermic response to food (Robinson et al., 1994) and insulin-induced thermogenesis (IIT) (Ravussin & Zawadzki, 1987) as compared to normoglycemic individuals. It has been proposed that these changes occur in the early development stages of T2DM with reduced glucose disposal rate (Weyer et al., 1999). Cold exposure, whether acute or chronic, may help in improving insulin sensitivity (IS). In support, acute cold exposure increased the oxidation of plasma glucose from 39.4 ± 2.4 to 93.9 ± 5.5 mg/min (~138%), of muscle glycogen from 126.6 ± 7.8 to 264.2± 36.9 mg glucosyl units/min (~109%), and of lipids from 46.9 ± 3.2 to 176.5 ± 17.3 mg/min (~376%) (Haman et al., 2002). A longer-term exposure to cold acclimation of one month improved the insulin sensitivity along with energy expenditure (Lee, Smith et al., 2014).

2.2.10 Cold Exposure, Thermogenesis and Vascular Responses

Determining whether a participant is in the TNZ is practically very difficult. One suggested way forward is to monitor peripheral skin temperature gradients (such as between the forearm to fingertip of one hand exposed to the environment) or monitor finger digit blood

flow through Doppler or thermography. Within the TNZ it would hence be expected that there should be no net vasoconstriction or vasodilation (Rubinstein & Sessler, 1990). However, 0°C represents initiation of thermoregulatory vasoconstriction when blood flow starts to decrease as determined by Plethysmography and represents fingertip perfusion (Sessler, 2003). On exposure to cold, there is reduced blood flow due to vasoconstriction. This hinders heat transfer from the core to the skin, followed by decreased heat loss from the skin. This is called an insulative vasomotor response. In the instance of a metabolic response, heat is produced and this results in increased RMR. Greater insulative vasomotor response and low metabolic response create a risk for potential weight gain. The layer of adipose tissue around the abdominal region acts as insulation for heat loss at low temperatures.

Thermoregulatory response is equally shared by core and skin temperatures to provide thermal comfort. Skin temperature changes precede any variation in core temperature in response to cold or food. On comparing the temperature in the trunk region between lean and obese participants, obese subjects had significantly lower abdominal temperature due to the presence of fat that acts as insulation for that region. Animal studies have shown lower temperatures at the preoptic area which then exhibits cold-induced thermogenesis by increasing metabolic activity in brown adipose tissue (Boulant & Gonzalez, 1977) and increased levels of thyroxine (Dauncey, 1981; Vybiral, Lesna, Jansky & Zeman, 2000), catecholamine and glucocorticoids (Gale, Jobin, Proppe, Notter & Fox, 1970). Human studies also show an increase in metabolic activity in response to cooling (Tikuisis, Bell & Jacobs, 1985). Cold-induced thermogenesis can be influenced by two pathways, namely the sympathetic nervous system and the hypothalamic-pituitary-thyroid axis (Lowell & Spiegelman, 2000).

2.2.11 Role of Irisin in Thermogenesis

Irisin is a myokine that is released in response to Peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) activation upon an acute increased physical activity (Huh et al., 2012; Löffler et al., 2015). Irisin, as currently understood, is capable of converting

white adipose tissue to BAT along with influencing glucose and energy homeostasis as seen in animal studies (Bostrom, Wu & Jedrychowski, 2012) via activation of SNS, heart and skeletal muscle (Sanchez-Delgado et al., 2015). Lower levels of irisin in circulation have been associated with T2DM, obesity and MetS in some studies (Yan et al., 2014) whereas other studies report that irisin levels increase with BMI, occurrence of diabetes, MetS (Park et al., 2013), increasing fat mass. They might decrease with age due to reduced muscle mass (Huh et al., 2012). More studies are needed for consistent conclusions. When considering the difference in the amounts of BAT in both species, Irving et al. (Irving, Still & Argyropoulos, 2014) suggest that one needs to be extra careful in interpreting animal study data for human irisin assessments. However, Choi et al. (Choi et al., 2014) reported no difference in serum irisin levels in BAT negative and BAT positive groups. Klangjareonchai et al. (2014) in their study on adults with prediabetes came up with a strong association between BMI and irisin levels among males, irrespective of age. However, no significant relation was found among females. Fasting insulin, HbA1c, waist circumference and serum A/G ratio have been reported to be negatively associated with serum irisin after adjusting for covariates. Thus, low irisin levels can be possibly linked with obesity, MetS, CVD, and insulin resistance.

Both exercise (Huh et al., 2012) and cold (Lee, Linderman et al., 2014) are understood to increase irisin levels in animal as well as human studies (Celi et al., 2010; Lee, Linderman et al., 2014) although some studies suggest neither resistance training, endurance training (Timmons, Baar, Davidsen & Atherton, 2012), aerobic interval training or strength training (Raschke et al., 2013) increase the expression of FNDC5 (Fibronectin type III domain-containing protein 5, a precursor of irisin). Going further, another RCT has shown no effect on increasing irisin levels after 26 weeks of resistance and endurance training. There are a few evidences to indicate that the shivering response to cold varies directly with irisin concentrations (Celi et al., 2010; Lee, Linderman et al., 2014). Following weight loss, there was a decrease in circulating irisin levels which is reversible with a possible weight regain (Crujeiras et al., 2013). A direct association between irisin levels and fat oxidation have been described recently (Calton, Soares et al., 2016). Also, doubts have been expressed over the validity of all previous data obtained from currently-available ELISA kits for circulating irisin levels in blood(Albrecht et al., 2015). They argue that all antibodies had prominent cross-reactions with non-irisin proteins in serum or plasma of different species, both animals and humans. These arguments have however been contraindicated and the validity of irisin assays have been defended in an editorial by Polyzos & Mantzoros (2015). In a study on obese

subjects with MetS, those with high baseline irisin levels showed greater reductions in glucose (P=0.022) and insulin (P=0.021) levels, following an 8-week weight loss intervention trial (Lopez-Legarrea et al., 2014).

In a study by Lee et al. (2014) the temperature was reduced gradually from 27º to 12ºC in just one hour and it was observed that circulating levels of both irisin and fgf21 increased on exposure to cold where the former is expressed in a shivering state and the latter in NST. However, the results can be different if measured on two separate days as the body gets acclimatised to the temperature in latter, and these hormones usually react faster to a sudden change of thermal states.

2.2.12 Fibroblast Growth Factor 21 (FGF21) as Metabolic Thermoregulator

FGF21 is one of a family of peptides that have pleiotropic effects as metabolic regulators. FGF21 controls glucose and lipid homeostasis in the liver. FGF21 can be a potential therapeutic factor for correcting dyslipidaemia and glucose levels (Dostálová, Haluzíková & Haluzík, 2009) as it increases heat production through NST and reduces body weight in animal models (Kharitonenkov et al., 2005). Even after 24 hours of administration in mice FGF21 plays a key role in converting white adipose tissue to BAT upon chronic exposure to cold (Fisher et al., 2012). Results from another animal study have shown that FGF21 is capable of correcting hyperglycaemia in diabetic mice by increasing glucose uptake in brown fat, converting WAT into BAT and increasing energy expenditure via BAT activation (Emanuelli et al., 2014).

Serum FGF21 levels can potentially be a biomarker for the presence of metabolic syndrome. It has been established that FGF21 increases in MetS (Zhang, 2008b) and other related conditions such as obesity, IGT and a raised lipid profile (Iglesias, Selgas, Romero & Dı´ez, 2012). This was supported in a follow-up study over 3 years where the authors confirmed that FGF21 can be a strong independent predictor of metabolic syndrome and T2DM. They reported circulating FGF21 was significantly related to BMI, WHR, blood pressure, triglycerides, fasting and post-prandial glucose, most of which are also predictors of MetS

(Bobbert et al., 2013). In a weight loss study on children, FGF21 was significantly reduced after following either a low carbohydrate or low fat diet for 2 months (Ibarra-Reynoso, Pisarchyk, Pérez-Luque, Garay-Sevilla & Malacara, 2015). Increased levels of FGF21 are negatively correlated with high density lipid cholesterol (HDLc) and adiponectin in MetS-positive dyslipidemic individuals (Novotny et al., 2014). In a cross-sectional study in Japan, increased levels of FGF21 was associated with raised triglyceride concentration, systolic and diastolic BP and higher BMI (Jin et al., 2014).

Activation of the sympathetic nervous system drives the browning of adipose tissue and this may be mediated by irisin and FGF21. Basal levels are not affected by sympathetic activity although acute sympathetic activation can increase, while sprint interval training can decrease, circulating levels of fgf21, irisin responses differing among males and females (Scalzo et al., 2014). FGF21 is raised in muscle and hepatic insulin resistant states including obesity, IGT, IFG and T2DM (Chavez et al., 2009). FGF21 also increases glucose uptake which points toward its possible role in correcting glucose metabolism. In obesity, FGF21 is elevated in the insulin-stimulated state, which demonstrates higher lipid oxidation and lower carbohydrate oxidation as compared to lean counterparts in the fasting and fed state. Lower delta RQ in obese subjects illustrates metabolic inflexibility among obese individuals (Straczkowski et al., 2013).

On exposure to cold, FGF21 regulates PGC1α protein and enhances thermogenic capacity by browning of WAT via altering thermogenic gene expression. There is no change in FGF21 levels with cold temperature, thus suggesting its action is via autocrine or paracrine signalling on adipose tissue (Fisher et al., 2012). One assumption is when BAT is activated it results in an increase in the release of FGF21 and its autocrine action further increases BAT activation (Hondares et al., 2011).

2.3 Vitamin D and Energy Metabolism

2.3.1 Background

Vitamin D is a generic name given to two molecules – cholecalciferol and ergocalciferol, which are biologically inert. The liver is the main site for its metabolism. Vitamin D is converted into its active form via a two-step hydroxylation process. First, it gets converted to 25 Hydroxy vitamin D3 or calcidiol. This reaction takes place in the liver prior to further hydroxylation to produce 1,25 dihydroxy vitamin D3 or calcitriol in the kidneys (Bikle, 2014).

Low vitamin D status has been identified as an emerging public health issue in Australia despite the availability of abundant sunshine. Living indoors, having dark skin, wearing sun cream and covering exposed skin with protective clothing are some of the reasons for its deficiency. A minimum of 6–7 minutes of daily exposure during mid-morning or mid-afternoon in summer for moderately fair-skinned people with exposed arms, or 7–40 minutes (depending on latitude) at noon in winter, is sufficient to maintain adequate vitamin D levels in the body. When sun exposure is not sufficient, the recommended vitamin D dosage is at least 600 IU (15 µg) per day for people aged <70 years and 800 IU (20 µg) per day for those aged >70 years either from dietary sources or supplementation (Holick, 2011). Older adults, people with chronic disorders or physical disabilities, dark-skinned or obese people and adults working indoors for most of the day are at higher risk of vitamin D deficiency.

Vitamin D status has been defined according to the following levels of serum 25-(OH)D which are based on the review of recently available evidence:

Table 2.4 Vitamin D status based on levels of serum 25(OH)D

Vitamin D status	Levels of serum 25(OH)D
Adequate	≥50 nmol/L at the end of winter (level may need to be 10–20 nmol/L higher at the end of summer, to allow for seasonal decrease).
Mild vitamin D deficiency	30–49 nmol/L
Moderate vitamin deficiency	12.5–29 nmol/L
Severe vitamin D deficiency	< 12.5 nmol/L

The function of Vitamin D is not limited to calcium homeostasis and skeletal health (Boyle, Miravet, Gray, Holick & Deluca, 1972; Tanaka & Deluca, 1973) but recent evidence shows its major role in adiposity, energy metabolism and functioning of the cardiovascular, renal and immune systems as well (Chen et al., 2011; Liu et al., 2006; Wang et al., 2004; Zhang, Kong, Deb, Chang & Li, 2010) (Figure 2.4). Association of vitamin D status and body fat is now well established (Beydoun et al., 2010; Cheng, 2009; Hypponen & Power, 2006; Konradsen, Ag, Lindberg, Hexeberg & Jorde, 2008; Lenders et al., 2009; Looker, 2005; Parikh et al., 2012; Snijder et al., 2007; Young et al., 2009). At the same time, it may vary with ethnicity (Sulistyoningrum, Green, Lear & Devlin, 2012), age (Janssen, Samson & Verhaar, 2002), lean mass, gender, body fat percentage (Looker et al., 2008) and regional adiposity (Sukumar et al., 2015).

Figure 2.4 Health conditions associated with vitamin D deficiency.

The nuclear vitamin D receptor (nVDR) mediates the biological functions that are regulated by vitamin D (Haussler et al., 2008). The active form of vitamin D ($1,25(OH)_2D$) binds to nVDR with high affinity and specificity. These receptors are present in many non-skeletal tissues including the brain, pancreas and immune cells (Bouillon, 2011). Although the specific mechanism by which vitamin D affects these conditions remains unclear, the following mechanisms are likely important: Low 25(OH)D levels lead to increases in PTH which then increases intracellular calcium in adipose tissues which further encourages lipogenesis (McCarty & Thomas, 2003; Zemel, Shi, Greer, Dirienzo & Zemel, 2000). Vitamin D receptor (VDR) present in insulin-producing beta-islet cells regulates cell proliferation and differentiation (Haussler et al., 2008). Obese individuals have enzymes that are capable of degradation of Vitamin D and over-expresses 25 D and 1,25 D which infers hydroxylation is impaired in obesity (Wamberg et al., 2013).

In this thesis, we focus on the potential roles of vitamin D status in regulation of resting and glucose induced thermogenesis energy metabolism and its relationship to metabolic syndrome characteristics such as adiposity, insulin resistance and lipid profile.

2.3.2 Vitamin D and Obesity

The possibility of a link between vitamin D and obesity was first proposed in 1971 by Rosenstreich, Rich & Volwiler (1971). Since then the serum concentration of 25(OH)D has been found to be negatively associated with body weight (Zitterman, Ernst, Gummert & Börgermann, 2014), BMI (Cabral et al., 2016), waist circumference (Jafri et al., 2016; McGill, Stewart, Lithander, Strik & Poppitt, 2008), and total body fat mass (Jafri et al., 2016; Kremer, Campbell, Reinhardt & Gilsanz, 2009). Furthermore, high levels of the 25(OH)D contribute to low visceral and subcutaneous adipose tissue measurements (Caron-Jobin et al., 2011). In a recent study, participants were randomised to receive either orange juice (supplemented with 350 mg Calcium and 100 IU vitamin D) or unfortified orange juice (control) for a period of 16 weeks. Both experimental and control groups exhibited a significant reduction in weight (~ 2.5 kg) from respective baselines but this did not differ between the groups. Interestingly, the group receiving the vitamin D+ Ca supplemented orange juice exhibited a significant decrease in visceral fat compared to the control group receiving only orange juice (Rosenblum, Castro, Moore & Kaplan, 2012).

Several potential effects of vitamin D metabolism that relate to obesity have been postulated by experts in this area of research. Researchers (Ding, Gao, Wilding, Trayhurn & Bing, 2012) have explained several potential roles of Vitamin D in reducing obesity such as the involvement of adipocytes in the synthesis or degradation of 25(OH)D, modulating adipokine production and inflammation in adipose tissues, insulin release and B-cell function. Several other researchers (De Pergola et al., 2013; Rajakumar, de las Heras, Lee, Holick & Arslanian, 2012; Sergeev, 2012) propose that calcium and vitamin D supplementation can even reduce adiposity-favouring adipocyte death (apoptosis), adipogenesis and lipid metabolism. The mechanism involves activation of 25OHD during increased intracellular calcium. Interestingly, a recent publication suggests increased fecal fat excretion of metabolites is possible after weight loss in obese individuals (Figure 2.5).

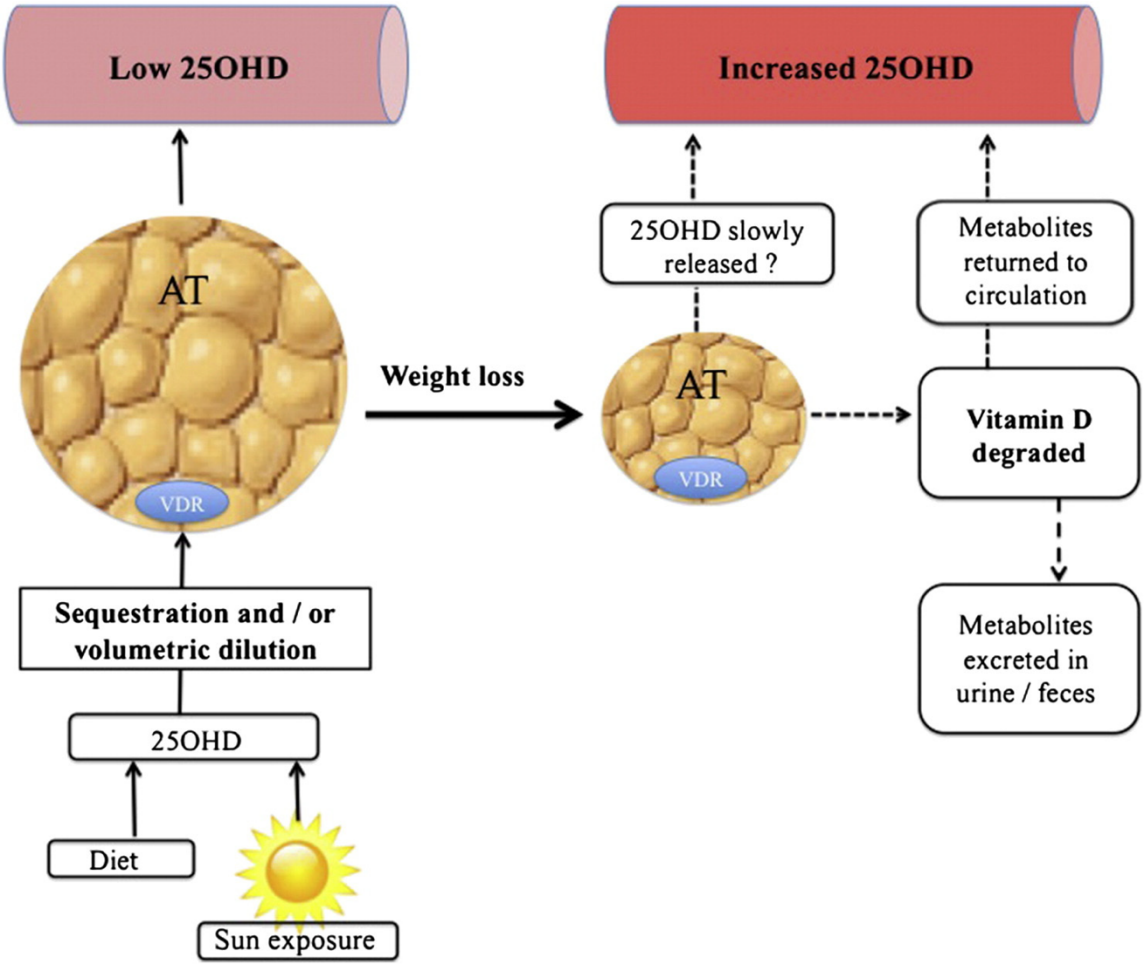

Figure 2.5 The potential pathways influencing 25OHD metabolism in obese individuals before and after weight loss.

Solid lines in arrows indicate established mechanisms; dashed lines in arrows indicate potential mechanisms.

Figure Adapted from Pannu et al. 2016, with permission (Appendix C).

A bidirectional Mendelian analysis on a big cohort with 12 BMI-related SNPs (combined in an allelic score) concluded that the presence of obesity may cause low 25(OH)D levels, but that low serum levels do not necessarily result in increased BMI (Vimaleswaran et al., 2014). There is evidence of a close association between low levels of vitamin D and gain in fat mass (Leenders et al., 2011). A recent meta-analysis clearly indicated that any loss in weight and body fat may result in increased serum levels of vitamin D (Pannu, Zhao et al., 2016).

For maximal benefits on weight loss and visceral fat loss, vitamin D supplementation may require calcium and caloric restriction (Mason et al., 2014; Pathak, Soares, Calton, Zhao & Hallett, 2014; Zhu et al., 2013; Zittermann et al., 2009). Moreover, few studies suggest its indirect effect in response to reduced PTH levels and body fat mass (Leenders et al., 2011; Salehpour, Shidfar et al., 2012). There are many studies that have examined the effect of varying vitamin D supplementation with caloric restriction on weight loss. Usually, long term supplementation from 6 weeks to one year showed more documented success in lowering weight (Shahar et al., 2010; Sukumar et al., 2015), fat mass (Ibero-Baraibar, Navas-Carretero, Abete, Martinez & Zulet, 2015) and visceral fat (Gangloff et al., 2015) with a few studies quoting no effect (Holecki et al., 2008; Zittermann et al., 2009). The possible reason why vitamin D supplementation fails to achieve desired results in an obese population may be due to an accumulation of vitamin D in adipose tissue following its administration (Wortsman, Matsuoka, Chen, Lu & Holick, 2000). Hence, it is important to consider the dilution effect of supplementation in obesity (Zitterman et al., 2014). Thus, the relevance of the percentage body fat of the individual when deciding on the supplementation dose (Arunabh, Pollack, Yeh & Aloia, 2003).

2.3.2a. Vitamin D supplementation and body weight status: A systematic review and meta-analysis of randomized controlled trials.

Pathak, K, Soares, MJ, Calton, EK, Zhao, Y and Hallett, J.

This review is a published paper in Obesity reviews: 15 (6); 528-537

Received NSA Emerging Researcher Award for the paper in NSA annual scientific meeting, 2014.

Introduction

Vitamin D inadequacy is a worldwide problem with potential consequences for many chronic diseases, including obesity, cardiovascular disease and type 2 diabetes mellitus (T2DM) (Holick & Chen, 2008). Emerging evidence suggests that the clustering of poor calcium intake, inadequate vitamin D status and chronic disease may represent causal associations. However, after reviewing the available evidence, a recent expert committee report has endorsed calcium and vitamin D only for bone health (Ross et al., 2011). The importance of calcium in the regulation of body weight and composition is emerging (Onakpoya, Perry, Zhang & Ernst 2011; Soares, Chan She Ping-Delfos et al., 2011). Two confirmed mechanisms now include the stimulation of whole-body fat oxidation and an increase in faecal energy loss (Christensen et al., 2009; Gonzalez, 2012; Soares, 2012). These actions offer a small but consistent shift towards a negative energy balance. Hence, an improvement in vitamin D status would improve calcium absorption and could have an indirect effect on body weight.

The role of vitamin D *per se* in the aetio-pathogenesis of obesity and chronic diseases is an area of tremendous importance to clinical nutrition and public health (Norman, 2008; Peterlik et al., 2009). There are numerous cross-sectional studies that have assessed the relationship between vitamin D status and measures of body fat. Most of them confirm an inverse association between 25(OH)D (25-hydroxyvitamin D) and total body fat (Soares, Ping-Delfos et al., 2011). Explanations for such outcomes have included the possibility that this association reflects low intakes of the vitamin in the obese, and that obese individuals are less likely to venture outdoors to engage in physical activity so their exposure to sunlight is low (Harris & Dawson-Hughes, 2002). The prevalent opinion, however, is that the fat-soluble vitamin is sequestered in the expanded adipose tissue mass, resulting in lower vitamin D status (Wortsman et al., 2000). Proof of such a view comes from studies which show that with weight (fat) loss, there is an improvement in vitamin D status (Mason et al., 2014; Rock et al., 2012).

The vitamin D receptor (VDR) is a nuclear receptor that mediates the actions of 1,25(OH)2D (1,25 dihydroxy vitamin D3), the active form of the vitamin. It is expressed in several tissues not directly involved in calcium metabolism, so the possibility arises that it serves other functions. Studies on VDR-null mice maintaining normal calcium intake demonstrated a lesser weight and fat gain on normal or high-fat diets when compared with wild-type mice (Narvaez et al., 2013; Wong et al., 2009). This was due to a higher rate of energy expenditure, an increased β oxidation of fatty acids and up-regulation of uncoupling protein (Wong et al., 2009). Since such a model results in a global absence of the receptor, it was important to understand site-specific action. Wong and colleagues developed a transgenic mouse model that had targeted expression of human VDR in adipocytes (Wong et al., 2011). In comparison to wild-type mice, a decrease in energy expenditure and an increase in whole body fat was observed in the VDR-null mice. Data from a human adipose tissue cell line suggest a non-genomic action of 1,25(OH)2D. This also included a stimulation of enzymes controlling fat synthesis and reciprocal inhibition of lipolysis (Shi, Norman, Okamura, Sen & Zemel, 2001). Overall, the data implies that 1,25(OH)2D, acting through VDR, predisposes to a gain in fat mass (FM) (Adams & Hewison, 2010). Hence, normalizing 25(OH)D levels in vitamin D-insufficient subjects may act as a brake on 1,25(OH)2D, either through lower parathyroid hormone (PTH) or other mechanisms, and so prevent weight gain. However, extrapolating data from cellular and animal models to human obesity is not straightforward. There is a need for greater clarity on how the endocrine and autocrine pathways of vitamin D action impinge on tissues controlling human energy and fat metabolism (Heaney, Recker, Grote, Horst & Armas, 2011).

In this article, we revisit an earlier proposition that vitamin D status *per se* may have an effect on obesity (Soares, Chan She Ping-Delfos et al., 2011; Soares & Pathak, 2012). Our main question was whether an improvement in vitamin D status reduced indices of body fat in the absence of caloric restriction. We embarked on a systematic review of high-quality, randomized controlled trials (RCTs) that had supplemented vitamin D but without an imposed deficit in energy intake.

Methods

A systematic search of the literature from 1995 to date was conducted in March 2013. We assessed the potential effect of vitamin D supplementation without caloric restriction on different measures of body fat. Two researchers (KP and EKC) independently searched the databases PubMed, Science Direct, Wiley, Web of Knowledge, Scopus and Springer using the key words: vitamin D, vitamin D supplementation, PTH, body fat, body weight, fat free mass, fat mass, adiposity, fat distribution, body fat regulation, BMI, weight loss and body composition. Criteria for inclusion were RCTs of high quality (based on score of >3 as proposed in the guidelines of Jadad et al. (1996), at least one arm of the RCT used vitamin D, conducted on adult non-pregnant, non-lactating women, included some measure of body weight status or fat distribution and in the English language. Those studies designed for measures of body composition as one of the main objectives were termed as primary and where obesity markers were part of the many outcomes, were termed as secondary studies. Data extraction was carried out by two investigators (KP and EKC) on an excel spreadsheet generated with the help of the statistician (YZ) to include all relevant information. KP contacted individual authors of studies where published data were inadequate for meta-analysis. The change in mean and respective standard deviation was calculated for some studies that provided pre-and post values. All the vitamin D data was converted where necessary to IU for intake and nmol L^{-1} for 25(OH)D status. MJS and EKC independently cross-checked all raw data in the spreadsheet and areas of potential error were corrected through mutual discussion.

Statistical Analysis

We assessed the effect of vitamin D on the reduction of the following outcomes: (i) weight (kg) (ii) waist circumference (WC) fat mass (FM, kg); (iv) percentage fat mass (%FM); (v) body mass index (BMI; kg m^{-2}); and (vi) lean body mass (LBM). The meta-analysis compared the post-value minus pre-value of vitamin D group vs. that of the placebo group. A negative value implied a greater reduction in the relevant obesity outcome following vitamin D. The effect sizes were generated using Hedges's g score for each study. The overall effect sizes were estimated using both fixed and random effects models, but only the latter model

is presented. Test for heterogeneity used the I^2 statistics and Galbraith plot (data not provided for brevity). Potential publication and small sample size bias was assessed by visual inspections of funnel plots and Egger's test.

To explore the effect of the two main factors of interest (vitamin D status and changes in vitamin D status) on predicting standardized mean difference (SMD), and to check for unknown sources of confounding, a meta-regression (random effects model only) was conducted. A backward elimination process was used to build a parsimonious regression model that identified those explanatory variables that were significant predictors of SMD. All data analyses were carried out with Stata version 11 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX, USA: StataCorp LP).

Results

The PRISMA (Liberati et al., 2009) search strategy and outcomes are presented in Fig. 2.6. We obtained 18 high quality studies from the systematic review. They are presented in Table 2.5 as those primarily designed to examine body weight and composition (Carrillo et al., 2013; Salehpour, Hosseinpanah et al., 2012; Sneve, Figenschau & Jorde, 2008; Wamberg et al., 2013; Zittermann et al., 2009), and those that had other endpoints or were a secondary analysis of evidence (Ardabili, Gargar & Farzadi, 2012; Belenchia, Tosh, Hillman & Peterson, 2013; El-Hajj Fuleihan et al., 2006; Gallagher, Fowler, Detter & Sherman, 2001; Gallagher, Sai, Templin & Smith, 2012; Grimnes, Figenschau, lmas & Jorde, 2011; Jorde & Figenschau, 2009; Nagpal, Pande & Bhartia, 2009; Nikooyeh et al., 2011; O'Sullivan et al., 2011; Petchey et al., 2013; Shab-Bidar et al., 2011; von Hurst, Stonehouse & Coad, 2010). Relevant raw data could only be extracted from 12 studies, either through author contact or directly from the publication.

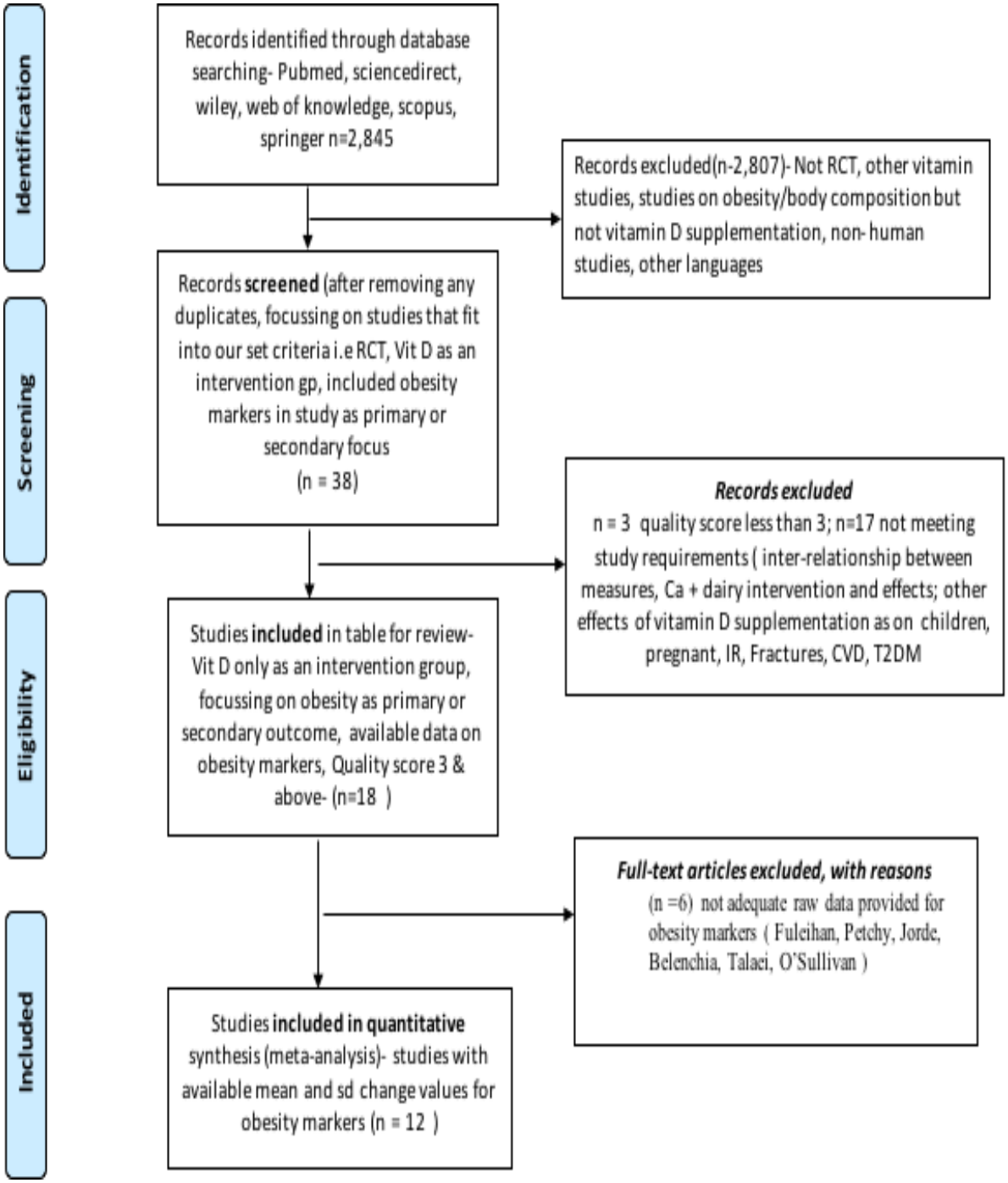

Figure 2.6 PRISMA flow diagram: Search strategy and selection of high quality RCTs for vitamin D supplementation and body weight status.

PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT, randomised controlled trial; Vit D, vitamin D; Ca, calcium; IR, insulin resistance; CVD, cardiovascular disease; T2DM, type 2 diabetes.

Table 2.5 Salient features of good quality randomised controlled trials of vitamin D supplementation on obesity markers.

Authors & year	Study location	Study Details	Nature & study quality score[1]	Vitamin D Status achieved nmol/L	Reported measures of body composition				
					BW	BMI	WC or WHR	FM or %FM	FFM
(Wamberg et al., 2013)	Denmark	Dosage: 7000 IU/d D_3 Subjects: 18-50 y, n=21placebo, n=22 vit D. Duration: 26 weeks	Primary, 5	Placebo Pre:34.0 ± 9.0 Post :46.8 ± 17.3 Vit D Pre: 33.0 ± 10.8 Post:110.2 ± 21.2	✓	✓	✓	✓	✓
(Salehpour, Hosseinpanah et al., 2012)	Tehran, Iran	Dosage: 1000IU/d D_3 Subjects: Premenopausal OW/OB, n=38 placebo, n=39 vit D. Duration: 12 weeks	Primary, 5	Placebo Pre: 46.9 ± 32 Post: 51.5 ± 31 Vit D Pre: 33.0±10.8 Post:110.2 ± 21.2	✓	✓	✓	✓	-
(Carrillo et al., 2012)	USA	Dosage:400IU/d D_3 Subjects: OW/OB, n= 13 placebo, n=10 vit D. Duration: 12 weeks	Primary, 4	Placebo Pre:45.18±16.22 Post: 58.66± 14.98 Vit D	✓	✓	✓	✓	✓

				Pre:51.92± 8.3 Post:33.4 ±.20.72					
(Zittermann et al., 2009)	Leipzig, Germany	Dosage:3320IU/d D_3 or placebo during caloric restriction. Subjects: n=82 intervention gp, n=83control gp; all with BMI >27 kg/m^2 & 25OHD < 30 nmol/L. Duration: 12 months	Primary, 5	VitD Pre- 30 Post: 85.5	✓	✓	✓	✓	-
(Sneve et al., 2008)	Tromsø, Norway	Dosage: gp1=20,000 IU D_3 twice a week, gp 2= 20000 IU once a week, gp3= placebo twice a week. Subjects: n= 116- vit D gp1; n=106 gp2; n=112- placebo OW/OB men and women Duration: 12 months	Primary, 5	gp1 Pre: 54.5 Post:114.5 gp2 Pre: 51.4 Post: 89.2	✓	✓	✓	✓	-
(Petchey et al., 2013)	Brisbane, Australia	Dosage: 2000IU/d D_3 or placebo Subjects: n=11 treatment gp; n=14	Secondary, 5	Vit D Pre: 95 ± 37 Post: 146 ±25	-	✓	-	✓	-

		placebo gp. Adults with stage 3-4 chronic kidney disease(CKD) Duration: 6 months							
(Belenchia et al., 2013)	Columbia, US	Dosage: 2000IU x2/d D_3or placebo Subjects: Obese adolescent n = 18 vit D, n=17 placebo; BMI (in kg/m2): 39.8± 6.1; 25(OH)D: 19.6± 7.1 ng/mL Duration: 6 months	Secondary, 5	<u>Vit D</u> Pre: 47.92 ± 15.72 Post: not reported	-	✓	✓	-	-
(Ardabili et al., 2012)	Iran	Dosage: 50 000IU/d D_3 or placebo every 20 d Subjects: 50 F with PCOS and vitamin D deficiency. 20-40 years. n= 24 for D3, n =26 for placebo Duration: 8 weeks	Secondary, 5	<u>Vit D</u> Pre :17.22 ± 6.99 Post: 58.41 ± 15.23	✓	✓	-	✓	-
(Gallagher et al., 2012)	Omaha, US	Dosage: low (400 and 800 IU/d), medium (1600 and 2400 IU/d) and high (3200, 4000 and 4800 IU/d) D_3 or Placebo	Secondary, 5	Not reported	-	-	-	✓	✓

		Subjects: n= 163, 57-90 y Duration: 1 year							
(Grimnes et al., 2011)	Tromso, Norway	Dosage: 20,000 IU /d D_3 or placebo, twice per week Subjects: n= 49- vit D gp; n= 45 placebo, healthy Caucasians, 30-75 y Duration: 6 months	Secondary, 3	<u>Vit D</u> Pre :42.2 ± 13.9 Post:142.7 ± 25.2	-	✓	-	-	-
(Nikooyeh et al., 2011)	Tehran, Iran	Dosage: gp 1: no vitamin D 150mg Ca BD; gp 2- 500IU vitD_3 + 150mg Ca BD; gp 3 - 500 IU vitD_3 + 250mg Ca BD. Subject: n=60, T2DM 30-60 y Duration: 12 weeks	Secondary, 3	<u>Vit D</u> Pre:44.4 ± 28.7 Post: 77.7 ± 28.6	✓	✓	✓	✓	-
(Shab-Bidar et al., 2011)	Tehran, Iran	Dosage: 500 IU/250ml D_3 twice a day or placebo Subjects: n=100 T2DM adults 25-70 y of age Duration: 12 weeks	Secondary, 3	<u>Vit D</u> Pre-38.5 ± 20.2 Post - 72.0 ± 23.5	✓	✓	✓	✓	-

(O'Sullivan et al., 2011)	Ireland	Dosage: 600IU/d D_3or placebo Subjects: n=64 placebo, n=62 vit D, free living Caucasian 18-63 y, all women premenopausal Duration: 4 weeks	Secondary, 5	<u>Vit D</u> Pre- 59.7±23.0 Post - 78.1±20.0	-	✓	-	-	-
(von Hurst et al., 2010)	Auckland, New Zealand	Dosage: 4000 IU/d D_3 or placebo. Subjects: n=42 vit D,n=39 placebo South Asian women, with D3 deficiency and IR. Duration: 6 months	Secondary, 4	<u>Vit D</u> Pre: 21.0 Post: 80.0	-	✓	-	-	-
(Nagpal et al., 2009)	New Delhi, India	Dosage: 120,000 IU D_3 or placebo X 3 Fortnightly Subjects: n=35 vit D,n=36 placebo over 35 y Duration: 6 weeks	Secondary, 5	<u>Vit D</u> Pre:36.5 Post: 71.6.	✓	✓	✓	-	-
(Jorde & Figenschau, 2009)	Tromso, Norway	Dosage: 20,000 IU/week D_3, 40,000 IU/week D_3 and placebo. Subjects: n = 441 OB adults	Secondary, 5	<u>Vit D</u> Pre:55.2 Post(40000IU): 112.2 (20,000IU): 87.8	-	✓	-	-	-

		Duration: 12 months							
(Fuleihan et al., 2006)	Beirut, Lebanon	Dosage: 1,400 IU/d D_3, or 14, 000 IU/d D_3 or placebo Subjects: n= 58 placebo; n=58 low dose; n=55 high dose, 10-17 y girls Duration: 12 months	Secondary, 4	<u>Vit D</u> Pre: 34.94 ± 19.97 Post – 94.85±31 (overall)	✓	✓	-	-	✓
(Gallagher et al., 2001)	Omaha, US	Dosage:20 IU/d Subjects: n= 123 (calcitriol), n=123(placebo), 57-90 y healthy white women Duration: 3 year	Secondary, 5	<u>Placebo</u> Pre:80.63±27.35 Post:63.25±25.02 <u>Vit D</u> Pre:78.14±21.57 Post:60.63±20.83	-	-	-	✓	✓

Study quality score: 0–2 = low; 3–5 = high (Jadad et al., 1996)

†Calcium given to both groups.

25(OH)D, 25-hydroxyvitamin D; BD, twice a day; BMI, body mass index; BW, body weight; FFM, fat-free mass; FM, fat mass; IR, insulin resistance; IU, international units; OB, obese; OW, overweight; T2DM, type 2 diabetes

Meta-analysis

Only the results of meta-analysis performed for BMI (kg m^{-2}), FM and %FM are reported, as the available information on waist circumference and LBM had too few studies for a meaningful analysis. The meta-analysis on BMI (Figure 2.7) included 605 subjects in the placebo group (210 male/390 female, aged 44 ± 9.5 years) and 605 in the vitamin D group (228 male /377 female, aged 43 ± 9.0 years). The plot indicates that 8 of 12 studies favoured a reduction in BMI following vitamin D, but overall this did not reach statistical significance (12 studies, overall SMD= −0.097, 95% confidence interval [CI]: [−0.210, 0.016], $P = 0.093$). No significant heterogeneity ($\chi^2 = 4.96$, $P = 0.933$, $I^2 = 0\%$) or publication bias was found.

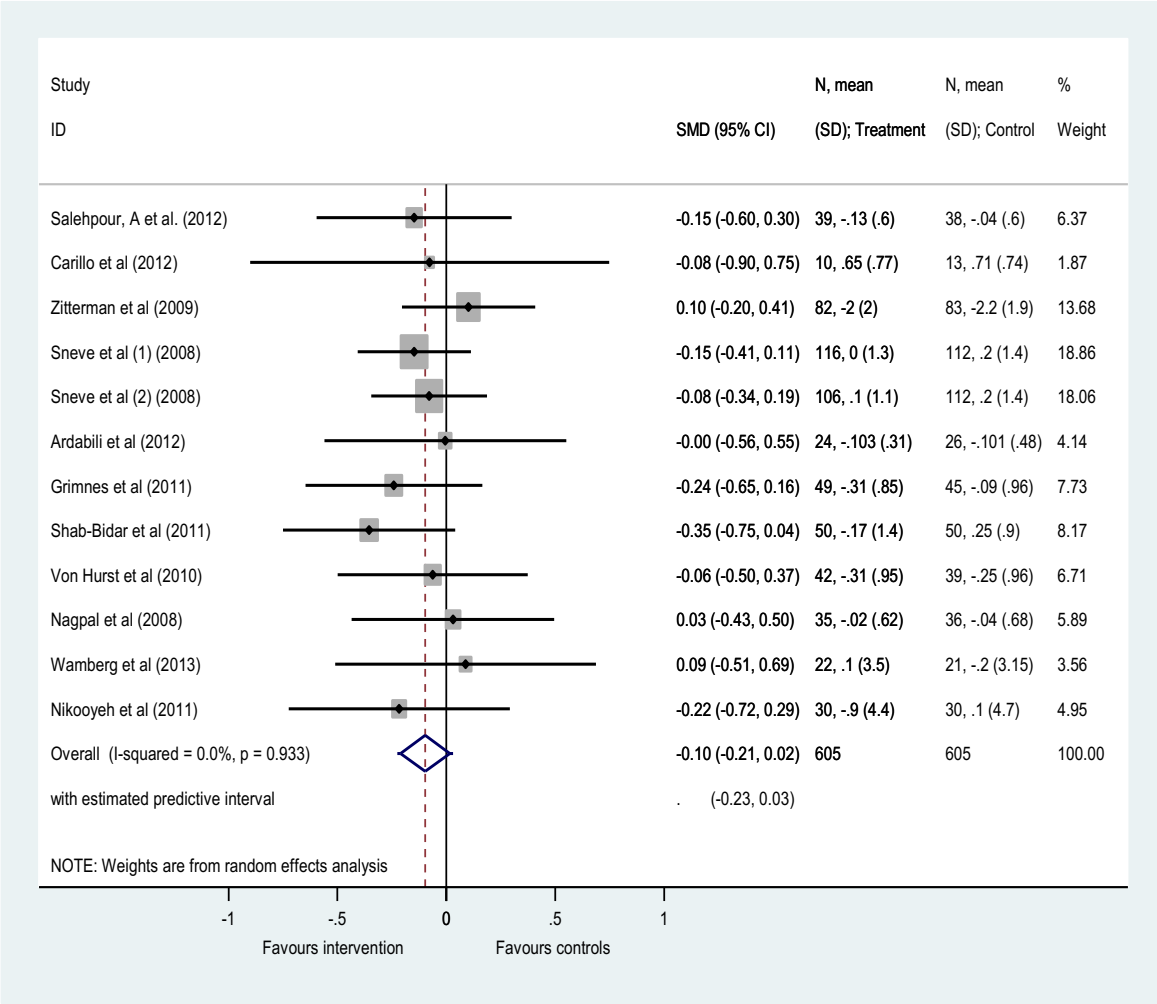

Figure 2.7 Forest plot of the effect of vitamin D supplementation on body mass index.

The meta-analysis on FM (Figure 2.8) included 268 subjects in placebo group (30 male /238 female, aged 59 ± 11.8 years) and 269 in the vitamin D group (39 male/230 female, aged 59 ± 12 years). There was no significant difference in FM (10 studies, overall SMD = −0.014, 95% CI: [−0.355, 0.308], $P = 0.934$). A moderate and significant heterogeneity in effect sizes was found across the studies ($\chi^2 = 28.44$, $P = 0.001$, $I^2 = 68.4\%$). After inspection of a Galbraith plot (not reported), the study of Salehpour *et al.* (25) was the potential source of the heterogeneity. Egger's test did not indicate a significant small study effect ($P = 0.904$).

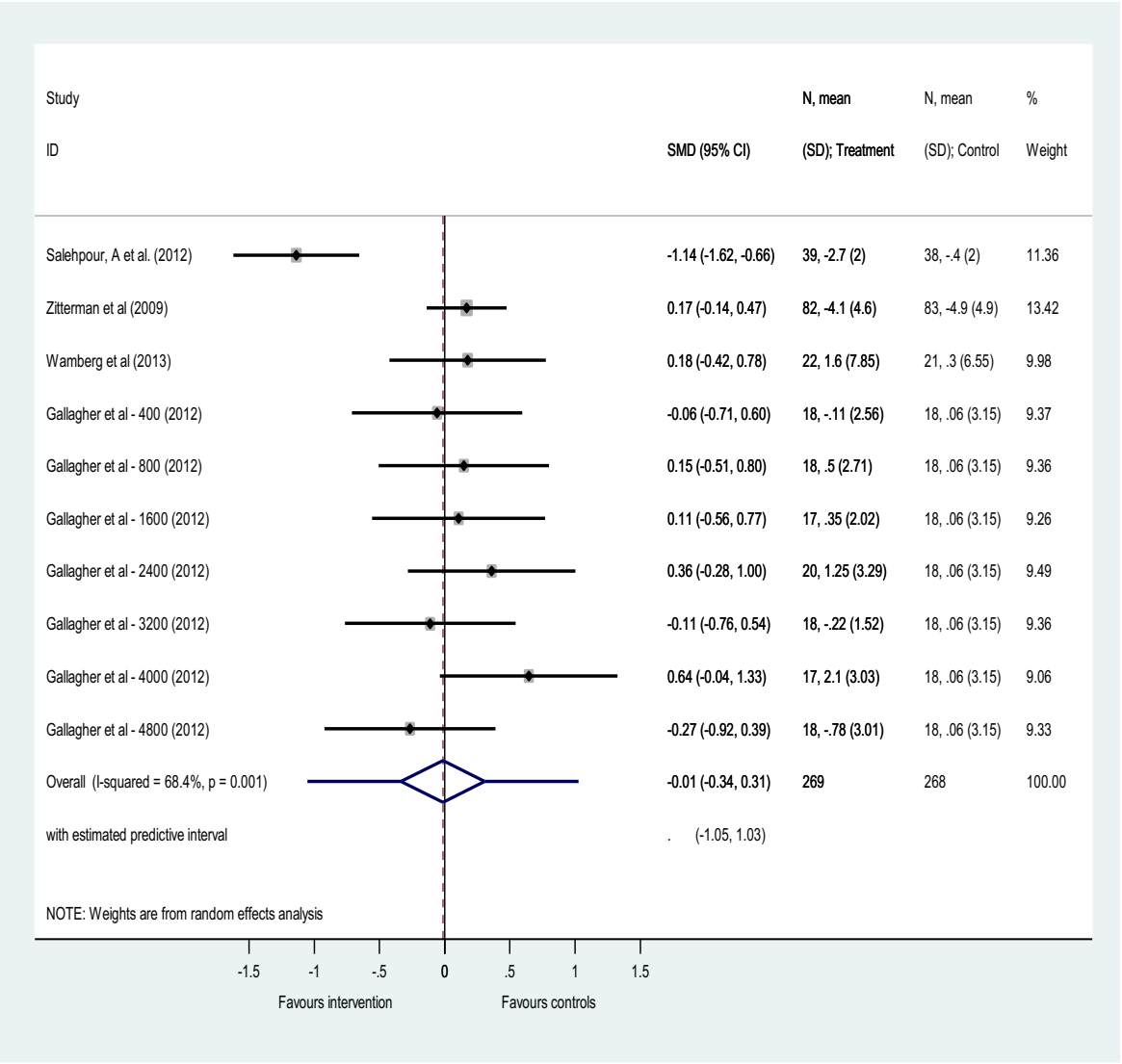

Figure 2.8 Forest plot of the effect of vitamin D supplementation on reduction in fat mass.

The meta-analysis on percentage FM included 443 subjects in placebo group (122 male/321 female, aged 62 ± 7.0 years) and 438 subjects in the vitamin D group (131 male/ 307 female, aged 62 ± 7.8 years). There was no significant effect for percentage change in fat mass (%FM) (12 studies, overall effect SMD = 0.051, 95% CI: [−0.098, 0.200], $P = 0.503$). A weak non-significant heterogeneity in effect sizes ($\chi^2 = 12.51$, $P = 0.327$, $I^2 = 12\%$) was noted. The study of Shab-Bidar et al (2011) was the source of the heterogeneity.

BMI did not show any trend for reduction when absolute status achieved was <85 nmol L^{-1} (seven studies, SMD = −0.139, 95% CI [−0.322, 0.043], $P = 0.135$) or when status achieved

was ≥85 nmol L^{-1} (five studies, SMD = −0.097, 95% CI: [−0.210, 0.016], *P* = 0.336). Similar observations were obtained for FM (four studies with <85 nmol L^{-1}; SMD = −0.262, 95% CI: [−0.940, 0.417], *P* = 0.450 and six studies with ≥85 nmol L^{-1}, SMD = 0.161, 95% CI: [−0.048, 0.371], *P* = 0.132). Percentage fat mass also did not show any difference <85 nmol L^{-1} (six studies, SMD = 0.007, 95% CI: [−0.311, 0.325], *P* = 0.966) or ≥85 nmol L^{-1} (six studies, SMD = 0.060, 95% CI [−0.101, 0.222], *P* = 0.464). In a similar analysis, the change in vitamin status (<60 or ≥60 nmol L^{-1}) did not affect any of these endpoints (data not shown).

Meta Prediction

The results of four models of meta-regression are presented in Table 2.6. Duration of intervention did not enter any of the models tested. Neither vitamin D status nor change in vitamin D status was found to significantly predict the SMD. However, age was positively associated with SMD for FM in two models (Models 3 and 4), whereas the percentage of women in each study was negatively associated with SMD for absolute FM (Table 2.6).

Table 2.6 Effect of vitamin D status and change in vitamin D status on SMD controlling for age and gender.

Outcome	Variable	Estimated Coefficient	95% CI	P Value
BMI	Vitamin D status			
Model 1	<85 nmol/L	-0.139	(-0.347, 0.069)	0.166
	>=85 nmol/L	0.069	(-0.195, 0.333)	0.574
	Change in Vitamin D status			
Model 2	<60 nmol/L	-0.092	(-0.228, 0.045)	0.165
	>=60 nmol/L	-0.046	(-0.451, 0.360)	0.807
Fat mass (FM)	Vitamin D status			
	<85 nmol/L	-0.314	(-2.093, 1.464)	0.681
Model 3	>=85 nmol/L	0.193	(-0.463, 0.849)	0.499
	Age (years)	0.035	(0.008, 0.063)	0.020
	Female (%)	-0.021	(-0.044, 0.003)	0.075
	Change in Vitamin D status			
Model 4	<60 nmol/L	0.158	(-2.092, 1.464)	0.761
	>=60 nmol/L	0.292	(-0.203, 0.787)	0.199
	Age	0.034	(0.011, 0.058)	0.011
	Female (%)	-0.026	(-0.041, 0.010)	0.006

Fat Mass (%)	Vitamin D Status			
	<85 nmol/L	0.027	(-0.274, 0.329)	0.844
Model 5	>=85 nmol/L	0.027	(-0.363, 0.416)	0.882
Model 6	Change in Vitamin D status			
	<60nmol/L	0.063	(-0.149, 0.274)	0.525
	>=60nmol/L	-0.061	(-0.476, 0.353)	0.748

BMI, body mass index; CI, confidence interval.

Discussion

There is a great interest in uncovering preventative roles of vitamin D beyond its traditional influence on skeletal health. The findings of an inverse relationship of vitamin D and measures of body fat is now consistent, but clarity is required in regards to whether this is cause or effect (Soares, 2011). Current evidence does not mechanistically support a role for the vitamin in human obesity (Boon et al., 2006; Soares & Pathak, 2012; Teegarden et al., 2008). A recent paper using novel analysis concluded that a higher BMI would result in a low vitamin D status, but a lower status was unlikely to increase BMI (Vimaleswaran et al., 2014). We have addressed the latter proposition by determining whether an improvement in vitamin D status, through supplementation, had an effect on measures of body fat. We excluded the effect of an imposed caloric deficit to maximize the possibility of an intrinsic effect of the vitamin. Our analysis was based on the selection of good quality RCT that had provided the vitamin in a variety of dosages and over durations from 6 to 52 weeks. A potential reduction in BMI following vitamin D supplementation (P = 0.092) was noted. Statistically, such an effect would be considered as small as the SMD was <0.2 (Cohen, 1988), but these effects were not confounded by heterogeneity nor small sample bias (Fig. 2.7). Although the acceptable level of probability was not reached, the data are intriguing in that they occurred in the absence of caloric restriction. However, from the data available and extracted, such observations of a potential reduction in BMI did not translate to reductions in absolute or %FM (Fig. 2.8). In all but two studies, body composition had been measured by DEXA (dual-energy x-ray absorptiometry), a high quality method, so methodology may not be the issue. There is evidence from acute trials that increases in dietary calcium at breakfast reduced *ad libitum* energy intake at lunch (Al-Mana, Lanham-New & Robertson, 2012; Chan She Ping-Delfos & Soares, 2011) as well as 24 hr energy and carbohydrate intake (Chan She Ping-Delfos & Soares, 2011). Could improvements in vitamin D status, through enhanced calcium absorption, have mobilized glycogen stores rather than body fat? Although hypothetical, such a scenario may explain the apparent dichotomy between the potential decrease in BMI and the lack of change in adiposity seen in the present analysis.

In models of meta-regression for FM, age and gender made significant contributions to the SMD (Table 2.6). Essentially, this suggested that studies in older people were less likely to

show an effect, i.e. they favoured the placebo. In contrast, the greater the percentage of women, the better the effect of vitamin D on decreasing FM (Table 2.6). Another potentially crucial factor affecting extra-skeletal outcomes of vitamin D could be the absolute value of vitamin D status attained as well as the duration over which it was maintained. Von Hurst et al. showed that vitamin D supplementation reduced insulin resistance (from HOMA-IR) only when a status of ~80 nmol L^{-1} was achieved and maintained for 6 months (von Hurst et al., 2010). Although not universally ratified, the latter value may represent the cut-off for normality of vitamin D function (Adams & Hewison, 2010; Heaney, 2008). In this analysis, we found that 6 of 12 studies reporting on BMI and 8 of 10 studies reporting on FM had achieved a vitamin status of 80 nmol L^{-1} in participants by the end of their trial. Although duration of intervention did not make a significant contribution to SMD in any of the models tested (Table 2.6), we were unable to decipher from the available trials just how long each study had maintained their achieved level of 25(OH)D. This may prove to be an important facet of future research in the area.

In summary, RCTs of good quality indicated a possible small effect of vitamin D supplementation on reducing BMI, but this was not supported by changes in FM in the same direction. We conclude that in the absence of caloric restriction, vitamin D supplementation did not reduce human obesity.

2.3.3 Vitamin D, Metabolic Syndrome and Related Risk Factors

Hypovitaminosis may be a potential risk factor for the development of MetS (Alissa, Alnahdi, Alama & Ferns, 2014). However, results from studies investigating this association are inconsistent. Results from interventional and observational studies reveal either an inverse association between serum 25OHD levels and MetS (Gradillas-García, Álvarez, Rubio & de Abajo, 2015) or no association between the two (Bonakdaran et al., 2016; Kim, 2015). Recent studies on rheumatoid patients have reported a significant association between vitamin D serum levels and all components of the metabolic syndrome as a group, but this relationship was only consistent in males (Beaa et al., 2015) and for triglyceride and low density lipoprotein (LDL) cholesterol concentrations (Baker et al., 2012). Similarly, in few other studies, the association was more evident for fasting insulin, triglycerides and the Homeostatic Model of Assessment (HOMA-IR) when studied in children (Kelishadia, Saleka, Saleka, Hashemipoura & Movahedianb, 2014) and adults (Osati, Homayounfar & Hajifaraji, 2016) with MetS. Additionally, intervention studies have reported improvements in CVD risk factors following supplementation with vitamin D. In one such study involving vitamin D supplementation for 90 days, improvements in HDL-cholesterol, apoA-I concentrations and LDL-cholesterol, apoB-100 ratio were observed (Salehpour, Shidfar et al., 2012). However, following a 6-month supplementation of 20,000 IU vitamin D twice every week in 94 participants in a case-controlled nested study, no improvement in insulin sensitivity or triglycerides were noticed (Grimnes et al., 2011). Similar findings have been reported following longer-term intervention studies (Muldowney et al., 2011; Wood et al., 2012), thus suggesting that vitamin D may not be a consistently preventive measure for cardiovascular disease (CVD) risk markers and that lengthening dosage duration may not increase any benefit. In their paper, Pannu, Zhao et al. (2016) conclude that vitamin D levels are lower in obesity which might be a consequence of volume dilution effect and sequestration. As we know, metabolic flexibility is impaired in chronic diseases and so vitamin D levels are reduced. It is paramount that in situations such as obesity, MetS and T2DM (when inflammation is elevated), determination of the optimal level of plasma 25 OHD must be considered.

2.3.4 Vitamin D and Insulin Sensitivity

Although the association of low serum 1,25(OH) D levels with insulin sensitivity has been well established, it is unclear whether vitamin D supplementation consistently leads to improvements in insulin sensitivity. This requires further scope for future investigations to confirm. Nevertheless, vitamin D supplementation has been shown to improve glycaemic status (Belenchia et al., 2013; Nikooyeh et al., 2011; Shab-Bidar et al., 2011) both with or without calcium supplementation and even in the absence of weight loss. In contrast, there are also studies that report no influence on insulin sensitivity post vitamin D supplementation (Agbaht, Mercan, Kutlu, Alpdemir & Sezgin, 2014; Kampmanna et al., 2014; O'Sullivan et al., 2011). High doses of vitamin D (30,000 IU) also did not lead to any improvement in B cell function or insulin sensitivity (Fuleihan et al., 2016; Wagner, Alvarsson, Mannheimer, Degerblad & Ostenson, 2016). In contrast, with a study including known cases of T2DM, vitamin D supplementation of 50,000 IU weekly for 8 weeks showed marked improvements on fasting glucose, fasting insulin concentration and insulin sensitivity (HOMA-IR) (Talaei, Mohamadi & Adgi, 2013). In addition, one recent RCT reported an improvement in fasting glucose after correcting serum levels of 1,25 (OH)D in T2DM patients (Moreira-Lucas et al., 2016) while a cross-sectional study on elderly subjects (Table 2.1) (Ibrahim Issa et al., 2016) indicated raised fasting blood glucose levels among those with a 25OHD concentration < 75 nmol/L. This discrepancy in results may be due to the use of different measures to assess insulin sensitivity. One study included non-diabetic, centrally obese men receiving 3 fortnightly doses of 120,000 IU each for 6 weeks. Marked improvements in insulin sensitivity were noticed when calculated using oral glucose insulin sensitivity (OGIS) (Nagpal et al., 2009) and other measures such as HOMA-IR. However, no effect of vitamin D supplementation was observed on measures of insulin sensitivity such as the quantitative insulin sensitivity check index (QUICKI) and HOMA%B, HOMA%2B (Table 2.1) (Nagpal et al., 2009). This is because OGIS includes post-prandial glucose disposal in its calculation whereas others are based on fasting measurements. We may interpret from this that vitamin D supplementation affects post-prandial glucose uptake whereas fasting metabolism remains unchanged. Similarly, another RCT with 16-week vitamin D supplementation on non-Western residents in Netherlands found that insulin sensitivity as measured by HOMA-IR, Stumvoll index and insulin sensitivity index composite were not affected, but after excluding all known diabetic cases and only including subjects whose vitamin D concentration reached ≥

60nmol/L after 4 weeks supplementation a significant change in insulinogenic index was observed. This index involves the interpretation of insulin and glucose responses 30 minutes after the ingestion of a 75 g glucose drink and represents ß-cell function (Oosterwerff et al., 2014). However, intervention studies that combined weight loss with vitamin D supplementation found distinct improvements in insulin sensitivity (Norman, 1990; Sukumar et al., 2015; Thibault et al., 2015).

2.3.5 Vitamin D, Mitochondrial Function and Energy Metabolism

Vitamin D status is emerging as a key player in the maintenance of energy balance (Calton, Pathak et al., 2016; Soares, 2012). The pathways by which vitamin D is linked to metabolism include upregulating oxidative phosphorylation in skeletal muscle, upregulating uncoupling proteins, initiating BAT and via VDR. Oxidative phosphorylation in mitochondria generates ATP production and impairments to this process can result in fatigue. Fatigue is also a common feature of vitamin D deficiency. This can be corrected with supplemental vitamin D, if it is the main reason for the fatigue (Roy, Sherman, Monari-Sparks, Schweiker & K, 2014). The maximal rate of oxidative phosphorylation was enhanced following 12 weeks of supplementation with 20,000 IU of cholecaliciferol on alternate days as part of a longitudinal study using adults with fatigue and myopathy who were severely vitamin D deficient (Sinha, Hollingsworth, Ball & Cheetham, 2013). These improvement in symptoms of fatigue and myopathy were accompanied with an improvement in mitochondrial function as measured by P-magnetic resonance spectroscopy (P-MRS). In an animal study, global VDR-null rats that were fed a high fat diet for 5 weeks had increased energy expenditure (Wong et al., 2009) compared to wild type rats. This was accompanied by an increased expression of uncoupling proteins (UCP- 1, 2 & 3) which is the protein responsible for activation of brown adipose tissue (Wong et al., 2009). Thus, separating oxidative phosphorylation from ATP production can result in increased heat production.

Vitamin D and calcium possess synergistic effects to each other. In another study, supplementation with a daily dose of 2000 IU of vitamin D on a low calcium diet (349 mg/d $Ca^{2+)}$) for one week resulted in no changes in RMR or fat oxidation as measured by indirect

calorimetry despite an increased serum 1,25(OH)D level. It was concluded that the relationship of calcium/dairy diets to body weight may not be mediated by vitamin D but by increased protein intake and reduced fat absorption (Boon et al., 2006). Similarly, in another supplementation study with 15 µg vitamin D and a very low calcium (600 mg elemental calcium weight-reducing diet for 15 weeks in females, no change in RMR was reported (Major, Alarie, Dore, Phouttama & Tremblay, 2007). Alternatively, these studies may imply that there is a certain minimal level of calcium required to be maintained in the body to activate vitamin D dependent mechanisms for energy metabolism. More research is required to derive conclusions.

All human and animal studies done on vitamin D supplementation and energy metabolism, including those discussed above, suggest a strong association between the two (Figure 2.5) (Pannu, Zhao et al., 2016). In addition, only one study has reported fuel utilization post vitamin D supplementation. After supplementing 15,000 IU/kg of food for 10 weeks RQ was lowered post vitamin D supplementation (Marcotorchino et al., 2014), thus suggesting high fat oxidation on a high fat diet. To our knowledge, there is no data on post-prandial influences on energy metabolism post vitamin D supplementation or vitamin D status. To translate this into human energy metabolism it will be interesting to examine the post-prandial effects of vitamin D.

Thus, a few factors need to be considered while analysing the effect of vitamin D on adiposity and energy metabolism. These factors include the presence of obesity, metabolic syndrome or any related metabolic disorder, gender, age, place of residence, caloric restriction and calcium status, in addition to dosage, its frequency and duration.

The following table (Table 2.7) includes RCTs, systematic review (SR), meta-analysis (MA) and cross-sectional studies associating vitamin D levels with either body composition measures or cardio-metabolic risk markers such as insulin sensitivity, blood pressure and lipid profile published in the past 10 years in different parts of the world. Few but not all are mentioned earlier in the text.

Table 2.7 Relevant studies on vitamin D status and its influence on obesity and associated cardiometabolic risk markers among adults.

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
RCTs						
(Moreira-Lucas et al., 2016)	Canada	T2DM Dosage: 28,000IU/ week Duration: 24 weeks	Increased	-	No change in IS markers, ↓ LDL (p=0.03)	Long term vitamin D supplementation has no effect on glycemic index
(Jafri et al., 2016)	Iran	Postmenopausal, obese women Dosage: fruit yoghurt; 2000 IU in 100g/d Duration: 12 weeks	Increased	↓ WC, WHR, FM and no change in weight and BMI	No change in HbA_{1C}	Vitamin D has positive effect on body composition and metabolic profile
(Gangloff et al., 2015)	Canada	Caloric restriction: less 500 kcal/d and PA Duration: 1 y	Increased	↓ visceral and abdominal fat (p<0.0005)		Weight loss increase circulating serum 25(OH) D
(Ibero-Baraibar et al., 2015)	Spain	Caloric restriction: less 15 % estimated energy restriction (EER) Duration: 4 weeks	Increased	↓ fat mass (p=0.007)	↓ SBP(p=0.021), ↓IL-6 (p=0.014)	Weight loss increase circulating serum 25(OH) D

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Vogt et al., 2015)	Germany	Longitudinal Duration: 3 y	Increased	None	↓Body fat gain in women	<3% of body fat gain in women
(Thibault et al., 2015)	Canada	Caloric restriction Duration: 1 y	Increased	↓ Weight loss	Improved IS following weight loss	Serum 25 (OH)D levels increase does not contribute to improvement in weight loss or IS
(Sukumar et al., 2015)	USA	Caloric restriction to one arm Dosage: 2500 IU/d (both arm) Duration: 6 weeks	Increased greater in weight loss group	The calorie restricted group ↓ weight	Improved markers of insulin sensitivity	Vitamin D supplementation favours IS in obesity
(Sadiya et al., 2015)	UAE	Dosage: 60000 IU/d D3 for 3 months- active arm 30000 IU/d for next 3 months- active arm 22000 IU/d for 6 months- both arms Duration: 1 y	Increased	None	-	No effect seen

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Mason et al., 2014)	USA	Dosage: 2000 IU/d ER to both groups 225 min/wk Subjects: OW/OB women Duration: 12 months	Increased in supplemented group	None	None	Vitamin D replete gp reduced more weight (p=0.05), waist (p=0.02), and body fat percentage (p=0.04)
(Wamberg et al., 2013)	Denmark	Dosage: 7000 IU/d D3 Duration: 26 weeks	Increased	None	None	No effect on body composition and metabolic risk markers post vitamin D supplementation
(Kampmanna et al., 2014)	Denmark	Dosage: 280 µg daily for 2 weeks, 140 µg daily Duration: 10 weeks	Increased		No change in IS, lipid profile, BP and HbA1C	Vitamin D increase has no influence on improvement in metabolic profile
(Petchey et al., 2013)	Brisbane, Australia	Dosage: 2000IU/d or placebo Subjects: Adults with stage 3-4 chronic kidney disease(CKD) Duration: 6 months	Increased	-	No improvement in insulin sensitivity, fuel utilisation, energy expenditure	Vitamin D supplementation has no metabolic effect in presence of CKD

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Belenchia et al., 2013)	Columbia, US	Dosage: 2000IU x2/d or placebo Subjects: Obese adolescent Duration: 6 months	Increased	No change in weight and BMI	HOMA-IR(p=0.033), QUICKI (p=0.016) show improvement in IS, reduce in fasting insulin (p=0.026)	Significant change in insulin markers observed after 6 months
(Salehpour, Hosseinpanah et al., 2012)	Tehran, Iran	Dosage: 25 µg/d D3 Subjects: Premenopausal OW/OB, Duration: 12 weeks	Increased		Improved levels of HDL in Vitamin D group	
(Carrillo et al., 2013)	USA	Dosage: 400IU/d Subjects: OW/OB Duration: 12 weeks	Increased	None	None	Calcium-500mg/d was given to all participants to control with sunscreen and exercise training for 12 weeks No significant change in IS markers as well body composition
(Gallagher et al., 2012)	Omaha, US	Dosage: low (400 and 800 IU/d), medium (1600 and 2400 IU/d) and high (3200, 4000 and 4800 IU/d). or Placebo	Not reported	No change in body fat, lean mass	-	Women with BMI<25kg/m^2 increased serum levels of vitamin D levels greater than those higher than 25 kg/m^2

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
		Subjects: 57-90 y Duration: 1 y				
(Gallagher et al., 2012)	Omaha, US	Dosage:0.25mcg twice/d Subjects: 57-90 y healthy white women Duration: 3 y	Increased	None	-	PTH reduced by 15% in calcitriol gp
(Ardabili et al., 2012)	Iran	Dosage: 50 000IU/d D3 or placebo every 20 d Subjects: Females (20-40 years) with PCOS and vitamin D deficiency. Duration: 8 weeks	Increased	-	No improvement in HOMA-IR and QUICKI	Not effective in PCOS and
(Nikooyeh et al., 2011)	Tehran, Iran	Dosage: Group 1: no vitamin D 150mg Ca BD; Group 2- 500IU vitD + 150mg Ca BD; Group 3 - 500 IU + 250mg Ca BD. Subject: T2DM 30-60 yr Duration: 12 weeks	Increased	↓ BMI and waist ($p<0.001$)	Improved IS (HOMAIR), $P<0.001$, reduced HbA1C ($p<0.001$)	Group 2 > group 3> Group 1

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Shab-Bidar et al., 2012)	Tehran, Iran	Dosage: 500 IU/250ml twice a day or placebo Subjects: T2DM adults 25-70 yrs of age Duration: 12 weeks	Increased	-	Improved IS markers (HOMAIR, QUICKI), HbA1C, lipid profile	Improvement in total metabolic in T2DM
(O'Sullivan et al., 2011)	Ireland	Dosage: 15µg/d or placebo Subjects: free living Caucasian females, premenopausal, 18-63 y, Duration: 4 weeks	Increased	-	None	No effect on MetS markers
(Shahar et al., 2010)	USA	Duration: 2 yrs	Increased	Increased wt loss in groups with high vitamin D levels		Higher vitamin D levels associated with greater wt loss
(Zittermann et al., 2009)	Leipzig, Germany	Dosage: vitamin D3 (83 µg/d) or placebo during caloric restriction. Subjects: all with BMI >27 kg/m^2 & 25OHD < 30 nmol/L. Duration: 12 months	Increased	None	Increased LDL, ↓ triglyceride ($p<0.001$) and inflammation ($p=0.049$)	Significant improvement in cardiovascular risk markers

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Talaei et al., 2013)	Tromsø, Norway	Dosage: Gp 1=20,000 IU D3 twice a week, Gp 2= 20 000 IU once a week ,Gp3= placebo twice a week. Subjects: OW/OB men and women Duration: 12 months	Increased	-	↓PTH	Large sample, long duration, good retention. 500 mg calcium to all.
(Holick & Chen, 2008)	Poland	Dosage: 0,25 µg/d or placebo during caloric restriction. Subjects: obese Duration: 3 months	Increased	None	None	No effect of vitamin D with food restriction
(von Hurst et al., 2010)	Auckland, New Zealand	Dosage: 100 µg/d (4000 IU) D3 or placebo. Subjects: South Asian women, with D3 deficiency and IS. Duration: 6 months	Increased	None	-	No relationship between change in vitamin D concentration & BMI 26% of intervention group achieved vitamin D >80 nmol/L
(Nagpal et al., 2009)	New Delhi, India	Dosage: 120,000 IU D3 or placebo X 3 Fortnightly Subjects: over 35 yrs	Increased	-	No improvement in IS, lipid profile	Vitamin D supplementation improves IS only in men (centrally obese, non-diabetic)

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
		Duration: 6 weeks				
(Fuleihan et al., 2006)	Beirut, Lebanon	Dosage: 1,400 IU/d, or 14, 000 IU/d or placebo Subjects: 10-17 y girls Duration: 12 months 500 mg calcium given to all	Increased	Increase in lean mass	-	Increments in lean mass and bone mineral content was more evident in pre-menarchial gp than post menarchial gp. Higher dose had much beneficial effects over low dose
Systematic Reviews						
(Pannu, Zhao et al., 2016)	Australia	SR & MA	Increased	↓ Weight, ↓ fat	-	Serum vitamin D levels increase with weight loss
(Zitterman et al., 2014)		SR on body weight and vitamin D status and supplementation				Body weight strong predictor for deciding the dose of vitamin D supplementation
Cross sectional studies						

Author & year	Study location	Study Details	Vitamin D Status	Effect on body composition measures	Effect on cardio metabolic risk markers	Comment
(Ibrahim Issa et al., 2016)	Brazil	Elderly adults	Decreased	-	Raised triglycerides and very low density lipids (VLDL) with ↓ serum 25 OHD. Whereas increased levels OF 25OHD improve blood glucose levels.	Adequate vitamin D status has benefit on cardiometabolic profile
(Cabral et al., 2016)	Portugal	Adolescents	Decreased	Increased BMI	Raised total cholesterol and LDL	Not associated with MetS
(Beaa et al., 2015)	USA	Association of 1,25(OH)D and MetS	Decreased	Raised waist circumference (p trend<0.04)	Increased TAG(P<0.001), ↓ HDL (P<0.001)	Reduced serum levels associated with MetS (p trend-<0.01)
(Kraemer, Semba, Eggersdorfer & Schaumberg, 2012)	USA	Women	Decreased	Raised body weight, fat, regional adiposity	↓ IS	
(Leenders et al., 2011)	Boston, USA		Decreased	Gain in fat mass		25(OH)D decreased by 0.46 ± 0.22 ng/mL per 1% increment in FM (P = 0.05)

RCT, randomized controlled trial; T2DM, type 2 diabetes; LDL, low density lipoprotein; SR, systematic review, MA, meta- RCT, randomized controlled trial; T2DM, type 2 diabetes; LDL, low density lipoprotein; SR, systematic review, MA, meta-analysis; VLDL, very low density lipo protein, BMI, body mass index; PA, physical activity; EER, estimated energy requirement; SBP, systolic blood pressure; IL-6, Intraleukine factor 6; IS, insulin sensitivity; ER, energy restriction; TAG, triglycerides; HDL, high density lipoprotein; CKD, chronic kidney disease; QUICKI, quantitative insulin sensitivity check index; PTH, pituitary thyroid hormone; PCOS, polycystic ovarian syndrome; IU, international unit; BD, twice a day; MetS, metabolic syndrome; Wt, weight; LDL, low density lipoprotein; OW/OB, overweight/obese.

2.4 Leucine and its Effect of Body Composition and Energy Metabolism

2.4.1 Background

The protein mass of the body undergoes continuous breakdown and resynthesis; this is termed protein turnover. The energetic cost of protein turnover is high and it accounts for a substantial portion of RMR; thereby constituting an adaptive pathway in energy balance (Soares, Piers, Shetty, Jackson & Waterlow, 1994). Leucine, a branched-chain amino acid (BCAA) like many other amino acids, functions as a hormone regulator in addition to being of nutrient value. The role of leucine may extend from acting as a stimulus for synthesis of new proteins via activation on mammalian target of rapamycin (mTOR), signalling in skeletal muscle (Wang & Proud, 2006), to modulating insulin/PI3-kinase signalling (Patti, Brambilla, Luzi, Landaker & Kahn, 1998). It is a fuel for skeletal muscle (Wagenmakers, 1998) and for supplying nitrogen for alanine and glutamine production in skeletal muscle (Ruderman, 1975). It is also a primary nitrogen donor for production of alanine and glutamine in skeletal muscles. Since leucine is one of the nine indispensable amino acids, the efficacy for above-mentioned roles is dependent on dietary intake and increasing leucine concentration in skeletal muscles.

Population Intakes

The NHMRC guidelines suggest minimum protein intake for adults within a range of between 0.75-1.1g/kg/d (*Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes*, 2006). According to the WHO guidelines (2007 #875), the estimated leucine requirements for an adult is 14mg/kg/d. This data was based on the highest-achieved nitrogen balance. In a tracer study using stable isotopes and measurement of whole-body leucine metabolism, the daily leucine usage was suggested to be 40 mg/kg/d>6 g/d, which is approximately 3 times higher than the recommended dietary

allowance values based on nitrogen balance (El-Khoury et al., 1994). In a recent eminent study (Pencharz, 2012a), the authors have reported an upper tolerable level of leucine intake as high as 39g/d in acute studies, beyond which leucine can be toxic. This level is close to the estimated values proposed by El-Koury et al. as 38.3 mg/kg/d (1994) and Kurpad, Raj, El-Khoury, Beaumier & Kuriyan, as 40 mg/kg/d (2001).

Leucine Sources and Action

Major sources of leucine are red meat and dairy, while fair amounts are found in eggs, poultry, fish, cereals, grains, beans and nuts. The richest sources of leucine are some cereals such as maize, sorghum and milk proteins, specially β-casein (10.5%), and the whey proteins, a-lactoglobulin (10.6%), and β-lactoglobulin (13.5%).

BCAA can play a role in metabolic functions as protein anabolism, cell growth and metabolism especially in restoring metabolic imbalance (Anthony et al., 2001; Dardevet et al., 2002; Lynch et al., 2002). Of all BCAA, leucine is the most widely studied. At the molecular level, leucine activates the mTOR, resulting in activation of p70S6 kinase and increased serine phosphorylation of IRS-1 (Wang & Proud, 2006), which restrains insulin signalling and insulin-stimulated glucose transport in the muscle (Tremblay & Marette, 2001). However, in his elegant study, Macotela et al. (Macotela, Emanuelli & Bang, 2011) proposed that despite an increase in phosphorylation of p70S6 kinase by leucine, doubling the dietary amount in mice fed a high-fat diet caused improvement in the glucose tolerance. In addition, leucine has also exhibited insulin signalling in adipose tissue in insulin-resistant mice (Hinault, Mothe-Satney, Gautier, Lawrence & Van Obberghen, 2004). The potential roles of leucine including influencing protein synthesis (Kimball & Jefferson, 2001), as a fuel source for skeletal muscle, insulin signalling (Baum et al., 2004) and as a nitrogen donor for production of alanine and glutamine (Ruderman, 1975) rely on dietary intake and increasing leucine concentration in skeletal muscle (Harper, Miller & Block, 1984; Layman et al., 2003). There is evidence that high protein and low carbohydrate diets are successful in weight (Piatti et al., 1994) and fat loss (Skov, Toubro. S., Ronn, Holm & Astrup, 1999) while maintaining lean mass and improving glycemic control (Layman et al., 2005).

2.4.2 Leucine and Body Composition

The loss of muscle in the elderly because of ageing is commonly termed sarcopenia. More recently, the loss of muscle function and muscle mass must also be present to diagnose sarcopenia, i.e., there must be a change in structure and function. Many recent studies support the fact that leucine in sufficient amounts can slow down the process of muscle loss in older adults (Verreijen et al., 2015). Leucine, when fed with adequate meals, can accelerate muscle protein synthesis without making any change in whole body protein kinetics (Rieu et al., 2006). This was possible only when leucine was available above its regular post-prandial levels in the body (Dardevet et al., 2002) and can also restore protein breakdown (catabolism) in old rats (Combaret et al., 2005). Since leucine stimulates insulin secretion, the increase in muscle protein synthesis could be an indirect consequence of an increase in plasma insulin. Thus, it may be beneficial for older adults to have good amounts of leucine to maintain muscle mass.

A reduced carbohydrate and increased protein intake may result in increased energy expenditure, reduced weight and fat mass (Layman et al., 2003), as well as improved insulin sensitivity (Patti, 1998). This weight loss may be mediated by leucine along with other BCAA present in a high protein diet. A study comparing high-protein (HP), adequate protein (AP) and AP supplemented with leucine diets reported leucine demonstrated intermediary effects between HP and AP diets. Leucine had a significant role in energy expenditure, insulin sensitivity, food intake, body composition and liver triglycerols. All diets had similar skeletal muscle protein synthesis. Unexpectedly, leucine showed no alteration in mTOR pathway (Freudenberg, Petzke & Klaus, 2012).

There have been many human and animal studies that show no contribution of leucine in altering lean mass, fat mass or total body weight (Eller, Sahab, Shearera & Reimera, 2013; Leenders et al., 2011; Tong, Li, Xu, Han & Qin, 2014; Balage et al., 2011). On the other hand, significant reductions in fat mass and appendicular lean mass have been noticed in several studies (Bauer et al., 2015; Ispoglou et al., 2016; Verreijen et al., 2015) where this increase in muscle mass was observed in older adults and only in those receiving high doses of leucine

(40% of energy requirement) as compared to standard doses (20% of energy requirement) (Table 2.5) (Ispoglou et al., 2016). In a 6 year longitudinal study among older adults, the group with higher leucine intake in the diet had reduced loss of lean body mass over the period when compared to those who consumed less amounts of leucine-rich foods in their diet. This association was stronger in adults over 65 years of age (McDonald et al., 2016) in comparison to the younger group.

With caloric restriction, leucine could not yet attenuate body weight and fat mass but augmented the appendicular lean mass (Verreijen et al., 2015) which was coherent with adding vitamin D in the diet (Bauer et al., 2015). Under catabolic conditions such as fasting or energy restriction, leucine has a protein-sparing effect by increasing glucose uptake in skeletal muscle via glucose-alanine action. Thus, leucine could be a potential treatment of obesity and metabolic syndrome (Layman & Walker, 2006). In a 6 week supplemental study with caloric restriction, they found that a 47% decrease in body fat and improved protein synthesis capacity in rats receiving a diet containing 1.77% leucine (50% increase in amount of leucine as compared to the control diet) (Donato, Pedrosa, Cruzat, Santana de Oliveira Pires & Tirapegui, 2005). Similar to this, another study with 6 weeks of supplementation of 15 g/l of L-Leucine in drinking water did not find any change in caloric intake or weight gain rates between the groups, although there was reduction in body fat in the leucine-supplemented group (Zampieri, Torres-Leal, Campaña, Lima & Donato, 2014). Leucine has to be accompanied with caloric restriction to target weight and fat loss from the body. A 24 week supplementation study did serial measurements during the trial, and in their investigation they found that the group with calorie restriction showed an 82% greater decrease in weight, two-fold reduction in body fat and three-fold decrease in waist circumference which sustained to two-fold until the end of the 24 week trial as compared to the maintenance group (Zemel & Bruckbauer, 2011).

In contrast, in an animal study, rats fed on a high fat diet developed obesity even more when continuing feeding on 15g/L leucine in drinking water. This was explained by leucine's mimicking ability of a gene expression that favours adiposity (Zampieri et al., 2014). In obese rats on HFD, leucine has been evidenced to increase epididymal adipocyte volume (Torres-Leal et al., 2011) and increased perirenal adipose tissue mass by 45% in another study (Table 2.3) (Zeanandin et al., 2012).

2.4.3 Leucine Action on Cardio-metabolic Variables

Leucine action on insulin sensitivity (IS) also needs confirmation. Animals fed with leucine HFD had increased insulin resistance (Tong et al., 2014; Newgard, 2012) and doubling the dose to what is present in serum improved the insulin sensitivity. Similarly, increasing the duration of supplementation also presented bi-directional outcomes showing improvement in one study (Zhang, 2007) to no change in others (Nairizi, She, Vary & Lynch, 2009). This change in IS is possible without any change seen in body composition or energy expenditure (Macotela, 2011). Leucine supplementation has been linked not only to reduced total and low density cholesterol but also could revert glucose and fat metabolism abnormalities (Zhang, 2007). Addition of casein may possess additional benefit (Eller et al., 2013).

2.4.4 Leucine and Energy Balance

There are plenty of animal but few human intervention and cross-sectional studies on leucine and energy metabolism. However, the results show positive to neutral to negative effects on metabolic rate and substrate utilization. Many *in vivo* and *in vitro* studies signal the role of leucine in energy balance (McAllan, 2013). Increased leucine in the diet is invariably capable of reducing diet-induced obesity and increasing muscle mass (Zhang, 2007). There may be various mechanisms on leucine action on weight loss. Leucine may alter the energy expenditure, energy intake, lipid metabolism and the effect on the cellular activity in the brain (hypothalamus) as well as in peripheral tissues (gastro-intestinal tract, adipose tissue, liver and muscle) regulating metabolic processes.

2.4.4 a Animal Studies on Role of Leucine in Energy Balance

Mice fed on high-fat diet (HFD) plus leucine had reduced food intake after 8 week supplementation where an increase was observed for those on chow+leucine feed without any change in body weight and body composition, thus approving the potential role of leucine in energy expenditure/balance (Zhang et al., 2007). Leucine deprivation may regulate UCP-1 expression. The increased amount of BAT in leucine deprivated mice result in increased thermogenesis as evident by increased oxygen consumption. RQ was lower in the leucine-fed group thus suggesting greater fat oxidation (Cheng et al., 2010).

Evidence in animal studies suggests that two hormones, GLP-1 regulate catabolic activities (Osakaa, Endoa, Yamakawaa & Inouea, 2005) and ghrelin induces anabolic functions (Mano-Otagiri et al., 2010) in the process of altering energy expenditure and hence adiposity. Rats fed on leucine have increased levels of both plasma and cerebrospinal fluid, thus demonstrating its approach to peripheral as well cerebral centres that are involved in maintaining energy balance (Ropelle & Dias, 2008). In addition, L-leucine supplementation over-expressed hypothalamic genes that encode enzymes metabolizing the BCAA. There is evidence eliciting various mechanisms of leucine action on energy balance where leucine can regulate the production of GI tract associated hormones. It is capable of decreasing ghrelin production via activation of the mammalian target of rapamycin (mTOR) (Xu et al., 2009). Leucine may also increase cellular GLP-1 release *in vitro* (Chen & Reimer, 2009). There was no significant difference in substrate oxidation between adequate protein (AP + leucine) and control group, while there was increase in fasting fat oxidation in HP group as evident by lower RQ (Freudenberg et al., 2012).

The other well-known mechanism of leucine is its action on reducing the size of a meal (satiation) as well as the frequency of food intake (satiety). Simultaneously, a study by Bong et al. (2010) concluded that rats on low fat diet supplemented with leucine (161 g casein and 36.3 g leucine/kg diet) have similar food intake to rats on an un-supplemented diet. The absence of the satiation and satiety effects of leucine could be due to the fact that this study used a much lower casein and leucine content to that used by other (Ropelle et al., 2008). In

contrast, it was observed that leucine-deficient mice had reduced energy intake, adiposity and increased EE by upregulating BAT (Cheng et al., 2010). This led to further investigation where leucine was injected cerebroventricularly, and it was argued that the increased expression of corticotropin-releasing hormone (CRH) during leucine deficiency may have contributed to the decreased food intake (Arase, York, Shimizu, Shargill & Bray, 1988).

2.4.4 b Human Studies on Role of Leucine in Energy Balance

One of the ideal situations after weight loss is reduction in fat mass and enhancement in lean mass. Increased lean mass means higher energy expenditure and higher metabolic rate. Involvement of situations such as increased physical activity (Votruba, Horvitz & Schoeller, 2000) and intake of protein in the diet (Farnsworth et al., 2003) in conjunction with caloric restriction definitely result in increased weight loss, fat loss (Layman et al., 2003) and building of muscle mass (Farnsworth et al., 2003). Donato et al. (2005) advocated that 5.91 g (~ 6 g) of leucine supplementation for 6 weeks with 50 percent caloric restriction may result in more fat loss and improve the protein synthesis in liver and muscle. Sun & Zemel (2009) in their work showed that leucine likely mediates these beneficial effects via an increase in mitochondrial mass, as demonstrated in C2C12 myocytes and 3T3-L1 adipocytes during energy flux (Sun & Zemel, 2009). Mediatory effects of leucine on energy balance as reduced energy intake, increased energy expenditure and increased satiety led to the hypothesis that leucine deficiency must do the reverse (McAllan, 2013).

2.4.5 Link Between Leucine and Vitamin D

The idea of a leucine-vitamin D interaction was recently generated in a cellular study where the authors found a synergistic effect of insulin and leucine on mTOR-dependent pathway, which was further stimulated by 1,25(OH)2D3 (Salles et al., 2013). To our knowledge, there have been two clinical trials that have supplemented vitamin D with leucine in their trials to explore its effect on muscle mass in the elderly. Vitamin D was supplemented because of its

strong association with muscle mass (Gordon, Sakkas, Doyle, Shubert & Johansen, 2007) and strength (Gloth, Gundberg, Hollis, Haddad & Tobin, 1995). Both studies supplied leucine plus vitamin D as a drink for a 13 week period, the authors reported a significantly increased muscle mass (Bauer et al., 2015) and appendicular lean mass (Verreijen et al., 2015). We have carried forward the concept and statistically examined the effect of leucine only, the change in vitamin D status during weight loss and the combined effect of leucine and vitamin D status on body composition and metabolic indicators.

The following table (Table 2.8) presents prominent studies, mainly RCTs focussing on the effect of leucine on body composition, insulin sensitivity and lipid profile on both human and animal subjects. As we can see, most of the recent studies included also show it as recent area of interest for research.

Table 2.8 Significant studies relevant to the thesis on leucine supplementation/association to body composition and metabolic health

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
(Ispoglou et al., 2016)	2016	Double Blind (DB), placebo controlled Randomised control trial (RCT)	Older adults 65-75 y	Gp A: 20% Leucine, 11 g Gp B: 40% Leucine, 21 g Gp C: Lactose (control) Duration: 12 wks Diet restriction: None	Lean tissue mass, functional performance(FP)	Improved FP, lean mass increase only in higher percentage leucine group	High dosage of leucine might be needed to show change in lean mass
(Verreijen et al., 2015)	2015	DB, RCT	Obese older adults > 55 y	Gp 1: 150 kcal, 21 g protein, leucine 2.8g, Vitamin D 20µg per 150 ml serve Gp 2: Control Duration: 13 wks Diet restriction: lower 600 kcal than estimated EE Resistance training 3/7	Mass preservation during weight loss and resistance training	Both gp reduced weight and fat from initial but no difference between groups in wt & fat mass Appendicular Mass (AM) increased in treatment gp	Leucine has no added effect except for AM in presence of high protein and vitamin D in diet
(Bauer et al., 2015)	2015	Multicenter, DB, Placebo controlled RCT	Older adults	Gp 1: 20 g whey protein, 3 g total leucine, 9 g carbohydrates, 3 g fat, 800 IU	Primary - Handgrip strength, physical performance, Secondary - AM	Active gp gained more AM, improvement in lower extremity function	Leucine might have additional benefit when provided with vitamin D

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
				vitamin D, and a mixture of vitamins, minerals, and fibers Gp 2: Isocaloric drink with carbohydrates, fats and trace minerals Dosage: Twice daily Duration: 13 weeks Diet restriction: None			
(Tong et al., 2014)	2014	RCT	Non-obese insulin-resistant rats	Fed on high fat diet (HFD) for 16 weeks to develop obesity Gp 1: 0% whey pro Gp 2: 5% WP Gp 3: 15% WP Gp 4: 1.5% LEU Duration: 8 weeks	Insulin sensitivity (IS), lipid profile, antioxidant activity	No change in body wt, body composition, food intake with leucine diet. 15% WP and leucine gp reduced AUC after OGTT, fasting insulin and HOMA-IR	High dose of WP and leucine improves IS
Zampieri et al.	2014	RCT	Obesity-developed Wistar rats	Fed on HFD for 10 weeks Gp 1: Chow diet Gp 2: Cow + leucine Gp 3: HFD	Gene expression, fat mass	Leucine mimic hypothalamic pattern of gene expression that favours adiposity	Leucine may favour fat accumulation in obesity even more on HFD

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
				Gp 4: HFD + leucine Dosage: 15g/L in drinking water Duration - 6 weeks			
(Zemel, 2013).	2013	RCT	Obese adults	Gp 1: Hypocaloric diet (less 500 kcal) A: 2.25 mg leucine + 30 g B6 (Nushape capsules) B: Placebo Gp 2: maintenance diet A: leucine + B6 (Nushape) B: Placebo Duration – 24 weeks Diet restriction: less 500 kcal/d in diet for leucine group	Body composition	Fat and weight loss in leucine supplemented gp which even increased with hypocaloric diet	Leucine with combination of low calorie diet may benefit more in altering body composition

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
(Eller et al., 2013)	2013	RCT	Sprague-Dawley Rats	Gp 1a: casein (14%)+ high Ca Gp 1b: casein (14%)+ low Ca Gp 2a: sk milk Pd +high Ca Gp 2b: skimmed milk Powder +low Ca Gp 3a: Leucine (4%)+ Casein (10%) + high Ca Gp 3b: Leucine (4%) + Casein (10%) + low Ca Duration: 8 weeks	Body composition, IS, gene expression in liver and muscle	Leucine improved IS Gene expression related to IS was altered Leucine gp reduced wt and FM in high calcium gp	Leucine alone has no effect on improving body composition but may affect IS
(Freudenberg et al., 2012)	2012	RCT	Male C57BL/6 mice	Gp 1: 20% fat+ 10% pro (adequate protein, AP) Gp 2: 20% fat + 50% protein (high protein, HP) Gp 3: AP+L (6%) Duration: 20 wks	Metabolic syndrome (MetS) and related-EE, Body composition, IS, liver lipogenesis, expression of mRNA	AP+L gp decreased BW($p<0.01$) and fat gain, increased EE, the prevention of high-fat-diet-induced hepatic steatosis, increased insulin sensitivity and increased post absorptive muscle protein synthesis. Otherwise, leucine supplementation did not	Leucine can duplicate some but not all metabolic responses of high protein diet

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
						affect LBM, hepatic lipogenesis and white fat lipolysis	
(Balage et al., 2011)	2011	RCT	4 months aged adult rats	Gp 1: 15% pro diet + 4.5% Leucine (Leung) Gp 2: 15% pro diet (C) Duration: 5 weeks	IS, body comp	No difference between groups on weight, lean mass, insulin sensitivity, plasma lipids, food intake Leucine had benefit on early steps of insulin signalling (30 min) which vanished after 1 h Increased glucose transport in skeletal muscle but no change in whole body glucose metabolism	Leucine may induce local adiposity as in this case, it increased visceral and IR in healthy state
(Macotela et al., 2011)	2011	RCT	8 week aged C57BL/6J mice	Mice first fed on high fat diet for 8 weeks to develop MetS characteristics Gp 1: Chow diet Gp 2: Chow + leucine Gp 3: HFD	Insulin sensitivity and other metabolic functions	Improved IS, characteristics of MetS reverted, reduced inflammation in adipose tissue and hepatic steatosis. No additional effect of leucine on body weight	Increasing dietary leucine may improve IS in obese population

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
				Gp 4: HFD + leucine Dosage: 1.5% w/v Duration: 8 wks			
(Leenders et al., 2011).	2011	RCT	Elderly males with T2DM	Gp 1: 2.5 G Leucine Gp 2: Placebo Duration: 6 months Diet restriction: None	Primary: Change in muscle mass, glycemic control Secondary: Fat %, muscle strength, muscle fibre type, lipidemia	No beneficial effect of prolonged supplementation in MM, FAT %, muscle strength, glycemic control, lipidemia	Extended duration of leucine supplementation may not benefit
(Zhang et al., 2011)	2011	RCT	Male C57BL/6J-5 months of age	Gp 1a: chow fed diet+Whey 1b: chow + Leucine Gp 2a: HFD +Whey 2b: HFD+ Leucine Dosage: Leucine (1.5%)-55 mg via drinking water Duration: 10 weeks	Insulin sensitivity, energy metabolism, expression of mRNA, body composition, blood parameters	Reduction in wt gain, increased EE, increases expression of UCP-3, BAT & WAT, improved diet-induced glucose and cholesterol metabolism IN HFD fed mice	No positive effect on control diet with leucine supplementation in any of the metabolic consequences

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
(Vianna et al., 2012)	2011	RCT	6 months aged rats	Gp 1: Adult Gp 2: Leucine Gp 3: Control Dosage: 4% Duration: 40 weeks	Body weight, food intake, chemical carscass composition, indicators of acquired chronic disease and protein status	Body weight and fat in leucine gp was lower than control but higher than adult gp. No difference in glycemic and lipid status between the groups	Leucine may prevent body fat gain with increasing age but cannot protect from metabolic consequences and retaining of lean mass due to aging
(Verhoeven et al., 2009)	2009	RCT	Elderly males	Gp 1: Placebo (n=15) Gp 2: leucine (n=15) Duration: 3 months Dosage: 7.5 g/d	Muscle mass and strength	No change in muscle mass or strength post-supplementation	No long-term supplementation effect observed on muscle mass or strength
(Ropelle et al., 2008)	2008	RCT	Normal and ob/ob rats	Gp 1: High protein diet, normal rats Gp 2: Leucine diet, normal rats Gp 3: High protein diet, ob/ob rats	Food intake, weight loss	Decreased food intake, body weight. Increased fat mass, no change in lean mass among leucine fed groups	Leucine has similar effect to high protein diet

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
				Gp 4: Leucine diet, ob/ob rats			
(Rieu et al., 2006)	2006	RCT	Older male adults	Acute study: Basal period: 240 min Next 5 h: diet with leucine or placebo Gp 1, control diet: 10.2 kcal, 0.4 g protein (in the form of casein), 1.3 g carbohydrate (dextrine maltose) and 0.36 g fat per kg body weight Gp 2, control diet +Leucine (0.052 g kg−1) + isoleucine (0.0116 g kg−1) + valine (0.0068 g kg−1) Duration: 540 min.	Effect of leucine supplementation on muscle protein synthesis and whole-body protein kinetics	Increased muscle synthesis	Potential importance for older adults

Author	Year	Type of Study	Target Population	Intervention	Outcomes	Major Results	Lessons Learnt
(Donato et al., 2005)	2006	RCT	Male Wistar rats on 50% caloric restriction	Control gp - AIN-93M diet Experimental gp - AIN-93M diet + 5.91 g leucine Duration: 6 weeks	Body composition and protein status	No difference in weight. Leucine gp lost more fat, improved liver protein status and muscle protein synthesis No effect on lean mass	Upon calorie restriction, moderate dose of leucine may reduce body fat and encourage protein synthesis
(Jensen & Haymond, 1991)	1991	Cross-sectional	Pre-menopausal obese women	Gp 1: UB Obese Gp 2: LB obese Gp 3: Control (NO) Diet restriction: None	Leucine turnover in obesity	Basal leucine carbon flux was greater in both obese groups than NO, but during insulin clamp suppressed only in LB obese and NO women	Increased proteolysis in obesity. Body fat distribution may affect glucose metabolism

DB, double blind; RCT, randomized controlled trial; FP, functional performance, Gp, group, Wt, weight; AM, appendicular mass, EE, energy expenditure; HFD, high fat diet, IS, insulin sensitivity; AUC, area under the curve; HOMA-IR, homeostasis model assessment- insulin resistance; MetS, metabolic syndrome; FM fat mass, LBM, lean body mass; MM, muscle mass; Wks, weeks; UCP, uncoupling protein; BAT, brown adipose tissue, WAT, white adipose tissue; UB, upper body, LB, lower body, NO, non-obese.

2.5 Conclusion of the Review

The first part of this review concludes that the thermal neutral temperature range for the Australian population might differ to those residing in much colder countries and thus needs to be reconsidered. The metabolism and adaptation ability of people living in Australia might differ to people living in Europe where colder ambient temperatures are the norm. Furthermore, the emerging ethnic mix of our population may complicate the definition of such temperature ranges. This suggests that we also need a better explanation on why obese subjects react differently to the same temperature than the non-obese population and the alternate pathways they follow to maintain their core body temperature. This can enhance our knowledge on future therapeutic targets to treat obesity and related disorders. Further evidence is needed on the mediatory role of hormones such as irisin and FGF21 on energy metabolism, especially non-shivering thermogenesis. To our knowledge, there is no study associating metabolic syndrome status with their response to CIT and DIT. In this thesis, we have addressed this angle by studying a Western Australian population group with due attention to skin blood flow and the thermal neutral range.

Secondly, comparing the data from the text above and that of last 10 years (Table 2.8) with additional evidence, we foresee the discrepancy that exists regarding the causal relationship of vitamin D status with obesity and related risk factors such as CVD and insulin sensitivity. The minimum levels of serum vitamin D to be termed as 'sufficient' is also still in debate. Place of residence may affect vitamin D levels and consequently determine obesity and insulin sensitivity occurrence. There is also a need for more in-depth studies to understand the mechanisms by which vitamin D affects human metabolism. Post-prandial effects of vitamin D may differ to fasting metabolism. There are some recent cross-sectional studies associating MetS with vitamin D available as discussed in the above text but more studies are still needed, especially targeting population residing in different geographical locations. Further information on the role of vitamin D in human metabolism and its interactions with other nutrients such as calcium, vitamin B6 and leucine need to be explored further to ascertain the combined effect of nutrients.

This review has identified a number of areas with limited or debated evidence that need further research. Some of these areas include: investigating the role of vitamin D levels

on adiposity measures, insulin sensitivity, cardio metabolic variables and to examine the effect of vitamin D status in fasting post-prandial energy metabolism and the combined effect of a BCAA, leucine and vitamin D in the presence of weight loss among individuals with obesity/ MetS. Hitherto, there is not much data for Australian population regarding the optimal levels of vitamin D or its deficiency outcomes on energy metabolism in either obese, CVD, IR or MetS positive individuals.

Lastly, despite the availability of an array of data among humans and animals, a discrepancy on the role of leucine on body composition and metabolic function remains (Table 2.5). This still demands for more high-quality RCT to confirm the positive association of leucine among human subjects predominantly on reducing fat mass and improving lean mass with a variation in age groups, with different physical activity levels and at different stages of onset of obesity and diabetes. We also need to understand its cellular action and other associated pathways in leucine metabolism. In addition, to ascertain if leucine might have added benefit in the presence of other nutrients such as protein, vitamin D and vitamin B12. Thus, more clinical trials focussing on these aspects are required.

2.6 Limitations of the Review

Since there was insufficient comparable data for the Australian population on cold acclimatisation, it was necessary to rely on studies conducted in much colder countries. Additionally, there was not enough literature on energy metabolism among MetS population. Due to contradictory studies on either leucine or vitamin D status/role on energy metabolism, obesity and insulin sensitivity clear conclusions could not be drawn and further research is directed.

2.7 Identified Knowledge Gaps

1. Australia has a population mix of various ethnicities who have been residing here for a short/long duration. Perth has a mean annual temperature of ~25°C. Winter temperatures in Perth could be closer to the warm days of some European countries. This may have altered the thermal comfort zone as well thermoneutral zone for most of the inhabitants. This requires a new definition for TNZ for the Australians.

2. Secondly, it is well understood that there are individual variations in TNZ and hence this may differ in presence of metabolic syndrome. To our knowledge, there is no study on Australian population with MetS determining their TNZ and their response to cold on energy metabolism.

3. Despite abundant sunshine, vitamin D deficiency among the Australian population is a concern. The potential role of vitamin D status in reducing obesity, fat gain/loss or improving insulin sensitivity and lipid profile is still debated. We have enough evidence regarding the above associations but discrepancy around optimal vitamin D levels, dosage of supplementation, duration and food interaction still exists. We need to ascertain whether vitamin D has a role in reducing obesity and understand the mechanism by which it might be possible.

4. Vitamin D status may vary with presence of obesity, chronic diseases or lifestyle factors and needs important consideration when planning to treat any of these. Again, we have countable studies assessing vitamin D status and its association with energy metabolism and chronic diseases in the Australian population.

5. Leucine, being a component of BCAA, and abundantly present in our daily dietis well known for its beneficial role in weight management but we still need to establish its role in human energy metabolism, insulin sensitivity and its action on altering body composition. For example, it might not be directly reducing fat mass but might result in decreased fat % by increasing lean mass with the right amount and duration of supplementation. The presence of caloric deficit may play a role.

6. A recent study has suggested a possibility of association between muscle strength, and both leucine and vitamin D, and we have tried to explore their combined effect on weight loss success. Sustainability of weight loss for a longer term is an option for future research.

The following chapters aim to bridge the gaps in understanding energy metabolism in obesity/MetS and determine if exposure to cold temperature, weight loss and vitamin D status can be potentially used as treatment options.

Chapter 3 Methods

3.1 Techniques/Equipment Used in the Study

3.1.1 Environmental Chamber

All metabolic measurements described in this thesis were carried out in an environmental chamber. The chamber is housed within a large room of the School of Public Health. It measures 5 x 3 meters with a volume of 57.75 m^3 and is insulated on all sides, with an independent control system for a temperature range from 4°C to 50°C. Humidity was set at 40% for all measurement days. The system logs temperature and humidity every 10 minutes and this data is continuously displayed on a built-in monitor screen.

3.1.2 Anthropometrics and Blood Pressure

The height was measured using a calibrated, portable, wall-mounted stadiometer (Seca, Hamburg, Germany). The participants are measured with bare feet, feet touching together against the wall. Weight was measured by a calibrated digital scale, after they had voided and changed into the gown. The weight was subtracted from the final weight. Waist measurements were done in standing position using a steel tape at the mid-point of lowest rib margin and iliac crest. The measurements were done twice and the mean was recorded. Except for height, other anthropometric measurements were repeated on each visit. Blood pressure was measured using an automatic (arm cuff) blood pressure monitor (Omron, Omron, HEM 7121standard Upper Arm Blood Pressure Monitor). Two blood pressure readings were measured at each time and the means recorded.

3.1.3 Body Composition Using DEXA

Dual Energy X-ray Absorptiometry (DEXA), (Prodigy™, Lunar USA) or DEXA scanning, is currently the most widely used gold standard to measure bone mineral density and body composition for research purposes. Quality assurance tests were conducted and logged each

morning as per manufacturer guidelines. During the test, a patient lies down on an examining table, wearing only a gown over undergarments. All metal jewellery is removed prior to testing. The DEXA scanner rapidly directs the x-ray energy from two different sources towards the body being examined in an alternating fashion at a set frequency. DEXA can accurately track regional changes in appendicular and non-appendicular lean tissue mass, fat mass and distribution from the android to Gynoid ration as well as whole-body bone mineral density and content.

3.1.4 Body Composition Using BIA

Body composition was measured for its components such as fat mass, fat percent, lean body mass and fat free mass (FFM) using, InBody 3.0, multi frequency Bio Impedance Analyser (InBody Co. Ltd, Korea) for the leucine supplementation study on each fortnightly visit. Since DXA emits a small amount of radiation there is a limit to its use in tracking body composition in a trial through repeated use. BIA-based body composition has good precision (Kyle et al., 2004; Segal, Van Loan, Fitzgerald, Hodgdon & Van Itallie, 1988).

3.1.5 Temperature Measurements

Skin temperature was measured continuously during the experiment by means of iButtons (type DS1921H; Maxim/Dallas Semiconductor, Dallas, TX) which was attached to the skin using fixomull tape (BSN,Hamburg, Germany) at mid-point of forearm and fingertip of middle finger of left arm. The mean skin temperature was calculated as the average temperature at both sites, and forearm to fingertip gradient (FFG) was calculated to ascertain body response on exposure to different temperature. *In the ear temperature (IET)* was measured using an Omron ear thermometer before and after each BMR measurements (Omron, model MC-510, Japan). Recordings were conducted in triplicate and the lowest two of three readings were noted down.

3.1.6 Indirect Calorimetry, Resting Metabolic Rate (RMR)

After an overnight fast of 10 hours and minimum eight hours of sleep, energy expenditure through indirect calorimetric was measured on the Deltatrac II indirect

calorimeter (DATEX-Ohmeda, Helsinki, Finland) using a ventilated hood system with the participant lying on the bed in supine position. Participants were requested to maintain minimal physical activity in the morning and to refrain from taking a shower in the morning or rushing to the study site. Participants were requested to arrive between 6.00 - 7.00 am and all RMRs were completed before 9 am. After resting for a minimum of 30 minutes at the temperature to be measured, RMR was recorded twice for 25 min with a 10 min rest over the hour. Post-glucose measurements were conducted on each half hour for a 15 minute duration for each half hour. Over the 2 hour period 4 consecutive readings were made. EE was calculated using the equation of Weir (1949) given by EE (kcal/min) = 3.9 + [1.1 x RQ]. Respiratory quotient (RQ) was calculated as the ratio of carbon dioxide production to oxygen consumption. The machine was calibrated each morning with a mixture of gases of known concentration (95% oxygen and 5% carbon dioxide). Alcohol burns were conducted intermittently throughout the study to confirm the validity of oxygen and carbon dioxide sensors. The theoretical value of RQ with alcohol combustion is 0.66. The mean RQ (± SD) of 13 burns was 0.66 ± 0.012, with a CV of 1.88 %.

The Deltatrac machine used to measure BMR broke down suddenly in the middle of visit 2 following the leucine supplementation study (phase 2). We therefore had to use another available metabolic monitor called TrueOne metabolic cart (Parvomedics, USA). 12 participants of the leucine trial were measured on this new monitor for their post measurements. Following repairs of the Deltatrac and at the end of our trial, we ran a validation study to determine the difference between the machines. 12 participants (5 from the leucine trial and 7 new participants from the community) were recruited.

3.1.6 a Conversion of TrueOne Values to Deltatrac Values - A Cross-validation

The Parvomedics system TrueOne (Parvomedics, USA) is one such system that shares the same make of oxygen and carbon dioxide analysers as the Deltatrac II. It comes with the option of both a canopy or a nose-clip and mouthpiece set up for the collection and

conduction of respired gases to the gas analysers. In this study, we tested the validity of the Deltatrac II in canopy mode to the TrueOne in mouth piece and nose clip mode.

The equation developed was used to convert TrueOne measurement gases (O_2 and CO_2) to Deltatrac gas units.

Study Design

Repeated measures with duplicates with balanced order of machines (Figure 3.1). Total of 4 measures in each subject (2 repeats x 2 machines) were made.

OR

Figure 3.1 Order of measurements

N=6 started on one order and N=6 on the other. Subjects were given a 10 min rest between each measurement.

Participant Recruitment

Selected participants ranged from normal to obese across the body fatness range. Adult men and women, aged 20 yrs and over in good health and weight stable (± 2 kg over last 6 months) were selected from amongst the staff, students of the University and from the local community. They arrived at Curtin University after an overnight fast of 10-12 hrs and rest for a mandatory 30 min before undergoing RMR measures in the random order assigned. At the end of the trial, the body composition from Bio-impedance analysis (SECA mBCA, Germany) was determined. All measurements were conducted in the clinical Room 209 of the School of Public Health. No blood specimens were collected.

Sample Size

Based on an effect size of 0.61 (difference to be detected = 30 kcal and an SD of difference of 80 kcals), a power of 0.95, α=0.05, we needed N=10 for repeated measures analysis of RMR (GPower version 3.1.9.2) (Faul, Erdfelder, Lang & Buchner, 2007). As this was a single day trial we did not expect a large drop out, so a 15% nominal value was used to calculate the final sample of N=12.

Ethics

All participants completed an informed consent form after the ethics approval was obtained (Approval Number SPH- 46-2014).

Equations used for converting gases from TrueOne system to Deltratrac (Leucine supplementation study):

Equation for converting TrueOne oxygen to Deltratrac oxygen:

Equation 1: Deltratrac oxygen = 107.9 – (age x 0.989) + (gender x 35.7) + (fat mass x 1.879) + (Trucal oxygen x 0.267)

Equations for converting TrueOne carbon dioxide to Deltatrac carbon dioxide [backward elimination]:

Equation 2: Deltatrac CO2 = 91.4 + (0.272 x TrueOne CO2) + (15.84 × gender)

Equation 3: Calorific values of oxygen = 3.9 + 1.1 × RQ

Equation 4: Hence EE kcal/min = oxygen consumption x [3.9 + 1.1 × RQ]

The original measurements on the TrueOne during the last phase of the leucine trial were then converted to 'Deltatrac' measurements for compatibility with the rest of the data collected on the leucine trial.

3.1.7 Measurement of 'Metabolic Switch'

The respiratory quotient was measured at the fasting and as the total area under the curve (AUC) during the post-prandial period. 'Metabolic switch' was calculated as the difference (or change) between the mean RQ as measured during the 30-min basal steady state period and the mean RQ post-glucose meal. Analysis of AUC adjusted for baseline was also used as an alternative, more robust statistical approach, since in some experimental situations the baseline [fasting] RQ could be different within and between participants.

3.1.8 Blood Assays

All blood samples were collected in BD vacutainer lithium separator tubes (BD Vacutainer® Serum Separator Tubes (BD Vacutainer® SST™ II Advance, Franklin Lakes, NJ, USA)- draw- 5 ml; size 13x100, green top)) and EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA, cat# 367873, purple top) as per the analysis needed using a butterfly needle (BD-Vacutainer Safety-Lok, Ref # 367282). Finger-stick blood samples were also performed immediately for glucose levels using a glucose monitor (Accucheck Performa, Blood glucose system). The LDL-cholesterol was calculated as: [Total cholesterol-HDL-(Triglycerides/5)]. Two venous blood samples were collected under aseptic conditions from a peripheral vein into appropriate vacutainers by trained phlebotomists. In total, 12 ml blood was collected fasting and 6 ml post-prandially for substrate/hormones responses to test meal ingestion. All samples were immediately centrifuged for 10 minutes at 1,500 x g (where g is the gravitational force) and the plasma was stored, labelled and coded in accordance to participants' assigned ID number prior to storage at -80ºC, until they were sent for analyses at the Royal Perth Hospital. The blood was tested for glucose, insulin, lipids and vitamin D status and liver function. Liver function as measured by serum concentration of AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) was used to rule out liver disease and major non-alcoholic fatty disease of the liver as exclusion criteria that might affect vitamin D metabolism.

3.1.9 Measurement of Insulin Resistance

We administered an oral glucose tolerance test (OGTT) which is approved by WHO criteria for diagnosis of Diabetes Mellitus and hyperglycemic states as advocated by Australian expert committees (Colman et al., 1999) and American Diabetes Association [ADA], 2016. For comparing post-prandial responses, the area under the curve (AUC) was calculated according to the trapezium rule.

3.1.10 Physical Activity Record

Physical activity was recalled using the International Physical Activity Questionnaire (IPAQ - short version) (Lee, Macfarlane, Lam & Stewart, 2011). The amount of physical activity was recorded for over the last 7 days and classified as either vigorous, moderate or walking

activities and given in units of metabolic equivalents (MET) minutes per week, where METs are multiples of the resting metabolic rate. These questionnaires have been validated in earlier studies (Lee et al., 2011).

3.2 Participant Recruitment

Participants were recruited via (a) flier advertisement situated around Curtin University Bentley Campus; (b) radio advertising on Curtin FM; (c) contacting General Practitioners in the local vicinity to gain approval for placing fliers in clinic waiting rooms; (d) advertisement in community newspapers, and (e) word of mouth. Curtin Media was approached to assist with the recruiting process. Interested participants were asked to complete a short screening questionnaire over the phone with the first co-investigator or online to ensure the interested participants fulfil the inclusion criteria for the study. Screened participants were then asked to arrive at the laboratory for orientation where they were made familiar with the experimenter, the chamber and the equipment to be used for the study. They were then required to sign consent forms after they have read the participant information sheet for the study, agreed and expressed interest to participate in the program.

3.3 Protocol Followed on All Measurement Days

The protocol was common to both the clinical trials (Chapters 4, 5, 6 & 7). Any addition/variation to the protocol is clarified in the respective chapters (Figure 3.2).

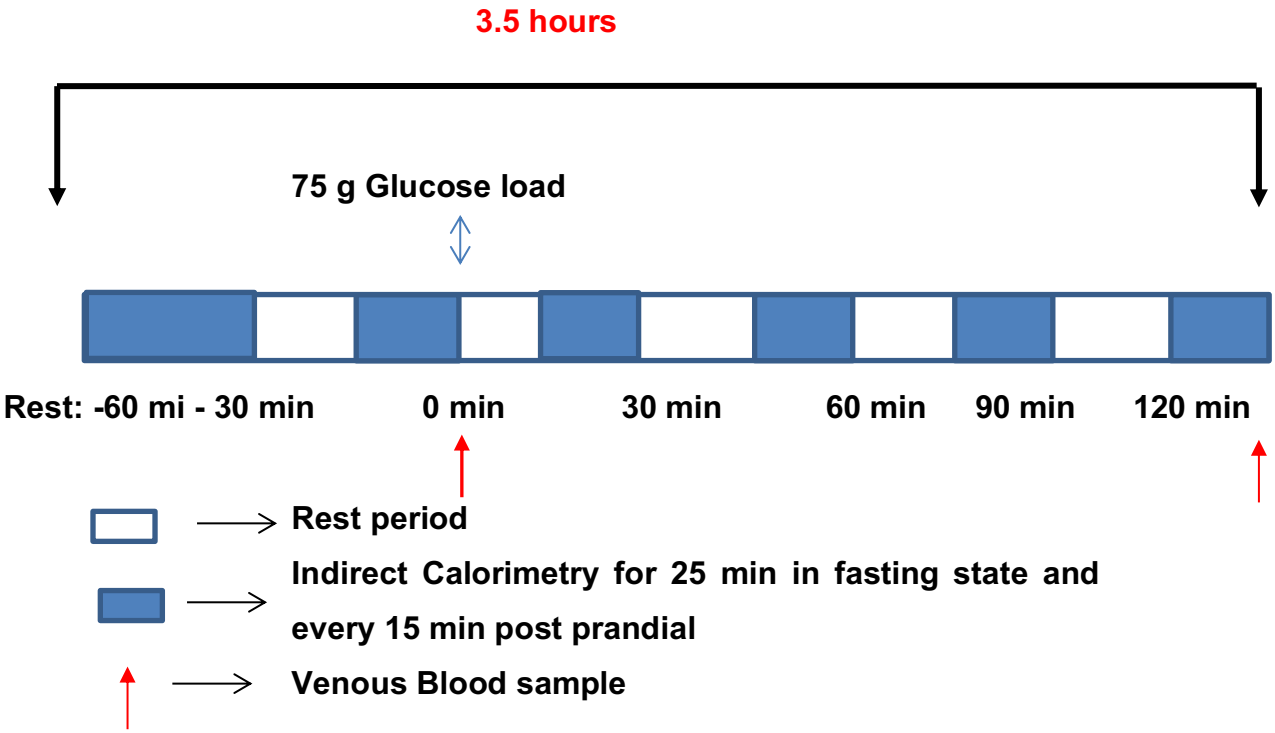

Figure 3.2 Protocol of Experimental Day

The study was conducted in the School of Public Health, Curtin University, Australia. All participants were being measured in a specialized temperature-controlled chamber set to the required temperatures at least 12 hours prior. Participants arrived at the laboratory in a post-absorptive state, after a minimum of 10-12 hours of overnight fasting and 8-hour sleep, and abstinence from heavy physical activity and alcohol at least 36 hrs prior to the trial day. All participants consumed a low fat, standardized frozen dinner meal (~ 450 kcal, 15-20 g protein and ~ 15 g of fat) on the nights prior to the experiment. They had an option to choose from beef, chicken or a vegetarian dish. Upon arrival, they were asked to void and change into a standardized gown (~0.46 clo). The insulation of clothes is often measured in the unit 'Clo', where 1 Clo = 0.155 m^2K/W. Clo = 0 corresponds to a naked person. The females wore their own underwear and bra (~0.04 clo) and males wore own underwear (~0.04 clo). In total the garments were 0.5 clo. Height was measured using a wall-mounted stadiometer. Waist circumference was measured using a steel tape around the mid-way of lowest point of rib cage and iliac crest. Body weight was measured using a digital calibrated scale after correction for gown weight. Forearm to fingertip skin temperatures were serially monitored by placing two (thermistors) iButtons™ (Texas, USA) using fixomull tapes, one on the mid-

point of left forearm and the other on the fingertip of the middle finger of the same arm. Subjects then entered an insulated chamber where the temperature of the experimental day had been randomly selected and set minimum 12 hours before. Detailed body composition was measured using a Dual X-ray absorptiometry DEXA (Prodigy™, Lunar USA) as required during the study. Following a mandatory 30-minute rest in a supine position on a bed, the baseline metabolic rate was measured using indirect calorimetric (DELTATRAC II, Datex, Finland) for the following hour. They were measured twice for 25 minutes each RMR. A fasting blood sample (12ml) was collected on conclusion by a trained phlebotomist. An oral glucose tolerance test (75 g glucose in 300 mL water, CarboTest, Australia) was administered and a venous sample (6ml) was collected after 2 hr. Indirect calorimetric was continued after the glucose load in an intermittent fashion (i.e. for 30 min in 1st hour and for another 30 min in 2nd hour) in all participants. Meanwhile, the blood pressure (Omron, HEM 7121standard Upper Arm Blood Pressure Monitor), and the inner ear temperature (Omron, model MC-510, Japan) were measured each time after the hood was taken off. Blood pressure and endothelial function were measured twice and the average of the two was noted. The ear temperatures were done a third time to get consistent readings, and the lowest two of three were noted and averaged. They were allowed to listen to light music or read as that involved minimal physical movement. Small quantities of water were permitted if at all required until lunch time. This amount was noted and kept constant for all trials in that participant. At the end of the study, a buffet-style meal was served for the participants. All participants were asked to fill in the IPAQ (short version) for physical activity. These questionnaires have been validated in earlier studies (Lee et al., 2011).

3.4 Statistical Analysis

We used different statistical approaches for RCTs and cross-sectional analysis to answer the research question, and their details are indicated in the relevant chapters (4-7).

SECTION A

Ambient Temperatures, Skin Temperature Gradients and Energy Metabolism

Chapter 4 Fasting and Glucose-induced Thermogenesis in Response to Three Ambient Temperatures: A Randomized Crossover Trial in the Metabolic Syndrome

Objectives:

1. To examine whether an acute cold exposure could stimulate adaptive thermogenesis and alter basal metabolism and glucose-induced thermogenesis.
2. To explore if metabolic hormones such as irisin and Fibroblast Growth Factor 21 (FGF21) played a role in these processes.

This chapter is an expanded version of a manuscript by the same title accepted for publication as: Pathak K, Woodman RJ, James AP, Soares MJ. Fasting and glucose induced thermogenesis in response to three ambient temperatures: a randomized crossover trial in the metabolic syndrome. European Journal of Clinical Nutrition. 2018. doi:10.1038/s41430-017-0058-x.

4.1 Abstract

Background: Cold exposure increases thermogenesis and may improve insulin sensitivity, but this has not been tested in the metabolic syndrome (MetS).

Methods: Twenty older adults 59 ± 10.4 y, (with MetS (MetS+, n=9)) and without MetS (MetS-, n=11), completed a randomized crossover design where each participant underwent a 3.5 hour exposure to 20°C, 25°C and 27°C in random order, on different occasions. After an hour's rest at the desired temperature, resting metabolic rate (RMR), respiratory quotient (RQ), forearm to fingertip gradients (FFG) and in-the-ear temperature (IET) was measured over 30 min. An oral glucose tolerance test followed and serial metabolic measurements continued for 2 hrs. Venous blood was sampled for clinical chemistry, irisin and fibroblast growth factor 21(FGF21). A mixed-model ANCOVA adjusted for age, gender, season, fat mass and fat free mass detected statistical significance. Post-prandial outcomes were additionally adjusted for fasting values.

Results: There was a significant MetS x temperature interaction where RMR increased significantly in MetS+ [20°C > 25°C> 27°C] but not in MetS-. FFG increased and IET decreased with decreasing temperature to the same extent as MetS groups. No temperature effects in irisin or FGF21 were observed. Adjusted post-prandial RQ and insulin to glucose ratio were significantly higher at 20°C relative to 25°C. Partial correlations indicated significant positive associations between changes from 27°C to 20°C, in incremental post-prandial RQ with respective changes in irisin and FGF21.

Conclusions: NST was only evident in those with the metabolic syndrome. Irisin and FGF21 may influence glucose oxidation.

4.2 Introduction

Modern humans live in a comfortable indoor thermal environment which reduces the opportunity for thermogenesis in response to low temperatures. This increase in thermogenesis in response to cold is non-shivering thermogenesis (NST). However, if the temperature is too low and the maximum capacity for NST is reached, shivering thermogenesis (ST) is seen. The recent documentation of the presence of brown adipose tissue (BAT) in adult humans has renewed an interest in NST and its potential to attenuate obesity (Cannon & Nedergaard, 2004). Classical non-shivering thermogenesis (NST) is both facultative (i.e. it can be turned on and off within minutes) and adaptive (i.e. that it needs weeks to develop its full capacity) (Cannon & Nedergaard, 2004; van Marken Lichtenbelt & Schrauwen, 2011). NST may induce an increase in RMR by 14% following long-term exposure (days) and by 30% in short term (few hours), acute exposure (Busiello, Savarese & Lombardi, 2015; Celi et al., 2010; Claessens-van Ooijen, 2008; Van Ooijena et al., 2004; Warwick & Busby, 1990), though some individuals show no increase in NST (Calton, Soares et al., 2016; van Marken Lichtenbelt & Schrauwen, 2011). The response however is highly variable (Kingma, 2011; van der Lans, 2013; Van Pelt, 2001; Wijers, 2010b) and a number of factors can influence its measurement in man (Brychta, 2017). These include the methods used for calorimetry, the precise ambient temperatures compared (warm vs. cold) and the manner of cold exposure (air vs. water cooling). Investigators have also noted either no change or a decrease in thermogenesis on cold exposure (Calton, 2016b; Wijers, 2010b); a response ascribed to the Q_{10} effect (Gisolfi, 2000; Lemieux, 2008). The recent confirmation of brown adipose tissue (BAT) in adult humans has renewed the interest in NST and its potential to attenuate obesity (Cannon, 2004). In fact, small changes in ambient temperatures within a range normally experienced in daily life (24^{o}C vs. 19^{o}C) elicited a thermogenic response determined in part by age, gender and BAT responses of the individuals studied (Chen, 2013). Emerging evidence also implicates two newer candidates, irisin and fibroblast growth factor 21 (FGF21), that may play a role as well in mediating ST and NST respectively (Broeders et al., 2015; Lee, Linderman et al., 2014).

Irisin, is a myokine that was originally discovered in response to increases in physical activity (Daskalopoulou et al., 2014). As currently understood, the shivering response to cold

varies directly with irisin concentrations (Lee, Linderman et al., 2014). Irisin is also capable of converting white adipose tissue to BAT (Choi et al., 2014). On the other hand, FGF21 is a member of a family of peptides that have pleiotropic effects as regulators of lipid and glucose metabolism. Recent data suggest that FGF21 may also convert white adipose tissue to BAT upon chronic cold exposure in mice (Fisher et al., 2012). Of interest to our study are the findings of Lee et al. (2014) who suggest that NST increases FGF21, while ST increases irisin.

There are many studies investigating the effects of cold exposure on energy expenditure of lean and obese humans (Celi et al., 2010; van Marken Lichtenbelt & Schrauwen, 2011; Warwick & Busby, 1990). To our knowledge there is no study on thermoregulatory responses to cold exposure in presence of the metabolic syndrome, and none have attempted to elucidate the potential role of irisin and/or FGF21 in this process. This is important since the global prevalence of MetS has increased tremendously (Cameron, 2007; Ford, 2005; Waterhouse, McLaughlin A.M, Sheehan & O'Shea, 2009; Sawant, Mankeshwar & Shah, 2011; Zhao, 2014) and in general terms, MetS reflects a metabolic dysfunction in addition to obesity. The former is seen as a defective adaptive thermogenesis (Bonet, 2006), altered post-prandial fuel utilization (Pathak et al., 2017), an FGF21 resistance (Novotny, 2014; Zhang, 2008) and lower irisin (Yan, 2014). Consequently, we hypothesized that while NST would be detected, those without MetS (MetS-) would respond with a greater increase in NST and an improved insulin sensitivity on cold exposure. The possibility that post-prandial increases in FGF21 may partly account for such metabolic effects was also investigated.

In this randomized control trial, we chose three temperatures: a mild cold of 20°C and two temperatures (25°C, 27°C) within the accepted thermo-neutral zone (TNZ).

4.3 Methods and Materials

4.3.1 Study Design

The study had a randomized crossover design testing exposure to three ambient temperatures, with a 1-3 week interval between visits. All participants were required to attend our laboratory on three separate days for measurements at three different

temperatures assigned in a random order. Two out of twenty participants chose not to complete the measurement visit at 27 °C. (Figure 4.1)

4.3.2 Sample Size

Based on a repeated measures ANOVA for within (3 measurements), two between subjects (2 group) and their interaction, an effect size of 0.35, an assumed correlation between repeat measures of 0.5, α =0.05 and power of 80%, we required a sample of n=16 (G power version 3.1.9.2) (Faul et al., 2007). We recruited 20% more participants to allow for drop-outs. Similar sample sizes have been used by other researchers in this field to detect changes in cold-induced thermogenesis (Celi et al., 2010; Westerterp-Plantenga, 2002; Wijers, 2010a).

4.3.3 Participant Recruitment, Selection and Randomisation

We advertised for adult Australians of European origin who met the following inclusion criteria: aged 30-70 y, weight stability (< ±2 kg) in the previous 6 months (with no intention for losing weight in the next 6 months), absence of thyroid disease and polycystic ovarian syndrome (by history), non-smokers; non-pregnant, non-lactating, not on hormonal contraception or testosterone replacement therapy or hormonal replacement therapy. Type 2 diabetics on good glucose control as judged by HbA1c <6.5% were included and judged as having the metabolic syndrome.

Allocation to the start of each treatment was through a computer-generated sequence using random allocation software (Saghaei, 2004). Metabolic syndrome (MetS) was determined from the current consensus definition of Alberti et al (2006) which includes presence of central obesity and cardiovascular risk factors such as decreased HDL-cholesterol (HDL-C) concentration, raised triglycerides, elevated blood pressure and elevated fasting plasma glucose concentration. Individuals with 3 or more of the above characteristics were

considered MetS+. Anyone with two or less characteristics were still considered MetS. A flow chart of recruitment, allocation and completion is shown in Figure 4.1.

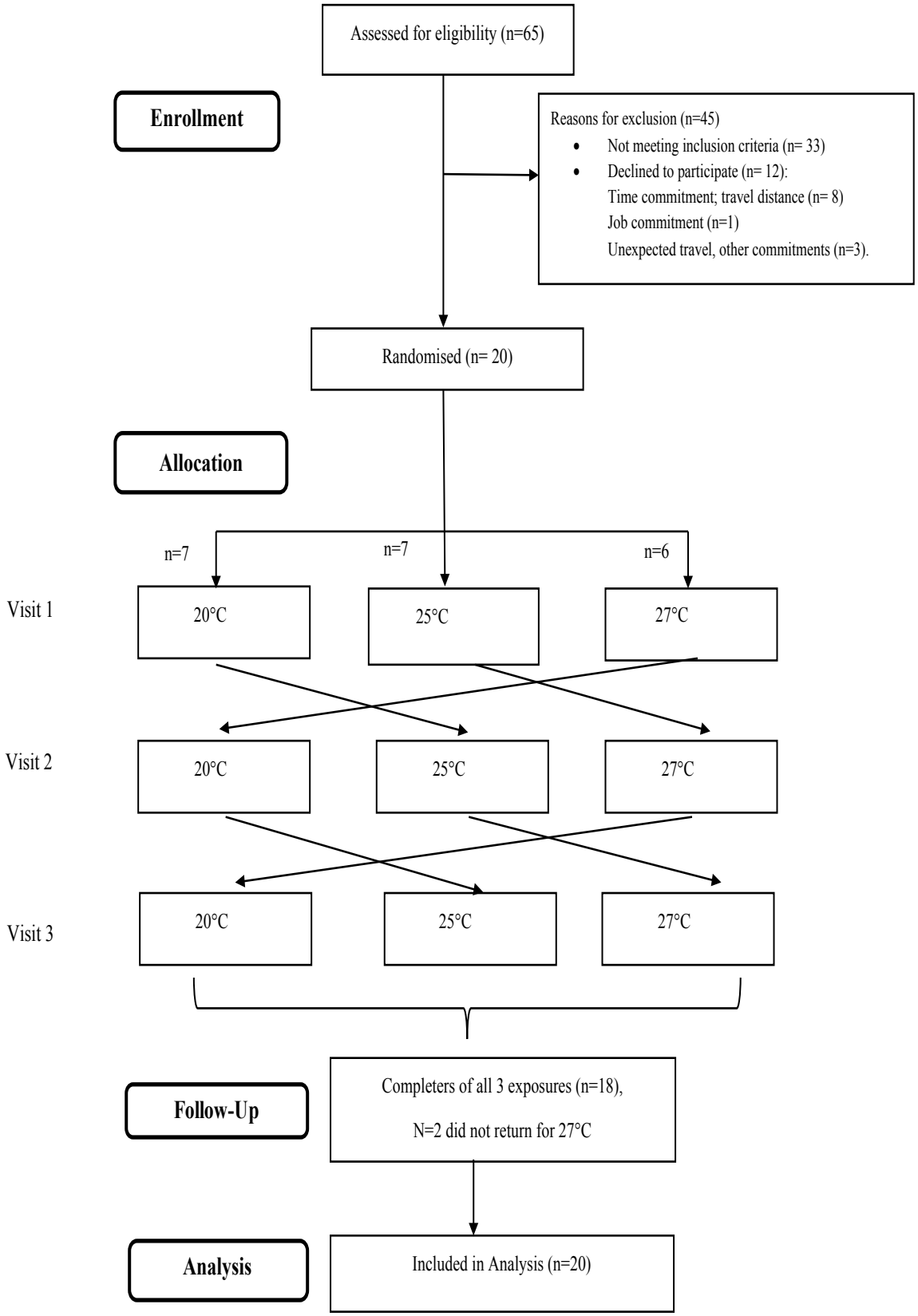

Figure 4.1 CONSORT flow chart: Participant recruitment, randomization and allocation.

4.3.4 Ethics

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by Curtin University's Human Research Ethics Committee; approval number HREC: HR 103/2012. Written, informed consent was obtained from all participants. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 12615000084583).

4.3.5 Study Day Protocol

The study was conducted in a temperature-controlled chamber between 7 am to 1 pm within a period of 18 months that included all seasons except summer. All RMR measurements were completed before 9 am. Timing for the protocol was kept similar for each participant at each temperature. Subjects arrived at the laboratory after a minimum of 12 h of overnight fasting and 8 hrs sleep, and abstinence from heavy physical activity and alcohol intake, for at least 36 hrs prior to trial day. As is the practice in our laboratory, all participants were provided with a low-fat, low calcium, frozen dinner meal of their choice to negate effects of uncontrolled dinner on next morning's nutrient oxidation (Nsatimba et al., 2016; Soares & Chan She Ping-Delfos, 2008). Each subject was studied during a 3.5 hr exposure to 20°C, 25°C or 27°C. On arrival at the laboratory in the morning, volunteers emptied their bladder and changed into a dressing grown. Clothing worn by participants was standardized to within 0.5 clo (clothing insulation) as detailed in another study by our group (Calton et al., 2016). Height was measured using a wall-mounted stadiometer. Waist circumference was measured using a steel tape around mid-point of lowest point of last rib and upper part of pelvic bone. Body weight was measured using a digital calibrated scale after correction for gown weight. We monitored forearm to fingertip skin temperatures by placing two iButtons™ (Texas, USA) using fixomull tape, one on the mid-point of left forearm and the other on the fingertip of the middle finger of the same arm. Subjects then entered an insulated chamber where the temperature of the experimental day had been randomly selected and the temperature set the evening before. Details of the chamber and its precision of maintaining temperature have been published elsewhere (Pathak, Calton et al., 2017). Following a mandatory 60-minute rest in a supine position at the selected temperature, resting metabolic rate was measured using an indirect calorimetry ventilated hood system

(Deltratrac II, Datex, Finland) for the next 30 minutes. A glucose tolerance test (75 g glucose in 300 mL water, CarboTest, Australia) was then administered. Indirect calorimetry was continued after the glucose load in an intermittent fashion (i.e. for 15 min every 30 min for 2 hrs post glucose) in all subjects (Ping-Delfos & Soares, 2011). Inner ear temperature was recorded using an Omron tympanic thermometer at RMR and every 30 mins thereafter until 2 hrs after glucose load. All these measurements were made with hood off. Multiple readings were taken at each time point and the average of the lowest two was recorded. In-between calorimeter recordings, subjects were de-hooded and kept comfortable. They relaxed and conducted activities with minimal physical effort (reading, listening to music, etc.) while lying down. They were allowed small quantities of water until lunch time and the amount of water consumed at first visit was noted and kept constant for all future visits in that participant. A venous blood sample was collected at fasting and 2 hrs post-glucose for measurements of insulin, glucose, irisin and FGF21. The insulin to glucose ratio (IGR) was calculated from both fasting and 2 hr measurements as an index of insulin resistance in fasted and fed states (Ping-Delfos & Soares, 2011; Soares & Chan She Ping-Delfos, 2008). Body composition was measured after the trial using dual energy x-ray absorptiometry (DEXA, Prodigy™, Lunar USA) that provided fat mass (FM) and fat free mass (FFM). On completion of the protocol, a buffet-style meal was served.

4.3.6 Clinical Chemistry Analysis

Plasma glucose was determined in an accredited Pathwest Laboratories Perth, using routine automated procedures on an Architect c16000 analyzer that used specific enzyme-based colorimetric reagents (Abbott Diagnostics, CV<2%). Insulin was determined on an Architect i2000SR Analyzer (Abbott Diagnostics, CV<3%). Plasma irisin was measured using an ELISA kit (AdipoGen, Switzerland. Cat. No. AG-45A-0046PP-KI01; inter-assay CV<7%). Plasma FGF21 was measured using an ELISA kit (AdipoGen, Switzerland; Cat. No. AG-45B-5001-KI01; inter-assay < 10.2 %).

4.3.7 Repeatability Trial

To gain an appreciation of the repeatability of our methods and protocol followed, 5 participants repeated the entire protocol at 25° C, within a period of 4-8 weeks of the first. That data was used to assess coefficient of variation (CV) for RMR and RQ at fasting and post-glucose. 4 participants (1 male, 3 females) repeated the entire trial at 25°C, while one female completed a repeat trial at 20°C. All return visits were completed within 4-8 weeks of the first. The combined data (n=5) was used to assess typical within-subject error (WSE) at fasting and post-glucose given as, the SD of the difference between visits ÷√2 (Hopkins, 2000). The WSE for RMR, RQ, FFG and IET was 11.4 kJ/h, 0.015, 1.55°C and 0.21°C respectively. If restricted to those measured at 25°C (n=4), the corresponding values were 6.6 kJ/h, 0.014, 1.4°C and 0.24°C. The change in post-prandial integrated area under the curve (iAUC) for resting metabolic rate (RMR), respiratory quotient (RQ) and forearm fingertip gradient (FFG) had a WSE of 12.4 kJ/h, 0.012, 1.38°C and 0.23°C respectively. Restricted to those measured at 25°C the corresponding values were 9.6 kJ/h, 0.014, 1.57°C and 0.23°C.

4.3.8 Statistics and Data Analysis

Data is expressed as mean (standard error) for all measurements. All data was tested for normality and analysed using SPSS 21.0 (SPSS. Inc., Chicago) and STATA 14.0 (StataCorp,

Texas, USA). Random effects multiple linear regression was used to assess the effects of temperature on fasting and post-prandial responses in metabolic rate, RQ, FFG, IET, FGF21, irisin and IGR. The model included a fixed effect for temperature as a categorical variable, a fixed effect for MetS status, a temperature X MetS interaction term and a random intercept effect for subject id. We assessed the global effect of temperature using a Wald test with 2 degrees of freedom (df) based on the 3 temperature-indicator variables. Similarly, the overall MetS X temperature interaction was assessed using a Wald test with 2 df and the MetS effect was based on 1 df and compared the 2 groups at 20 degrees C. For post-prandial responses, a fixed effect for the fasting value was also included besides the three covariates. When the overall temperature effect was significant, the LSD procedure was used to adjust for two pairwise comparisons between the three temperatures (20°C vs. 25°C and 25°C vs. 27°C). Total area under the curve was based on the trapezoid rule and was used for all post-prandial measures with an adjustment for respective fasting values. In all models the effect of season of measurement was tested. We also performed partial correlational analysis to assess associations between changes in thermoregulatory and metabolic responses between 20^{o}C and 27^{o}C in both fasted and post-glucose state. These correlations were adjusted for age, gender, season, fat mass (FM) and fat free mass (FFM). A p value of <0.05 was considered statistically significant.

4.4 Results

Sixteen women and four men completed the study. Mean and standard deviation (SD) among MetS- and MetS+ groups were: age (57 ±13.5 and 62 ± 3.8) y, DXA weight (68.9 ±10.83 and 81.4 ± 22.08) kg, BMI (25.1 ±3.01 and 30.1 ± 5.90) kg/m^2, fat (25.7 ± 6.22 and 32.3 ± 15.50) kg and fat free mass (42.0 ±7.28 and 48.0 ± 10.96) kg respectively. The study included nine MetS+ and eleven MetS- individuals in the study. Three out of the 20 were known cases of T2DM but on good glucose control as judged by their $HbA1_c$ <6.5%. These subjects were categorized as having MetS. The main characteristics of the participants are detailed in Table 1. The mean ± SD of the environmental chamber temperatures between measurement days was 19.98^{o}C ± 0.36, 24.95^{o}C ± 0.38 and 26.96^{o}C ± 0.42 with CV of 1.8%, 1.5% and 1.6%. Humidity was maintained at 40%.

Table 4.1 Characteristics of the participants in the study.

Variables	MetS- Group	MetS+ Group
Age (y)	57 ±13.5	62 ± 3.8
Gender	10F, 1M	6F, 3M
DXA Weight (kg)	68.9 ±10.83	81.4 ± 22.08*
BMI (kg/m^2)	25.1 ±3.01	30.1 ± 5.90**
Fat Mass (FM)	25.7 ± 6.22	32.3 ± 15.50*
Fat Free Mass (FFM)	42.0 ±7.28	48.0 ± 10.96*
Fat Percentage	38.5 ±6.01	40.0 ± 7.9

Data are mean ± standard deviation (SD), independent t-Test comparing 2 groups

* P<0.05; ** P<0.001

MetS-, without metabolic syndrome; MetS+, with metabolic syndrome; y, year; DXA weight, weight as measured by DXA machine; BMI, body mass index.

4.4.1 Thermogenesis and Body Temperature

We did not detect any seasonal effect on either metabolic rate or fuel utilization in this study. There was no significant effect of temperature on RMR for the group as a whole but the MetS+ group had a significantly higher adjusted RMR compared to the MetS- group across all temperatures (Table 4.2). However, this main effect was modified by a significant temp x MetS interaction, where MetS+ responded with a significant stepwise increase in RMR of 10%, but the MetS- group had a insignificant increase of 2.7% (Table 2). In effect, the MetS+ group showed a higher RMR at 20^{o}C and 25^{o}C relative to the MetS- group, while they were comparable at 27^{o}C (Table 4.2). Three MetS- individuals showed a negative response between 27^{o}C and 20^{o}C. There were no differences in fasting RQ within temperatures or between MetS groups. FFG increased with decreasing temperature in a stepwise fashion with significant vasoconstriction at 20ºC and significant vasodilation at 27ºC (Table 4.2). IET was significantly reduced as temperature dropped, with no difference in the response of the two MetS groups (Table 4.2).

Table 4.2 Exposure to three ambient temperatures in the metabolic syndrome: Effects on fasting energy metabolism and temperature regulation.

Variables	20° C	25° C	27° C	Overall P Value for Mixed-model ANCOVA		
				Temperature Effect	MetS Effect	Temp. X MetS Effect
RMR(kJ/h)						
MetS-	221 (4.5)	220 (4.5)	217 (4.5)	NS	<0.001	<0.004
MetS+	247 (5)*	234 (5)**	224 (5)			
RQ						
MetS-	0.84 (0.01)	0.83 (0.01)	0.82(0.01)	NS	NS	NS
MetS+	0.84 (0.01)	0.85 (0.01)	0.85 (0.01)			
FFG (°C)						
MetS-	5.2 (0.40)	1.5 (0.40) †	-1.0 (0.44)† ‡	<0.001	NS	NS
MetS+	4.2 (0.44)	0.3 (0.44) †	-1.4 (0.44)† ‡			

Variables	20° C	25° C	27° C	Overall P Value for Mixed-model ANCOVA		
IET (° C)						
MetS-	35.5 (0.11)	35.8 (0.11) †	35.9 (0.12)†	<0.001	NS	NS
MetS+	35.5 (0.12)	35.7 (0.12) †	35.8 (0.12)†			
FGF21 (pg/ml)						
MetS-	115.4(32.00)	134.3(32.00)	89.8 (32.93)	NS	NS	NS
MetS+	156.9(35.58)	145.9(35.58)	131.2(35.58)			
Irisin (μg/ml)						
MetS-	1.3(0.14)	1.2(0.14)	1.20(0.140)	NS	<0.001	NS
MetS+	0.53(0.15)	0.48 (0.15)	0.44(0.15)			
IGR						
MetS-	1.0(0.11)	1.0(0.11)	0.9(0.11)	NS	NS	NS
MetS+	1.1(0.118)	1.0(0.12)	0.9(0.12)			

Data are means (SE), based on mixed-model ANCOVA adjusted for age, gender, season, FM and FFM. Temperature Effect: † P<0.05 vs. 20°C; ‡ P<0.05 vs. 25°C. Temp x MetS interaction:

* P<0.05 vs. MetS- at 20°C; ** p<0.05 vs. MetS- at 25°C.

SE, standard error; RMR, resting metabolic rate; RQ, respiratory quotient; FFG, forearm to fingertip gradient; IET, inner ear temperature; FGF21, fibroblast growth factor; IGR, insulin glucose ratio.

There were no differences across temperatures or between MetS groups for most post-prandial data once adjusted for their respective fasting value and other covariates (Table 4.2). We did, however, find a significant difference in PP_RQ between temperatures, with lower RQ at 25^{o} C compared to 20^{o} C (Table 4.3).

Table 4.3 Change in post-prandial metabolic and temperature variables on exposure to three ambient temperatures in the metabolic syndrome.

Variables	20° C	25° C	27° C	Overall P Value for Mixed-model ANOVA		
				Temperature Effect	MetS Effect	Temp* MetS Effect
TAUC_RMR (kJ/hr)						
MetS-	247 (3)	247 (3)	247 (3)	NS	NS	NS
MetS+	248 (4)	249 (3)	250 (3)			
GIT %						
MetS-	1.8 (0.24)	1.8 (0.24)	1.8 (0.27)	NS	NS	NS
MetS+	1.2 (0.27)	1.6 (0.27)	2.0 (0.27)			
TAUC_RQ						
MetS-	0.90 (0.008)	0.88 (0.008)†	0.90 (0.009)	0.048	NS	NS
MetS+	0.89 (0.009)	0.88 (0.009)†	0.88 (0.009)			
TAUC_FFG (°C)						
MetS-	3.0 (0.51)	2.3 (0.33)	1.9 (0.46)	NS	NS	NS
MetS+	3.0 (0.46)	1.8 (0.39)	1.7 (0.48)			
TAUC_IET (°C)						
MetS-	35.7 (0.106)	35.8 (0.08)	35.8 (0.06)	NS	NS	NS
MetS+	35.7 (0.06)	35.8 (0.06)	35.9 (0.06)			
PP_FGF21(pg/ml)						
MetS-	138.5 (15.9)	163.4 (15.9)	142.7 (17.5)	NS	NS	NS
MetS+	119.9 (17.81)	120.8 (17.72)	125.42 (17.7)			
PP_Irisin (µg/ml)						

Variables	20° C	25° C	27° C	Overall P Value for Mixed-model ANOVA		
MetS-	0.38 (0.030)	0.44 (0.029)	0.36 (0.030)	NS	NS	NS
MetS+	0.40 (0.031)	0.41 (0.032)	0.43 (0.032)			
PP_IGR						
MetS-	2.7 (0.39)	2.1 (0.39)†	2.2 (0.35)	0.02	NS	NS
MetS+	1.9 (0.38)	1.8 (0.38)†	1.8 (0.38)			

Data are mean (SE), based on mixed-model ANCOVA adjusted for respective baseline value, age, gender, season, FM and FFM.

Temperature effect: † P<0.05 vs. 20°C

SE, standard error; TAUC, total area under the curve; RMR, resting metabolic rate; IET, inner ear temperature; RQ, respiratory quotient; FFG, forearm to fingertip gradient; IGR, insulin glucose ratio.

4.4.2 Substrates and Hormones

There was no difference in fasting insulin glucose ratio (IGR), irisin and FGF21 between the three temperatures. However, the MetS- group had higher adjusted irisin than the MetS+ group (Table 4.2). In response to oral glucose there was a significant increase in adjusted PP_IGR at 20^{o} C compared to 25^{o}C and this was accompanied by significant increases in PP_RQ between these temperatures (Table 4.3). Presence of MetS+ did not alter any of these outcomes (Table 4.3).

4.4.3 Correlation of Biomarkers

Partial correlation analysis adjusted for age, gender, season, FM, FFM and MetS was carried out. Differences in the fasting RQ between 20°C and 27°C was associated with differences in FGF21 and irisin. In the post-glucose period, the differences in iAUC of RQ between 20°C and 27°C were related to difference in post-prandial increase (2 hrs value minus fasting) in FGF21, and in irisin. Changes in IGR were related to change in RQ at fasting but not in the post-prandial state (Table 4.4).

Table 4.4 Partial correlations of the change between 27°C and 20°C in metabolic variables measured in the fasted and post-prandial period.

Variable	Δ Irisin	ΔFGF21	ΔIGR
Fasting			
ΔRMR (kJ/hr)	0.01 (0.99)	-0.46 (0.14)	-0.026 (0.28)
ΔRQ	0.75 (0.005)	0.60 (0.044)	0.63 (0.027)
Post-glucose			
	- 0.02 (0.95)	0.39 (0.22)	- 0.18 (0.59)
GIT (%)			
ΔMR (kJ/hr)	-0.04 (0.91)	0.18 (0.58)	-0.16 (0.62)
ΔRQ	0.60 (0.041)	0.65 (0.022)	-0.28 (0.38)

Data are partial correlation coefficients (p values) adjusted for age, sex, FM, FFM and presence of MetS.

FGF21, fibroblast growth factor 21; IGR, insulin glucose ratio; RMR, resting metabolic rate; RQ, respiratory quotient; GIT, glucose induced thermogenesis; MR, metabolic rate.

4.5 Discussion

There is increasing interest in the hypothesis that living within a comfortable thermal range may increase our susceptibility to obesity. From an energy balance perspective, such behaviours negate the opportunity for thermogenesis to occur in response to low temperature (van Marken Lichtenbelt & Schrauwen, 2011). The human body responds to cold via a combination of vasoconstriction, decreased core body temperature and increased heat production. Both acute and chronic cold exposure increases BAT activity in human adults and this is evident more in lean and insulin-sensitive individuals (Cannon & Nedergaard, 2004; Vijgen, 2013). In the present trial, we examined the response of a group of older, overweight and obese individuals exposed to three temperatures; two within the thermo-neutral zone (TNZ), and one just below (20°C). The thermo-neutral zone for each individual depends on their cold capacity which, to an extent, relies on the ambient temperature of their habitual residence, as well as habitual clothing, age and other (Kingma

et al., 2012). For most of the year Perth has a Mediterranean climate. The mean annual temperature in Perth, WA is ~25°C (Bureau of Meteorology) and so we used 20°C as mild cold temperature for our study while the other two temperatures were within the conventional TNZ of 22 -27°C for a lightly clothed person (Kingma, 2012b). The latter could be a summer temperature for some parts of the world. We monitored physiological variables, hormones and substrates that respond to an acute mild cold stress within a protocol that provided tight control over ambient temperature and clothing worn.

We noted a stepwise increase in RMR as temperature decreased from 27°C to 20°C with significant differences between each temperature, but this response was significantly modified by presence of a MetS x temperature interaction. However, this was true only of the MetS+ group (Table 4.2) who showed ~10% increase, while the MetS- groups showed a insignificant overall effect of ~2% Adjusted RMR was significantly higher in MetS+ participants compared to MetS- participants at 20^{o}C and 25^{o}C, while at 27^{o}C the two groups were similar (Table 4.2). None of our subjects complained about shivering, nor was it observed. Since irisin is expected to increase with shivering, the insignificant changes between temperatures would confirm that shivering did not occur (Table 4.2). However, since electromyography (EMG) recordings were not made we cannot discount small increases in muscle tone that precede shivering (Meigal, 2002; van Marken Lichtenbelt and Schrauwen, 2011). Overall, we conclude that NST was observed only in MetS+ participants.

The absence of a detectable NST in MetS- group was contrary to our expectation. Three of nine in this group responded with a decrease in RMR between 27^{o}C and 20^{o}C that resulted in an overall insignificant increase of 2.7% (Table 2). On their deletion, there was a significant increase in RMR of ~7% (data not shown). Previous investigators have also noted a negative response in NST in some of their participants (Calton, 2016b). Such observations are ascribed to the Q10 effect where a decrease in tissue temperature is accompanied by a decrease in energy expenditure (Gisolfi, 2000; Lemieux, 2008). In contrast, all MetS+ individuals in this study showed a positive response that resulted in a significant 10% increase in RMR on average (Table 2). Most of our participants were older, and with ageing, the defence of core body temperature upon cold exposure can be a challenge. Older adults also demonstrate nil (Kingma, 2011) or blunted NST (DeGroot, 2007) and instead respond through increased vasoconstriction (DeGroot, 2007) or increased blood pressure (Kingma, 2011).

Another reason could be a reduction in physical activity with ageing, where a lesser skeletal muscle mass or function may act to reduce uncoupling protein 2 activity (Van Pelt, 2001).

Kingma et al. (Kingma, 2014) have proposed a biophysical model that relates behavioural changes, factors influencing body insulation and thermo-regulatory physiology (autonomic nervous system activity) in the defence of core body temperature. Inherent to this model are inputs from skin and core body temperature (Kingma, 2014). In the current experimental situation, we statistically adjusted data for potential individual factors like age, gender, FM, FFM and season of measurement; since they potentially influence the TNZ (Kingma, 2014). We standardized clothing and measured each participant in the supine position in beds of the same make and model; so we controlled external insulation.

Individual behavioural changes (expected in free-living humans exposed to cold) were restricted, due to the need for compliance to the trial protocol over the 3.5 hr period. The observation of a significantly higher adjusted RMR in MetS+ participants at 25^{o}C was hence contrary to expectation (Table 4.2). Both these temperatures are currently accepted as within the TNZ for humans, and so RMR should not differ between the two temperatures. In parallel, FFG indicated a significant vasodilation at 27°C relative to 20°C in both groups, but were not different between MetS+ and MetS- groups (Table 4.2). Hence MetS- individuals maintained body temperature at 25°C and 27°C (no difference in IET at these temperatures) mainly through vasoconstriction and vasodilation respectively (Table 4.2), while MetS+ participants also responded through changes in RMR. As a randomized within-subject design, these outcomes are less likely to be a chance finding since the observations on RMR coincided with appropriate physiological changes in IET and FFG. Overall, it raises the possibility that the range of ambient temperatures that typify the TNZ may have shifted upwards in the MetS+ group. This would explain the greater adjusted RMR in that group but importantly would signal that the RMR responses observed at 25^{o}C had a percentage of NST in its measurement. We acknowledge the small sample size and that future studies are needed to place such findings on a firmer footing. The practical implications, if confirmed, would be that studies on RMR comparing MetS+ and MetS- may need to critically consider the ambient temperature conditions of such measurements.

There are several studies in the literature that have examined the ability of cold exposure to increase energy expenditure (Celi, 2010; Lee, Linderman et al., 2014; Lee, Smith, 2014; van Marken Lichtenbelt et al., 2014; W. D. van Marken Lichtenbelt, 2011; Warwick and Busby, 1990; Westerterp-Plantenga, 2002; Wijers et al., 2010). These embrace a wide disparity in lowest temperature used, duration and method of cold exposure, each of which could affect the thermoregulatory and hormonal response to a cold temperature. For example, the early study of (Dauncey, 1981) used a whole-body calorimeter where subjects stayed for 30 hours at 22°C and 28°C, on two separate days. They noted an increase in RMR, increased vasoconstriction and decreased body temperature at the cooler temperature. This is similar to the results observed in our study that used an environmental chamber to house the participants on each visit and allowed a one-hour equilibration before measurements commenced. In another study (Claessens-van Ooijen, 2008), lean and obese individuals only had their legs exposed to cold air (15ºC) and then rewarmed using a duvet. The obese participants demonstrated blunted heat production whereas lean participants had a larger change in heat production from baseline to rewarming. Yoneshiro et al., 2011 suggested that BAT positive individuals show a greater increase in energy expenditure and less of a drop in supraclavicular temperature as opposed to BAT negative ones, who instead had more of a reduce in temperature and a negligible increase in energy expenditure. They had used intermittent placement of feet onto ice blocks with room temperature maintained at 19ºC. Their findings could also stem from absence of standardized clothing and the use of ambient room temperatures with only immersions of feet to a 24ºC and 27 ºC stimulus during energy expenditure measurements in that study.

Recently, in a series of elegant studies Lee et al (Lee, Linderman et al., 2014) rapidly transitioned their subjects from warm (27°C) to cold (12°C). The changes in temperature exposure were induced through each subject wearing water-infused thermoblankets to achieve the desired temperature. Hence in a short experimental protocol, Lee et al could examine the entire spectrum of cold-induced thermogenesis (CIT) responses from NST to shivering. When the temperature dropped to shivering, irisin concentration increased to meet the demand for heat production in defence of core body temperature. In a sub-study, they proposed that in response to a lowered ambient temperature, NST varied directly with FGF21 (Lee, Linderman et al., 2014). Our study was designed to stimulate NST, not shivering thermogenesis. The lack of an increase in irisin with mild cold would support that our strategy worked for both groups studied.

Reports suggest that both irisin (Park et al., 2013) and serum FGF21 levels are raised in those with metabolic syndrome (MetS+) and increase in parallel with the numbers of components of MetS (Zhang et al., 2008).We found that the MetS+ group in this trial had significantly lower adjusted irisin levels compared to the MetS- group. This outcome is similar to that reported by Yan et al. (Yan et al., 2014) but is direct contrast to Park et al. (2013) and Fukushima et al. (2016) who concluded the reverse. Some reasons for these opposite findings could stem from sample size, study design, the non-adjustment for important covariates and possibly the different assays used for irisin. Our participants described themselves as sedentary to moderately active. However, we cannot discount a difference in habitual physical activity in these groups as we lack direct measurements of the same. Interestingly a recent meta-analysis of a small number of randomized controlled trials concluded that chronic exercise training decreased irisin levels (Qiu et al., 2015).

In the present study, FGF21 did not vary significantly across temperatures but the expected two- to three-fold increase in FGF21 following glucose was clearly observed in all sub-sets of the data. So, while our outcomes indicate a NST response to mild cold, we found no role for FGF21 in the process. One possibility could be the narrower range of temperatures used here and the longer duration at each temperature, when compared to studies by Lee et al. (2014) where each subject was transitioned through the desired temperature range on one visit and within 2 hours (Lee, Linderman et al., 2014). Instead, we found changes in FGF21 as well as irisin to be directly related to increases in fasting RQ; signalling a potential role of glucose oxidation following cold exposure.

Modern humans live in a post-prandial state for most of the day due to the consumption of several meals over a 24 hr period. To understand the transition from fasted to fed state and to gain an appreciation of the limits of thermogenic capacity in the cold, we monitored our participants after a standard oral glucose tolerance test (OGTT). This simple meal stimulus avoided too long a cold exposure and the risk of shivering thermogenesis (ST), but also afforded the opportunity to examine glucose tolerance across the temperature range. In the light of fasting differences in metabolic variables we approached the analysis of all post-prandial data through adjustment for baseline effects. The outcomes in Table 4.3

indicate that there was no additional thermogenic component at 20°C. Calculation of glucose-induced thermogenesis (GIT) responses were within the 2-7% theoretical thermic cost of glucose (Table 4.3). The latter depends on whether glucose is directly oxidized or initially stored as glycogen and then mobilized for oxidation (Flatt, 1995). Dauncey et al. (1981) also concluded that in the presence of cold-induced thermogenesis, meal ingestion does not increase metabolic rate. This raises the question as to whether there is a limit to the total capacity for thermogenesis that is dictated by the need to either respond to a drop in temperature or to meal ingestion.

FGF21 belongs to a family of peptides involved in metabolic regulation, and is expected to improve insulin sensitivity and glucose tolerance. FGF21 can drive the uptake of glucose into BAT, increase thermogenesis and cause browning of white adipose tissue, even in the absence of an insulin effect (Emanuelli et al., 2014). FGF21 levels increases with obesity and may affect insulin sensitivity (Olszanecka-Glinianowicz, Madej, Wdowczyk, Owczarek & Chudek, 2014). Following an oral glucose load, FGF21 responds in a biphasic manner. After an initial decrease in circulating levels up to 1 hr post-glucose, FGF21 increases significantly after 2 hrs and beyond; well above fasting values (Korwutthikulrangsri et al., 2014; Lin et al., 2012). We noted a significant stimulation of FGF21 following glucose in both groups and at all temperatures; which fits in with these findings. However, no differences were observed between the temperatures studied. Overall, the data implies that FGF21 responds to glucose administration in the cold in much the same fashion as to normal room temperatures (Fisher et al., 2012; Straczkowski et al., 2013).

There is some evidence to suggest that cold exposure may improve insulin sensitivity both in the fasting and post-prandial state [32]. We could not detect this based on IGR, a valid surrogate marker for insulin resistance, at fasting and following OGTT (Lorenzo et al., 2010). Instead we noted a significant increase in IGR at 20^{o}C compared to 25^{o} C in the post-prandial period, suggestive of an increase in post-prandial insulin resistance. The SNS plays an important role in cold-induced thermogenesis (Calton, Soares et al., 2016; Soares & Chan She Ping-Delfos, 2008), and catecholamine-induced lipolysis may contribute to raised free fatty acids in the cold as noted by Celi and group (Celi et al., 2010). Although not measured in this study, raised FFA can cause a transient insulin resistance, which would require a higher surge in insulin to clear the same glucose load administered. This could explain the IGR and higher

RQ at 20°C relative to 25°C (Table 4.2). Interestingly, changes in FGF21, but not IGR were associated with changes in RQ across the temperatures employed. A statistically-similar effect was also seen with irisin and RQ. Collectively, the data may argue for a physiological role for the two in glucose oxidation under these conditions. Previous investigators have also implicated both irisin and FGF21 in glucose metabolism in the obese (Celi et al., 2010; Emanuelli et al., 2014; Lee, Linderman et al., 2014; Lopez-Legarrea et al., 2014; Semba, 2012).

4.6 Limitations & Strengths

A limitation of this study was the absence of blood measures at intermediate time-points following glucose. This would have enabled the determination of area under the curve of the post-prandial excursions in hormones and substrates. However, we observed a striking 1.5-2-fold increase in FGF21 at 2 hrs following glucose, across all temperatures. There was a good association between adjusted changes in FGF21 and RQ in response to cold, for both the fasted and the fed state. We did not use relatively low temperatures of 12-16°C as reported in the literature, since the plan was to mimic the range encountered in Perth, Western Australia. Moreover, as our participants were under free-living conditions between visits, we were unable to control their exposure to environmental temperature and diet between visits. It is probable that we did not elicit the maximal thermogenic potential of NST in our participants. Further, due to sample composition, we cannot comment on gender differences in thermoregulation. We, like others, have reported a greater thermogenic and lesser vasoconstrictive effect in men compared to women (Graham, 1988; Pettit, Marchand & Graham, 1999), but are unable to endorse these findings here. We also understand that the use of IET to monitor core temperature has been validated by some (Chamberlain, 1995; Childs, Harrison & Hodkinson, 1999) but we acknowledge this is not universally accepted (Casa, 2007). However, its precision was good with typical within-subject error of 0.21°C, that allowed us to detect subtle changes in this crossover design (Calton, Soares et al., 2016).

There are several strengths to our trial. We examined 3 temperatures in each person to assess their impact on basal and glucose-induced thermogenesis. Our study design provided adequate power to detect medium-sized effects; that would allow easier

translation to practice. We monitored thermoregulatory responses, whole body metabolism and determined the potential influence of irisin and FGF21 in the transition from the fasted to the fed state. Our protocol was strictly adhered to and we used a chamber to control temperature. We also standardized other factors that could influence results, including the clothing worn and prior diet.

4.7 Conclusions

We document an NST response in MetS+, but not in MetS- individuals; the latter outcome possibly arose from the Q_{10} effect in some individuals. For the first time, we document a significantly higher RMR at 25^{o}C compared to 27^{o}C in those with the metabolic syndrome. This could be confirmed in future studies with a larger sample size, since it would have significant implications for the definition of TNZ in the metabolic syndrome. We did not uncover a role for FGF21 in NST, perhaps due to the milder nature of our cold stimulus and the mode of cold exposure, i.e water cooled thermoblankets vs. ambient room temperature. Our data instead suggests a potential role for FGF21 and irisin in glucose oxidation at fasting and post-prandial upon exposure to a cold ambient environment.

Chapter 5 Potential Influence of Forearm to Finger-tip Skin Temperature Gradients in the Thermoneutral Zone on Resting Energy Metabolism

Objective:

1. To investigate if variations in FFG contribute to RMR and RQ measured in TNZ.

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5.1 Abstract

Background: Resting metabolic rate (RMR) should be measured in the thermoneutral zone (TNZ). Forearm to fingertip skin temperature gradients (FFG) could serve as an objective measure of this pre-condition.

Subjects/Methods: Eighty-six adult Australians were studied at 25^{o}C in a temperature-controlled chamber. Measurements of overnight fasted RMR, RQ and FFG were complemented by clinical biochemistry. McAuley's index of insulin sensitivity (McA_ISI) and presence of metabolic syndrome (MetS) was determined. Physical activity (PA) was estimated from the short version of the International Physical Activity Questionnaire (IPAQ). Fat mass (FM) and fat free mass (FFM) were obtained from DEXA. Twenty-nine participants were assessed for changes in RMR (ΔRMR), RQ (ΔRQ) and in FFG (ΔFFG) following a six months free-living period. Multiple linear regression analysis of RMR and RQ on FFG, and of ΔRMR and ΔRQ on ΔFFG were conducted after the controlling of twelve known determinants of energy metabolism, respectively.

Results: There were wide between-subject variations in unadjusted FFG ranging from 4.25 to +7.8^{o}C. The final parsimonious model for cross-sectional observations of RMR included age, FM, FFM, McA_ISI and FFG (β=63 kJ/d [95%CI: 14.2, 112.1, P=0.012]). However, FFG was unrelated to RQ. In the longitudinal cohort, adjusted ΔRMR significantly associated only with ΔFFG (β =100kJ/d [95% CI: 10.3, 189.1; P=0.030], while adjusted ΔRQ associated with ΔFFG (-0.003 [95%CI: -0.005, 0.0002, P=0.038]), age and McA_ISI.

Conclusions: Sizeable between-subject variations in FFG at 25^{o}C were associated with RMR and RQ. Monitoring FFG may serve as an objective assessment of the TNZ during RMR measurements.

5.2 Introduction

Basal metabolic rate (BMR) is the least amount of energy expended to main body functions in the awake state, and is dependent on mitochondrial function, thyroid status and activity of the sympathetic nervous system (SNS)(Clapham, 2011). Resting metabolic rate (RMR) is generally measured under the same exacting pre-conditions as for BMR, except for the individual traveling to place of measurement and undergoing a mandatory 30 min rest prior to measurement. RMR is therefore closely related to BMR and is generally accepted to be about ~5% higher (Soares, 1989). There are many factors that contribute to the total variance in RMR/BMR and these include age (Johnstone et al., 2005) gender (Cunningham, 1980), physical activity (Van Pelt, Dinneno & Seals, 2001), fat mass (Johnstone et al., 2005; Landsberg, Young, Leonard, Linsenmeier & Turek, 2009) and fat free mass (FFM) (Johnstone et al., 2005). Once adjusted for these traditional contributors, including the composition of FFM (Muller, Bosy-Westphal, Later, Haas & Heller, 2009), ethnic differences in RMR are still evident (Gallagher, 2006; Hayter & Henry, 1993; Kimm et al., 2002; Nsatimba et al., 2016). Recently, vitamin D status and insulin sensitivity were shown to be novel independent predictors of RMR (Calton, Pathak et al., 2015). While increasing vitamin D status (as judged by circulating 25 hydroxycholecalciferol, 25OHD) resulted in higher RMR, greater insulin sensitivity independently reduced RMR. Clearly, in population groups where higher prevalence of low-grade inflammatory conditions like obesity and metabolic syndrome (MetS) are prevalent, determining additional contributors to RMR are important. This was seen in studies where gender and their potential interaction with MetS played a role in influencing energy metabolism and substrate utilisation (Blaak, 2001; Soares, Cummings et al., 2011).

Approximately 50% of RMR is directed towards maintaining homeothermy (Landsberg L, Saville & Young, 1984), while the remainder is the energetic cost of vital processes such as Na/K ATPase pump activity, protein turnover and substrate cycling (Van-Den-Berg, Berga, van Marken Lichtenbelt, van Dijka & Schrauwen, 2011). Current techniques for RMR measurement are based on indirect calorimetry, which utilizes measurements of the respiratory gas exchange of oxygen, carbon-dioxide and ventilation volumes. These measures are used to calculate heat production by the body, which only equates to energy

expenditure if all measurements take place in the thermo-neutral zone (TNZ). The TNZ is a range of ambient temperatures where core body temperature can be maintained through modulation of non-evaporative heat loss and without compensatory changes in RMR (Claessens-van Ooijen, 2008). When temperatures fall below the lower limit, RMR will rise and together with increased peripheral vasoconstriction will offset any change to core body temperature. Such thermoregulatory changes are seen in the non-shivering response to a mild cold temperature (Calton et al, 2016; Landsberg et al., 1984; Roth & Sheard, 1948; Van Ooijena, 2004). At temperatures above the TNZ, RMR will also rise and be accompanied by evaporative heat loss (Kingma, Frijns A, Schellen & van Marken Lichtenbelt, 2014; Roth & Sheard, 1948). Determining whether a participant is in the TNZ is practically very difficult. The literature only rarely provides information on compliance to TNZ conditions (Nsatimba et al., 2016) since it is assumed that if the measurement is conducted between 23-27^{0}C, the latter pre-condition has been met. One suggested solution is to monitor peripheral skin temperature gradients such as between the forearm to fingertip of one hand exposed to the environment. Other approaches are to monitor finger digit blood flow through Doppler or the use of thermography, and to monitor core body temperature using telemetric pills. Within the TNZ all these approaches should show no net vasoconstriction or vasodilation (Kingma, 2012) and core body temperature should be constant.

As part of our ongoing work in this area, we have noted a considerable variation in forearm-fingertip gradients (FFG) at 25^{o}C, a temperature that is well within the TNZ (Henry, 2006; Kingma et al., 2014; Roth & Sheard, 1948). We found that factors like FFM, gender and insulin sensitivity made independent contributions to FFG (Pathak & Soares, unpublished). These factors are known predictors of RMR and RQ. The question arose whether large variations in-between subject FFG signalled non-compliance to TNZ pre-conditions, and if so, whether such values were accompanied by variations in resting energy metabolism measured under the same controlled conditions. We have addressed our objective through a collation of data obtained from a standardised protocol in a group of adults who varied in age, body composition and metabolic status but were all measured at 25^{o}C.

5.3 Methods and Materials

5.3.1 Study Design

This is a secondary analysis of cross-sectional data from 86 adult residents of Perth, Western Australia. The data was obtained from two clinical trials performed in the same laboratory following the same protocol.

5.3.2 Participant Selection

All participants were of European descent, weight stable (< ± 2 kg) in the previous 6 months with no intention of losing weight in the next 6 months, absent of thyroid disease and polycystic ovarian syndrome, non-smokers, non-pregnant, non-lactating, not on hormonal contraception or testosterone replacement therapy or hormonal replacement therapy and residing here for 2 years or more. The studies were approved by Curtin University's Human Research Ethics Committee via approvals HR103/2012 and RDHS-13-15. All participants had a prior orientation visit to the laboratory and equipment, before attending the study day, and all provided written, informed consent.

5.3.3 Sample Size Calculation

Sample size calculations were based on a partial r^2 of 0.20 for FFG on dependent variables, RMR and RQ, α=0.05 and power of 80%. The effect size was 0.25 for 12 potential predictor variables, and the required sample size was 82 (G Power version 3.1.9.2) (Faul F, Erdfelder E, Lang AG & Buchner, 2007).

5.3.4 Measurements

Standardized conditions for RMR measurement were followed (Nsatimba et al., 2016). The participants arrived after a 10-12 hr overnight fast, minimum eight hours of sleep and at least 36 hours of abstinence from vigorous activity and alcohol. RMR as measured here is closely related to classical BMR except it allowed the volunteers to come to the place of measurement from home (i.e. outpatient). In this study, we have measured overnight fasted RMR since we did not have the capacity to accommodate participants for an overnight sleep at our institute. RMR was measured between 7-9 am. All volunteers were awake and rested in a supine position for a 30-60 min period, to allow equilibration to an especially designed climate chamber (volume = 57.7 m^3) set at 25^oC (40% humidity). On arrival, all participants emptied their bladder and changed into a gown provided by the lab (~0.46 clo), worn over their own undergarments (~0.04 clo), except n=37 who wore light clothing with exposed extremities. The thermal insulation of clothes is measured in the unit 'Clo', where 1 Clo = 0.155 m^2K/W and Clo = 0 corresponds to a naked person. Height was measured using a wall-mounted stadiometer. Waist circumference was measured using a steel tape around mid-point of lowest point of last rib and upper part of pelvic bone. Body weight was measured using a digital calibrated scale after correction for gown weight. We collated data from two studies where RMR and RQ were measured using two different machines (n = 49; Deltatract II, Finland and n=37; TrueOne system, Parvo Medics, USA) but each followed the same protocol and pre-requisites. While the Deltatrac II is the gold standard for indirect calorimetry, it is not manufactured anymore, so newer systems like the TrueOne are gaining significant worldwide use. Continuous monitoring of forearm and fingertip temperatures was achieved through the use of adhesive thermistors (iButtons; type DS1921H; Maxim/Dallas Semiconductor, Dallas, TX). One thermistor was placed on the mid-point of the dorsal aspect of the left forearm and the other on the fingertip of the middle finger of the same arm, using fixomull tape. Minute to minute readings were averaged over the 30 mins of RMR recording. After RMR recordings were completed, inner ear temperature (IET) was recorded in triplicate using a tympanic thermometer (Omron, MC-510, Kyoto, Japan) and the lowest two readings were averaged. Duplicate blood pressure recordings in the upper arm (Omron, HEM 7121, Kyoto, Japan) were made when the canopy of either machine was removed following RMR. Following BP, a venous blood sample was drawn for the determination of fasting glucose, insulin and triglycerides to calculate McAuley's index of insulin sensitivity (McA_ISI) (McAuley

K et al., 2001). Vitamin D status [25OHD] was measured using the chemiluminescent immunoassay method (Liaison, DiaSorin and Architect, Abbott). Presence of the metabolic syndrome (MetS) was ascertained from the most recent guidelines (Alberti et al., 2006). Following energy metabolism measures, the participant's body composition was measured using dual energy x-ray absorptiometry (DEXA, Prodigy™, Lunar Corp. Madison USA). Fat mass (FM) and fat free mass (FFM) were used in this analysis. Physical activity was determined from the short version of IPAQ (Lee, Macfarlane, Lam & Stewart, 2011). Fifteen volunteers underwent a repeat protocol under the same conditions and within 3 weeks of the first. This data was used for the calculation of repeatability statistics. In a second longitudinal study, twenty nine subjects from this sample returned for a repeat protocol, after a free-living period of approximately 6 months from the first visit to detect any seasonal effect on vitamin D status and metabolism, and to observe if change in FFG was related to RMR and/or RQ.

5.3.5 Repeatability

Fifteen participants provided data to assess coefficients of variation (CV) of repeat measurements within 3 weeks of the first trial. The values obtained were: RMR = 3.8% and RQ = 2.8%, while FFG had a typical within-subject error of 1.28^{o}C (CV couldn't be calculated due to negative-to-positive range). These data compared very favourably with values in the literature and our previous study, (Calton EK et al. 2016) where the within-subject error in FFG was similar for men (1.3^{o}C) and women (1.2^{o}C) or between younger (≤35 y; 1.8^{o}C) or older individuals (1.1^{o}C). The performance of each indirect calorimeter included daily calibration of gas analysers with gases of known chemical composition and calibration of the flow sensors. In addition, repeated alcohol burns over the study period had a mean ± SD for RQ of 0.66 ± 0.012 for the Deltatrac, and 0.67 ± 0.01 for TrueOne. These data are equal to the theoretical RQ of alochol of 0.66.

5.3.6 Statistical Analysis

In this study, the primary outcome variables were RMR and RQ, obtained from the cross-sectional study (n=86), the secondary outcome variables were the change in RMR (ΔRMR) and in RQ (ΔRQ) obtained from the longitudinal study (n=29). The main research exploratory variable of interest was FFG and change in FFG (Δ FFG) for each respective study.

Multiple linear regression analysis was performed to explore the association between the dependent variables RMR and RQ with the following explanatory variables: age, gender, FM, FFM, waist circumference (WC), season, physical activity (PA), McAuley's Index (McA_ISI), IET, vitamin D status (25OHD) and MetS status. A catergorical variable, instrument type, was also included in the regression analysis to account for potential differences in the two indirect calorimetry systems used.

In the multiple linear regression analysis, both stepwise and backward elimination methods were applied with the aim of achieving a parsimonious model that provided the highest adjusted coefficent of determination for each dependent variable. Two-way interactive effects were tested when two or more categorical variables entered the model. Standardized β coefficients and estimated regression β coefficients with 95% confidence intervals of all signifcant predictors in the final model are reported. Multicollinearity was assessed by examining variance inflation factors (VIF) and tolerance for individual variables. A value of VIF that exceeds 10 (a tolerance value less than 0.1) is often regarded as indicating multicollinearity. Assumptions (normality, linearity, homogeneity of variances) of the regression analysis were assessed based on standardized residuals. The appropriateness of fit of the final models was assessed by adjusting the coefficients of determination (adjusted r^2). All statistical analysis was performed on SPSS for Windows software (version 22; SPSS Inc., Chicago, IL, USA).

5.4 Results

The study included 86 participants (58 women, 28 men), in an age range of 19 to 69 years. Other salient features are reported in Table 5.1. The mean day-to-day ambient temperature of the chamber was 24.97 ± 0.063°C.

Table 5.1 Subject Characteristics

Variable	Mean ± SD	Range
Age (y)	46.2 ± 17.0	(19, 69)
Gender (F/M)	58 / 28	
BMI(kg/m2)	27.8 ± 5.1	(19.4, 27.8)
FM(g)	30.3 ± 11.9	(11.3, 71.5)
FFM(g)	50.2 ± 11.1	(31.8, 79.8)
RMR(kJ/d)	6153 ± 1329	(3422, 9757)
RQ	0.82 ± 0.04	(0.73, 0.90)
FFG(ºC)	0.91 ± 2.71	(-4.25, +7.80)

BMI, body mass index; FM, fat mass; FFM, fat free mass; RMR, resting metabolic rate; RQ, respiratory quotient; FFG, forearm to fingertip gradient.

Association between RMR and FFG; RQ and FFG

There were wide subject variations in unadjusted RMR (range: 3422 to 9757 kJ/d) and in FFG (-4.25 to +7.8°C). Tables 5.2 and 5.3 shows the multiple regression outcomes of RMR and RQ on FFG respectively. The final parsimonious model of RMR included age, FM, FFM, McA_ISI and FFG (β=63 kJ/d [95%CI: 14.2, 112.1, P=0.012]), which indicated that for each

degree increase in FFG, RMR increased by 63 kJ/d on average. However, FFG was not found to contribute signficantly to RQ (Table 5.3). Instead, we noted a significant MetS x gender interaction in RQ (Table 5.3).

Table 5.2 Predictors of RMR measured at 25° C in a group of Australian adults.

	[1]RMR (kJ/d)		
	β Coefficient	**(95% CI)**	**P Value**
Constant	2188	(929, 3447)	0.001
Age (y)	-13.1	(-20.5, -5.7)	0.001
FM (kg)	31	(18.41, 43.23)	0.001
FFM (kg)	88	(76.2., 100.5)	0.001
McA_ISI	-100.5	(-174.0, -27.0)	0.008
Instrument type			NS
True One			
Deltatrac II (ref.)			
Gender			NS
Male			
Female (ref.)			
MetS			NS
With			
Without (ref.)			
MetS* Gender			NS
FFG, °C	63	(14.4, 112.1)	0.012

[1]R=0.911; adjusted R^2=0.82; SEE=564.08.

FM, fat mass; FFM, fat free mass; McA_ISI, McAuley's insulin sensitivity index; MetS, metabolic syndrome; FFG, forearm fingertip gradient; NS, non-significant.

Table 5.3 Predictors of RQ measured at 25° C in a group of Australian adults.

	[2]RQ		
	β Coefficient	**(95% CI)**	**P Value**
Constant	0.9	(0.859, 0.953)	0.001
Age (y)			NS
FM (kg)			NS
FFM (kg)	-0.002	(-0.003, -0.001)	0.003
McA_ISI			NS
Instrument type	0.026	(0.011, 0.040)	0.001
True One			
Deltatrac II (ref.)			
Gender	0.054	(0.027, 0.081)	0.001
Male			
Female (ref.)			
MetS	0.032	(0.013, 0.052)	0.002
With			
Without (ref.)			
MerS* Gender	-0.039	(-0.069, -0.009)	0.011
FFG, °C			NS

R=0.549; adjusted R^2=0.258; SEE=50.031

Legend: Tables 5.2 and 5.3.

Estimated regression coefficient with its 95% CI of significant predictors from a parsimonious model based on GLM backward regression approach. Each dependent variable was tested initially for the effects of age, gender, season, PA, FM, FFM, waist, 25OHD, McAuleys' index, MetS and FFG. Use of two machines was also included for RMR and RQ.

RMR, resting metabolic rate; RQ, respiratory quotient; MetS, metabolic syndrome; PA, physical activity; FM, fat mass; FFM, fat free mass; FFG, forearm to fingertip gradient; Deltatrac II and True One indirect calorimeter systems by Datex, Finland and Parvo Medics respectively; McA_ISI, index of insulin sensitivity [18]; ref., reference category; NS, not significant at 5% significance level; SEE, standard error of estimate.

In a sub-analysis, we restricted the observations to those without MetS (n= 57), and re-ran the multiple regression analysis for both RMR and RQ respectively. The variables that signficantly contributed to RMR were the same as obtained in Table 5.2 and were given by age (β = -14.9 kJ/d, 95% CI= -24.1, -5.73, p=0.002), FM (β = 32.4 kJ/d, 95% CI= 15.6, 49.3, p=0.001), FFM (β = 90.2 kJ/d, 95% CI= 75.2, 106, p=0.001) and McA_ISI (β = -123.4 kJ/d, 95% CI= -223, -16.8, p=0.024) with FFG retained as a significant preditor of RMR (β =70.5 kJ/d, 95% CI= 12.3, 128.8, p =0.019). The overall model had an r value of 0.90; adjusted r^2 of 0.79; SEE = 583 kJ/d.

In this restricted sample the model for RQ included FM (β = -0.001, 95% CI= -0.002, -0.001, p=0.001), FFM (β = -0.001, 95% CI= -0.003, -0.0002, p=0.028) and gender (0=women, 1 = men) (β = 0.036 95% CI= 0.004, 0.068 p=0.028). However FFG and instrument type did significantly contribute to RQ. This model had an r value of 0.57, adjusted r^2 of=0.29; SEE = 0.03.

Association between ΔRMR and ΔFFG; ΔRQ and ΔFFG

In the longitudinal study (n=29), we observed that the ΔFFG was the only factor significantly associated with the change in RMR (ΔRMR) of a free-living cohort (Table 5.4). No other variables were retained in the final parsimonius model. In contrast to the cross-sectional data, ΔRQ was predicted by ΔFFG and other variables (Table 5.5).

Table 5.4 Observational associations between changes in RMR after a 6 months free-living period.

	ΔRMR (kJ/d)		
	β Coefficient	**(95% CI)**	**P Value**
Constant	205	(-130, 539)	0.22
Age, y			NS
Δ FFG, ºC	100	(10.3,189.1)	0.03
Δ McA_ISI			NS

Estimated regression coefficients with its 95% CI of significant predictors from a parsimonious model based on GLM backward regression approach. Each dependent variable was tested for the effects of age, gender, ΔPA, ΔFM, ΔFFM, Δwaist, Δ25OHD, ΔMcA_ISI, ΔMetS components and ΔFFG.

FFG, forearm to fingertip gradient; McA, McAuley's Index; ISI, insulin sensitivity index [18]; NS, not significant at 5% significance level.

Table 5.5 Observational associations between changes in RQ after a 6 months free-living period.

	Δ RQ		
	β Coefficient	**(95% CI)**	**P Value**
Constant	-0.072	(-0.095, -0.049)	0.001
Age,y	0.001	(0.001, 0.002)	0.001
Δ FFG, ºC	-0.003	(-0.005, 0.000)	0.038
Δ McA_ISI	-0.015	(-0.022, -0.008)	0.001

Estimated regression coefficients with its 95% CI of significant predictors from a parsimonious model based on GLM backward regression approach. Each dependent variable was tested for the effects of age, gender, ΔPA, ΔFM, ΔFFM, Δwaist, Δ25OHD, ΔMcA_ISI, ΔMetS components and ΔFFG.

FFG, forearm to fingertip gradient; McA, McAuley's Index; ISI, insulin sensitivity index; NS, not significant at 5% significance level.

Assessments of Multicollinearity

For all models (both cross-sectional and longitudinal studies), there was no concern of multicollinerity in the final models of RMR and RQ. The collinearity between FM and FFM was R^2= 0.127 allowing us to enter both at the same time in the final model, as all tolerance values for FM and FFM were >0.1 (0.677 & 0.825) and VIF was always <5 (1.476 & 1.211) for variables in the model.

Assessments of Regression Assumptions

For all models (both cross-sectional and longitudinal studies), the normality of standardised residuals was assumed since the Shapiro-Wilks test for the four models was p=0.641 for RMR, p= 0.34 for RQ; p= 0.11 for ΔRMR and p= 0.46 for ΔRQ (Tables 5.2 – 5.5).

In addition, the R^2 alternation for the variables that remained in the model were FFM = 0.67, FM = 0.10, age = 0.032, McAuleys = 0.01 and FFG = 0.014.

However we did note some slight departures from the assumptions of linearity and homogeneity of variances. This encouraged us to further investigate whether relationships other than linear (a quadratic or cubic fit) would provide a better model of adjusted RMR and adjusted RQ (Ravussin & Bogardus, 1989) on FFG. We conducted this investigative analysis for both the cross-sectional (Figure 5.1) and longitudinal data (Figure 5.2 & 5.3). Adjusted RMR was linearly related to FFG (Figure 5.1, r^2 = 0.065, p =0.018), while a quadratic fit (not shown, r^2 = 0.065, p = 0.063) was insignificant. However a cubic fit showed that FFG explained a greater proportion of the variance in RMR in the cross-sectional data (Figure 5.1; r^2 = 0.107, p = 0.025).

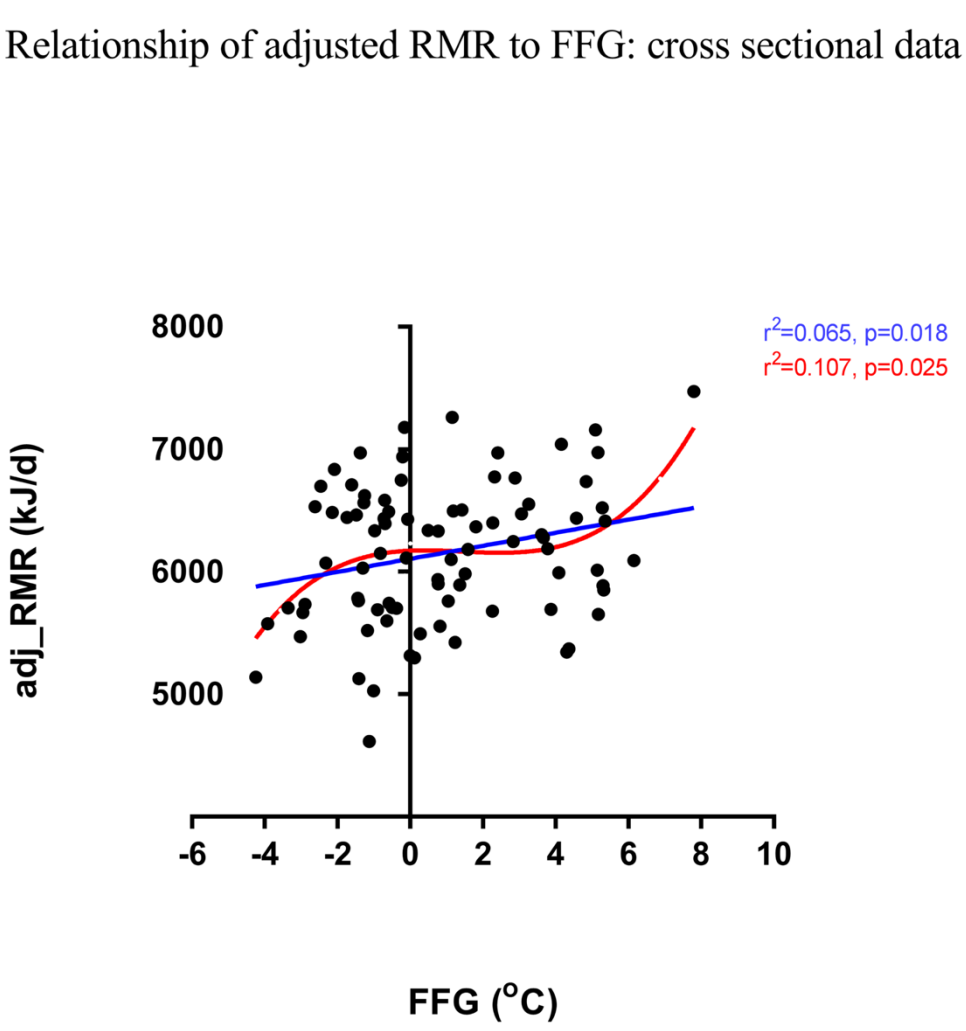

Figure 5.1 Relationship of adjusted RMR to FFG: Cross-sectional data.

Legend:

Blue line: linear regresison line with r^2 = 0.065, p= 0.018

Red line: cubic regression line with r^2 = 0.107, p= 0.025

RMR was adjusted for all variables in Table 5.2 except FFG by the method of (Ravussin & Bogardus, 1989).

In the longitudinal study over 6 months (Figure 5.2), while ΔRMR was significantly and linearly related to ΔFFG (r^2 = 0.168, p =0.027), a quadratic fit (not shown, r^2 = 0.204, p = 0.052) and a cubic fit (Figure 5.2, r^2 = 0.236, p = 0.077) provided non-sigificant but slightly superior coefficients of determination. In a similar analysis, adjusted ΔRQ was significantly and linearly related to ΔFFG (r^2 = 0.137, p =0.048), a quadratic fit (not shown; r^2 = 0.138, p = 0.148) and a cubic fit (Figure 5.3, r^2 = 0.178, p = 0.192) both provided insignificant outcomes.

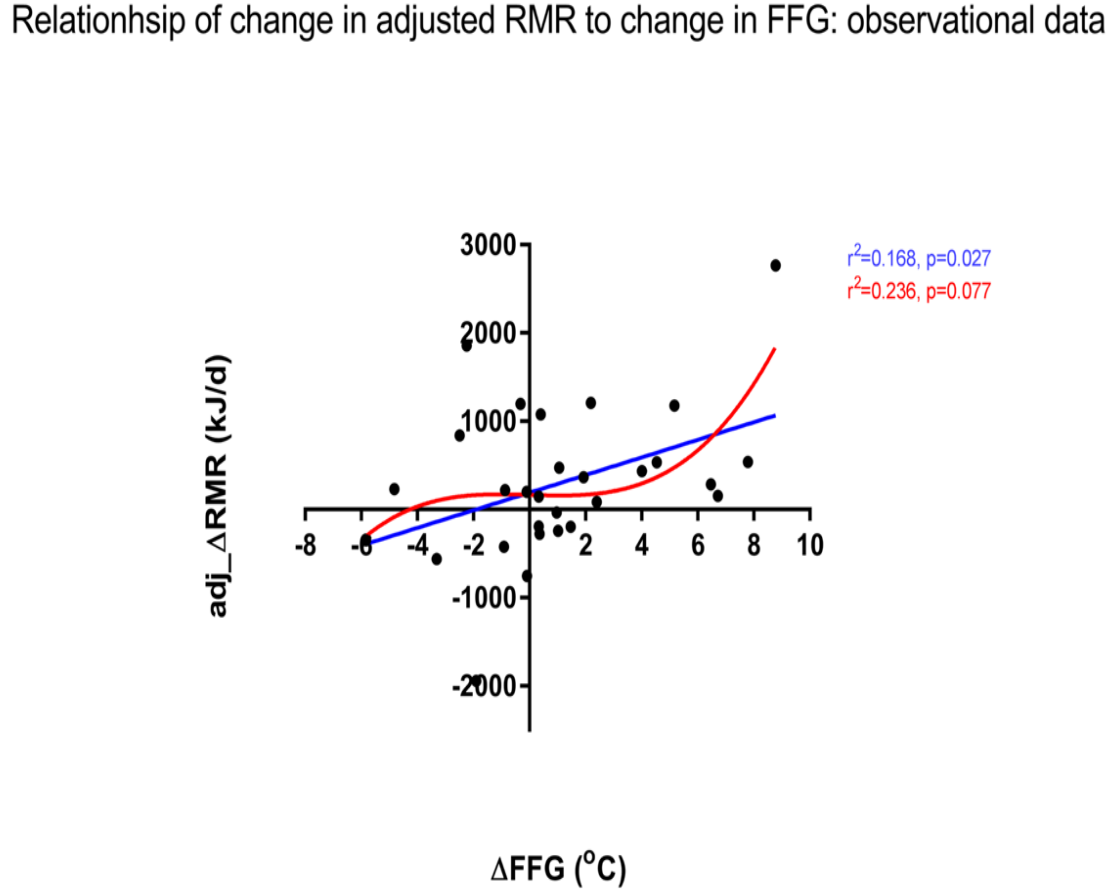

Figure 5.2 Relationship of adjusted change in RMR to change in FFG: Observational data.

Legend:

Blue line: linear regresison line with r^2 = 0.168, p= 0.027

Red line: cubic regression line with r^2 = 0.236, p= 0.0.077

RMR was adjusted for all variables in Table 5.3 except FFG by the method of Ravussin & Bogardus, 1989.

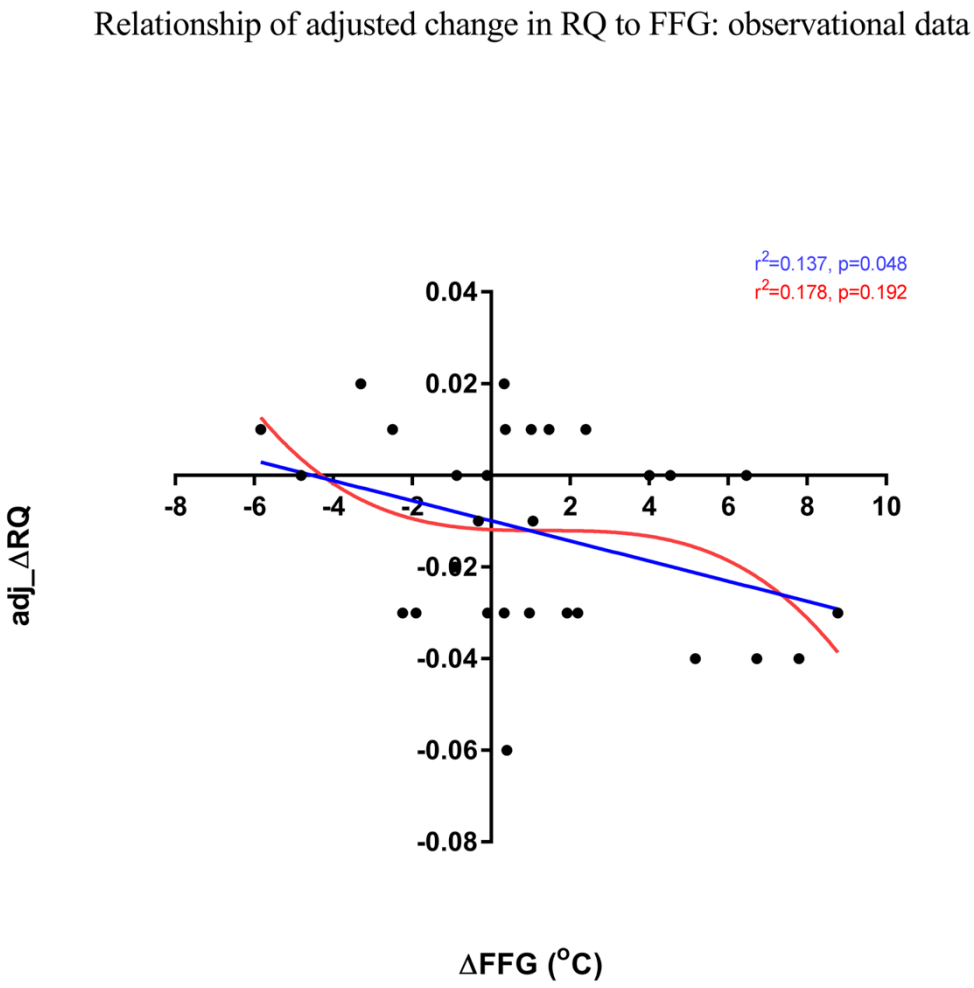

Figure 5.3 Relationship of adjusted change in RQ to change in FFG: observational data.

Legend: Blue line, linear regresison line with r^2 = 0.137; p= 0.048

Red line, cubic regression line with r^2 = 0.178 ; p= 0.192

RQ was adjusted for all variables in Table 3 except FFG by the method of Ravussin & Bogardus, 1989.

5.5 Discussion

The thermoneutral zone is a range of ambient temperature between 23ºC - 27ºC for a lightly clothed person (Du Bois, 1921 in Henry C, 2006). Within this range, the human body should maintain homeothermy without recourse to changes in metabolic rate (Kingma, 2012a). This is a key requirement for the valid measurement of RMR in humans. The hand and fingers, when compared to other areas of the body, are quickest to respond to a change in ambient temperature; including those within the TNZ (Caldwell, Matsuda-Nakamura & Taylor, 2014; Roth & Sheard, 1948). Changes in skin surface temperatures have been used to determine the redistribution of blood flow in reponse to cold and heat (Caldwell et al., 2014; Claessens-van Ooijen, 2008; Roth & Sheard, 1948) and measurements of FFG can accurately mimic such responses (Rubinstein E & Sessler, 1990).

In this study, we found a wide range of FFG values (-4.25 to +7.8 °C, Table 5.1), which indicated that even at 25^{o}C, the mid-point of TNZ, there were indviduals who significantly vasoconstricted (FFG >2°C) and others who significantly vasodilated (FFG < 0°C) (House & Tipton 2002). This could suggest that despite being measured in the accepted TNZ, there were many individuals who were not in their thermal comfort zone (TCZ). After including factors that influence thermoregulation (age, gender, FM, FFM, season, fat distribution) (Kingma, 2012a; Muller et al., 2009), there remained an intrinsic positive relationship between FFG and RMR measured at 25^{o}C (Figure 5.1). Such an outcome was unexpected for measurements made in the TNZ and was obtained after due consideration of 12 known predictors of RMR. In contrast, RQ and FFG were unrelated. While gender and presence of MetS made a contribution to RQ, those effects were modified by a significant MetS x gender interaction (Table 5.2). Men with MetS had lower RQ as compared to women with MetS; an outcome that adds to previous findings that the presence of MetS influenced gender differences in RMR (Soares, Cummings et al., 2011). Gender differences in substrate oxidation have been described (Blaak, 2001). However the potential role of testosterone, which is thermogenic and differentially infleunces MetS in men and women[13]; needs further evaluation.

The estimated regression coefficient suggested an increase in RMR of 63 kJ/d on average for every one degree C increase in FFG (Table 5.2), though the limits of this value ranged from +14 to + 112 kJ/d. Clearly at the lower limit of the 95% CI, this increase would be difficult to detect in studies with small sample sizes even if, for instance, a two degree change in FFG was observed. In contrast, the upper limit of the 95% CI was sizeable and could account for significant between-subject differences in RMR. As a proof of concept, we then evaluated whether longitudinal changes in free-living RMR and RQ were indeed associated with FFG. The outcomes in Table 5.3 clearly show that changes in FFG significantly impinged on measurements of RMR in a model that replicated all the factors used in the cross-sectional comparison (Table 2). We also found that unlike the results in Table 5.2, change (Δ) in RQ was also significantly predicted by ΔFFG (Table 5.3). Overall, these results support the contention that variations in FFG, even at the mid-point of the TNZ, could make sizeable contributions to RMR and possibly RQ. Clearly, a temperature of 25ºC was not a thermo-neutral temperature for many individuals in this study. If FFG measurements had not been conducted, we would have assumed the measurement of RMR and RQ was precise for all.

A relationship between RMR and FFG is expected, although changes in both have been demonstrated by many investigators when studying the thermoregulatory response to cold and warm temperatures (Maeda, Fukushima, Ishibashi & Higuchi, 2007; Roth & Sheard, 1948; Wijers & Saris, 2010). We are unaware of studies in human nutrition and metabolism that have reported direct relationships between adjusted RMR or RQ to FFG at a temperature within the TNZ. Essentially, the data suggests that even at a warm temperature of 25^{o}C, each individual responded with a characteristic vasomotor response which ranged from frank vasoconstriciton (cold response) to vasodilation (hot response). Importantly, such changes were associated with a change in RMR (and RQ) in the direction of the vasomotor change.

In Figures 5.2 & 5.3 we expanded our analysis to include the possibility of non-linear associations between RMR and RQ to FFG. Though in most cases a cubic fit accounted for a greater r^2 than the linear relationship, they did not all achieve statistical significance (Figure 5.2 & 5.3). Perhaps future studies with larger sample sizes are required to clarify this aspect. This would be important since confirmation of either a quadratic or cubic fit would confirm a range of FFG when no discernable change in RMR or RQ is seen. Identification of such a range would assist investigators in determining those individuals who meet the requirement

of TNZ for RMR measurements. From a thermoregulatory perspective, it could also mean that for a given temperature within the TNZ, there is a range of thermal comfort where no measureable change in RMR or RQ is detectable. However outside these limits (which may depend on the population under study) FFG and energy metabolism may be related.

Modern housing, work places and even modes of transportation now provide the opportunity to always reside at a thermally comfortable temperature, despite wide variations in environmental conditions. Future studies need to understand how shifts in each person's TNZ may influence their total energy expenditure (TEE). RMR is the largest component of TEE and almost ~50% of RMR is directed towards homeothermy. There is an ongoing interest in how variations in core body temperature may underscore the propensity to a positive energy balance and hence obesity (Landsberg et al., 2009). However, in our study IET [a surrogate for core body temperature] did not associate with RMR or RQ, once FFG entered the model.

We acknowledge that these outcomes did not stem from a randomised controlled trial, and so do not offer a causal pathway. However, the demonstration here of a relationship from a cross-sectional study, and its confirmation through observational change in the same variables, is a good indicator that this relationship is possibly true. As elegantly argued by Kingma et al. (2014) there needs to be a reassessment of the crtieria to judge the TNZ for thermal physiology and measurements of human energy metabolism. Untill then, and from a practical perspective, our data would argue for measurements of FFG to be included in the protocol for resting energy metabolism. Such information would allow investigators to evaluate which participants are in the TNZ. Alternatively, measurements of FFG would allow for statistical adjustment of differences in RMR and RQ between groups of individuals (Nsatimba et al., 2016).

5.6 Conclusions

In a diverse group of Australian adults, we demonstrated that wide fluctuations in skin surface temperature gradients were observed at 25^{o}C (a temperature within TNZ), suggesting that some individuals were outside their TNZ. Adjusted for known confounders, such variations in FFG were significantly associated in the same direction as changes in RMR and inversely with RQ measured at that temperature. The data would argue for the inclusion of FFG measurements when studying energy metabolism, so as to detect individuals who deviate from thermoneutrality. Alternatively, measurements of FFG could be used toward statistical adjustment of data to ensure that accurate conclusions are reached in comparisons of RMR and RQ between groups of individuals.

Section B

Vitamin D Status and Energy Metabolism

Chapter 6 Post-prandial Changes in Glucose Oxidation and Insulin Sensitivity in the Metabolic Syndrome: Influence of Fibroblast Growth Factor 21 and Vitamin D Status

Objective:

1. To understand influence of prevailing vitamin D status on basal and post-prandial energy metabolism in overweight and obese adults.

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6.1 Abstract

Background: Metabolic inflexibility due to insulin resistance has been reported in metabolic syndrome (MetS). Fibroblast growth factor 21 (FGF21) and vitamin D status may improve insulin sensitivity.

Methods: Forty eight overweight and obese older adults (14M, 34F), aged 51±15 years were studied. Resting metabolic rate (RMR) and respiratory quotient (RQ) were measured before and intermittently for 2h after an oral glucose tolerance test (OGTT). The total area under the curve (TAUC) was calculated. Insulin sensitivity index (ISI) was determined as 10^4/(insulin x glucose) for fasting and 2h venous blood. Fat mass (FM) and fat free mass (FFM) was measured by DEXA. Participants were grouped by metabolic syndrome (MetS+ = presence; MetS- = absence) and by median 25OHD concentration as VD_low and VD_high. 25OHD was also tested as a continuous variable. A parsimonious 2x2 ANOVA included age, FM, FFM, MetS and gender interaction.

Results: RMR was similar between groups but an interactive effect of MetS and gender was noted. Fasting RQ was significantly different between vitamin groups (VD_low: 0.835 ± 0.008 vs. VD_high: 0.810 ± 0.008, P= 0.024) and fasting ISI was greater in the MetS- individuals compared to MetS+ individuals, but not significantly. Post-glucose increases in thermogenesis, RQ and FGF21 were significant, but ISI decreased. Adjusted post-prandial TAUC_RQ (VD_low: 1.71±0.01; VD_high: 1.74±0.01, P=0.041) and ISI_2h (VD_low: 35.41±0.21; VD_high: 101.90±0.21, P= 0.001) were significantly different. Adjusted FGF21 was similar across all comparisons before and after OGTT.

Conclusions: Higher vitamin D status, but not FGF21, was associated with greater post-prandial glucose oxidation and improved insulin sensitivity.

6.2 Introduction

Metabolic syndrome (MetS) is an escalating health concern all over the world. Presence of MetS greatly increases the risk of type 2 diabetes and cardiovascular disease. Our modern eating pattern of several meals per day places us in a post-prandial state for most of the 24 hours. Depending on meal composition, exaggerated glucose and lipid levels can lead to increased inflammation, dyslipidaemia and vascular dysfunction (Bonora et al., 2001; O'Keefe, Gheewala & O'keefe, 2008). Altered post-prandial metabolism is hence central to many diet-related chronic conditions from atherosclerosis to obesity to insulin resistance.

After an overnight fast, the mobilisation of fat is the main source of fuel and this is reflected in a lower respiratory quotient (RQ). Following mixed-meal ingestion, there is a change in fuel use to favour carbohydrate as the primary source, and RQ rises. This capacity to switch between available fuels is termed metabolic flexibility (Galgani, 2008; Kelley et al., 2002; Storlien et al., 2004). Metabolic flexibility (MF) is usually determined as the change between the RQ in the fasting (basal) state and that following insulin stimulation (Van Oostrom et al., 2007). The gold standard for investigating MF has been the use of the euglycemic-hyperinsulinemic clamp (Faerch & Vaag, 2011). However, studies have also reported MF after an oral glucose tolerance test (OGTT) (Stumvoll et al., 2000) and a high fat challenge (Kardinaal et al.; Wu & Yu, 2004).

Metabolic flexibility is impaired in obesity (Blaak et al., 2006), insulin resistance (Musso, Gambino, Pacini, De Michieli & Cassader, 2009), type 2 diabetes (Wu & Yu, 2004) and metabolic syndrome (Van Oostrom et al., 2007). Moreover, those with impaired fasting glucose (IFG) exhibited reduced glucose oxidation and a slightly elevated fatty acid oxidation rate during insulin infusion, despite having normal total peripheral glucose disposal (Faerch & Vaag, 2011). This impaired mitochondrial function can be either due to change in amount and density of mitochondria (Abdul-Ghani & DeFronzo, 2008) or oxidative capacity (Ritov et al., 2005). This compromised function is more evident in the post-prandial state because those with insulin resistance fail to increase their mitochondrial activity or oxidative

phosphorylation capacity during normoglycaemic and hyperinsulaemic clamps. Thus reducing levels of glucose 6 phosphate and glucose transport (Szendroedi et al., 2012). Following a high fat diet for 4 weeks, MetS+ individuals had a high RQ and demonstrated a delayed switch from fat to carbohydrate utilisation (~ 60 min) as compared to a healthy group that took only 30 min (O'Keefe et al., 2008). Lifestyle modification and weight loss can reverse metabolic inflexibility to a certain extent (Corpeleijn et al., 2009).

FGF21 belongs to a family of peptides involved in a plethora of metabolic functions. FGF21 is produced in the liver and is capable of promoting a change in white adipose tissue to allow conversion to brown adipose tissue (Fisher et al., 2012) and has hence been implicated as a cold-induced stimulator of non-shivering thermogenesis (Lee, Werner, Kebebew & Celi, 2014). Following glucose ingestion, FGF21 shows a biphasic excursion in plasma; an initial decline within 30 min followed by a >2 fold increase by 2 hrs. Hence, FGF21 may have a role in energy expenditure, glucose and lipid metabolism (Mashili et al., 2011; Semba et al., 2012; Straub & Wolfrum, 2015) and may influence the metabolic inflexibility seen in MetS.

Low concentrations of 25OHD, an index of vitamin D status, are of global occurrence and are potentially linked to many chronic conditions including greater adiposity, metabolic disorders, altered cardiovascular, renal and immune system disorders (Chen et al., 2011; Liu et al., 2006; Wang et al., 2004; Zhang et al., 2010). Vitamin D has a potential mechanistic role in human energy metabolism and balance (Soares, 2012) with or without calcium, and this is observed across human and animal studies (Calton, Keane & Soares, 2015; Mason et al., 2014; Nagpal et al., 2009; Nikooyeh et al., 2011; Pathak et al., 2014; Wong et al., 2011; Wong et al., 2009; Zittermann et al., 2009). Moreover, emerging data suggests an effect of 25OHD on glycaemic control and weight (Parikh et al., 2012; Zitterman et al., 2014), waist circumference (McGill et al., 2008), total fat mass (Kraemer et al., 2012; Rosenblum et al., 2012) and visceral and subcutaneous adipose tissue measurements (Caron-Jobin et al., 2011). It is unclear whether vitamin D influences the post-prandial energy metabolism of humans (Soares et al., 2012; Soares, Pathak & Calton, 2014).

Due to associated benefits of both FGF21 and vitamin D on energy homeostasis and insulin sensitivity, we were interested to explore their individual and combined effect in improving metabolic flexibility among those with MetS.

Objectives

The objective of this study was to investigate glucose-induced thermogenesis and oxidation in MetS, and to examine whether changes in FGF21 or prevailing vitamin D status modulated defined metabolic parameters.

Hypothesis

We hypothesized that FGF21 would facilitate an improved switch in fuel utilization from fasted to post-glucose state as evidenced by greater post-prandial glucose oxidation and improved insulin sensitivity. Adequate 25OHD may in turn play a permissive role in the beneficial changes envisaged.

6.3 Methods and Materials

6.3.1 Recruitment

Participants were recruited via flyers in and around the University campus, through radio advertising and advertisement in community newspapers. Interested participants completed a short screening questionnaire over the phone with the first author or online. Eligible participants were then provided with the Participant Information Sheet and consent forms to be signed.

6.3.2 Subject Description

Forty eight Australians of European origin and residents of Perth were recruited. Eligible participants between 18-65 years of age were without thyroid disease and polycystic ovarian syndrome (by history), non-smokers, non-pregnant, non-lactating, not on hormonal contraception or testosterone replacement therapy and not suffering from any GI tract disorder or metabolic disease affecting energy metabolism. Four type 2 diabetics with good glucose levels through lifestyle modification (as judged by HbA1c <6.5%) were included and classified as having metabolic syndrome. Salient anthropometric and metabolic features of the volunteers studied are provided in Table 5.1.

6.3.3 Study Design

This was a secondary analysis of a collation of cross-sectional data from two studies following the same protocol. To the best of our knowledge there were no acute studies that had examined between-group differences in vitamin D on post-prandial metabolism. Twenty four out of 48 participants had MetS and they were designated as MetS+ (≥ 3 characteristics) and the rest as MetS- (without MetS, ≤2 characteristics) based on the recommendation of Alberti et al. (2009). A second grouping based on the median 25OHD of 65.7 nmol/L was used to categorize VD_low and VD_high groups. The analysis studied each grouping separately together with an interaction term to uncover statistically significant outcomes.

6.3.4 Ethics Approval

This study was approved by the Curtin University Human Research Ethics Committee (Approval numbers: HR 103/2012 and HR 108/2013). All participants provided written, informed consent before joining the study.

6.3.5 Sample Size Calculation

Our previous studies had indicated that a sample size of 7-11 detected within-subject differences in post-prandial thermogenesis, RQ and endothelial function in response to calcium and vitamin D (Cummings, James Soares, 2006; Soares, Chan She Ping-Delfos et al., 2011; Soares & She-Ping-Delfos, 2010). Because this was planned as a between-subject design, we used a x 4 multiplier. Consequently, the current sample size of 48 with 5 covariates achieved a study power of 80% to detect a moderate effect size of 0.5 at 5% significance level (GPower version 3.1) (Faul et al., 2007) .

6.3.6 Data Collection

The study was conducted in the School of Public Health, Curtin University. All measurements were performed over 4 hrs in a specialized temperature-controlled chamber. Operating temperature was set to 25^0C on the day prior to the experiment day. As described previously, the chamber had a volume of 57.75 m^3, an insulated roof, walls and sliding door (Calton, Soares et al., 2016, Nsatimba, 2016). The temperature can be controlled from 4 - 50°C. The study required two visits. The first was an introduction to the laboratory, equipment and the researcher to avoid any anxiety occurring on the experiment day that may affect RMR. On the second visit, the study day, participants arrived at the laboratory after a minimum of 12 hrs of overnight fasting and 8 hrs sleep, and abstinence from heavy physical activity and alcohol at least 36 hrs prior to the trial. All participants consumed a standardized dinner (commercial meal) on the night prior to measurement. These were provided free of charge and participants chose from a selection that had been screened for their low fat and calcium content by the researcher to minimize carry-over effects on the next morning's macronutrient oxidation (Soares, Chan She Ping-Delfos et al., 2011; Soares & She-Ping-Delfos, 2010). Any leftovers were brought back to the lab. Upon arrival, participants had their anthropometry measured after having voided and changed into a gown provided (0.5 clo). Women were allowed to wear a bra and underwear (~0.04 clo), and all men wore their briefs (~0.04 clo). Following a mandatory 60-min rest in a supine position, baseline oxygen consumption and carbon dioxide production was measured using canopy-based

indirect calorimetry (Deltatrac II, Datex, Finland), for the next 30 min. Resting metabolic rate (RMR) was calculated from the last 25 min of continuous recording using the Weir equation (Weir, 1949). A glucose tolerance test (75 g glucose in 300 ml water, CarboTest, Australia) was then administered. Indirect calorimetric was continued after the glucose load every 30 min for the next 2 hrs in all participants. Venous blood samples were drawn at fasting and 2 hrs post-glucose and were tested for insulin, glucose, FGF21 and 25OHD. Vitamin D status [25(OH)D] was determined using the chemiluminescent immunoassay method (Liaison, DiaSorin and Architect, Abbott). FGF21 was measured using an ELISA kit (Adipogen(R), Life Science), where the intra-assay and inter-assay coefficients were 5.88% and 12.65% respectively, with a minimum detection limit of 47.82 pg/mL. Body compositional analysis was conducted on Dual Energy X-ray Absorptiometry ((DEXA, Prodigy™, Lunar Corp. Madison, USA) for components such as percent fat, fat mass, fat free mass and android/Gynoid ratio (A/G ratio). On completion of the protocol, a light buffet-style meal was served. ISI was determined as 10^4/(insulin x glucose) both at fasting and 2 hrs post glucose (Sluiter, Erkelens, Terpstra, Reitsma & Doorenbos, 1976).

All other details are elaborated on in 'Chapter 3 - Methods'.

6.3.7 Statistical Analysis

All data was entered and analysed by IBM SPSS statistics for windows version 22 (SPSS Inc., Chicago, IL, USA). Data is presented as mean $\pm$ SE for all metabolic and plasma variables.

Total area under the curve (TAUC) was calculated using the standard trapezoid method with time of start (t_0) as reference point until (t_2) as end of 2 hrs post-glucose. The unadjusted effect of MetS and vitamin D status on main outcomes (RMR, RQ, ISI and FGF21) were initially assessed by an independent samples t-test for comparing the difference between groups. Normalised variables were used to run the analysis and values were then back-transformed for data presentation. All post-prandial endpoints were adjusted for their corresponding fasting values and additional covariates (age, gender, FM, FFM and FGF21) in a two-step

model (Tables 6.3 and 6.4). A 2x2 ANCOVA with interaction was used to test for statistical differences. Partial correlation coefficients were assessed to confirm relationship between variables. A paired samples t-test was performed to examine the change between all fasting and post-prandial values. A p-value of less than 0.05 was accepted as being statistically significant for all analyses.

6.4 Results

34 females and 14 males participated in the study. The group had a mean age of 51 ± 15 yrs, BMI 30.6 ± 5.75 kg/m^2 and percent fat 41.8 ± 8.31(DEXA). Baselines characteristics of each group are presented in Table 6.1. The final parsimonious multiple linear regression model for RMR included age, FM, FFM, MetS x gender interaction, but excluded gender, season, and all other two-way interactions. There was no difference in adjusted RMR between groups (MetS+ vs MetS-; and VD_low vs VD_high), but a significant MetS x gender interaction was noted.

Table 6.1 Body composition and baseline metabolic characteristics of the study sample.

Variable	MetS- (n=24)	MetS+ (n=24)	VD_low (n=24)	VD_high (n=24)
Age, yr	47.1(3.24)	56.7(3.24)*	49.5(3.24)	54.4(3.24)
[1]BMI, kg/m^2	28.1(1.17)	31.7(1.17)	30.7(1.17)*	29.1(1.17)
FFM, kg	46.6(2.8)	51.8(2.8)	52.2(2.8)*	46.1(2.8)
[1]FM, kg	32.1(2.5)	37.3 (2.5)	35.7(2.5)*	33.7(2.5)
Fasting ISI	356.7(30.03)	232.6(30.03)	298.9(30.03)	290.4(30.03)
Fasting FGF21, pg/ml	118.9(39.03)	206.3(39.03)	178.9(39.03)	146.3(39.03)

Data are mean (SE). ANOVA between groups: * P <0.05; ** < 0.005; [1]A significant MetS *VD interaction was noted (P<0.05)

MetS+ = with the metabolic syndrome, MetS- = without the metabolic syndrome; BMI = body mass index; FFM = fat free mass; FM = fat mass; ISI= insulin sensitivity index; FGF21=fibroblast growth factor 21.

Table 6.2 Comparison of fasting energy metabolism grouped by metabolic syndrome and vitamin D status.

Variable	MetS- (n=24)	MetS+ (n=24)	VD_low(n=24)	VD_high (n=24)
RMR kJ/h				
Adjusted RMR (Model 1)	263 (5.3)	269 (5.4)	266 (5.3)	265 (5.3)
Adjusted RMR (Model 2)	263 (5.3)	269 (5.4)	266 (5.3)	265 (5.3)
RQ				
Adjusted RQ (Model 1)	0.82(0.010)	0.83 (0.008)	0.84(0.009)	0.81 (0.008)*
Adjusted RQ (Model 2)	0.82 (0.010)	0.83 (0.008)	0.84 (0.009)	0.81 (0.008)*
ISI				
Adjusted ISI (Model 1)	75.25 (21.97)	96.9 (11.54)	127.6 (15.70)	114.3 (13.72)
Adjusted ISI (Model 2)	151.0 (21.13)	99.7 (13.87)	130.9 (15.44)	115.0 (13.23)
FGF21 (pg/ml)				
Adjusted FGF21 (Model 1)	132.1(41.79)	154.9(36.67)	151.2(37.73)	135.0(34.79)
Adjusted FGF21 (Model 2)	-	-	-	-

Data are estimated mean (SE). 2x2 ANOVA

*P <0.05; for treatment effects

Model 1 = adjusted for age, gender, FM, FFM

Model 2 = adjusted for age, gender, FM, FFM plus FGF21

There was no MetS* vitamin D interaction for variables, except where fasting RMR (p=0.015) was detected.

MetS- = without metabolic syndrome; MetS+ = with metabolic syndrome; RMR= resting metabolic rate; RQ = respiratory quotient; ISI= insulin sensitivity index (10^4/fasting insulin x fasting glucose); FGF21 = fibroblast growth factor 21; FM = fat mass; FFM = fat free mass.

Adjusted RQ was significantly lower in VD_high compared to VD_low (Figure 6.1) and ISI was higher in MetS+ participants, although this is not statistically significant (Table 6.2).

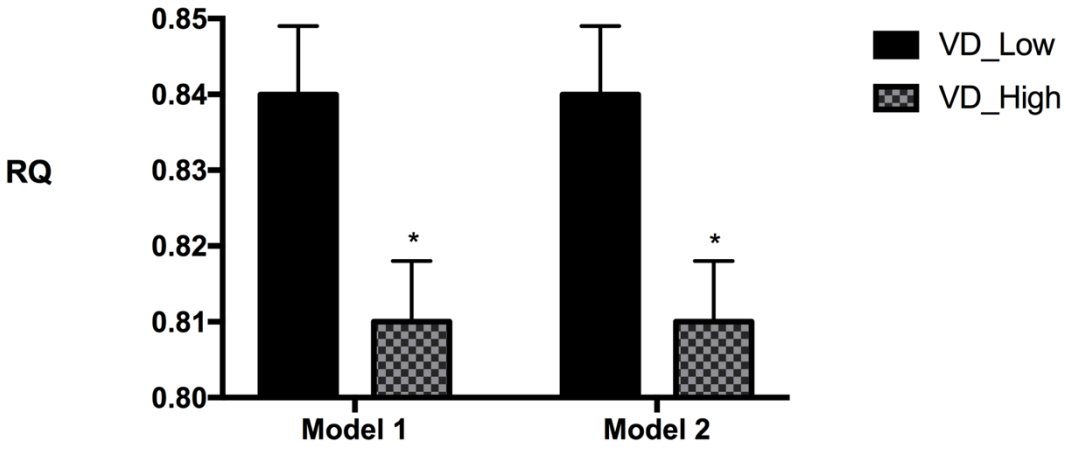

Figure 6.1 Fasting fuel utilisation and vitamin D status.

Model 1 = adjusted for age, gender, FM, FFM

Model 2 = adjusted for age, gender, FM, FFM plus FGF21

*P <0.05; for treatment effects, vit D_high vs vit D_low

Paired t-tests revealed that post-prandial values for RMR, RQ and plasma FGF21 significantly increased over-fasting, while ISI decreased post-glucose in the whole group (p=0.001) [Table 6.3].

Table 6.3 Comparison between fasting and post-prandial values of metabolic variables as a group (n=48).

	Fasting	Post-prandial (TAUC)/hr	P-value (2-tailed)
RMR	265 ± 59.4	283 ± 59	0.001
RQ	0.82 ± 0.037	0.86 ± 0.031	0.001
FGF21	11.40 ± 6.32	16.02 ± 6.52	0.001
ISI	2.8 ± 0.29	2.1 ± 0.64	0.001

RMR = resting metabolic rate; RQ = respiratory quotient; ISI = insulin sensitivity index (10^4/fasting insulin x fasting glucose); FGF21 = fibroblast growth factor 21; TAUC = total area under curve.

Change (Δ=(postprandial-fasting)) in RQ was higher in MetS- (MetS-: 0.11±0.06) vs. MetS+ (0.073 ± 0.50, p= 0.021) but not when adjusted for 25OHD as a continuous variable. Adjusted for fasting values (Model 1), post-prandial TAUC for RQ (Figure 6.2) and ISI (Figure 6.3) were significantly lower in VD_low compared to VD_high. These outcomes were not altered on further adjustment for change (Δ) in FGF21 (Model 2, Table 6.4). When postprandial RQ was treated as a change variable (Δ), we obtained the same result as for adjusted TAUC_RQ in Table 6.3.

We also re-analysed the models in Table 6.2 and 6.4 using plasma 25OHD as a continuous variable rather than a categorical one based on its median. The outcomes remained the same. Briefly, for fasting RQ the β coefficient for 25OHD was -0.001 (95% CI; -0.001, -4.446); for post-prandial RQ, the β coefficient for 25OHD was 0.001 (95% CI: 0.000, 0.001); and for post-prandial ISI, the β coefficient for 25OHD was 0.031 (95% CI: 0.016, 0.046). Partial correlation analysis adjusted for age, gender, season, FM and FFM indicated significant relationships between 25OHD and fasting RQ (r = -0.37, p= 0.012), fasting FGF21 and fasting ISI (r = -0.32, p= 0.032), and post-prandially between 25OHD and changes in RQ (r =0 .52, p= 0.005) and in ISI (r = 0.465, p= 0.002).

Table 6.4 Association of FGF21 and vitamin D status on post-prandial energy metabolism in the metabolic syndrome.

Variable	MetS- (n=24)	MetS+ (n=24)	VD_low (n=24)	VD_high (n=24)
PP_MR (kJ / hr)				
Adjusted PP_MR (Model 1)	274 (2.8)	280 (2.8)	276 (2.8)	279 (2.8)
Adjusted PP_MR (Model 2)	274 (2.8)	280 (2.8)	276 (2.8)	279 (2.8)
PP_RQ (RQ/hr)				
Adjusted PP_RQ (Model 1)	0.87(0.005)	0.86 (0.005)	0.86 (0.005)	0.87 0.005)**
Adjusted PP_RQ (Model 2)	0.87 (0.005)	0.86 (0.005)	0.86 (0.005)	0.87 (0.005)**
△RQ				
Adjusted for △FGF21	0.098(0.010)	0.086(0.010)	0.076(0.010)	0.11(0.010)**
ISI/h				
Adjusted ISI/h (Model 1)	34.67 (7.63)	26.05 (5.73)	17.71 (3.72)	50.95 (10.7) †
Adjusted ISI (Model 2)	34.71 (7.64)	26.02 (5.73)	17.74(3.73)	50.9 (10.69) †
FGF21 (pg/ml)				
Adjusted FGF21 (Model 1)	130.85 (13.10)	125.68 (12.84)	126.92(12.75)	129.61(12.88)
Adjusted FGF21 (Model 2)	-	-	-	-

Data are estimated mean (SE). Univariate analysis:

*P <0.05; ** P<0.01

† P <0.005 for treatment effects

Model 1: adjusted for corresponding fasting value.

Model 2: Model 1 plus △FGF21.

There was no MetS*VD interaction for any variable.

MetS- = without metabolic syndrome; MetS+ = with metabolic syndrome; RMR= resting metabolic rate; RQ = respiratory quotient; ISI = insulin sensitivity index (10^4/fasting insulin x fasting glucose); FGF21 = fibroblast growth factor 21; △FGF21 = post-prandial minus fasting FGF21.

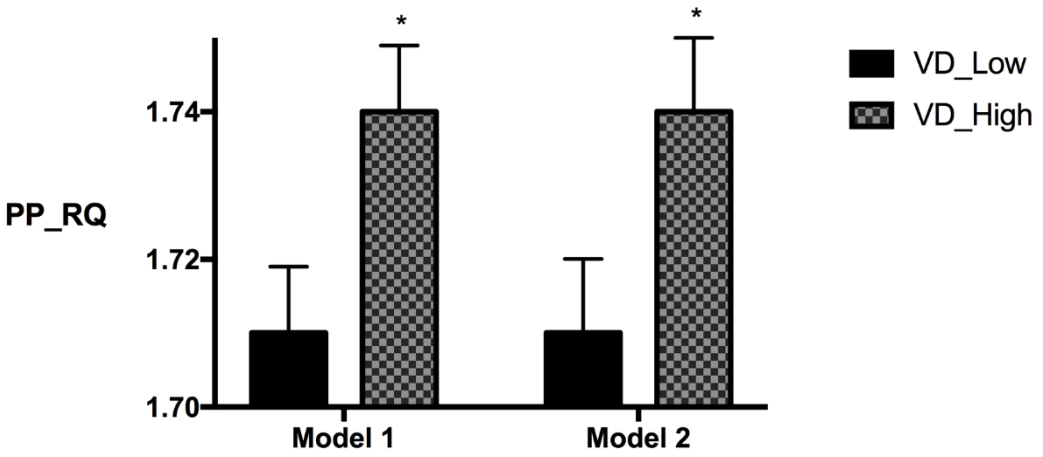

Figure 6.2 Post-prandial fuel utilisation and vitamin D status.

Model 1: adjusted for corresponding fasting value.

Model 2: model 1 plus ∆FGF21.

* P<0.01, vit D_high vs vit D_low

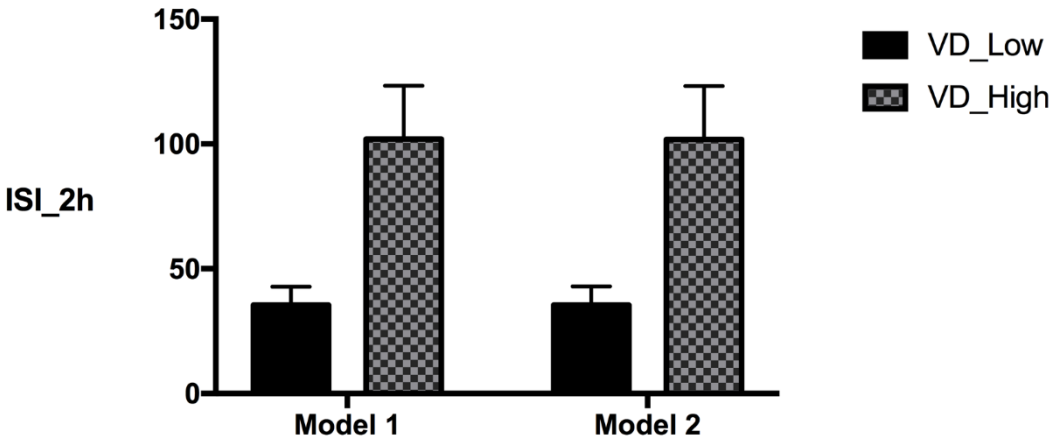

Figure 6.3 Insulin sensitivity and vitamin D status.

Model 1: adjusted for corresponding fasting value.

Model 2: model 1 plus ∆FGF21.

P <0.005, vit D_high vs vit D_low.

In addition, we found that those with metabolic syndrome had lower vitamin D status as compared to those without (Figure 6.4).

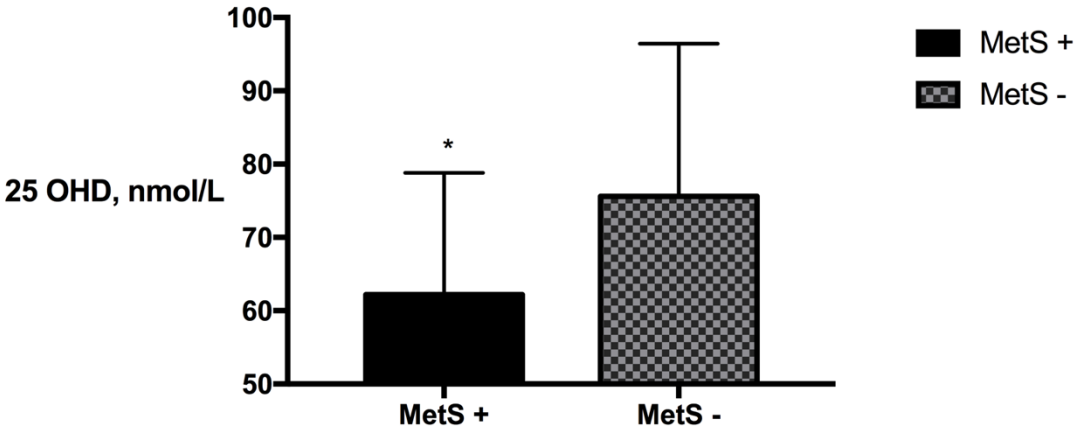

Figure 6.4 Vitamin D status in metabolic syndrome.

*P <0.05 for significance

6.5 Discussion

Previous studies have argued for a metabolic inflexibility in MetS by observing the switch in substrate utilisation after a high fat meal challenge (Faerch & Vaag, 2011; Kardinaal et al.) or a glucose load (Bonora et al., 2001). This inflexibility was also associated with a time delay where a MetS+ group took 60 min to switch to carbohydrate oxidation from fat oxidation, while a healthy group took 30 min (O'Keefe et al., 2008). Overall, a reduced utilization of glucose in the fed state possibly stems from the insulin resistance common to MetS and could account for the increased predisposition of MetS to incidence of T2DM. In the present study, we also observed a significantly lower ΔRQ following glucose in MetS+. Those with MetS+ also had lower 25OHD status. However, the significance disappeared when adjusted for 25OHD, but not FGF21. This initially suggested that vitamin D status may have, as yet, an unrecognised role in the metabolic inflexibility seen with MetS. Further analysis confirmed that vitamin D status (which was lower in MetS+, see Figure 6.4), was a greater contributor to the post-prandial changes in RQ and ISI than either MetS or FGF21 (Table 6.2

& 6.4). These outcomes were consistent when the effect of 25OHD was tested either as a continuous or categorical variable in the analyses. The overall outcomes were also consistent when we used the traditional approach of determining MF by examining ΔRQ (Sparksa et al., 2009; Stumvoll et al., 2000; Van Oostrom et al., 2007), or if we used a statistically robust approach where we adjusted all post-prandial outcomes for their respective baseline (Table 6.4) (Liu et al., 2016). Further we obtained significant partial correlations between 25OHD and RQ in the fasting and post-prandial state. Collectively, the demonstration that vitamin D modulates insulin sensitivity (as judged by a surrogate marker) and glucose oxidation in the post-glucose state is a novel finding. Previous reports of metabolic inflexibility in MetS did not control vitamin D status (O'Keefe et al., 2008, Buscemi, 2007, Corpeleijn et al., 2009).

It may be argued that our data does not reflect metabolic inflexibility since we did not use the euglycemic-hyperinsulinemic clamp technique to increase and maintain insulin levels (Sparksa et al., 2009; Stumvoll et al., 2000). However, there is now sufficient data to confirm that insulin sensitivity/resistance indices derived from oral glucose tolerance tests closely parallel similar measures from clamp techniques, both in the fasting and post-prandial state (Lorenzo et al., 2010; Muniyappa, Lee, Chen & Quon, 2008).

The differences we observed in RQ were not accompanied by significant differences in energy expenditure. At the fasting state there were no differences in RMR between groups studied which contrasts with the findings of Buschemi et al (2007), who reported lower RMR in MetS+ obese individuals. We have recently shown in a larger dataset that vitamin D status made a significant contribution to RMR (Calton, 2016d). In that study, MetS only entered the regression analysis if insulin sensitivity was omitted from the predictor list. We, however, do note a gender x MetS interaction in RMR in this study. This indicated that MetS+ women had a higher RMR than those without, while MetS+ men had a lower RMR than men without MetS. This interactive effect of MetS and gender confirms a similar observation in an independent study from our group (Brand, vander Tweel, Grobbee, Emmelot-Vonl, vander Schouw, 2011). One explanation for the latter may be that testosterone, which is thermogenic, shows a similar pattern of interaction as does RMR. MetS+ women have higher testosterone than their MetS- counterparts.

The rise in post-prandial metabolic rate did not differ between groups once adjusted for the fasting value. Overall, the lack of differences in energy expenditure in the fasted and fed state, despite alterations in fuel usage, is not unexpected (Geisler, 2011). Geisler et al (2011) have shown that the ratio of ATP produced to oxygen consumed (P:O ratio) is approximately equal whether the fuel oxidized is glucose or lipid. Hence, metabolic switching of fuel usage does not necessarily imply a change in energy expenditure or leanness (Hoehn et al., 2010). Similar to these acute results, RCTs on vitamin D supplementation did not demonstrate a mediatory effect of vitamin D on RMR (Major et al., 2007), but RQ was lowered post vitamin D supplementation (Marcotorchino et al., 2014).

FGF21 is a metabolic regulator that plays a role in energy expenditure, glucose oxidation and lipid metabolism. Specifically, FGF21 increases EE, decreases glycemia and increases fatty acid oxidation (Straczkowski et al., 2013). We noted a two-fold increase in FGF21 following glucose in all groups in this study, confirming their close link. However, FGF21 was not different in MetS or across vitamin D groups once adjusted for confounders (Table 6.4). This was in contrast to reports that the higher FGF21 seen in MetS may reflect a resistance to FGF21 (Novotny et al., 2014; Zhang et al., 2008). Except for an inverse relationship to fasting ISI (Model 2, Table 6.2), no other associations were evident. The latter may suggest that when ISI is high, as during fasting (due to lower glucose and circulating insulin), there is a decline in circulating FGF21. Following glucose ingestion, ISI declined and FGF21 dramatically increased. However, the increase in FGF21 observed with oral glucose was unrelated to any metabolic function in this study.

6.6 Conclusions

Vitamin D status determined fuel utilisation and insulin sensitivity irrespective of the presence of metabolic syndrome. Surprisingly, FGF21, a potential key regulator of energy and glucose metabolism, did not account for the improved metabolism seen in with greater 25OHD. Future long-term trials on vitamin D supplementation are essential to place these outcomes on a firmer footing, while attempting to understand the mechanistic basis of the observations.

SECTION C

Leucine and Energy Metabolism

Chapter 7 Alterations in Body Composition and Energy Metabolism Following 8 Weeks of L-Leucine Supplementation During Weight Loss: Unexpected Effect of Vitamin D Status

Objectives:

1. To ascertain whether leucine supplementation increased weight loss and modified body composition through changes in energy metabolism.

2. To examine the added effect of vitamin D status on the above parameters.

7.1 Abstract

Background and objectives: Increased loss of fat mass with higher protein intake has been reported. Leucine is an indispensable branched-chain amino acid that is present in large amounts in animal protein. Vitamin D status (serum 25OHD) could impact on insulin sensitivity and visceral adiposity, but could also interact with leucine intake on these endpoints.

Methods: Thirty-seven adults with abdominal obesity plus an additional characteristic of the metabolic syndrome were enrolled in an 8 week, double-blind placebo controlled trial. Participants were randomized to receive leucine (LEU, 3g/d) or placebo capsules (PL, cellulose, 3g/d) as part of an individually tailored weight loss program. Body composition (DEXA, Prodigy™) and clinical biochemistry were measured at the start and end of the study. To investigate the effect of a change in vitamin D status, a categorical variable was derived based on those who increased their 25OHD following weight loss (High_VDS) and those who decreased their status (Low_VDS). An intention to treat approach was followed for data analysis. Two-way ANCOVA with interaction was conducted after adjustment of the baseline values and other covariates. A p-value of <0.05 was accepted for statistical significance.

Results: Eight of nineteen from PL and eight of eighteen from LEU groups had a mean increase in 25OHD during weight loss of ~10 nmol/L. There were no significant effects on weight, fat mass, percent fat mass (%fat), waist circumference and Android to Gynoid ratio between LEU and PL. Change in vitamin D status however was associated with a significantly lower FM [data shown as mean (SE)] (High_VDS: 36.0 (0.60) vs. Low_VDS: 37.8 (0.52), p= 0.035), lower % fat (High_VDS: 40.5 (0.53) vs. Low_VDS: 42.4 (0.46), p= 0.010) and higher appendicular lean tissue mass (ALTM) (High_VDS: 23.0 (0.42) vs. Low_VDS 21.7 (0.37), p=0.025). There was a significant interaction between treatments (LEU vs. Pl) and change in vitamin D status (High_VDS vs. Low_VDS) for Stumvol's ISI and McAuley's index of insulin sensitivity after adjustment for final FM and ALTM.

Conclusion: Incidental increases in 25OHD had a beneficial effect on FM and ALTM and confounded LEU effect on fasting insulin sensitivity.

7.2 Introduction

High-protein diets are a promising approach for weight loss and maintenance as compared to isocaloric diets that manipulated fat and carbohydrates (Azadbakht, Izadi, Surkan & Esmaillzadeh, 2013; Layman et al., 2003). Milk proteins are casein (80%) and whey (20%) and they exert different gastrointestinal (GI) effects on post-prandial hormonal profiles. Whey leads to increased energy expenditure and reduces energy intake by its contribution to short and long-term feelings of satiety (Veldhorst et al., 2008). Importantly, whey also provides branched-chain amino acids (BCAA) that may have a positive effect on muscle mass and bone. Of the three BCAAs, leucine has received much interest due to its high abundance (~10%) in whey proteins. Leucine, which is anticipated to promote weight loss, accelerates protein anabolism, cell growth and metabolism (Anthony et al., 2001; Dardevet et al., 2002, Lynch et al., 2002). Leucine has a distinct role in protein metabolism as a key signal in the translation-initiation pathway of muscle protein synthesis. It is an indispensable amino acid, so humans are dependent on dietary sources like meat, dairy, eggs, cereals and grains for its intake. Experimental diets rich in leucine increase muscle mass (Farnsworth et al., 2003) and increase REE associated with increased UCP-3 expression in skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) (Zhang et al., 2007). This also increases satiety (Westerterp-Plantenga, Lemmens & Westerterp, 2012), reduces food intake (Ropelle & Dias, 2008) and may stimulate energy (McAllan, Cotter, Roche H. M., Korpela R. & Nilaweera, 2013) and lipid (Zhang, 2007) metabolism. Mechanisms involved are both central (hypothalamic activity) and peripheral involving the gastro-intestinal tract, adipose tissue, liver and muscle (McAllan et al., 2013). There is now strong evidence for leucine or its ketoacid to influence insulin secretion (Newsholme, 2006). It appears therefore that increased leucine during catabolic situations may have benefits for improving insulin sensitivity, cardiovascular risk markers and a reduction in metabolic syndrome status (Macotela, 2011). Emerging data from cellular studies indicate a role for leucine on mitochondrial function. Using muscle and adipose tissue cell lines, Sun & Zemel (2009) demonstrated a significant increase in mitochondrial mass and oxygen consumption following the treatment with leucine. It has been opined that feeding leucine at intakes at well above the current recommendations of 3 - 4 g/d or 40-50mg/kg/d for a 75 kg man may have many beneficial body compositional and metabolic effects (Layman et al., 2003; Pencharz, 2012b).

There is evidence associating vitamin D with obesity (Slusher, McAllister & Huang, 2015), insulin sensitivity (Osati et al., 2016), cardiovascular diseases (Baker et al., 2012) and metabolic syndrome (MetS) (Awad, AlappatL & Valerio, 2012). A formal model for a role in energy balance has also been described (Soares, 2012; Soares et al., 2014). Low serum 25(OH)D status is associated with increased body weight (Zitterman et al., 2014), BMI (Parikh et al., 2012), waist circumference (McGill et al., 2008), and total body fat mass (Kraemer et al., 2012). High 25OHD may contribute to low visceral (Rosenblum et al., 2012) subcutaneous adipose tissue (Caron-Jobin et al., 2011) and improved insulin sensitivity (Salehpour, Hosseinpanah et al., 2012). However, improving vitamin D in the absence of caloric restriction alone does not trigger weight loss (Pannu, Calton & Soares, 2016; Pathak et al., 2014) but possibly has a combined effect with dietary calcium during trials of weight loss where it reduced the visceral adipose tissue (Rosenblum et al., 2012; Zhu et al., 2013). One consequence of weight and fat loss is a small but significant increase in 25OHD even in the absence of vitamin D supplementation (Pannu, Zhao et al., 2016). However, whether such changes have biological effects is not clear. Interestingly, recent evidence suggests that the combination of increasing vitamin D status and leucine supplementation might improve appendicular mass and thus muscle strength in older adults with sarcopenia (Bauer et al., 2015).

The primary aim of this study was to examine the effect of leucine during weight loss on body composition and fasting metabolic markers, and to investigate the potential mechanisms in energy metabolism. We also explored, as a secondary outcome, whether incidental changes in vitamin D status during weight loss could negate or enhance any alteration in body composition and whole-body metabolism. We hypothesized that increased intake of leucine would assist in achieving a beneficial body composition while improving insulin sensitivity, thus reducing features of MetS. It may also impart better metabolic flexibility post-glucose. Vitamin D status during this period could contribute to the final outcome.

7.3 Methods and Materials

7.3.1 Study Design

Two arms parallel, double-blind randomized controlled trial of 8 weeks inclusive of 2 weeks run-on period of leucine supplementation (treatment) or placebo during caloric restriction.

7.3.2 Participants Inclusion/Exclusion Criteria

Adult Australian men and women with abdominal obesity and at least one other criteria for MetS as judged by recent consensus guidelines (Alberti et al., 2009), aged 20-65 years were included. All participants were of European origin, with no history of MI, stroke, T1DM, weight loss in previous 6 months, absence of thyroid disease (by history), polycystic ovarian syndrome (by history), non-pregnant, non-lactating, not on hormonal contraception or testosterone replacement therapy or hormonal replacement therapy. Type 2 diabetics on good glucose control as judged by HbA1c <6.5 % were included. Anyone on medication that affects body composition, energy expenditure or food intake were excluded. Those on calcium or vitamin D supplements were asked to stop taking them two weeks prior to the start of the program until the end of the 8 weeks. Medications like lipid-lowering therapy, metformin and hypertension were quantified and monitored.

7.3.3 Randomization & Blinding

The subjects were assigned to two groups using random allocation software (Saghaei, 2004). The subjects and the main investigator (KP) were blinded to treatment allocation.

7.3.4 Capsules Preparation and Manufacturing

The capsules of leucine and placebo were identical in shape and colour though a marginal difference in mean weight ((267 mg/capsule) of placebo was necessary to fill each capsule of the same size as those with the leucine). Both placebo and leucine capsules were prepared and manufactured by an external compounding pharmacy (Pharmacy 777, Applecross, Perth, WA), who were unaware of the treatment allocation. All capsules were coded and allocated by a third investigator who was neither involved in data collection nor in analysis. Codes were broken only after all measurements were completed and data was ready for analysis.

7.3.5 Study Diets

All participants were prescribed individualized diets. Energy requirements were assessed at baseline by measuring resting metabolic rate through indirect calorimetry and using an activity factor of 1.5 for men and 1.3 for women. Subjects were then prescribed 75% of their estimated energy requirements. Dietary composition was based on NHMRC dietary guidelines except for dairy products, which were restricted to 1 serve per day, and meat intake that was prescribed at no more than 3 serves/week for men, and 2 serves/ week for women. Our two-week rotational menu ranged from 1000 to 2800 Kcal/d to cover the needs of the population group under study. All diets/menus were prepared by the candidate. Calorie-calculated recipes with standard measurements of ingredients were provided to all participants to maintain a good control of portion sizes. Both groups received a calorie-restricted diet, whereas the placebo group received lactose supplements as capsules and the experimental group received control diet plus leucine (3 g/d). Both groups were required to ingest 6 capsules/day; 2 each with breakfast, lunch and with dinner. The leucine content in the daily diet was maintained at 3g/d. Compliance was judged as 85% capsule intake (judged by self-maintained records and capsule return) and >5% weight loss at the end of each month of the trial.

7.3.6 Methods/Procedures Used

The following measurements were performed in the environmental chamber set at 25 degrees, 40% humidity following the standard operating procedures. All measurements were made before and after 8 weeks of the intervention.

- Body composition from dual energy X-ray absorptiometry (DEXA; Prodigy, Lunar Corporation), BIA (In Body 3.0, multi frequency Bioimpedance Analysis) and anthropometry.
- Basal metabolic rate (BMR) – Indirect calorimetry (Deltatrac II) at rest and sequentially following OGTT.
- Oral glucose tolerance test OGTT (75 g glucose load).
- In house designed FFQ for dietary intake compliance and short version of IPAQ (physical activity questionnaire) were filled prior to and during intervention.
- Compliance to leucine supplementation was assessed using self-reported checklist forms, pill counts and changes in body weight on face to face visits every fortnight.
- Fasting blood for: LDL, HDL, TAGs, glucose, insulin, liver function tests.

7.3.7 Anthropometry and Blood Chemistry

Weight, waist circumference and body composition were measured using standardized and calibrated equipment pre-supplementation, at each fortnightly visit and post-supplementation. Height was measured once at the beginning. DXA measurements for regional adiposity were done at pre- and post- stages. Fasting and post-prandial venous blood was collected pre- and post-experiment for clinical chemistry. Integrated area under the curve (IAUC) was calculated using the trapezoid rule. Vitamin D status [25(OHD] was determined using the chemiluminescent immunoassay method (Liaison, DiaSorin and Architect, Abbott). Details are mentioned in Methods - Chapter 3 of this thesis.

7.3.8 Ethics Approval

This study has been approved by the Curtin University Human Research Ethics Committee (HR 108/2013). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616001528448).

7.3.9 Statistics

7.3.9 a Sample Size Calculations

Sample size was calculated using the software GPower version 3.1.9.2 (Faul et al., 2007). Based on an ANOVA for repeated measures (2 time points), with treatment effect (2 diets) and interaction, to detect a small effect size of 0.25, $\alpha = 0.05$, power = 80% and 0.5 correlation between measures, we needed a sample of N= 34, i.e. 17 per diet group.

7.3.9 b Statistical Analysis

All collected data was entered and analysed by SPSS version 21 (SPSS Inc., Chicago, IL, USA). Descriptive statistics was obtained for variables of interest. Multivariate linear regression (two-way ANOVA) was used to compare the mean values of the outcome variables before and after the 8-week intervention for both groups. An independent samples t-test was performed to explore between-group variation at baseline and at 8 weeks, respectively. To investigate the effect of leucine supplementation (treatment) on body composition and fasting metabolic parameters, an analysis of covariance (ANCOVA) was used to compare the

group difference, controlling for the baseline values. In addition, the influence of vitamin D status and the possible interactive effect between treatment and vitamin D status on body composition and risk markers of metabolic syndrome were examined by a two-way ANCOVA with an adjustment of the baseline values. Various equations of insulin sensitivity using fasting and post-prandial measures of glucose and insulin, BMI or age for calculations were tested to rule out any small possibility of overlooking any effect of leucine on insulin sensitivity. All blood parameters were then adjusted for change in fat mass and fat free mass during the weight loss period. A p-value of less than 0.05 was accepted as being statistically significant. Initial analyses reported followed an intention to treat (ITT) for the missing data. Further similar analyses were also conducted for compliers to check if that made any difference (see appendix for latter results, Table A1-3). Participants consuming at least 85% of pills and demonstrating > 5% weight loss were considered to be compliant.

7.3.10 Intervention Progression

The whole study was completed in two phases with n=18 in phase 1 and n=19 in phase 2. Participants were screened and those who met inclusion criteria and agreed to participate were required to come for an orientation visit. At that visit they were shown the laboratory, equipment to be used, parking facilities and planned diets and capsule intake was explained. All interested participants were then given consent forms to sign with the information sheet. We also advised them of the meal for the previous night prior to baseline measurements which was similar to our planned dinner to be consistent with the post-supplementation measurements. The participants then arrived for baseline measurements on another day after following all prerequisites for RMR measurements.

On the appointment day, they were measured for anthropometry, body composition, energy expenditure and blood variables in approximately 5 hours, following which they were served a light buffet meal. Energy requirements were prescribed to them based on 75% of their estimated energy expenditure. They were also asked to fill in diet records and physical activity questionnaires. Capsules sufficient for four weeks, two weeks rotation menu and recipe cards were provided to all participants. They were then required to visit the lab each

fortnight with their capsule container and capsule intake fill-in sheets provided beforehand to check for capsule compliance. The dietary intervention was carried out by the candidate (KP) who was blinded to treatment allocation. Body composition (BIA, Inbody 3.0) was measured every 2 weeks to determine diet compliance. Any participants demonstrating poor compliance in first two weeks underwent dietary counselling to ascertain reasons for poor compliance. All participants were requested not to change their physical activity pattern during the eight weeks of supplementation. A new container of capsules was provided at the end of 4 weeks. Regular phone calls were made to check for compliance and resolve any diet-related issues. At 8 weeks, post-supplementation, all measurements, diet and physical activity questionnaires were repeated as at the baseline. All participants returned their capsule containers at the end.

Fortnightly Appointments

Each participant was called every two weeks for repeating anthropometric measurements, body composition using (BIA, Inbody 3.0) and checked for capsule and diet compliance. Any issues related to food or capsule compliance were discussed and sorted out.

Equipment used, procedure followed and protocol of the measurement day are described in Methods - Chapter 3.

Calculations of insulin resistance index used is provided in Literature Review - Chapter 2.

7.4 Results

7.4.1 Subject Characteristics

Total of thirty seven subjects (25 females, 12 males) completed the study (Figure 7.1). Mean ± SD of their ages was 48.8 ± 14.69 years, 25(OH)D status 68.64 ± 19.20 nmol/L, total body weight 94.4 ± 21.8 kg and fat percent as 43.10 ± 8.69 g. Eight out of nineteen from the placebo group and eight out of eighteen from the leucine group increased their vitamin D status during weight loss, by +9.5 mmol/L (95% CI, 3.7-15.3 mmol/L) and +9.6mmol/L (95% CI, 3.8-15.4 mmol/L) respectively, while the remainder decreased their vitamin D levels. There were no significant differences in either demographic or biomedical parameters between the two groups of the study (Table 7.1) at baseline.

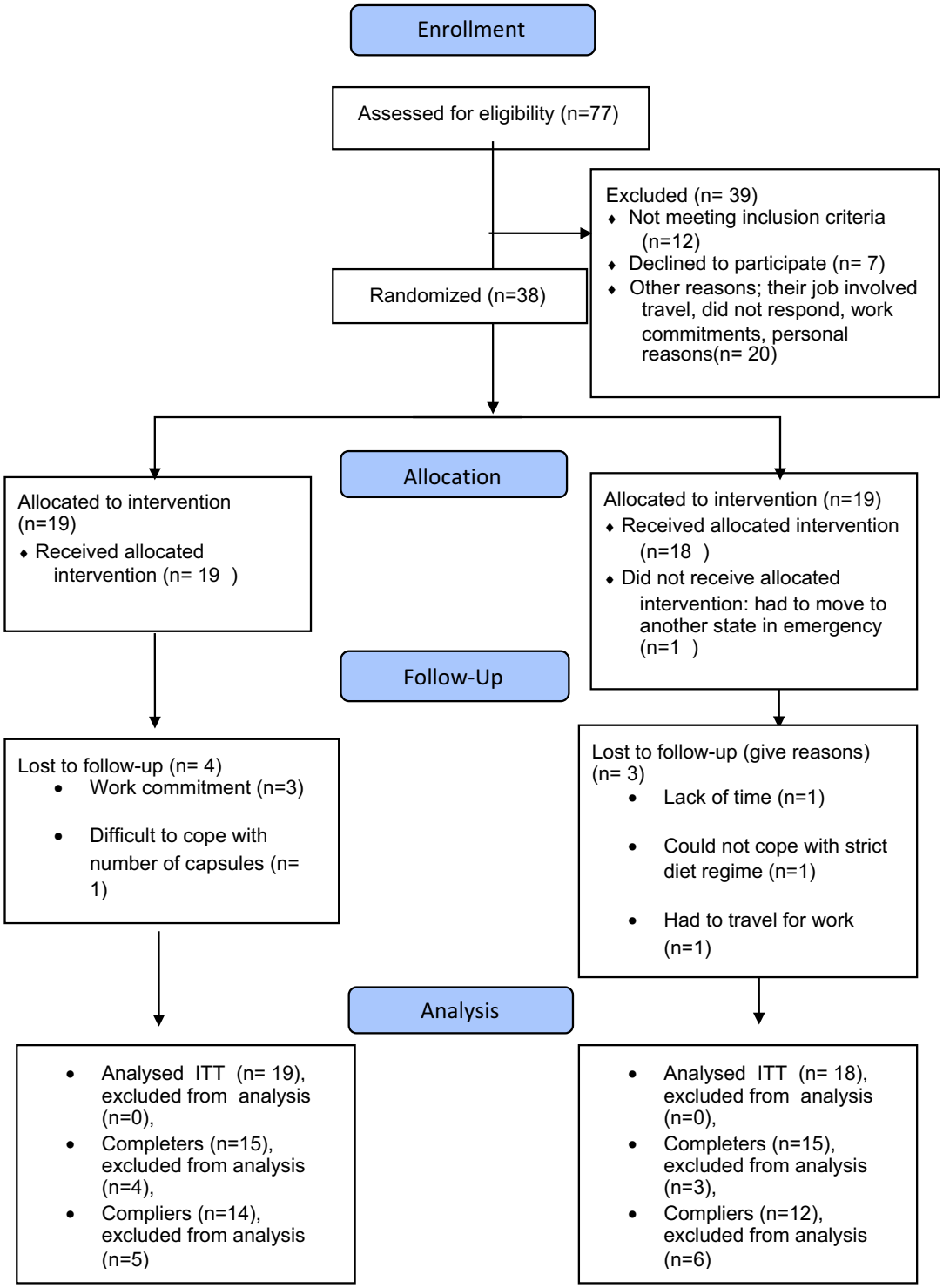

Figure 7.1 CONSORT Flow Diagram - Recruitment, allocation and intervention.

Table 7.1 Baseline characteristics defining the groups before supplementation.

Variable	Placebo (n=19)	Leucine (n=18)	Independent t-test
Age, y	47 ± 14.6	51 ± 14.8	NS
Gender (M/F)	7M, 12F	3M, 15F	
25 (OH) D, mmol/L	69.5 ± 19.52	67.8 ± 19.39	NS
ISI, Stumvoll	0.08 ± 0.03	0.09 ± 0.02	NS
FGF21, pg/ml	162.14 ± 151.52	168.31 ± 220.20	NS
Calorie deficit kJ/d	-2387 ± 616.1	-2336 ± 144.3	NS

M, male; F, female; AG Ratio, android-gynoid ratio; ISI, insulin sensitivity index, (Stumvoll et al., 2000); FGF21, fibroblast factor 21.

7.4.2 Leucine Supplementation and Body Composition

Weight loss was significant, as expected, but to similar extents in both groups. However, there were no significant differences for post-adjusted pre-values between the groups (Table 7.2).

Table 7.2 Whole-body and regional body composition at baseline and at the end of 8 weeks of caloric restriction with and without L-Leucine supplementation.

	Placebo (n=19)			Leucine (n-18)			ANOVA[a]
Variables	**Base**	**End**	**Change**	**Base**	**End**	**Change**	
Weight, kg	92.5 ±19.42	89.3 ± 0.76	- 4.3 ± 3.73**	94.0 ± 21.43	90.9 ± 0.79	-3.1 ± 2.74**	NS
FM, kg	38.1 ± 11.55	36.9 ± 0.57	- 3.1 ± 2.88**	41.8 ± 11.76	37.1 ± 0.59	- 2.7 ± 2.00**	NS
Fat percent	41.3 ± 9.89	41.0 ± 0.53	- 2.2 ± 2.62**	45.1 ± 6.96	42.2 ± 0.54	- 0.91 ± 1.74**	NS
FFM, kg	54.4 ± 13.00	52.2 ± 0.41	- 1.19 ±1.74*	52.2 ± 13.86	52.6 ± 0.42	- 0.73 ± 1.85*	NS
Lean, kg	51.5 ± 12.55	49.4 ± 0.42	- 1.1 ± 1.77*	49.5 ± 13.41	49.8 ± 0.43	- 0.7 ± 1.92*	NS
ALTM, kg	23.2 ± 5.51	21.9 ± 0.41	- 0.36 ± 1.02	21.0 ± 5.10	22.5 ± 0.43	+0.56 ±.58	NS
AG Ratio	1.10 ± 0.23	1.08 ± 0.022	- 0.04 ± 0.130	1.14 ± 0.20	1.12 ± 0.023	- 0.01 ± 0.08	NS
WC, cm	103.0 ± 14.8	99.3 ± 1.26	- 5.12 ± 6.73**	106.0 ± 15.6	99.5 ± 1.29	- 4.98 ± 3.43**	NS
WH RATIO	0.90 ±0.10	0.89 ± 0.11)	- 0.01± 0.07	0.91 ±0.09	0.89± 0.08	- 0.02 ± 0.04	NS

Data shown as adjusted mean ± SD ; *P=0.05; **P=0.005; paired t-test performed to test within group difference

[a]ANOVA was used to examine treatment effect where all post values were adjusted for pre-values.

FM, fat mass; FFM, fat free mass; ALTM, appendicular lean tissue mass; AG Ratio, android-gynoid ratio; WC, waist circumference; WH Ratio, wait to hip ratio; NS, non-significant

Base, before supplementation; End, after 8 weeks of supplementation.

7.4.3 Leucine Supplementation and Changes in Fasting Metabolic Variables and Blood Parameters

To understand whether leucine ingestion has any potential effect on energy expenditure, glucose or fat oxidation insulin sensitivity, lipid profile and liver function, the changes in all fasting metabolic variables measured in this study between post- and pre-intervention were calculated and compared (Table 7.3).

Table 7.3 **Fasting metabolic variables at baseline and at the end of 8 weeks of caloric restriction with and without L-Leucine supplementation**.

	Placebo (n=19)			Leucine (n = 18)			ANOVA
Variable	**Base**	**End**	**Change (End-Base)**	**Base**	**End**	**Change (End-Base)**	**Between the Two Changes**
RMR, kJ/hr	287 ± 58.07	252 ± 63.62	-34.7 ± 36.7 **	285 ± 58.81	257 ±64.81	-27.74± 37.66 *	NS
RQ	0.81 ± 0.040	0.82 ±0.050	+0.01 ± 0.04	0.81 ±0.04	0.80 ± 0.08	- 0.01 ± 3.87	NS
FFG, °C	0.34 ± 1.98	1.11 ± 2.23	+0.77 ± 1.99	0.74 ±2.40	0.82 ± 3.18	+0.08 ±3.87	NS
			BLOOD PROFILE				
FGF21, pg/ml	162.14±151.52	99.35 ± 96.95	-62.79 ± 105.72 *	168.30 ± 220.20	132.25 ± 187.19	-36.05 ± 76.39*	NS
25(OH)D (nmol/L)	69.5 ± 19.52	70.1± 23.91	+0.65 ± 11.33	67.8± 19.39	68.84± 17.68	+1.06 ± 10.76	NS
			LIPIDS				
Total cholesterol (mmol/L)	5.6 ± 0.10	5.0 ± 1.00	- 0.6 ± 0.69**	5.5 ± 0.86	5.2 ± 1.12	- 0.2 ± 0.83	NS
HDL(mmol/L)	1.3 ± 0.340	1.2 ± 0.30	- 0.1 ± 0.16*	1.3 ± 0.36	1.2 ±0.33	- 0.1 ± 0.19	NS
TAG (mmol/L)	1.8 ± 1.05	1.6 ± 1.01	- 0.24 ± 0.52	1.4 ± 0.60	1.3 ±0.79	- 0.13 ± 0.42	NS
			LIVER FUNCTION TESTS				

Alkaline Phosphatase (U/L)	69.8 ± 20.11	66.8 ± 21.52	- 3.1 ± 7.63	66.6 ± 0.79	56.9 ± 19.76	- 9.7 ± 22.01	NS
Albumin (g/L)	41.1 ± 2.68	41.4 ± 2.71	+0.3 ± 1.73	41.2 ± 2.10	40.6 ± 1.95	- 0.6 ± 1.54	NS
ALT (U/L)	37.4 ± 24.00	29.2 ± 17.61	- 8.2 ± 12.61*	39.2 ± 22.26	34.9 ± 18.01	- 4.28 ± 17.06	NS
GGT (U/L)	36.5 ± 47.48	31.0± 46.96	- 5.5 ± 6.25**	45.8 ± 51.00	42.1± 59.11	- 3.7± 22.30	NS
Total Protein (g/L)	70.5 ± 4.40	69.5± 4.58	- 1.1 ± 3.49	71.3 ± 3.29	68.6± 3.84	- 2.7 ± 3.55*	NS
			INSULIN SENSITIVITY INDEXES				
HOMAIR	2.29± 1.542	2.00 ± 1.736	- 0.29 ± 0.665	2.06 ± 1.253	1.88 ± 1.196	- 0.18 ± 0.630	NS
Stumvoll[1]	0.08± 0.03	0.09 ± 0.03	-0.004± 0.011	0.09 ± 0.02	0.09 ± 0.02	-0.001 ± 0.015	NS
Stumvoll[2]	0.09 ± 0.02	0.09± 0.02	-0.004 ± 0.002	0.09 ± 0.02	0.09 ± 0.02	+0.001 ± 0.001	NS
McAuleys	6.93 ± 1.753	7.96 ± 2.67	+1.03 ± 1.69*	7.45 ± 1.455	8.02± 1.81	+0.58 ± 0.92	NS

Data shown as adjusted mean ± SD

*P=0.05

**P=0.005; paired t-test performed to test within group difference.

[a]ANOVA was used to examine treatment effect where all post values were adjusted for pre-values.

RMR, resting metabolic rate; RQ, respiratory quotient; FFG, forearm fingertip gradient; ISI, insulin sensitivity index; FGF21, fibroblast growth factor; HDL, high density lipoprotein; TAG, triglycerides; ALT, alanine amino transferase ; GGT, Gamma-glutamyl transpeptidase; ISI, insulin sensitivity index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; Stumvoll[1] equation using fasting values of insulin and glucose; Stumvoll[2] using equation with demographics as BMI and age; NS, not significant; Base, before supplementation; End, after 8 weeks of supplementation.

Table 7.3 shows that both groups displayed similar fasting energy expenditure, substrate oxidation and temperature gradient at baseline. The resting energy expenditure reduced significantly from baseline in both groups: placebo group (-34.7 ± 36.7, $p<0.001$) as compared to leucine group (-27.74± 37.66, $p<0.05$). There was a slight trend seen for vasodilation with weight loss among the placebo group although not significant. Both groups also had comparable fasting blood values of interest. There was a significant drop in fasting FGF21 in both groups ($p<0.05$) with weight loss. However, no significant differences emerged between the two treatment groups in all the parameters measured (Table 7.3).

We also investigated further to ascertain whether leucine makes a difference if we only carried out a compliers analysis (appendix Tables A1 and A2). Interestingly, it did not alter the conclusions based on intention to treat (ITT) analysis. No difference was detected in body composition, insulin sensitivity, most cardiovascular markers and liver function, other than liver enzyme. However, ALT was improved on placebo arm in compliers (P= 0.047) and LDL decreased with increase in vitamin D levels.

7.4.4 Post Hoc Analysis: Potential Effects of Change in Vitamin D Status During Weight Loss on Energy Metabolism, Body Composition and Cardio-metabolic Markers

Significant alterations in body composition and no evidence of leucine-related change suggested the possibility of some other confounding factor/s other than mere caloric restriction to be present and assisting weight and fat loss during the trial. In my previous published work (Pathak, Soares et al., 2017), I concluded a strong relationship between vitamin D status and obesity and proposed its role in post-prandial metabolism (Pathak, Soares et al., 2017). I was therefore interested in examining the potential role of vitamin D status with the consideration of an interaction between the original intervention. All data was controlled for the baseline values (Table 7.4). A 'vitamin D increase group' is described as those who elevated their serum 25 OHD levels from baseline and 'vitamin D decrease' group had a fall in their serum levels for 25 OHD compared to baseline. We found that those who were able to increase vitamin D status during the trial showed significantly lower fat

mass compared to their counterparts (p=0.035, Table 7.4, Figure 7.2), irrespective of the treatment received. Similarly, increased appendicular lean mass was found to be significantly associated with the increase in vitamin D status achieved (p=0.025), regardless of the treatment setting (Figure 7.3).

Table 7.4 Incidental effect of a change in vitamin D status on body composition following 8 weeks of Leucine supplementation on a calorie-restricted diet.

	Placebo (n= 19)		Leucine (n=18)		2 x 2 ANCOVA*		
Variable	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Only Effect**	**Vitamin D Only Effect**	**Treatment x Vitamin D Status Interaction**
Weight (kg)	87.36(0.88)	85.63(0.86)	88.24(0.88)	85.63(0.86)	NS	NS	NS
FM(kg)	37.6(0.63)	35.8(0.73)	38.0(0.68)	36.2(0.70)	NS	P = 0.035	NS
FFM(kg)	52.1(0.49)	52.3 (0.54)	52.5 (0.50)	52.7 (0.55)	NS	NS	NS
Lean Mass (kg)	49.3 (0.50)	49.6 (0.55)	49.7(0.51)	50.0 (0.56)	NS	NS	NS
ALTM(kg)	21.4 (0.46)	22.6 (0.50)	21.9 (0.47)	23.2 (0.51)	NS	P = 0.025	NS
WC(Tippetts, #455)	99.5 (1.49)	99.1(1.70)	99.7 (1.57)	99.3 (9.66)	NS	NS	NS
AG Ratio	1.1 (0.03)	1.1(0.03)	1.1(0.03)	1.1 (0.03)	NS	NS	NS
WH Ratio	0.89 (0.02)	0.90 (0.02)	0.88 (0.02)	0.89 (0.02)	NS	NS	NS

Data presented as mean (SEM). *2 x 2 ANCOVA of values at end of trial adjusted for respective baseline values.

Natural log transformation was applied for weight which was then back-transformed in the table.

FM, fat mass; FFM, fat free mass; ALTM, appendicular lean tissue mass; WC, waist circumference; AG ratio, android-gynoid ratio; WH Ratio, waist to hip ratio; Kg, kilograms

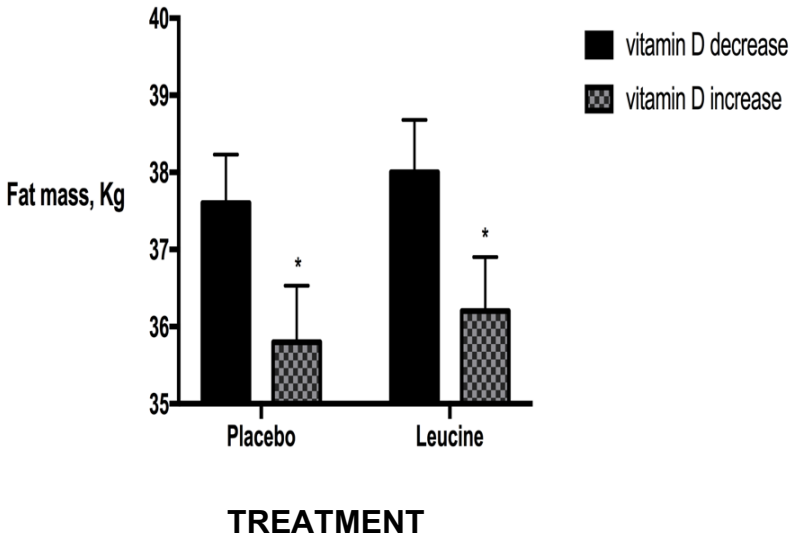

Figure 7.2 Change in fat mass due to treatment and vitamin D.

Legend: * P<0.05 versus vitamin D decrease.

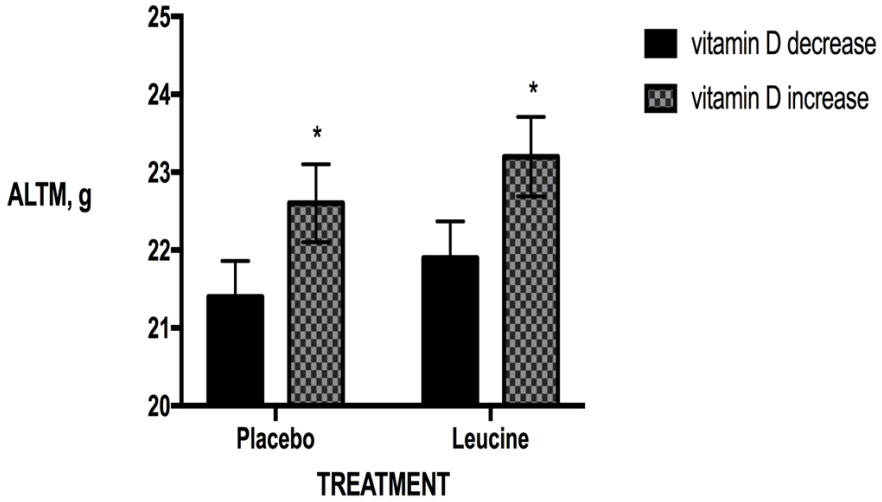

Figure 7.3 Change in ALTM due to treatment and vitamin D.

Legend: * P<0.05 versus vitamin D decrease.

In this weight loss trial, we then tested the main effect of 'treatment' and 'vitamin D status', and the interactive effect of 'treatment' x 'vitamin D status' on all these blood variables adjusting for change in fat mass and fat free mass. Table 7.5 shows that the main effect of 'treatment' and the interactive effect were found not to be associated with all blood variables after adjusting for change in fat mass and fat free mass, while 'vitamin D status' retained to be negatively associated only with HDL(P=0.048) and total protein (P=0.004) (Table 7.5). At the same time, we found no positive association between leucine or vitamin D status with energy metabolism, use of substrate or temperature gradient.

Table 7.5 Incidental effect of a change in vitamin D status on a fasting biomedical and metabolic parameters following 8 weeks of Leucine supplementation during weight loss.

	Placebo (n=19)		Leucine (n=18)		2 x 2 ANCOVA		
Variable	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Only Effect**	**Vitamin D Only Effect**	**Treatment x Vitamin D Status Interaction**
RMR, kJ/hr	252 (9.19)	258 (10.55)	253 (9.66)	259 (10.36)	NS	NS	NS
RQ	0.80 (0.016)	0.84 (0.019)	0.79 (0.017)	0.83 (0.019)	NS	NS	NS
FFG, ° C	1.3 (0.80)	0.9 (0.91)	1.0 (0.82)	0.6 (0.91)	NS	NS	NS
FGF21(pg/ml)	110.06 (19.54)	88.91 (22.46)	139.75 (20.57)	118.60 (22.01)	NS	NS	NS
			LIPIDS				
Total Cholesterol(mmol/L)	5.1 (0.17)	4.75(0.19)	5.4(0.18)	5.0(0.19)	NS	NS	NS
HDL (mmol/L)	1.2 (0.04)	1.1(0.05)	1.3 (0.04)	1.2 (0.05)	NS	P = 0.039	NS
LDL	3.6 (0.14)	3.4 (0.16)	3.8 (0.15)	3.6 (0.16)	NS	NS	NS
TAG (mmol/L)	1.5 (0.12)	1.3 (0.14)	1.6 (0.13)	1.3 (0.14)	NS	NS	NS

			LIVER FUNCTION TESTS				
Alkaline Phosphatase (U/L)	65.5(4.14)	67.2 (4.72)	56.7 (1.32)	58.5(4.67)	NS	NS	NS
Albumin (g/L)	41.8 (0.43)	41.0 (0.51)	40.9 (0.46)	40.1 (0.49)	NS	NS	NS
ALT (U/L)	33.2 (3.15)	25.2 (3.68)	37.8 (3.33)	29.8 (3.55)	NS	NS	NS
GGT (U/L)	40.2 (4.29)	31.1 (5.04)	40.4 (4.61)	31.4 (4.83)	NS	NS	NS
Total Protein (g/L)	71.0 (0.82)	68.0 (0.96)	70.0 (0.88)	66.5 (0.93)	NS	P = 0.004	NS
			INSULIN SENSITIVITY INDEXES				
HOMAIR	1.91 (0.180)	1.93 (0.205)	1.95 (0.187)	1.98 (.204)	NS	NS	NS
Stumvoll[1]	0.12 (0.002)	0.12 (0.002)	0.12 (0.002)	0.11 (0.002)	NS	NS	NS
Stumvoll[2]	0.092 (0.001)	0.091 (0.001)	0.091 (0.001)	0.091 (0.001)	NS	NS	NS
McAuleys	8.2 (0.35)	8.1 (0.38)	7.9(0.35)	7.8 (0.40)	NS	NS	NS

Data presented as mean (SEM). 2 X 2 ANCOVA of values at end of trial adjusted for respective baseline values., change in fat mass and change in fat free mass.

RMR, resting metabolic rate; RQ, respiratory quotient; FFG, forearm to fingertip gradient; ISI, insulin sensitivity index; FGF21, fibroblast growth factor; HDL, high density lipoprotein; TAG, triglycerides; ALT, alanine aminotransferase; GGT, Gamma-glutamyl transpeptidase; ISI, insulin sensitivity index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; Stumvoll[1] equation using fasting values of insulin and glucose; Stumvoll[2] using equation with demographics as BMI and age; NS, not significant; SEM, standard error mean.

7.4.5 Post-prandial Metabolism Following Weight Loss with Leucine Supplementation

We have discussed fasting metabolic variables such as RMR, RQ, FGF21 earlier in the chapter and the same variables were studied post-prandially over two hours post 75 g glucose ingestion adjusted for any changes in fat and fat free mass. We were interested to see if vitamin D status was contributing to all these changes in addition to weight loss. We witnessed a greater delta RQ in the leucine group as compared to the one with placebo. Other metabolic variables had no effect of either leucine or vitamin D on post-prandial metabolism (Table 7.6, Figure 7.4).

Table 7.6 Post-prandial measures following 8 weeks of Leucine supplementation during weight loss.

	Placebo (n=19)		Leucine (n=18)		2x2 ANCOVA		
Variables	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Effect**	**Vitamin D Effect**	**Interaction**
iAUC_EE, kJ/2hr	34.7 (10.91)	23.6 (12.37)	25.4 (11.32)	14.2 (12.31)	NS	NS	NS
iAUC_RQ	0.08 (0.016)	0.06 (0.019)	0.12 (0.017)*	0.10 (0.019)*	P= 0.044	NS	NS
GIT%	2.8 (0.87)	1.9 (0.99)	2.0 (0.90)	1.1 (0.98)	NS	NS	NS
iAUC_FFG, ° C	1.47 (0.809)	1.99 (0.902)	0.85 (0.830)	1.37 (0.878)	NS	NS	NS
Change in ISI (Stumwoll)	-15.4 (3.29)	-17.8 (3.77)	-13.94 (3.55)	-16.37 (3.72)	NS	NS	NS
Change in FGF21, pg/ml	101.8 (23.48)	91.8 (27.12)	110.0(24.87)	100.0 (26.43)	NS	NS	NS

Data presented as mean (SEM) , P<0.05 was considered significant

*P<0.05 versus placebo.

2 x 2 ANCOVA adjusted for respective baseline values, change in fat mass and fat free mass during weight loss.

iAUC, integrated area under the curve; TEM, total energy metabolism; RQ, respiratory quotient; GIT, glucose induced thermogenesis; FFG, forearm fingertip gradient; ISI, insulin sensitivity index; FGF21, fibroblast growth factor.

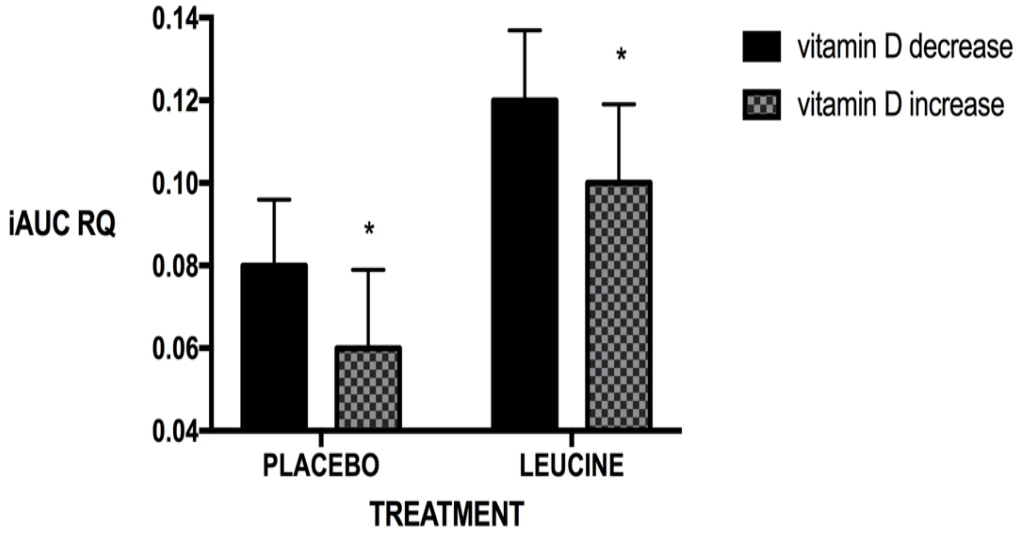

Figure 7.4 Change in post-prandial respiratory quotient due to treatment and vitamin D status.

* P < 0.05 versus vitamin D decrease

7.5 Discussion

We examined the effect of supplementation of leucine in a short-term calorie-restricted diet on body composition and metabolic parameters. Our hypothesis stated that leucine would be primarily responsible for a greater reduction in fat mass over energy restriction per se, retain lean mass, improve insulin sensitivity and related biomarkers of disease. Our results revealed that leucine by itself may not be accountable for improving either body composition or glycemic status more than caloric restriction alone. There have been few animal and human studies that have advocated a role for leucine in reducing fat mass (Donato et al., 2005; Eller et al., 2013) and improving glycemic control (Macotela et al., 2011; Tong et al., 2014). On the other hand, there are several studies that propose leucine supplementation has no effect on body weight, muscle mass or insulin sensitivity (Donato et al., 2005; Leenders et al., 2011; Verreijen et al., 2015). This discrepancy leaves the role of leucine in weight loss and maintenance still in question. In the present study, the only significant effect was an increase in post-prandial RQ following weight loss (Table 7.6). No

negative effects on body composition were observed as seen in animal studies where leucine increased epididymal adipocyte volume in rats on HFD (Torres-Leal et al., 2011) and increased perirenal adipose tissue mass by 45% in another study (Zeanandin et al., 2012).

The overall lack of effect made us rethink the possibility of a potential role of vitamin D status since we know that vitamin status improves the weight/fat loss (Pannu, Zhao et al., 2016). As a secondary analysis, we found that those who were able to increase their vitamin D levels during the trial period favoured a greater reduction in body fat and retained ALTM. As ALTM is mainly skeletal muscle (i.e. minus some skin and subcutaneous fat) (Kim, Wang, Heymsfield, Baumgartner & Gallagher, 2002), such outcomes indicate a greater loss of fat mass and preservation of muscle due to small incidental increases in 25OHD. We are unaware of similar outcomes in the literature on leucine trials, and overall this could indicate a potential confounding that has not been examined.

The only major finding of this study was for post-prandial substrate utilization adjusted for any changes due to weight loss. We observed a significantly higher RQ on the leucine arm. An increase in carbohydrate oxidation following oral glucose is indicative of an improved 'metabolic switch' activated by leucine during weight loss. Although animal studies (Freudenberg et al., 2012; Zhang et al., 2011) indicated that leucine supplementation increased energy expenditure and fasting fat oxidation, to our knowledge this has yet to be confirmed in humans, and hence is still an assumption. A change in fuel oxidation between fat and carbohydrate does not always have to result in an increased energy expenditure since the moles of ATP obtained from each substrate are roughly equal (Geisler, 2011). Accordingly, we noted a higher RQ but no difference in post-glucose metabolic rate or glucose-induced thermogenesis. The latter values, in fact, were close to the theoretical cost of glucose oxidation of 2-4% (Flatt, 1995) which varies depending whether glucose is directly oxidized, or initially stored as glycogen and then mobilized for oxidation. In this regard, we had monitored changes in FGF21 since it is implicated as a key protein that controls glucose and lipid metabolism (Bobbert et al., 2013; Novotny et al., 2014). There was a significant drop in fasting FGF21 for both groups post weight loss, which is the reverse of that observed with overfeeding (Heilbronn et al., 2013). However, we could not detect any difference between the arms of this trial. This may suggest that FGF21 either acts through an indirect pathway

not measured here or that changes in FGF21 are consequent to alterations in weight and fat loss, with no intrinsic link with leucine supplementation.

Weight loss has previously been shown to reduce liver weight and enzymes and improve liver function (Gasteyger, Larsen, Vercruysse & Astrup, 2008). Another significant observation in our study was that increased vitamin D status adjusted for FM and FFM lost was linked to diminished total protein, although it remained within the optimal limits (60-83 g/L). Low vitamin D levels are generally associated with poor liver function (Fisher et al., 2012; Kasapoglu et al., 2013). Our analysis on compliers where we also observed a reduction in serum ALT with an increase in vitamin D (Appendix Table A5). Most studies associating leucine and lipids did not find any link with HDL (Fu et al., 2015; Torres-Leal et al., 2011) but we did find a drop in HDL concentrations significantly with no effect on any other component of lipid profile. Firstly, both of these studies were on mice, and secondly, both were fed with high fat diets. In contrast, our diet was limited in fat content on a calorie-restricted diet. Clearly this is an area for further study. It is also relevant to note that HDL may not be a good marker of pro-atherogenic chylomicron remnants (apoB48) in the metabolic syndrome (Couillard et al., 2000; Irawati et al., 2016).

7.6 Limitations and Future Recommendations

Due to lack of funding, our sample size was perhaps not sufficiently large enough to detect differences in insulin sensitivity and cardiovascular markers. We used OGTT to measure insulin sensitivity which is less sensitive to short-term change when compared to clamp techniques. The availability of muscle biopsies could help investigate cellular pathways and the role of leucine in skeletal muscle metabolism. A dose response trial would also have been helpful and the possible combined effects of leucine with other nutrients such as calcium, vitamin D or pyridoxine are potential options for further studies in the future.

7.7 Conclusions

From our findings and analyses, we conclude that leucine had no demonstrable added effect on modifying body composition, improving insulin resistance, lipid profiles or liver function. The only significant effect of leucine we could detect was a greater switch from fat oxidation to glucose oxidation following weight loss. This metabolic flexibility may hint at improved insulin sensitivity not captured by the surrogate markers we used. We made the unexpected observation that increases in vitamin D status during weight loss resulted in retention of appendicular lean mass and greater loss of fat.

Chapter 8 Final Conclusions

This thesis focussed on a better understanding of human energy metabolism in overweight and obese Australian adults with or without metabolic syndrome. This was accomplished through a three-way approach: (a) the response in adaptive thermogenesis to mild cold temperature, (b) the role of vitamin D status in energy balance, weight regulation and glucose-induced thermogenesis, and (c) the effects of supplementation of a branched-chain amino acid, L-Leucine during weight loss with the incidental role of vitamin D status.

The main objectives of the study were:

1. To examine whether an acute cold exposure could stimulate adaptive thermogenesis and alter basal metabolism in obesity.

2. To explore the possibility of mediating the effect of two metabolic hormones, irisin and FGF21 in human metabolism.

3. To investigate the factors that potentially influence RMR and RQ at ambient temperature within TNZ.

4. To understand the relationship of vitamin D status with obesity.

5. To investigate the influence of vitamin D status on basal and post-prandial energy metabolism in overweight and obese adults.

6. To examine the added benefit of leucine on weight loss and body composition.

7. To understand whether leucine and or vitamin D modulated fasting and post-prandial glucose metabolism.

8.1 Overview of Three Target Areas Covered in the Thesis

We have contributed some novel and significant findings to the literature that are described as follows:

8.1.1 Cold Exposure and Energy Metabolism

To the best of our knowledge, we are the first to propose that the thermoneutral zone for older adults with MetS may need to be reconsidered, since we found significant vasoconstriction and increased RMR at 25°C as compared to 27°C. The latter two temperatures are within the current conventional TNZ (23°C-28°C). We were not able to detect a role for FGF21 in non-shivering thermogenesis as opposed to other studies that have used much lower temperatures and instituted a decrease in temperature over a few hours. However, we did observe a potential role for FGF21 and irisin in enhancing glucose oxidation on exposure to a cold temperature.

These outcomes are published in: Pathak K, Woodman RJ, James AP, Soares MJ. *Fasting and glucose induced thermogenesis in response to three ambient temperatures: a randomized crossover trial in the metabolic syndrome*. European Journal of Clinical Nutrition. 2018. doi:10.1038/s41430-017-0058-x.

In our findings, we also demonstrate that skin surface temperature gradients made a significant contribution to RMR and RQ measured at 25°C for Australian adults. This implies that measurements of FFG must always be included while measuring RMR and RQ to negate the possibility of incorrect assumptions in energy metabolism studies.

These outcomes are published in: Pathak K, Calton EK, Soares MJ, Zhao Y, James, AP, Keane K, Newsholme P. *Forearm to fingertip skin temperature gradients in the thermoneutral zone were significantly related to resting metabolic rate: potential implications for nutrition research.* European Journal of Clinical Nutrition. 2017. 71: 1074-1079; advance online publication, April 5, 2017; doi: 10.1038/ejcn.2017.30.

8.1.2 Vitamin D Status and Energy Metabolism

Vitamin D supplementation in the absence of caloric restriction does not influence adiposity status. This systematic review, meta-analysis and meta-regression analysis found no significant effect on fat mass, fat percent or lean body mass with increasing vitamin D status

in the absence of caloric restriction post vitamin D supplementation. The proportion of females in RCT studies and the age range of participants were potential confounders of the relationship between 25(OH)D and adiposity.

These outcomes have been published in: Pathak K, Soares MJ, Calton EK, Zhao Y, Hallett J. *Vitamin D supplementation and body weight status: A systematic review and meta-analysis of randomized controlled trials*. Obesity Reviews. 2014. 15(6):528-37 doi:10.1111/obr.12162.

We found that vitamin D status determined substrate oxidation and insulin sensitivity. Higher 25OHD status favoured a lower fasting RQ and improved the metabolic switch following oral glucose. This was accompanied by increased insulin sensitivity based on a surrogate marker. Surprisingly, FGF21, a potential key regulator of energy and glucose metabolism, did not play any role in this improved metabolism observed with increased levels of 25(OH)D.

These outcomes are published in: Pathak K, Soares MJ, Zhao Yun, James AP Sherriff JS & Newsholme P. *Postprandial changes in glucose oxidation and insulin sensitivity in the metabolic syndrome: influence of fibroblast growth factor 21 and vitamin D status*. Nutrition. 2017. 37:37-42. doi: 10.1016/j.nut.2016.12.007.

8.1.3 Leucine Supplementation, Weight Loss, Vitamin D Status and Energy Metabolism

Leucine supplementation had no added effect on body composition or improving insulin resistance, lipid profile and liver function on a calorie-restricted diet. All the positive changes that were observed were in fact due to incidental increases in vitamin D status. However, fat percentage did show an interactive effect suggesting that a lower fat percentage can be obtained by increases in vitamin D status in those assigned to the placebo arm. Such data may imply that leucine and vitamin D have similar pathways of action on adiposity and body composition, particularly skeletal muscle mass, and that once quenched by either there was no additional effect. We acknowledge the short duration of the trial and that the observed changes may well be transient in nature.

8.2 Limitations of the Study

The scope of methodologies available to support these studies was limited to that available in the institute as competitive research funding was not obtained and constrained by PhD allocations and minor internal grants. With no access to technologies like PET scans we could not ascertain the involvement of BAT in our cold and leucine trials, which would have been a great advantage. Secondly, additional time points of blood collection post-glucose would have given us a better picture of insulin sensitivity, as insulin concentrations change within 30 min of glucose responses/elevations and are more sensitive indicators for ß-cell function. The leucine trial could have benefited from a larger sample, though in our defence, significant treatment effects and interaction were detected.

8.3 Future Recommendations

Section A

- Total body energy expenditure can be studied where whole-room calorimeters are available and tissue biopsies can ascertain changes at tissue/cellular level in the same subjects for a clearer understanding of metabolic pathways and mitochondrial function.
- For studies on cold-induced thermogenesis, use of PET scan or thermal cameras is always an advantage, as they can demonstrate the presence and activation of BAT to support the results.
- More studies in future can confirm the range of TNZ in the older adults with the metabolic syndrome. This has implications for thermal physiology as well the practical process of measuring RMR which is used worldwide in determining the energy requirements of population groups and patients requiring enteral/parenteral nutrition support.

- FFG monitoring must be considered while conducting any metabolic studies to minimize the misinterpretation of BMR/RMR.

Section B

- Long-term trials designed to achieve a high vitamin D status (possibly >80 nmol/L) testing of both weight loss and weight regain are needed to causally relate vitamin D status and adiposity among the Australian population. This is of importance since overweight and older adults have reduced vitamin D levels and are also at high risk of developing metabolic syndrome, T2DM.

- Vitamin D supplementation studies should be complemented with direct objectives to understand the mechanisms involved that explain the causal relationship/effect of vitamin D status.

- It has been opined earlier that change in vitamin D status might be well associated with baseline and change in PTH concentrations, so this needs consideration while planning intervention studies with vitamin D.

- Larger diverse patient groups are always an advantage to randomized clinical trials which will achieve good statistical power and allow some translation of outcomes to culturally mixed populations.

Section C

- More studies are needed to compare the effect of leucine on the maintenance and improvements of muscle strength in an ageing population. Also, to ascertain whether its action varies with type and amount of fat and protein included in the diet.

- Future studies should also investigate the role of leucine in endothelial and cardiovascular functions.

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APPENDICES

Appendix A Supplementary Tables to Chapter 7

Table A1: Potential effect of vitamin D status in altering body composition following 8 weeks of Leucine supplementation for compliers

Table A2: Potential role of vitamin D status on adjusted fasting metabolic parameters following 8 weeks of Leucine supplementation for compliers

Table A3: Adjusted post-prandial effects of acute OGTT following 8 weeks of Leucine supplementation for compliers

Table A4: Potential role of vitamin D status in altering unadjusted fasting metabolic blood parameters following 8 weeks of Leucine supplementation

Table A5: Comparison of unadjusted post-prandial change of metabolic variables after OGTT at start and end of weight loss.

Table A.1 Potential effect of vitamin D status in altering body composition following 8 weeks of Leucine supplementation for compliers.

	Placebo (n= 26)		Leucine (n=26)		2x2 ANCOVA*		
Variable	**Vitamin D Decrease (n=14)**	**Vitamin D Increase (n=12)**	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=15)**	**Treatment Only Effect**	**Vitamin D Only Effect**	**Treatment x Vitamin D Status Interaction**
Weight(kg)					NS	NS	NS
FM(g)	32.6 (0.81)	32.74 (0.75)	33.2 (0.89)	33.30 (0.75)	NS	NS	NS
FFM(g)	51.0 (0.56)	51.5 (0.50)	50.9 (0.58)	51.5 (0.53)	NS	NS	NS
Lean Mass (g)	48.2 (0.57)	48.9 (0.51)	48.0 (0.60)	48.7 (0.55)	NS	NS	NS
ALTM(g)	21.2 (0.42)	22.4 (0.37)	21.7 (0.43)	22.9 (0.40)	NS	P = 0.015	NS
WC(cm)	95.0 (2.00)	97.6 (1.82)	95.4 (2.14)	98.0 (1.88)	NS	NS	NS
AG Ratio	1.1 (0.04)	1.1(0.04)	1.2 (0.04)	1.1 (0.04)	NS	NS	NS
WH Ratio	0.88(0.024)	0.90 (0.021)	0.88(0.025)	0.90 (0.022)	NS	NS	NS

Data presented as mean (SEM).

*2 x 2 ANCOVA on post-values adjusting for pre-values. Natural log transformation was applied for weight which was then back-transformed in the table.

FM, fat mass; FFM, fat free mass; ALTM, appendicular lean tissue mass; WC, waist circumference; AG ratio, android-gynoid ratio; WH Ratio, waist to hip ratio; KG, kilograms.

Table A.2 Potential role of vitamin D status on adjusted fasting metabolic parameters following 8 weeks of Leucine supplementation for compliers.

	Placebo (n=19)		Leucine (n=18)		2x2 ANCOVA		
Variable	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Only Effect**	**Vitamin D Only Effect**	**Treatment x Vitamin D Status Interaction**
RMR, kJ/hr	228 (12.3)	238 (11.0)	238 (12.8)	248 (11.7)	NS	NS	NS
RQ	0.80 (0.025)	0.85 (0.022)	0.79 (0.026)	0.83 (0.024)	NS	NS	NS
FFG, ° C	1.9 (1.24)	0.8 (1.06)	1.9 (1.26)	0.8 (1.16)	NS	NS	NS
FGF21(pg/ml)	88.7 (23.3)	78.1 (20.61)	99.4 (24.11)	88.8 (22.20)	NS	NS	NS
Total Cholesterol (mmol/L)	5.2 (0.21)	4.8 (0.18)	5.3 (0.22)	4.9 (0.20)	NS	NS	NS
HDL (mmol/L)	1.3 (0.06)	1.2 (0.05)	1.3 (0.06)	1.2 (0.06)	NS	NS	NS
LDL (mmol/L)	3.6 (0.16)	3.4 (0.14)	3.7 (0.16)	3.5 (0.15)	NS	0.05	NS
TAG (mmol/L)	1.6 (0.17)	1.3 (0.14)	1.4 (0.16)	1.1 (0.16)	NS	NS	NS
Alkaline Phosphatase (U/L)	64.8 (6.25)	64.5 (5.50)	52.42 (6.46)	52.1 (5.88)	NS	NS	NS
Albumin (g/L)	41.9 (0.65)	40.9 (0.60)	41.2 (0.70)	40.2 (0.62)	NS	NS	NS

	Placebo (n=19)		Leucine (n=18)		2x2 ANCOVA		
ALT (U/L)	29.7 (3.95)	22.7 (3.61)	40.1 (4.24)	33.09 (3.75)	NS	P=0.033	NS
GGT (U/L)	38.9 (6.7)	29.2 (6.52)	37.3 (7.71)	27.55 (6.40)	NS	NS	NS
Total Protein (g/L)	70.9 (1.18)	67.28 (1.11)	69.5 (1.32)	65.8 (1.11)	NS	P = 0.019	NS
HOMAIR	1.6 (0.27)	1.6 (0.23)	1.6 (0.27)	1.7 (0.26)	NS	NS	NS
Stumvoll[1]	0.10 (0.001)	0.10 (0.001)	0.09 (0.001)	0.09 (0.001)	NS	NS	NS
McAuleys	8.8 (0.57)	8.7 (0.48)	8.5 (0.56)	8.3 (0.53)	NS	NS	NS

Data presented as mean (SEM).

2 X 2 ANCOVA on post-values adjusted for pre-values, change in fat mass and change in fat free mass.

RMR, resting metabolic rate; RQ, respiratory quotient; FFG, forearm to fingertip gradient; ISI, insulin sensitivity index; FGF21, fibroblast growth factor; HDL, high density lipoprotein; TAG, triglycerides; ALT, alanine aminotransferase; GGT, Gamma-glutamyl transpeptidase; ISI, insulin sensitivity index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; Stumvoll[1] using equation with demographics as BMI and age; NS, not significant; SEM, standard error mean.

Table A.3 Adjusted post-prandial effects of acute OGTT following 8 weeks of Leucine supplementation for compliers.

	Placebo (n=19)		Leucine (n=18)		2x2 ANCOVA		
Variables	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Effect**	**Vitamin D Effect**	**Interaction**
iAUC_TEM, kJ/2hr	46 (12.6)	33 911.2)	19 (13.1)	6.5 (11.86)	NS	NS	NS
iAUC_RQ	0.06 (0.024)	0.04 (0.021)	0.11(0.025)	0.09(0.022)	NS	NS	NS
GIT	3.7 (1.00)	2.7 (0.89)	1.5 (1.04)	0.52 (0.95)	NS	NS	NS
iAUC_FFG, ° C	1.6 (1.35)	1.7 (1.15)	1.3 (1.37)	1.4 (1.20)	NS	NS	NS
$ISI_{(Stumwoll)}$	0.116 (0.002)	0.115 (0.002)	0.115 (0.002)	0.114(0.002)	NS	NS	NS
Change in FGF21, pg/ml	80.3 (20.33)	77.4 (18.20)	92.9 (21.31)	90.0 (19.25)	NS	NS	NS

Data presented as mean (SEM), P<0.05 was considered significant.

*P<0.05 versus placebo.

Multiple linear regression 2 x 2 ANCOVA to detect treatment (leucine) effect and change in vitamin D status with post weight loss values adjusted for pre-intervention values. All variables were also adjusted for change in fat mass and fat free mass during weight loss.

iAUC, integrated area under the curve; TEM, total energy metabolism; RQ, respiratory quotient; GIT, glucose induced thermogenesis; FFG, forearm fingertip gradient; ISI, insulin sensitivity index; FGF21, fibroblast growth factor.

Table A.4 Potential role of vitamin D status in altering unadjusted fasting metabolic blood parameters following 8 weeks of Leucine supplementation.

	Placebo (n= 19)		Leucine (n-18)		*2x2 ANCOVA		
Variable	**Vitamin D Decrease (n=11)**	**Vitamin D Increase (n=8)**	**Vitamin D Decrease (n=10)**	**Vitamin D Increase (n=8)**	**Treatment Only Effect**	**Vitamin D Only Effect**	**Treatment x Vitamin D Status Interaction**
FGF21 (pg/ml)	116.9 (19.20)	80.0 (21.46)	146.6 (19.91)	109.6 (21.41)	NS	NS	NS
Total Cholesterol (mmol/L)	5.2 (0.18)	4.6 (0.20)	5.6 (0.19)	4.9 (0.20)	NS	P = 0.006	NS
HDL (mmol/L)	1.2 (0.04)	1.1(0.04)	1.3 (0.04)	1.2 (0.04)	NS	P = 0.010	NS
LDL (mmol/L)	3.7 (0.16)	3.2 (0.17)	4.0 (0.16)	3.5 (0.17)	NS	NS	NS
TAG (mmol/L)	1.6 (0.12)	1.2 (0.13)	1.6 (0.12)	1.3 (0.14)	NS	P = 0.029	NS
Alkaline Phosphatase (U/L)	67.1(4.28)	63.5(4.72)	59.7(4.38)	56.13 (4.78)	NS	NS	NS
Albumin (g/L)	41.7 (0.42)	41.1 (0.47)	40.8 (0.43)	40.2 (0.46)	NS	NS	NS
ALT (U/L)	32.9 (3.00)	25.2 (3.36)	37.8 (3.12)	30.1 (3.34)	NS	P = 0.048	NS
GGT (U/L)	39.3 (4.30)	30.12 (4.92)	41.4 (4.54)	32.2 (4.81)	NS	NS	NS
Total Protein (g/L)	71.0 (0.81)	67.8 (0.92)	69.8 (0.85)	66.6 (0.90)	NS	P = 0.004	NS
QUICKI	0.42 (0.098)	0.17 (0.107)	0.50 (0.097)	0.25 (0.112)	NS	P = 0.065	NS
HOMAIR	1.95 (0.185)	1.81(0.204)	2.05 (0.187)	1.92 (0.209)	NS	NS	NS
Stumvoll[1]	0.116 (0.002)	0.116 (0.003)	0.115 (0.003)	0.114 (0.003)	NS	NS	NS
Stumvoll[2]	0.09 (0.001)	0.09 (0.001)	0.09 (0.001)	0.09 (0.001)	NS	NS	NS
McAuleys	8.1 (0.40)	8.4 (0.43)	7.6 (0.39)	7.9 (0.45)	NS	NS	NS

Data presented as mean (SEM). *2 X 2 ANCOVA on post-values adjusted for pre-values.

HDL, high density lipoprotein; TAG, triglycerides; ALT, alanine aminotransferase; GGT, Gamma-glutamyl transpeptidase; ISI, insulin sensitivity index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; Stumvoll[1] equation using fasting values of insulin and glucose; Stumvoll[2] using equation with demographics as BMI and age; NS, not significant; SEM, standard error mean.

Table A.1 Comparison of unadjusted post-prandial change of metabolic variables after OGTT at start and end of weight loss.

	Placebo (n =19)			Leucine (n=18)			Independent Samples T-Test Between the Two Changes
Variables	Pre	Post	Difference (Post-Pre)	Pre	Post	Difference (Post-Pre)	
TEM	29.54 ± 47.36	28.17 ± 47.88	-1.36 ± 51.22	40.8 ± 32.0	22.5 ± 32.8	-18.30 ± 34.14*	NS
GIT (%)	2.35 ± 3.77	2.25 ± 3.81	-0.11 ±4.08	3.25 ± 2.55	1.79 ± 2.61	-1.46 ± 2.72*	NS
RQ	0.10 ± 0.07	0.07 ± 0.07	-0.03 ± 0.06	0.08 ±0.05	0.11 ± 0.07	+0.03 ± 0.07	NS
FFG, °C	1.49 ± 2.60	1.64 ± 11.33	+0.15 ±3.43	2.58 ±3.57	1.14 ± 3.17	-1.44 ± 4.23	NS
ISI, $_{Stumvoll}$	0.08 ± 0.03	0.09 ± 0.03	-0.003 ± 0.015	0.09 ± 0.02	0.09 ± 0.02	-.0.001 ± 0.011	NS
FGF21, pg/ml	104.69 ± 114.14	96.56 ± 79.62	-8.13 ± 95.21	115.95 ± 110.34	106.68 ± 88.20	-9.28± 56.82	NS

Data are iAUC following OGTT or 2 hr post-prandial value minus fasting for blood parameters. All data as mean ± SD.

*represents significance within treatment on paired t-test

iAUC, integrated area under the curve; OGTT, oral glucose tolerance test; TEM, total postprandial change in energy metabolism; GIT, glucose induced thermogenesis; RQ, respiratory quotient; FFG, forearm fingertip gradient; FGF21, fibroblast growth factor.

- = decrease from baseline; + = increase from baseline.

Appendix B Tools Used for Data Collection

1. Consent form – A (used for temperature study)

2. Consent form – B (used for supplementation study)

3. IPAQ (International Physical Activity Questionnaire) - short version form

4. Capsule consumption record

5. Two-day sample diet plan

6. Sample recipe

7. In-house prepared food compliance form

Energy metabolism in obesity: effect of mild cold

Informed Consent Form- A

I, _____ residing at

_____

Hereby consent to be a volunteer for the above-mentioned study. I understand that as part of the study, I will allow myself to be screened for suitability as a volunteer.

I have read the *Information to volunteers form* and, have had the opportunity to ask questions regarding the research.

I confirm the following (please put a tick mark on the box):

☐ At this moment, I am not pregnant.

☐ I have not had an X-ray or CT scan in the last 3 months

☐ I have not had more than the average life time exposure to X rays.

I consent to the following (please put a tick mark on the box):

☐ To be fasting for at least 12 hours prior to the appointment time and to undergo the glucose tolerance test.

☐ To allow 3 Venous blood withdrawals (18 ml) for assessment of various components: fasting, 30 min, 1 and 2 hours after taking the glucose solution.

☐ To rest for 4 hours in temperature controlled chambers at 20 °C and 25° C on two occasions.

☐ To have my body composition measured using the DEXA machine. I understand that this method involves a very small dose of X-rays, and I consent to this procedure.

☐ To have my blood vessel flexibility measured using the non-invasive Pulse Trace machine (PT-2000).

☐ To complete a Food Frequency Questionnaire and Three day food Diary prior to the trial day and to note my physical activity pattern in a diary for 3 non-consecutive days.

☐ To complete the Physical activity and 3 day food diary questionnaires during the trial.

I fully understand all of the potential risks of these procedures as they have been explained to me. I also understand that my participation is purely voluntary and signing this form DOES NOT prevent me from withdrawing from the study at any time. I have been assured that confidentiality will be maintained at all stages throughout the study.

_____

Signature: _____ Date: _____

Witness statement:

I, the undersigned, certify to the best of my knowledge that the participant signing this informed consent form had the study fully explained in a language understood by him / her and clearly understands the nature, risks and benefits of his / her participation in the study

Witness: _____ Date: _____

Signature: _____

Energy metabolism post Leucine supplementation: a randomised controlled trial

Informed Consent Form- B

Mar 2014

I, _____residing at

_____

Hereby consent to be a volunteer for the above-mentioned study. I understand that as part of the study, I will allow myself to be screened for suitability as a volunteer.
I have read the *Information to volunteers form* and, have had the opportunity to ask questions regarding the research.

I confirm the following (please put a tick mark on the box):

☐ At this moment, I am not pregnant.

☐ I have not had an X-ray or CT scan in the last 3 months

☐ I have not had more than the average life time exposure to X rays.

☐ I am not on any calcium or vitamin D supplements.

I consent to the following (please put a tick mark on the box):

☐ To be fasting for at least 12 hours prior to the monitoring of the testing of glucose tolerance.

To rest for 4-5 hrs in temperature controlled chambers at 25°C during the measurements.

☐ To allow venous blood collections at 2 time point as follows: fasting (13 ml), and 2 hours after (3 ml) ingesting the glucose solution at each visit.

☐ To allow finger prick for measuring blood glucose and lipids in a situation of difficulty with venous blood.

☐ To allow measurement for Basal Metabolic Rate (BMR) on six occasions (30 min each time) during the study.

☐ To allow placement of iButtons to measure skin temperatures on forearm and fingertip of middle finger.

☐ To allow use of infra-red Camera, a non-invasive device, for measuring hot and cold spots in the neck region during the study.

☐ To have my body composition measured using the BIA and DEXA machine. I understand that DEXA involves a very small dose of X-rays, and I consent to this procedure.

☐ To have my blood vessel flexibility measured using the non-invasive

☐ To record my dietary intake as advised by the research team.

☐ To complete a Food Frequency Questionnaire and Physical activity Questionnaire (IPAQ) prior to the study

☐ To complete the VAS questionnaires to assess cold tolerance during the trial.

I fully understand all of the potential risks of these procedures as they have been explained to me. I also understand that my participation is purely voluntary and signing this form DOES NOT prevent me from withdrawing from the study at any time. I have been assured that confidentiality will be maintained at all stages throughout the study.

Signature: _____ Date: _____

Witness statement:

I, the undersigned, certify to the best of my knowledge that the participant signing this informed consent form had the study fully explained in a language understood by him / her and clearly understands the nature, risks and benefits of his / her participation in the study.

Witness: _____ Date: _____

Signature: _____

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(August 2002)

SHORT, LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of four questionnaires. Long (five activity domains asked independently) and short (four generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back-translation methods available on the IPAQ website. If possible, please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information:

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71. 14-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website. SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐

Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ **days per week**

☐ No moderate physical activities → ***Skip to question 5***

2. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐

No walking ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

	MONDAY Breakfast, lunch, dinner			TUESDAY Breakfast, lunch, dinner			WEDNESDAY Breakfast, lunch, dinner			THURSDAY Breakfast, lunch, dinner			FRIDAY Breakfast, lunch, dinner			SATURDAY Breakfast, lunch, dinner			SUNDAY Breakfast, lunch, dinner		
WEEK1																					
WEEK2																					
WEEK3																					
WEEK4																					
WEEK5																					
WEEK6																					
WEEK7																					
WEEK8																					
WEEK9																					

CAPSULE CONSUMTION RECORD

Instructions: Please place a tick in the first box when you have consumed the 2 capsules at breakfast, a tick in the second box when you have consumed the 2 capsules at lunch time and a tick in the third box when you have consumed the 2 capsules at dinner. If you did not consume the capsules, place a cross in the box. An example has been shown below. The subject consumed all capsules in week 1, except for lunch on Wednesday and breakfast on Sunday.

	MONDAY Breakfast, lunch, dinner			TUESDAY Breakfast, lunch, dinner			WEDNESDAY Breakfast, lunch, dinner			THURSDAY Breakfast, lunch, dinner			FRIDAY Breakfast, lunch, dinner			SATURDAY Breakfast, lunch, dinner			SUNDAY Breakfast, lunch, dinner		
WEEK1	✓	✓	✓	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x	✓	✓
WEEK2																					
WEEK3																					
WEEK4																					
WEEK5																					
WEEK6																					
WEEK7																					
WEEK8																					
WEEK9																					

Two-day Sample Meal Plan – Leucine Supplementation Study

	Day One	Day Two
Breakfast	**1 egg w mushrooms and tomato** **Black tea/coffee**	**Oats (50 g) w milk (50 ml) & cinnamon sprinkle** **Black tea/coffee**
Snack	**Yoghurt (100 g) + 1 Fruit**	**1 c of fruit salad**
Lunch	**Grilled chicken (60g) + rice (50g) & vegetables of your choice**	**Lean steak sandwich w vegetables (60g see recipe)**
Snack	**6-8 pcs mixed nuts**	**1 fruit + yoghurt (100 g)**
Dinner	**Lentil & potato (see recipe) patties + stir fry vegetables**	**Spinach & lentils (see recipe) soup+ roast potato (1 medium)**
Snack	**1 c of fruit salad**	**Diet jelly**

Sample Recipe Card

Spinach & Lentil Soup

Ingredients:

30 g Lentils

¾ c water

½ tsp olive oil

1 medium onion, finely chopped

1 clove garlic

20 English spinach leaves, finely chopped

¼ tsp ground cumin

½ tsp grated lemon rind

½ c vegetable stock

½ c water

Coriander finely chopped to sprinkle

Method:

1. Place the lentils in large pan with water, place the lid and allow to boil till cooked.

2. In a separate pan, heat oil, and add onion & garlic till golden. Add spinach and cook for another 2 minutes.
3. Add the lentils, cumin, lemon rind, stock & water to the pan. Simmer uncovered for at least 15 minutes. Add the coriander & stir through.
4. Serve hot.

P.S: Slow cooker or Pressure cooker can be used as per convenience.

Legend: c, cup; g, grams; tsp, teaspoon; tbsp= tablespoon

Food Compliance Recorded at the End - Supplementation Study

Food item	**Frequency of consumption per day**	**Frequency of consumption per week**	**Approximate serve size each time you consume the food item.** **Specify whether this is raw or cooked**
Red meat (lamb, beef, offal)			
Chicken/turkey			
Seafood (prawns, fish, other)			
Processed meat (ham, salami, sausage, bacon)			
Eggs			
Bread			
Bread rolls			
Rice			
Pasta			
Oats			
Cereal			
Milk			
Cheese			
Yoghurt			
Cream			
Custard			
Ice-cream			
Legumes/lentils/baked beans/peas/beans			
Nuts/seeds			

Appendix C Copyright and Permissions

To Whom It May Concern

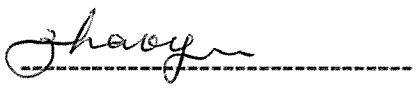
I, **Kaveri Pathak**, independently (conducted the literature search, assembled the initial tables, extracted the data, contacted authors, and co-wrote the manuscript) to the paper/publication entitled (Pathak K, Soares,,MJ, Calton EK, Zhao Y, Hallett J. **Vitamin D supplementation and body weight status: A systematic review and meta-analysis of randomized controlled trials**. Obesity Reviews. 2014, 5(6):528-37. doi:10.1111/obr.12162).

First Author Signature 

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

A/Prof Mario Soares 

Emily Calton 

Dr. Yun Zhao 

Dr Jonathan Hallett 

To Whom It May Concern

I, *Kaveri Pathak* collected data, conducted literature search, and co-wrote the manuscript to the paper/publication entitled **Pathak K**, Woodman RJ, James AP, Soares MJ. **Fasting and glucose induced thermogenesis in response to three ambient temperatures: a randomized crossover trial in the metabolic syndrome.** Eur J Clin Nutr 2017. Accepted Manuscript Number: 2017EJCN0276RR.

Kaveri

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Richard J Woodman

Anthony P James

Mario J Soares

To Whom It May Concern

I, *Kavori Pathak*, collected data, conducted literature search, co-analysed data, and co-wrote the manuscript to the paper/publication entitled **Pathak K**, Calton EK, Soares MJ, Zhao Y, James AP, Keane K and Newsholme P. *Forearm to finger-tip skin temperature gradients in the thermoneutral zone maybe causally related to resting energy metabolism: potential implications for nutrition research.* Eur J Clin Nutr 2017. 71: 1074-1079; advance online publication, April 5, 2017; 10.1038/ejcn.2017.30

Kavori (KAVERI PATHAK)

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Emily Kathleen Calton

Mario J Soares

Yun Zhao

Anthony P James

Kevin Keane

Phillip Newsholme

To Whom It May Concern

I, *Kaveri Pathak*,individually collected data, *conducted literature search*, co-analysed data, and co-wrote the manuscript to the paper/publication entitled. Pathak K, Soares MJ, Zhao Yun, James AP, Sherriff JS & Newsholme P *Postprandial changes in glucose oxidation and insulin sensitivity in metabolic syndrome: Influence of fibroblast growth factor 21and vitamin D status*. Nutrition. 37. 37-42. doi: http://dx.doi.org/10.1016/j.nut.2016.12.007.

(Signature of Candidate)

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Mario J Soares

Yun Zhao

Anthony P James

Jillian J Sherriff

Phillip Newsholme

Title: NON-SHIVERING THERMOGENESIS AND ITS THERMOREGULATORY SIGNIFICANCE

Author: L. JANSKý

Publication: Biological Reviews

Publisher: John Wiley and Sons

Date: Jan 21, 2008

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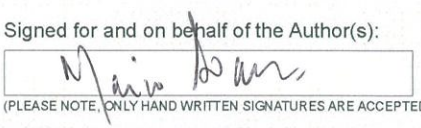
Signed for and on behalf of the Author(s): 

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