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Establishing a non-invasive protocol for estimating brown adipose tissue volume

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*A thesis submitted in partial fulfilment of the requirements for the degree of Master of
Health Science, The University of Auckland, 2017*

ABSTRACT

Background

Brown adipose tissue has emerged as an attractive therapeutic target for anti-obesity strategies. Currently, non-invasive techniques in the assessment of brown adipose tissue are limited, however, the use of infrared thermography imaging has gained recent recognition as a potential method.

Objectives

To 1) optimise a non-invasive, reproducible protocol for the assessment of skin temperature overlying the supraclavicular regions in human participants using infrared thermography imaging, 2) assess the changes in energy expenditure using a similar protocol, and 3) assess the relationship between energy expenditure and supraclavicular skin temperature change.

Study Design

Infrared thermography imaging was used in healthy volunteer participants to develop a protocol involving a cold stimulus for inducing an increase in the skin temperature of the supraclavicular region. The protocol was then optimised by assessing the detection in skin temperature under different conditions. The established protocol (submersion of the hand and forearm in water at 16°C) was used to assess the reproducibility of supraclavicular skin temperature change between repeated measures. A similar cold stimulus (submersion of the feet and calves in water at 16°C) was used to assess the elevation in energy expenditure. The relationship between skin temperature change, resting energy expenditure, and cold-induced elevations in energy expenditure were explored.

Results

We demonstrated increases in the skin temperature of the supraclavicular region using the established protocol. Furthermore, repeated measurements using this protocol yielded reproducible results. Increases in energy expenditure following cold stimulus exposure were also demonstrated. However, we were unable to show a relationship between cold-induced increases in supraclavicular skin temperature and cold-induced increases in energy expenditure or resting energy expenditure at thermoneutrality.

Conclusion

The cold-induced thermogenesis in the supraclavicular region can be consistently quantified with infrared thermography imaging. These findings suggest the potential use of infrared thermography imaging as a non-invasive technique in the assessment of brown adipose tissue function in humans. This technique may also be used in conjunction with the assessment of energy metabolism to further understand the role of brown adipose tissue thermogenesis in human energy balance and its potential exploitation in the prevention and treatment strategies of obesity.

*To my mother, Susan Hsieh, my grandparents, Shih-Nan and Tsai Sai-Hua Hsieh,
and to my partner, Sarah Herbert, for their ongoing love and support.*

ACKNOWLEDGEMENTS

Firstly, I would like to express my special thanks to:

Dr. Troy Merry, my supervisor, for his knowledge and incredible attention to detail in all aspects of the study, as well as his patience with my learning throughout my research journey. I am truly thankful for the opportunity to work on this research project.

Associate Professor Lindsay Plank, my co-supervisor, for his provision of the laboratory facilities and the training to operate the equipment, as well as his willingness to answer questions and share his advice at all times.

Shannon Adams, research assistant, Department of Medical and Health Sciences, for her assistance in the data collection of the study and with general administrative matters, as well as her contributions in the progression of the research project.

Dr Andrea Braakhuis, Department of Nutrition and Dietetics, for her endless support, encouragement, and inspiration. I am sincerely grateful for her invaluable mentorship and guidance through my years studying at The University of Auckland.

I would like to thank the previous and present dietetic teaching staff during my time in the programme, **Clare Wall, Rhodi Bulloch, Julia Sekula, Andrea Braakhuis, Rajshri Roy, and Luke Gemming**, Department of Nutrition and Dietetics, as well as **Lindsay Plank**, Department of Surgery, for enabling me to take part in this course.

I would also like to thank everyone who has guided and supported me through my research year, including my family and friends. In addition, I am grateful for the encouraging environment created by my peers and the assistance with my learning from my classmates at university.

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CHAPTER 1. INTRODUCTION

1.1 Overview

Obesity is characterised by excess body fat and is associated with many health implications such as diabetes, cardiovascular disease, metabolic syndrome, and overall mortality (1, 2). The size of the health condition has reached epidemic proportions globally, with the prevalence of obesity being 1 in 3 adults in countries such as the United States, Mexico, and New Zealand (3, 4). While there is a plethora of research in the field of obesity, the increasing prevalence of the condition illustrates that its multifactorial aetiology is more complex than once thought.

Currently, much of the strategies in the prevention and treatment of obesity resolve around managing energy intake, however these attempts have been futile on the population level. In contrast, strategies involving energy expenditure has been comparatively underutilised. Of the factors that influence energy metabolism, brown adipose tissue (BAT) appears to have a large capacity to contribute to energy expenditure through its thermogenic function (5). Therefore, BAT has emerged as an attractive therapeutic target in the prevention and treatment of obesity. However, there is a lack of understanding for the physiological behaviour of human BAT and its role in regulating energy metabolism. The gold standard of quantifying BAT volume is expensive, invasive, and exposes participants to a dose of radiation (6), while existing non-invasive strategies in the quantification of BAT volume are limited. The use of infrared thermography (IRT) imaging has recently emerged as a promising, non-invasive method (7-11). However, it remains a novel and nascent field with limited protocols and methods of processing. For this reason, the exploration of a protocol and methods to process imaging data should be considered a research priority, thus this study will investigate these considerations of IRT in the detection of human BAT volume.

1.2 The Obesity Epidemic

1.2.1 Defining Obesity

Obesity is a condition relating to one's body composition, characterised by a degree of excess body fat and may result in impaired health (12, 13). The acquisition of body fat is a function of the body's energy homeostasis. When energy intake in the form of food consumption exceeds the energy expenditure, the surplus energy becomes stored in the body primarily in the form of body fat (14, 15). Similarly, energy from adipose tissue is usually liberated first when energy expenditure is not met by energy intake (14, 15).

Body mass index (BMI) is a commonly used method of quantifying obesity in a clinical setting as it is cost-effective and relatively easy to use (16). The World Health Organisation (WHO) defines BMI as a weight to height reference used to classify the nutritional status of an individual (13). These values are based on the morbidity and mortality statistics of Dutch men over 32 years of age, where the lowest risk of mortality were observed between BMI values of 18.5-25kg/m² (4, 17). The weight of an individual in kilograms is divided by the square of their height in metres to calculate BMI (kg/m²), this value can then fall under the following classifications of underweight, normal weight, overweight, or obesity (18).

According to the Centers for Disease Control (CDC) and the WHO, the BMI ranges are <18.5 for underweight, 18.5-24.9 for normal weight, 25-29.9 for overweight, and ≥30 for obesity (18, 19). Moreover, obesity can be further classified into BMI 30-34.9 for class I, 35-39.9 for class II, and ≥40 for class III (19).

Table 1.1 World Health Organisation BMI classification cut offs

BMI Range (kgm²)	Classification
<18.5	Underweight
18.5-24.9	Normal weight
25-29.9	Overweight
30-34.9	Class I obesity
35-39.9	Class II obesity
≥40	Class III obesity

While BMI shows good correlation with more direct measures of body fat, such as bioelectrical impedance and skinfold thickness measurements (20-22), considerable underestimation of obesity and misclassification of adiposity using BMI may still occur (22-24). As BMI is only an index of total body mass, it does not provide much insight on individual differences in body composition. Therefore, a major limitation becomes that it is unable to differentiate lean mass, such as bone and muscle, from fat mass (25). For this reason, a high BMI for an individual with a muscular stature may not accurately represent adiposity and the individual may therefore be misclassified as being overweight (26). There is also a sex difference in measuring adiposity using BMI, with women showing 10% higher body fat than men across all BMI ranges, even in the severely obese (27). However, this finding is expected as men have significantly higher proportions of skeletal muscle mass than women (28). While there are age-specific criteria for BMI in children, older adults share the standard BMI classifications and consequently are considerably underestimated for obesity due to age-related changes in body composition (29, 30).

Due to the variability of body composition between individuals, BMI is merely a surrogate to reflect adiposity. While its accuracy to predict individual variability in adiposity is limited, its validity as a screening tool for the general population is justified as it demonstrates relationship with disease risk, such as for cardiovascular disease, diabetes and its complications; elevated BMI is associated with an increase in risk factors such as cholesterol levels and blood pressure (31-33). Furthermore, BMI is a strong predictor of overall mortality, demonstrating a J-shaped relationship between the variables (1, 2). However, it should be noted that these relationships are calculated from a population level and may not represent individual risk. One of its most significant advantage is the availability of extensive reference data both nationally and globally (34). Other advantages of BMI include its ease of use and accessibility due to height and weight being the only patient information needed to calculate this (16).

For children, weight-for-height reference ranges are based on growth charts such as the WHO Child Growth Standards, with overweight and obesity being 2 and 3 standard deviations above the age-specific median for children under 5, and 1 and 2 standard deviations for children between ages 5-19

(see Table 1.2) (13). The CDC's BMI-for-age growth charts are also used to define overweight and obesity as being above the 85th and 95th percentile respectively (see Table 1.3) (35).

Table 1.2 World Health Organisation weight for height reference range cut off criteria for overweight and obesity in children

WHO weight-for-height reference ranges for children	Overweight	Obesity
Standard Deviations above median (<5 years old)	2	3
Standard Deviations above median (5-19 years old)	1	2

Table 1.3 Centers of Disease Control BMI-for-age growth chart cut off criteria for overweight and obesity in children

CDC BMI-for-age growth charts for children	Overweight	Obesity
BMI-for-age value	>85 th percentile	>95 th percentile

To accurately quantify adiposity and body composition, other methods of more direct measures are can be used. A widely accepted non-invasive method is the dual-energy X-ray absorptiometry (DXA), due to ease of use and low radiation exposure (36). While DXA directly measures the different components of body composition (36), it does not necessarily provide an advantage over BMI in the accuracy of predicting disease risk (37). However, it is a valuable technique to use when the adiposity of individuals are of interest. Its limitations include: a small amount of radiation, equivalent of less than 10% of a chest radiograph; the inability of the machine to accommodate very large persons; and large fluctuations in hydration status are able to hinder its accuracy (36). Despite these limitations, DXA remains a widely used method for the direct quantification of body fat due to its availability, low radiation, good accuracy and reproducibility (36).

1.2.2 Consequences of Obesity

There are many medical consequences of excessive body fat accumulation. Obesity is strongly associated with the incidence of comorbidities such as type II diabetes mellitus (T2DM), cardiovascular diseases, asthma, gallbladder disease, cancers, osteoarthritis, and chronic back pain, as well as metabolic syndrome which is comprised of risk factors such as abdominal obesity, hypertension, dyslipidaemia, and elevated fasting blood glucose (12, 38). It is suggested that obesity further increases the risk of diabetes complications, including cardiovascular, ocular, renal, lower extremity, and cerebrovascular complications (32). Overall mortality is also associated with obesity, including vascular, diabetic, renal, and hepatic mortality (2).

In 2006, the overweight and obesity-attributed health care costs was estimated to be \$686 million New Zealand Dollars (NZD), which along with the lost productivity costs was between \$787-911 million NZD (39). This health care cost in 2006 equated to 4.5% of total healthcare spending in New Zealand (39). To further highlight the trajectory of the increased financial spending on obesity, in 1991, the healthcare

related costs of six obesity-related comorbidities (T2D, coronary heart disease, hypertension, gallstone disease, post-menopausal breast cancer, and colon cancer) in New Zealand was estimated to be only \$135 million NZD (40). Considering that obesity and its related risk factors and comorbidities is largely preventable (41), its significant financial burden and impact on population health illustrates the necessity of targeting the health issue at the stage of prevention rather than treatment. While there is an overwhelming body of research in the field of obesity, its increasing prevalence suggests that its aetiology from a genetics perspective is likely more complex than what is currently known. Therefore, there is a need to further understand the condition in order to better prevent and manage this health issue.

1.2.3 Size and Significance of the Growing Problem

The WHO estimates that in 2014, approximately 39% of the adults were overweight, with a further 13% being obese (13). A much higher prevalence is seen in New Zealand, which currently ranks third place among the OECD for obesity (3). According to the New Zealand's Ministry of Health, 35% of adults in New Zealand are overweight and a further 32% are obese, meaning that two in three adults are at least overweight (42). From these statistics, obesity appears to be an epidemic globally, with New Zealand being more so affected compared to the OECD average.

Since 1980, the prevalence of obesity has increased from 4.8% to 9.8% in men and 7.9% to 13.8% in women, at an average rate of 0.4kg/m² per decade in the last 30 years (43). In New Zealand, obesity seems to be increasing at a faster rate than the global average, where in just the last decade, the prevalence of obesity has increased from 27% to 35% in the adult population and from 8% to 11% in children (42). Based on these data, the trend of the increasing body mass shows an upward rising trajectory of overweight and obesity both globally and nationally for New Zealand.

New Zealand also has the third highest prevalence of childhood overweight and obesity in the OECD, following Greece and Italy (3). According to the New Zealand Health Survey 2015/16, the rate of children who are overweight in New Zealand stands at 32%, meaning that one in three Kiwi children are at least overweight (42). Comparing this rate to the mean of developed countries worldwide, only one in four children are overweight, at 23.8% for boys and 22.6% for girls (34). For childhood obesity alone, the rate in New Zealand is one in nine, while the prevalence of developed countries is one in twelve (34, 42). Moreover, childhood obesity is a major predictor of adulthood obesity; it is suggested that 80 percent of obese children will become obese adults, thereby also predicting the future health outcomes of the population (44).

In addition to the national high prevalence of obesity, there are also large disparities between ethnic groups in New Zealand. The Pacific Island population have the highest prevalence of obesity among any ethnic groups. 67% in adults and 30% in children; 47% in Maori adults and 15% in children (42). In concordance to the medical consequences of disease, Pacific and Maori are disproportionately represented in health outcomes. While there are evidence suggesting that Pacific and Maori populations exhibit differences in body composition, thereby affecting their BMI classifications, this alone may not explain the ethnic disparities observed.

From these data, it is clear that obesity is currently a significant health issue in New Zealand, given its relative burden globally. The trajectory of the increasing obesity rates in adults and children, both globally and in New Zealand, along with the growing ethnic disparities therefore highlights the urgency of the health problem.

1.2.4 Ethnic differences in Body Composition

There is increasing evidence suggesting the consideration of ethnic differences when interpreting BMI. This is due to the variations in body composition between different ethnic groups and that the reference population for BMI classifications are Caucasians (45). When examining these body composition differences between African and Caucasian Americans, African Americans show higher bone mass and skeletal muscle mass than Caucasian Americans after adjusting for age, sex, height, and weight (46-48). This increased ratio of fat-free mass to fat mass at a given BMI would therefore result in a greater number of African Americans to be misclassified as obese. A nation-wide cross-sectional study in the US also found that the relationship between increasing BMI and mortality to be weaker in African Americans than Caucasian American, especially in women, indicating BMI as a screening tool that should be implemented with caution across different ethnic groups (49). This is also seen in Pacific Islanders and Maori in New Zealand, where the same body fat percentage at BMI of 30kg/m² in Europeans equates to 31kg/m² for Maori and 34kg/m² for Pacific (50). While these differences in body composition appear likely to contribute to the overrepresentation of Maori and Pacific populations in New Zealand's obesity rates, it is unlikely for this to completely account for such disparities, suggesting other aetiological factors at play.

In contrast, Asian populations show higher percentages of body fat than Caucasians at a given BMI, resulting in the underestimation of obesity and the misclassification of a healthy body weight (51-56). There is also a difference in the body composition to BMI relationship between the three major Asian ethnic groups, with Asian Indians having the highest body fat percentage to BMI, followed by Malay, then Chinese (57). One study showed that at a "healthy" BMI classification of 24.5kg/m², Asian Indians demonstrate an abnormally high level of body fat percentage at 33% (58). Even between countries, New Zealand, USA, Singapore, and with their respective environmental influences, Asian Indians consistently demonstrate high body fat percentages for low BMI values (50, 56, 58). However, it is suggested that there are also differences within the same ethnic group as Singaporean Chinese demonstrate lower bone mass and higher body fat percentage than Beijing Chinese (52, 59). In concordance to the higher body fat percentage, Asian populations also show higher risks of comorbidities such as diabetes, dyslipidaemia, hypertension, and metabolic syndrome, as well as mortality (58, 60, 61).

These ethnic differences in body composition therefore leads to the misclassification of obesity if the standard BMI cut-offs are used. Until cut-offs are reviewed and re-established for these differences, BMI to predict adiposity on an individual level should be used with caution in regards to ethnicity.

1.3 Energy Metabolism

1.3.1 Law of Thermodynamics and Energy Balance

The First Law of Thermodynamics states that total energy is conserved; energy neither created nor destroyed, but rather transformed from one form to another (62). Changes in body weight is therefore underpinned by this principle, through the concept of energy balance. Energy balance describes the rate at which the body's energy storage changes as the net difference between energy intake and energy expenditure (63). The equation of energy balance is as follows:

$$\text{Energy Storage} = \text{Energy Intake} - \text{Energy Expenditure}$$

Applying this principle, to maintain body weight, energy intake must be equal to the energy expended. Any shifts in energy intake or expenditure away from energy balance will result in a change in body weight. In the case of energy imbalance where energy intake is greater than energy expenditure, weight gain occurs as this energy becomes stored in the body, and vice versa with weight loss (14). While this principle appears straightforward in explaining the mechanism of obesity development and weight control, its multifactorial aetiology is far more complex considering the current losing battle with the obesity epidemic as evidenced by the increasing obesity rates.

1.3.2 Energy Expenditure

The three components of total energy expenditure (TEE) are the basal metabolic rate (BMR), thermic effect of food (TEF), and physical activity (64). Physical activity accounts for both exercise and non-exercise activity, as well as any spontaneous activity such as fidgeting (65, 66). BMR is the largest contributor to TEE, followed by physical activity, then TEF (64). Although, as energy expenditure from physical activity is highly variable depending on the individual, the proportional contribution of these three components will have high inter-individual variability. Moreover, TEF is the energy expended in the processing of consumed food with the thermic effect dependent on the macronutrient composition, although this variation is insignificant as TEF accounts for only ~10% of TEE (66).

Basal Metabolic Rate and Resting Energy Expenditure

BMR is defined as the amount of energy required to sustain vital physiological functions, it is essentially the bare minimum energy cost of survival (67). As energy expenditure is influenced by many various stimuli, BMR must be measured under strict conditions to warrant its accuracy (67). These conditions are elaborated on below. Due to the difficulty of implementing these conditions in a practical sense, the measurement of resting energy expenditure/resting metabolic rate (REE/RMR) which entails a more flexible criterion is often used. RMR is defined as the energy expended at rest when the individual is in an awake, postabsorptive, thermoneutral state without having exercised in the last 12 hours (68). Moreover, as the interaction between the individual and the environment on a daily basis is dynamic, REE may be more indicative of energy expenditure in a free-living setting (69). However, BMR and REE are often used as interchangeable terms in research and in the clinical setting. As both BMR and REE represent the same component of energy expenditure, they will be used and discussed synonymously under the term "REE" for the purpose of this review.

Measuring Basal Metabolic Rate versus Resting Energy Expenditure

The measurement of BMR requires to be implemented under strict conditions. These conditions are: 1) the subject must be lying down, fully awake, yet completely relaxed before and during the measurement, 2) the subject must be at least 10-12 hours fasted prior to the measurement, 3) the environment must be thermo-neutral between the ranges of 22-26°C to minimise any thermogenic responses, and 4) the subject must not be under emotional stress and be familiar with measurement equipment (67). Whereas, the recommended conditions for measuring REE include: 1) a minimum of five hours abstinence after a meal, two hours for alcohol or nicotine, four hours for caffeine, 2) two hours after moderate or 14 hours after vigorous exercise, 3) rested and relaxed for 10-20 minutes prior to measuring, and 4) a thermo-neutral room temperature (69).

Factors Influencing Resting Energy Expenditure

REE is the largest contributor to TEE, accounting for at least 50-70% in free-living individuals (64, 70) and is determined by biological factors such as age, sex, body composition, hormone status, and ethnicity (71). The primary predictor of REE is fat-free mass, although this relationship is variable across the wide range of fat-free mass in part due to differences in the proportion of metabolically active tissue (72, 73). Fat-free mass may be accountable for up to 81% of the variance in the TEE of sedentary individuals, thus highlighting its contribution to energy expenditure (65). Fat-free mass can be further categorised into compartments namely: skeletal muscle, bone, and residual mass which refers to vital organs such as the brain, liver, heart, and others (74). However, fat-free mass is not metabolically homogenous at rest, with residual mass being the most active at 226kJ/kg, followed by skeletal muscle at 54kJ/kg, then bone at 10kJ/kg (75). Furthermore, it is proposed that the small portion of adipose tissue that is devoid of fat also has a slight metabolic activity at 19kJ/kg (75). While it is arguable that the variance of REE seen between other determinants such as age or sex, is a result of the underlying differences in fat-free mass, these factors also act independently of body composition changes. For example, after adjusting for body composition, there is a decline in REE with increasing age from adulthood (76, 77). There are also sex differences independent of body composition, with males demonstrating higher REE than females across different age groups (78).

The finding that REE decreases with increasing age is concordant with the age-related decreases in BAT found in humans (79). BAT is a thermogenic organ primarily observed in the cervical supraclavicular (SCV) region which produces heat when activated (7). It is suggested that BAT activity is positively correlated with REE under thermoneutral conditions and may contribute total energy expenditure in the energy balance equation (80), although this not conclusive (81, 82). Despite this, the activation of BAT thermogenesis in response to cold stress shows marked increases REE (80-83), thereby able to significantly contribute to total energy expenditure when activated to maintain body temperature. It is estimated that as little as 40-50g of BAT can contribute towards 20% of an individual's total energy expenditure when maximally stimulated (5).

In addition, thyroid hormone is a key regulator in BMR, where excess levels elevate REE as seen in hyperthyroidism and vice versa in hypothyroidism (84). It is suggested that there may be some

correlation between thyroid hormone and BMI within the normal euthyroid reference ranges (85, 86). In obese individuals, a high conversion rate of thyroid prohormone into its active form is observed, which may suggest a possible regulatory mechanism to attenuate fat accumulation by increasing energy expenditure (87). Similarly, weight loss in the obese appears to decrease and restore the elevated thyroid hormone levels to normal values (88, 89). However, the effects of thyroid hormones on REE in euthyroid individuals are unclear and require further investigation (72, 90-92).

Interestingly, the REE of formerly obese individuals (after weight loss) appears to be on 3-5% lower than body composition matched controls that have never been obese (93). While there may be genetic factors that contribute to this finding, the environmental factor of recent energy imbalance such as adaptive thermogenesis may also account for this (66). It is suggested that under conditions of underfeeding, REE decreases in both obese and lean individuals, with the extent of the reduction being greater in those that are lean (94). It is worth noting that the differences in the reduction in REE may represent the greater energy stores in the obese able to accommodate for greater deficits in energy balance. This adaptive thermogenesis is proposed to be attributable to decreased BAT activity. As BAT is activated with the integration of sympathetic nervous input and the activation of thyroid hormone receptors, the weight-loss related declines in sympathetic nervous system activity and circulating thyroid hormone may result in reduced BAT thermogenesis activity (95).

Relationship between Ethnicity and Resting Energy Expenditure

As discussed earlier on in this review, there is an appreciable difference in body composition of various ethnic backgrounds. Individuals of African descent demonstrate higher bone mineral and skeletal muscle mass than those of European descent at a given height and weight, increasing the proportion of fat-free mass to body mass (46-48). Despite this, a slightly lower REE in those with African ancestry is observed when compared to matched European counterparts, even in children (96). An explanation for this could be the effect of adjusting for height and weight, i.e. the greater skeletal muscle mass results in proportionately less of the other fat-free components by default, especially highly metabolically active residual mass, as seen in one study (74). Due to the lower metabolic activity of skeletal muscle compared with the other fat-free mass (75), the increased proportion of skeletal muscle in FFM results in a lower REE. Similarly, the increased bone density observed in individuals of African descent may also decrease the proportion of highly metabolic fat-free mass for weight-adjusted individuals and contribute to the findings of lower REE. It is also worth noting that perhaps this difference in the proportions of higher metabolically active fat-free mass is greater than the difference in fat-free mass versus fat mass, which is why greater fat-free mass does not necessarily translate to greater REE.

Currently, there is little information on the REE differences between Maori, Pacific Island, and Caucasian groups in New Zealand. In children aged 5-14 years, there appears to be no significant differences in REE between these three ethnic groups (97). On the contrary, it is suggested that young Pacific Island women show lower REE compared with their Caucasian counterparts (98) however, this does not appear to have a significant impact on total energy expenditure (99). Interesting, the correlation between REE and total energy expenditure of the non-obese Caucasian group was higher than that of

the non-obese Pacific group, indicating a higher activity related energy expenditure in the contribution of REE of the Pacific group (99). These findings indicate that to achieve and maintain a non-obese body fat status, Pacific populations in New Zealand may be required to do physical activity to achieve the same total energy expenditure as their Caucasian counterparts.

The reasons for these ethnic differences in REE may be explained by BAT. The plasticity of BAT, as evidenced by seasonal changes in its recruitment (100, 101), may infer that populations who come from a warmer climate have less BAT volume and subsequently a lower REE. Bakker et al. (102) reported that even under the same climate with the plasticity of BAT recruitment, ethnic differences in BAT activity, non-shivering thermogenesis, and REE are observed. In this study, healthy, lean Caucasian and south Asian Dutch nationals were compared. While these groups resided in the same environment, the south Asian population's ancestry were from a warmer climate than the Caucasian population's ancestry. These differences in ancestry may explain the variations in BAT volume and similarly may apply to the Pacific and Maori populations in New Zealand over-represented in obesity.

1.4 Brown Adipose Tissue

The function of BAT is heat production through non-shivering thermogenesis (103). Brown adipocytes are generally smaller than white adipocytes, and contain non-uniform multilocular lipid droplets inside their relatively larger cytoplasm (104). As well as accumulating lipid droplets that serve as substrate for thermogenesis, this thermogenic function is also facilitated by its characteristics of extensive vascularisation and high density of mitochondria (105). The rich vascularisation in the tissue provides additional routes of fuel delivery and heat exchange, while the high mitochondrial density provides the capacity of generating a large amount of heat; these phenotypic characteristics in turn result in the brown appearance of BAT (104).

In contrast, white adipose tissue (WAT) acts as an important energy storage and endocrine organ. The primary form of energy storage is fat, stored within adipocytes of white adipose tissue where there is a large capacity for energy storage (106, 107). Fats in white adipocytes are stored mainly in the form of lipid droplets, comprised of triglycerides and cholesterol esters, and through either *de novo* lipogenesis from glucose or direct uptake of fatty acids from the circulation (106, 108). As well as being an important reservoir for energy storage, WAT is also involved in the regulation of energy homeostasis (105). WAT is a metabolically dynamic, complex network comprised of adipocytes and other cells called the stroma-vascular fraction which include blood cells, endothelial cells, pericytes, and adipocyte precursor cells (104). Its regulatory function is achieved through the adipocytes' production of adipokines, a collective term used to describe the various secreted cytokines, hormones, and peptides (109).

1.4.1 Function and Mechanism of Action

The mitochondria play a large multi-purpose role within the cell, which includes their primary function of ATP regeneration (110). In BAT thermogenesis, the transfer of chemical energy into heat occurs within the mitochondria through the action of uncoupling protein 1 (UCP1), an integral membrane protein specific to BAT (111, 112). In the process of oxidative phosphorylation after substrate oxidation, electrons are transported down the electron transport chain while the protons are pumped across the

inner mitochondrial membrane (113). The uneven distribution of protons establishes an electrochemical gradient, creating a proton-motive force utilised to drive ATP synthesis (113). However, in BAT mitochondria where UCP1 is expressed, UCP1 acts as a channel to divert protons back across the membrane, rendering the inner mitochondrial member “leaky”; substrate oxidation and UCP1 in BAT therefore powers a futile proton cycle where chemical energy is not transferred into work but rather dissipated as heat energy (114). The primarily thermogenic function of BAT through the dissipation of energy as heat indicates its role in energy expenditure rather than energy storage, therefore appearing as a potential target in shifting the energy balance equation away from energy surplus.

1.4.2 Signalling of Thermogenesis

The thermogenic pathway of BAT is controlled by the sympathetic nervous system and the most important factor that signals for its activation is norepinephrine (103). Norepinephrine interacts with all three types of adrenergic receptors in the brown adipocyte which are: α_1 , α_2 , and β , the last of which being the most extensively studied pathways (103). The β -adrenergic receptors can be further subdivided into β_1 , β_2 , and β_3 . Of these receptors, the β_1 and β_3 -receptors are present within the brown adipocytes but not β_2 -receptors (115). Indeed, as β_3 -receptors are found in white and brown adipose tissue, and given the low capacity of thermogenesis in white adipose tissue, the thermogenic response following norepinephrine administration confirms the role of sympathetic nervous system input in BAT-thermogenesis (103). The adrenergic response to norepinephrine subsequently triggers downstream signals involving G_s protein, adenylyl cyclase, cyclic AMP (cAMP), and protein kinase A which phosphorylates the transcription factor, CREB which then initiates the transcription of thermogenic genes (103). One of these expressed genes is a iodothyronine deiodinase which converts thyroid prohormone T4 to thyroid hormone T3 within BAT, thus creating a localised hyperthyroid state; T3 in turn regulates the transcriptional control of UCP1, resulting in thermogenesis (116).

The central regulator of BAT biology is cold stress. The signal of cold stress is sensed by multiple mechanisms, such as thermoreceptors on the skin that regulates the sympathetic outflow to BAT through a neural thermoregulatory network (117). Cold stress also stimulates the alternative activation of BAT macrophages which in turn secretes norepinephrine (118). In addition, other factors that may activate and recruit BAT include exercise and capsaicin analogues, both of which activates the sympathetic nervous system (119, 120). These signals in turn activates thermogenesis through the signal transduction cascade as described above.

1.4.3 Brown Adipose Tissue Life Cycle

Despite sharing common characteristics with WAT, BAT appear to be developmentally closer to the myogenic lineage (121). Fate-mapping experiments show that BAT and skeletal muscle arises from the same common progenitor that expresses Myf5 and Pax7, two genes formerly thought to be exclusively expressed in committed skeletal muscle precursors (122, 123). Indeed, the skeletal myoblasts and brown adipocytes exhibits a bidirectional cell fate controlled by a transcriptional factor in vivo (122). Genetic fate mapping also show brown adipocytes arising from the central dermomyotome, which also gives rise to dermis and muscle (124). Similarly, gene profiles and mitochondrial proteomic signatures

of brown adipocyte precursors show relationships with skeletal myocytes, but not with white adipocyte precursors (125, 126). It is also worthy to note that the differentiation and proliferation of brown pre-adipocytes into mature adipocytes is facilitated by the β adrenergic receptors (127). Prolonged exposure to sympathetic signals may induce this proliferation and differentiation of BAT. This is observed in the cold acclimation of rats where BAT hyperplasia is induced, resulting in an increase in BAT volume and thermogenic capacity (128).

The differentiation process of brown and white adipocytes share a similar transcriptional control cascade, including key transcription factors peroxisome proliferator-activated receptor- γ (PPAR γ) and CCAAT/enhancer binding proteins (C/EBP) (129). PPAR γ is a master regulator of adipogenesis capable of initiating the entire adipogenic programming of non-adipogenic fibroblasts, resulting in cells with much of the mature adipocytes functions (130). While the hypomorphism or deficiency of the PPAR γ gene results in severe lipodystrophy (131). C/EBPs are a family of transcriptional factors, of these C/EBP α is essential for the development of white adipocytes, including the insulin signalling pathway and maintaining PPAR γ expression (132); although it is not involved in brown adipocyte development (133). The other subtypes, C/EBP- β and δ are important in the terminal differentiation of both brown and white adipocytes, allowing for normal lipid accumulation (134). Other important transcriptional factors exclusively involved in brown adipocyte differentiation include: Forkhead box C2, PPAR γ -coactivator-1 α , co-repressor RIP140, retinoblastoma protein, retinoblastoma-like protein, and PRD1-BF-1-RIZ1 homologous domain containing protein-16 (121, 135-140). Indeed, some of these transcriptional factors are capable of inducing BAT differentiation or brown adipocyte-like characteristics from myogenic progenitors and WAT pre-adipocytes (122, 136, 138, 139). The total thermogenic capacity of BAT is dependent on the total number of brown adipocytes and its degree of differentiation, such as its mitochondrial density and UCP1 levels (103). For this reason, further understanding of BAT differentiation and proliferation is essential in the research of BAT as an anti-obesity target.

1.4.4 Physiological Significance to Energy Metabolism

BAT or brown fat is an organ unique to all mammals which has the function of producing non-shivering thermogenesis, allowing for survival under cold stress in evolutionary processes (103). This is seen in mice models where the inactivation of BAT activity results in marked sensitivity to cold, demonstrating the absence of BAT thermogenesis (141). In the breeding of BAT-inactivated mice, failure of maintaining a warm room temperature (27°C) results in the death of the progeny, indicating the significance of BAT in maintaining body temperature under cold stress (142). This thermogenic function of protection against the cold also in turn regulates energy homeostasis and body weight through the generation of heat from energy dissipation (114). Overfeeding in rats results in diet induced thermogenesis that is related to BAT activity, indicating a mechanism for the preservation of energy balance (143). In support of this notion, mice genetically manipulated to have decreased BAT volume show increased susceptibility to diet-induced obesity while wild-type mice with more BAT volume are more resistant (144). This suggests an increase in metabolic efficiency in mice with decreased BAT volume, leading to the subsequent increases in energy surplus that results in body fat gain (144).

On the contrary, other mouse models of more severe UCP1-deficiency showing sensitivity to cold have been reported to maintain body weight and resist the development of diet-induced obesity (141, 145). As UCP1 facilitates thermogenesis through allowing energy to dissipate as heat, deficiency in the expression of this protein should decrease energy expenditure through the lack of BAT thermogenesis. It is suggested that alternative mechanisms of thermogenesis that require more energy expenditure are utilised in order to maintain body heat under cold animal housing conditions (142, 146-148). Once this cold stress is removed, BAT-inactivated mice continue to demonstrate increased susceptibility to obesity (142, 148). These findings suggest that the protective effect of BAT in regulating body weight is unmasked and evident in the absence of cold stress. This has significant implications for the BAT regulation of body weight in modern humans, as we live predominantly in thermoneutrality due to the advancements in clothing and shelter protecting against the outside environment.

As energy intake in these BAT-inactivate mice was not the primary cause for weight gain, the weight changes must be attributed to alterations in energy expenditure to obey the law of thermodynamics. In the absence of cold stress, the REE and physical activity of BAT-inactive mice are no different to wild type mice with functional BAT (148), therefore this increased metabolic efficiency in BAT-inactive can only be attributed to the diet induced thermogenesis component of energy expenditure. Diet-induced thermogenesis was originally thought as simply the metabolic cost of processing the consumed meal, however, there appears to be a true thermogenic component following a meal (103). Post-prandial examinations of BAT show increases of blood flow by twofold (149) and norepinephrine turnover rate by threefold (150), suggesting BAT activity and its sympathetically mediated involvement. While these speculations do not necessarily equate to BAT being responsible for post-prandial thermogenesis, the magnitude of the thermogenic response is associated with the capacity of BAT (103).

Moreover, the macronutrient composition of the diet can also recruit BAT and modulate the response to adrenergic stimulation. Diets that lead to overeating and obesity (high fat, high carbohydrates, low protein) show the recruitment of BAT and the postprandial induction of its activity in mice (151-156), therefore earning the term as “recruiting diets” (103). It is suggested that a long-term high carbohydrate diet may produce greater BAT thermogenic capacity than standard chow diet in mice, through palatability-induced sensory stimulation resulting in enhanced sympathetic activity (153). A degree of postprandial thermogenesis in humans may also be contributed to by sympathetic activity associated with the palatability of food (157). Furthermore, carbohydrate overfeeding in humans results in increased carbohydrate oxidation and total energy expenditure, while fat oxidation and total energy expenditure is unaffected by fat overfeeding (158). This upregulated oxidation of carbohydrates may be a result of the insulin-stimulated glucose uptake in BAT which increases by 5-fold as observed in humans (159). Moreover, greater BAT volume may increase the glucose disposal and consequently improve glucose tolerance, insulin resistance, and obesity (159). Indeed, BAT activity is suggested to be involved in the diet-induced thermogenesis of healthy humans (160). While these findings suggest a possible role of BAT in regulating body weight through postprandial energy metabolism, future research to further understand diet induced BAT thermogenesis in the regulation of body weight is needed.

The findings from these mice studies illustrates the role of BAT in the protection against obesity, however, this relationship in humans is unknown due to the limited research in this field. For this reason, the concept of BAT in human obesity can only be currently maintained from a theoretical perspective. Furthermore, as human BAT has a large capacity to contribute to energy expenditure (5), BAT appears to be an attractive potential therapeutic target for the prevention and treatment of obesity. Therefore, it is important that future research is dedicated to understanding of BAT physiology in humans.

1.4.5 Brown Adipose Tissue in Humans

The relationship between BAT thermogenesis, regulation of body weight and energy balance led to its interest in human subjects, particularly as a therapeutic target to combat obesity (120). BAT in humans is located in the interscapular, neck, axillae regions with depots also seen in renal, suprarenal, para-aortic deposits; in early childhood there is wide-spread distribution of BAT around the body which regresses with age, with those situated in more peripheral areas being the first to regress, such as the interscapular region and the abdominal wall (79). Since the 1960s, BAT in human infants is suggested to contribute significantly to metabolism through non-shivering thermogenesis to maintain core body temperature (161). Similarly, early studies have demonstrated the presence of BAT in adult humans, even in the eighth decade of life (79, 162-164). Although, other studies suggested that there are no BAT in human adults or at least in physiologically significant amounts to affect body weight (165). This belief that BAT was absent in relevant amounts in human adults became widely adopted and resulted in emerging clues indicating the presence of BAT being overlooked for many years (166). This was the case until more recently where a paradigm shift occurred when imaging, immunohistochemistry, gene and protein expression techniques provided evidence to show the presence of significant and functional BAT in adults (167, 168). In addition, magnetic resonance imaging (MRI), IRT and lipid metabolism imaging are a few of the emerging methods that have also been identified for the measurement of BAT in humans.

Furthermore, a study of 1972 patients with a combined number 3640 whole-body combined positron emission tomography (PET) and computed tomography (CT) scans were analysed and provided evidence for functionally active BAT in adult humans (169). These scans, with the combined use of histology and immunohistochemical assays for UCP1, the study identified substantial depots of BAT in the anterior neck to thorax region. It was identified that the detection of BAT activity was higher in women, and the detectability of BAT was inversely correlated with age, outdoor ambient temperature, beta-blocker use, and BMI (169). These findings indicated the potential of BAT having a physiologically significant role in the metabolism of adult humans.

There is evidence suggesting that human BAT volume may be acquired and recruited. This is seen where the detectability of BAT and thermogenesis increased during the winter seasons compared to the summer, indicating cold adaptation (100, 101). Similarly, the presence of BAT activity is detected in a group of outdoor workers in northern Finland that are regularly exposed to cold ambient temperatures, while compared with indoor working controls that show no activity (163). In cold acclimation studies, daily cold exposure was able to induce an increase in BAT thermogenic activity (120, 170, 171). While

BAT can be recruited under cold acclimation, this recruitment can also regress through returning to a thermoneutral environment (172). Pharmacological strategies such as hormonal manipulation have also demonstrated recruitment of BAT functions in isolated human pre-adipocytes (173). Furthermore, subcutaneous WAT may also be recruited to express brown-like thermogenic genes (174). These findings support the notion of cold-adapted recruitment of BAT and provides potential strategies for the recruitment of BAT as a therapeutic target in preventing and treating obesity.

1.4.6 Methods for Measuring Brown Adipose Tissue Volume

Position Emission Tomography and Computed Tomography

The current gold standard for estimating BAT volume is the use of combined PET and CT (PET/CT) imaging of its activity (175). The use of PET/CT to measure BAT arose as an unexpected finding of tracing tumour metastasis with a labelled marker, fluorodeoxyglucose (FDG) used in PET scans (176). The localisation of tumours in nuclear medicine can be detected through examining cellular activity by using these labelled glucose markers; as tumours are generally glycolytic, areas of high glucose uptake may indicate its presence. FDG follows the pathway of general glucose uptake, however it cannot be metabolised and results in a temporary accumulation within the cell. This accumulation of FDG thus allows for the tumour to be identified by PET imaging, along with other tissues with high glucose uptake such as the brain. Symmetrically distributed unexpected areas of high uptake is also detected, although this was initially attributed to muscular uptake as tumours are unlikely to have symmetrical distribution (177). Upon combining CT with PET, the density and composition of tissues that show high FDG uptake could then be visualised. The combined use of PET/CT therefore identified these symmetrical areas of high uptake as tissues with similar characteristics to adipose tissue, leading to the “rediscovery” of functional BAT in human adults (178). To further confirm its identity as BAT, histological examinations and immunostaining for UCP1 using biopsies from the regions of high FDG uptake were also implemented to provide such evidence of BAT presence (100, 169). This provided strong evidence that BAT is present and active in adult humans.

The uptake of glucose in the brown adipocyte is mediated by adrenergic stimulation (179). Due to the thermogenic function of BAT being induced through sympathetic activity, stimulated BAT should result in greater FDG uptake as detected by PET/CT imaging. Therefore, exposure to cold stress should result in the adrenergic activation of BAT and consequently improve the accuracy of the measurement. The effects of cold stress on the imaging of BAT is seen in when subjects are measured under cold conditions compared to thermoneutral conditions. The uptake of FDG in BAT is observed under cold exposure with stimulus temperature of 6-19°C, while this activity was absent under thermoneutral conditions at 22-27°C (80, 100, 180). Although it should be noted that adrenergic stimulation of glucose uptake can also occur in the absence of UCP1 (181), suggesting this uptake may not completely represent the metabolic rate of the tissue but nevertheless indicating that the tissue is metabolically active.

While FDG-PET/CT has demonstrated its accuracy in the quantification of BAT activity, its use in healthy individuals is not warranted for a number of reasons. Firstly, the cost of a single examination has been

estimated to range from up to \$900-2300 New Zealand Dollars (NZD), with the cost of the radiopharmaceuticals being up to \$300-400 alone (182). Secondly, it is an invasive procedure as it requires the FDG to be administered via intravenous injection. Lastly, subjects are exposed to radiation due to mainly the radioactively labelled FDG and a minor part from the CT scan. The radiation emitted by PET/CT tracers has been described to be the most energetic of any radiation that is used in medical diagnostic procedures (6). Together, the mean effective dose from a PET/CT examination is approximately 10mSv, which is three times more than the average yearly natural background radiation exposed to the general population at 3mSv/year (6, 183). While its use remains justified in oncology patients to detect tumours, its implementation in healthy subjects appears extreme, and certainly in the case of identifying BAT which currently has little practical relevance in health.

Magnetic Resonance Imaging

The use of MRI to quantify BAT has gained recognition as an alternative imaging technique to PET/CT due to its elimination of radiation exposure and the need for a cold stimulus (184). Additionally, combined PET/MRI are also becoming a popular alternative to PET/CT as it eliminates the ionisation radiation component of CT (185). MRI measures BAT independently of its metabolic state through its physiological differences to WAT, which is its greater water-fat-ratio, mitochondrial density, and blood vessel density (186). In mice, these differences between BAT and WAT have facilitated in the detection of their contrasting signals through comparing MR properties such as spectral interactions, fat fraction, T1, T2, and T2* relaxation times, triglyceride saturation, and blood perfusion (187). While MRI can indeed measure BAT independently of metabolic state, MR signals such as fat fraction may vary depending on its activity which may require adjusting for to accurately quantify BAT (188-190). The decreased fat fraction signal in active BAT upon cold stimulation is attributed to an increase in water content from the increased perfusion; and a decrease in fat content due to lipid consumption (190). However, the decrease in fat fraction signal are maintained after reheating from the cold exposure, suggesting that lipid consumption, rather than increased perfusion, is the primary contributor to this sustained decrease (189). On the contrary, after the exclusion of vascular structures and thereby minimising the effects of altered perfusion, cold exposure did not significantly affect the fat fraction signal compared to the resting state (184). Similarly, dynamic T2* imaging also show signal fluctuations in response to BAT activity which correlated with those findings from PET/CT (191). While MR signals may vary based on the metabolic state of BAT, accurate imaging at its resting state may proceed with precautions to ensure the absence of BAT stimulation. These findings suggest that MRI is a promising technique for the quantification of BAT. However, MRI requires specialised expensive equipment, specialised training to operate the apparatus, lacks portability, and is expensive to run. Therefore, there is a need to investigate other methods of estimating BAT volume that are still non-invasive, but are low-cost and low-burden.

Infrared Thermography Imaging

As the primary function of BAT is thermogenesis, the magnitude of its activation can be quantified by measuring its temperature change when a stimulus is introduced. In rodents, temperature changes may

be measured by directly inserting thermistors into BAT depots (192), however this is highly invasive and presents with ethical problems for human studies. An alternative method of measuring BAT activity in humans is the use of thermistors on the overlying skin of BAT depots, which has been validated with the FDG-PET/CT imaging of BAT activity (193). As previously reported in PET/CT studies, the neck region of adult humans is a common location of BAT detection due to its substantial FDG uptake, this includes the cervical SCV area and SCV fossae (100, 169, 194). Due to the thermogenic function BAT, it was hypothesised that the temperature of the skin overlying these areas changes in response to BAT stimulation and could therefore be detected by heat-sensing devices (9). The use of IRT has recently gained popularity as a non-invasive method to measure SCV skin temperature changes in humans (7-11). The temperature change findings from IRT imaging has so far been concordant with that of FDG-PET/CT imaging, demonstrating an inverse relationship between BAT activity and age (11, 169) and BMI (10, 100), while being positively correlated with energy expenditure (7, 83, 120). IRT has also been directly validated against the gold standard that is FDG-PET/CT imaging. A study by Jang et al., (8) showed that a temperature change of 0.9°C using IRT imaging conferred positive predictive value of 85% for BAT activity in SCV depots as detected by FDG-PET/CT in a group of 17 individuals, validating the use of IRT as a method of estimating human BAT volume.

The use of IRT imaging requires a BAT to be stimulated as it is the thermogenic function that is measured. These stimuli may include capsinoid ingestion (7), a meal challenge (9), or cold exposure (7-11). The latter of which being heavily favoured due to its ease of use, practicality, and non-invasiveness. Cold stimulus in IRT imaging of BAT can be delivered through different ways, including the exposure of limbs to cold water (7, 10, 11), and manipulation of room temperature through air-conditioning (8). However, as the control of room temperature requires access to an air-conditioning unit, the limb-in-water method of cold exposure may have an advantage over this for accessibility and practicality reasons. The compatibility of IRT imaging to detect BAT with the use this simple limb-in-water stimuli illustrates its practical advantage over other methods of measurement such as FDG-PET/CT imaging or MRI. IRT provides several advantages over FDG-PET/CT scanning, including the lack of radiation exposure, ability to track BAT activity over time, portability, and ease of use (8). Another advantage of IRT over the current gold standard is its time-efficiency, where the it can be done within minutes while the procedure of FDG-PET/CT imaging requires at least 2 hours to perform (8).

One limitation of IRT is the thickness of the subcutaneous adipose tissue that overlies the region of interest, thereby influencing the change of skin temperature (195). However, this confounding factor could perhaps be mitigated by adjusting for the adiposity of the individual. Furthermore, the sensitivity of IRT in BAT detection at a single time point is reduced as a result of the variations in skin thickness impacting heat exchange as well as the lack of control to measure the extent of BAT activity. Therefore, BAT should be quantified through measuring the change in temperature over the course of its stimulation in order to make meaningful comparisons between its inactive and active state.

1.5 Research Rationale

The current statistics of obesity reveals a concerning global health issue that results in the development of many comorbidities. Its upward rising trajectory not only highlights the magnitude of the issue at present but also predicts the health of the future population. The development of obesity abides by the law of thermodynamics through the concept of energy balance, where the rate of change in body weight is a result of the net difference in energy intake and expenditure. While this is true, the aetiology appears far more complex as evidenced by the current increasing prevalence of obesity despite the advancements made in science research. Currently, many of the intervention strategies to combat obesity are nutritionally-focused and orientated around energy intake, while the energy expenditure side of the energy balance equation remains relatively underutilised. Moreover, current strategies revolving around energy intake appears futile in the prevention and treatment of obesity. Therefore, the emergence of the rediscovery in human BAT has provided an attractive anti-obesity target which exploits the largest component of energy expenditure, REE.

In New Zealand, Maori and Pacific populations are disproportionately affected by obesity, indicating differences in energy balance from other ethnic groups. These statistics combined with the potential of BAT activity in manipulating energy expenditure and biological differences in BAT volume, we therefore hypothesise that BAT volumes are lower in Maori and Pacific populations than in New Zealand Europeans. The investigation of these differences in BAT activity will not only contribute to the growing understanding of BAT as an anti-obesity target, but also provide insight on the disproportionately high obesity rates between the most affected ethnic groups, allowing for these disparities to be rectified. As humans exhibit an age-related decline in BAT mass and the greatest extent of BAT activity is seen in children rather than adults, we propose that estimating BAT volume in children optimises the sensitivity of detecting these ethnic differences. However, due to the novelty of human BAT and the methods of quantifying its volume, there is a lack of literature to support an exact protocol to produce an accurate result. To proceed with our study of investigating the relationship between BAT and ethnicity in children and to potentially exploit BAT activation as a means to prevent and treat obesity, we first need to develop and validate a cost-effective, non-invasive method of measuring BAT volume.

1.6 Aim

The aims of our study are:

1. To develop and validate non-invasive protocol for the quantification of BAT volume in human subjects using IRT imaging with an appropriate stimulus
2. To investigate the relationship between BAT and REE in human subjects
3. To investigate the cold-inducible changes in REE that is attributable to non-shivering thermogenesis in human subjects

CHAPTER 2. METHODS

2.1 Study Design

The study was a series of single and multiple participant experimental trials conducted at the University of Auckland Grafton campus and the Body Composition Laboratory of the Department of Surgery at Auckland City Hospital in Auckland, New Zealand. Since the aim of this thesis was to establish a practical, non-invasive protocol for estimating BAT volume, a series of preliminary experiments were conducted to optimise a protocol and this protocol was then tested for reproducibility. The study took place between April 2017 and September 2017. The University of Auckland Human Participants Ethics Committee (UAHPEC) approved the study protocol (reference number 018504).

2.2 Participants

Participants in this study included residents in the Auckland region between the ages of 18 and 32. The average age across participants was 22.37 +/- 2.3 years. There were 11 female participants and 16 male participants. Participants were volunteers recruited via recruitment posters and word of mouth. Consent forms and a health questionnaire were obtained with testing. Any participants presented with a history of metabolic diseases, pertinent beta-blocker medications, or exceeded the age range were excluded from the study. As we wished to investigate the relationship between BAT and energy metabolism, metabolic diseases may lead to confounding results, hence the exclusion criteria. Similarly, beta-blocker use was also excluded as its pertinent use is inversely correlated with BAT detectability (169). There were no other exclusion conditions.

All participants were required to be overnight fasted from midnight prior to the day of the experiment to avoid any resulting changes in energy metabolism and BAT activation resulting from diet-induced thermogenesis (103). During the fasting period, the participants were instructed to abstain from all food, alcohol, caffeine, and drugs from the night before. Water was permitted and encouraged during the fasting period. As BAT metabolism may also be influenced by exercise, participants were restricted from performing any exercise or strenuous physical activity during the fasting period (119).

2.3 Materials and Procedures

2.3.1 Cold stimulus

Previous studies have demonstrated activation of adult human BAT using a cold stimulus, as measured by IRT imaging and indirect calorimetry (7, 8, 11, 80, 83, 120). This indicates the possibility of inducing BAT activity in the clinical research setting. In our study, the immersion of the participants' limbs in a bucket of cold water maintained at 16-18°C, as previously demonstrated (7, 11). This method of delivering cold stimulus was chosen as it was easy to implement and was non-invasive technique. Water temperature was adjusted and maintained using ice, tap water, and a digital thermometer.

2.3.2 Infrared Thermography Imaging

The overarching goal of the IRT imaging was to establish a standard protocol for estimating BAT volume that is both optimal and reproducible. More specifically in regards to the cold stimulus, the aim is to compare the overlying skin temperature of BAT before and after the cold exposure. The use of cold exposure in the IRT estimation of BAT has been previously validated with PET/CT scan by altering ambient room temperature (8).

To optimise the protocol, a series of experiments exploring and comparing different conditions were conducted to determine this. The thermal images were manually taken and saved onto memory card. Moreover, as the outcome variable was temperature change over time and a video would perhaps best reflect this. However, the methods of analysis to process video data were limited. Therefore, single time point images over time was the primary technique of obtaining radiometric data.

Region of interest

The regions of interest were the SCV fossae and cervical SCV regions, as these regions contain BAT depots that show substantial FDG uptake (100, 169, 176, 194) and have previously been used for IRT imaging (7, 10, 11). For the purpose of this manuscript, these regions will collectively be referred to as the SCV region. Participants were therefore required to wear a singlet that were able to expose these regions. In addition, while the mediastinal region has previously been considered a depot of human BAT (176), the overlying skin temperature is not maintained upon cold exposure, or at least maintained as well as the overlying skin temperature of the SCV (9). This suggests that BAT from the mediastinal region is unlikely to impact on the overlying skin temperature during cold exposure, therefore it was suitable to be used as the control region. For this reason, by comparing the difference between the changing temperatures of the SCV and the mediastinal region in response to stimuli, we can account for changes in the skin temperature that are not attributable to BAT e.g. any possible reduction in blood flow or cooling of the venous blood flow in response to cold exposure. See Figure 2.1 for an illustration of our defined regions of interest.

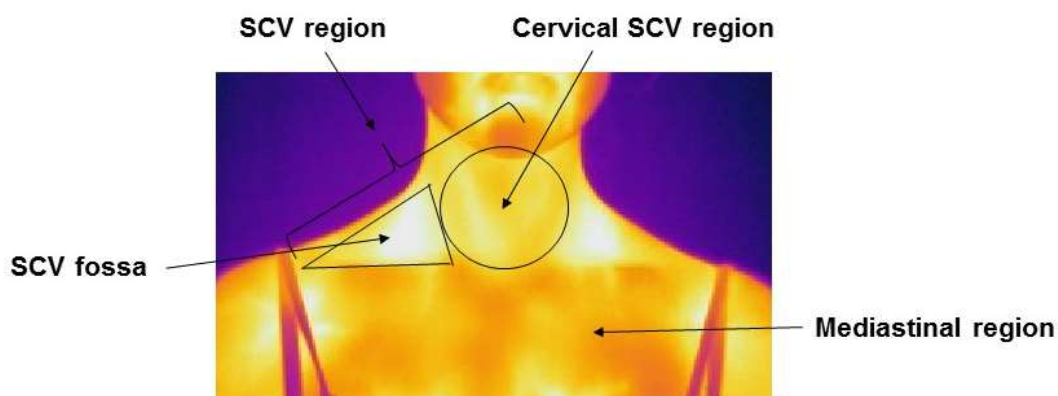


Figure 2.1 Illustration of the regions of interest on an IRT image

Measurement Parameter Settings

Thermal images were obtained using a FLIR model e60 thermal camera (Wilsonville OR, USA, FLIR Systems) fastened to a tripod (model Manfrotto Compact Action Tripod; Cassola, Italy, Lino Manfrotto + Co. Spa). Measurement parameters of emissivity, reflectivity, atmospheric temperature, and distance were set accordingly to either the conditions. We were unable to determine relative humidity, thus it was set at the default recommended settings (Table 2.1). It was important to turn the camera on 5 minutes prior to any imaging to allow for the time needed for its automatic calibration.

Of the known conditions: emissivity was set to 0.96 for human skin as that was the main region of interest; reflected temperature was calculated using the thermal camera itself by obtaining the background temperature when the emissivity is 1.0; atmospheric temperature was determined with a digital thermometer. The atmospheric temperature of the clinical rooms where we conducted these experiments ranged from 21-24 degrees Celsius, depending on the time of day and air conditioning of the building. This was adjusted accordingly to daily fluctuations. We did not have control over the atmospheric temperature due to central air conditioning systems of the building. The height of the camera was adjusted from the tripod to be horizontally in line with the region of interest. The best distance of the camera to the participant in measuring brown fat was unknown, and was therefore one of the conditions explored in the study.

Table 2.1 Default recommended conditions, adapted from FLIR Systems, 2016 (196)

Atmospheric temperature	+20°C (+69°F)
Emissivity	0.95
Relative humidity	50%
Reflective temperature	+20°C (+69°F)
Distance	1.0 m (3.3 ft.)

Data Analysis

A necessary component of developing a protocol for IRT detection of BAT is the technique for interpreting the radiometric data. A variety of different software and analysis techniques were used to interpret the data obtained from the thermal images. These techniques were explored in this study to identify which methodology was most suitable. The software programmes include: FLIR Tools (Wilsonville OR, USA, FLIR Systems), ThermaCAM Researcher Pro 2.10 (Wilsonville OR, USA, FLIR Systems), ImageJ (Bethesda MD, USA, National Institutes of Health), and Microsoft Excel and PowerPoint 2013 for Windows.

Due to the nature of BAT having a thermogenic function, its activity results in an increase in mean skin temperature of the surrounding local area. While looking for the highest temperature can give insight on the thermogenic capacity of BAT, it does not account for the transfer and spread of heat on the overlying skin. Therefore we had to measure the mean temperature of a set area which included some of the surrounding local area such as the neck region between the SCV fossa. However, to analyse

two-dimensional images, there was a strong need for the images to be the same. Large movements of the participant forwards or backwards would change the plane of the region of interest, affecting the size of the area recorded. Movements from side to side would also cause asymmetry of the region of interest and result in difficulties in analysis, depending on the software and technique used. Therefore it was crucial for participants to be in the same position to allow for the analysis to be consistent.

The following data analysis techniques were explored in the preliminary experiments:

Threshold analysis

The threshold analysis technique was developed as a means to systematically measure temperature change by quantifying each pixel within a selected range of area. In this method, the images were imported into the FLIR Tools software, where radiometric data can then be exported into a comma-separated values (CSV) file as temperature units. A rectangular area of the SCV region on the image was selected to be exported. The lateral border was defined by the acromion, the superior border was defined by the chin, and the inferior border was defined by approximately three centimetres below the sternal end of the clavicles (see Figure 2.2). This selected area was exported into a CSV file where the temperature of each pixel corresponded to a cell i.e. the numbers of row and columns of the CSV file matched to the dimensions of the area of the image exported. This process was repeated for all of the images of the subsequent time points.

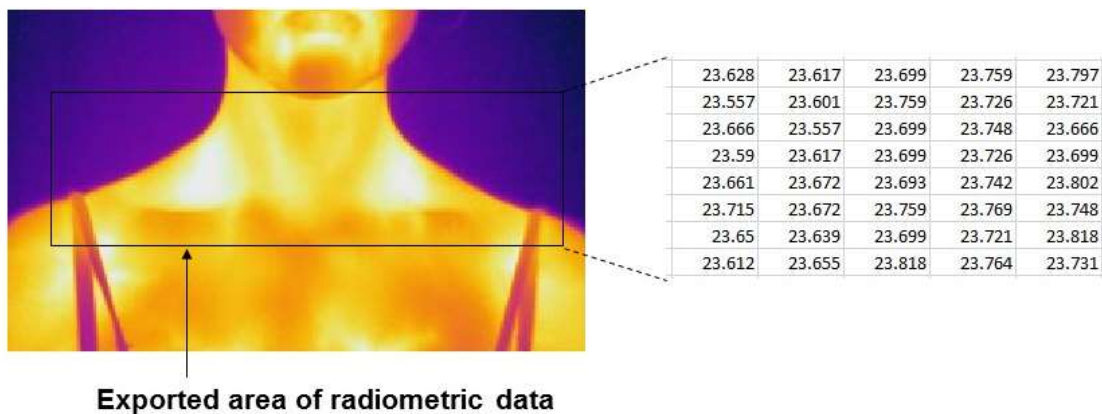


Figure 2.2 Area of radiometric data selected for export as CSV file

The set of data at the first time point was used to determine the threshold. On the participant, the hottest temperature readings came from the SCV region regardless of the condition or time point. The threshold temperature was arbitrarily determined at the 90th percentile on the assumption that the hottest 10% of skin would be from the SCV region and that temperatures exceeding this threshold of subsequent data sets would likely be a result of BAT heating this region. This is supported by the notion that SCV temperature is higher than that of surrounding skin temperatures such as the chest (8). For each of the data sets, the total number of cells with temperatures which met the threshold would then be counted as calculated as a fold change from that of the first data set. This area change over time would therefore account for the heat transfer from BAT to any surrounding tissue. It is also worthy to note that for the data sets following the first time point, the interest was the total number of pixels where the temperatures

met the defined threshold. Therefore, the accuracy of the area exported from FLIR Tools was less important as long as it included the hottest regions that was the SCV region.

ResearcherPro Analysis

The ResearcherPro analysis uses the ThermoCAM Researcher Pro 2.10 software. In this method, the images were directly imported and the analysis was able to take place on the software programme. The freehand tool was used to outline the area of the SCV fossa (see Figure 2.3). The mean temperature of the selected area for each data set was then compared to calculate the change in temperature of the SCV region. These changes were entered and compared on Microsoft Excel.

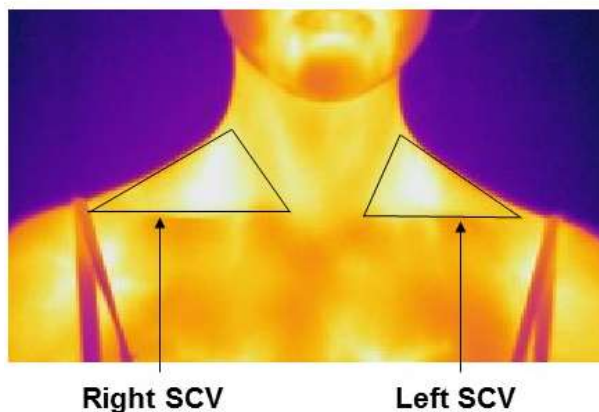


Figure 2.3 Freehand outline of SCV fossa using the ThermoCAM Researcher Pro 2.10 software package

ImageJ Analysis

The ImageJ analysis technique required a three-step process which included changing the temperature scale of the images on FLIR Tools followed by compiling the images on a tagged image file format (TIF) file to be finally analysed on the ImageJ software. The images were first imported onto the FLIR Tools software to ensure that the temperature scales were consistent across all images at 20°C to 40°C. These images were then compiled on a Microsoft PowerPoint slide to be converted into a TIF file. It was crucial for these image dimensions to be identical to ensure that the area measured on each image maintained consistent. The TIF files were then opened with the ImageJ software where a fixed area was defined to capture the mean value. The shape of the measurement area tool was determined by preliminary experiments described in the results section below. An oval shaped area was determined by having the outline of the oval meet the outline of the trapezium while including both the sternal and acromial ends of the clavicle. It was important to not include any background as the background temperature was much lower than the skin and would have therefore affected the mean of the values taken. An oval shaped area resulted in some skin outside of the SCV region to be included which therefore accounted for the transfer of heat from BAT to the surrounding skin. As ImageJ data are in units of brightness based on the colour scale, a calibration curve was conducted as part of the preliminary testing. These values were then converted into units as degrees Celsius using the equation from the calibration.

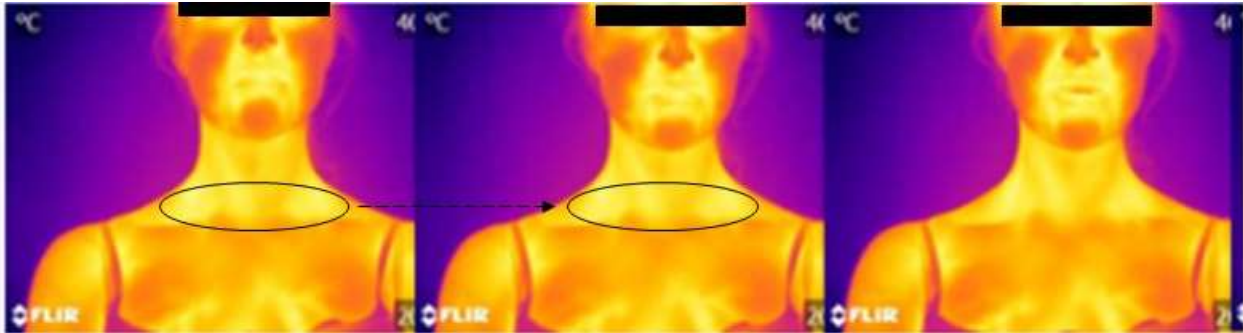


Figure 2.4 Compiled images on TIFF file with a fixed defined area of measurement

2.3.3 Draft Infrared Thermography Protocol

Preliminary Testing

The preliminary experiments were conducted with the intention to develop a base protocol that could then be optimised and test for its reproducibility. Due to the nature of protocol development, the preliminary experiments segment will be further described in detail in the results section of the manuscript. The first base protocol is as follows:

The participants were required to be in clothing with the regions of interest exposed. They were instructed to be seated in a comfortable position which they were able to maintain for 30 minutes. The thermal camera was placed one metre in front of the participants and the height adjusted to align with their neck and chest region. The measurement parameters were manually entered on the camera settings.

The participants were required to remain seated for 10 minutes prior to measurement to allow for the body temperature to stabilise. Single time point images were then taken every 30 seconds for 10 minutes prior to the introduction of the cold stimulus and a further 10 minutes after. These images were then analysed using the ImageJ analysis with an oval shaped area of analysis.

Optimisation of Protocol

The aim of optimising the protocol was to compare whether there is a difference between: 1) different room temperatures, 2) different water temperatures, 3) different distances 4) different areas for analysis, and 5) exposing the arm or both feet to cold water. The protocol determined from the preliminary experiments described above was used for comparing the different stimulus locations only. The other optimisation experiments were conducted prior to the establishment of the first base protocol, therefore no standardised protocols were used.

For the comparison of different stimulus locations, participants were required to undergo two experiments on separate days as the amount time needed for activated BAT to return to its inactivated state was unknown. The cold stimulus was exposed to either the right arm or both feet. The arm was submerged in cold water from the hand to up to the elbow, and the feet were submerged to up to the calves.

Reproducibility of Protocol

As described in the results section, the right arm was selected as the preferred limb for cold exposure. This protocol was repeated three times in total to assess its reproducibility.

2.4 Indirect Calorimetry

Energy expenditure was measured using respiratory gas analyser, Parvo Medics' TrueOne 2400 metabolic measuring system (East Sandy, UT, USA, Parvo Medics). The oxygen consumption and carbon dioxide output of the participant at rest was collected using the TrueOne 2400 canopy (East Sandy, UT, USA, Parvo Medics) and delivered to through a tube attachment to the measuring system for analysis. The gaseous exchange of the participant and the environment was continuously sampled, calculated and expressed as daily energy expenditure. Prior to the measurement of the participant, a calibration was done as detailed in the standard operating procedure as detailed in appendix F.

In this experiment, the parameters of interest were the REE at baseline (REE_{baseline}) and REE with cold exposure to both feet with the cold water stimulus described above ($REE_{\text{cold exposed}}$). Firstly, REE_{baseline} was measurement first for a minimum of 15 minutes to ensure stabilisation of the measurements. An introductory explanation of the apparatus and experiment was given to the participant to familiarise them with the procedure. This was done to facilitate the participant to remain calm and relaxed through the experiment to allow for stable breathing and consequent results. The participant was instructed to lie in the supine position on the measuring bed where the canopy was placed over their head and neck to start the measurement. The participants' feet were then placed in the cold water at the end of the REE_{baseline} recording for a further minimum of 15 minutes. For both segments of the experiments, if the measurement readings had not stabilised, the participant were kept under the condition until a stable five minute reading can be taken. The mean of the most stable five minutes each condition was taken as the participants' REE. The feet location was selected for cold exposure for practicality reasons, as the metabolic cart did not accommodate for the arm to be exposed during the measurement.

REE was used to demonstrate a relationship with temperature change. Temperature change data was used from the IRT experiments conducted with the arm exposed to cold water. Temperature change was determined from the difference between the 0-5 minutes before and the 5-10 minutes after cold exposure. For participants that underwent repeated testing, temperature change of the repeated tests were averaged and the mean was used for comparison.

2.5 Statistical Analysis

No sample size was calculated due to the developmental nature of the research. All statistical analyses were performed with Microsoft Excel 2013 or GraphPad Prism version 7 for Windows. A P-value of <0.05 was considered statistically significant.

Preliminary Experiments

Due to the developmental nature of the research, the preliminary experiments were not statistically analysed.

Optimisation of Protocol

Data was expressed as mean +/- the standard error of the mean (SEM). Normality testing was conducted by plotting a histogram of the arm and feet data which followed a Gaussian distribution. A Two-way Analysis of Variance (ANOVA) with time and cold exposure location as the independent variables was used to analyse time- and cold exposure location-dependent differences in SCV temperature change during the experiment. Paired parametric t-tests were conducted to analyse the temperature change from baseline between 0-5 minutes before cold exposure and 5-10 minutes after cold exposure.

Reproducibility of Protocol

Data was expressed as mean + SEM. Normality testing was conducted using the D'Agostino & Pearson normality test which followed a Gaussian distribution. The mean area under the curve (AUC) for the three repeated tests were calculated. A One-way ANOVA was conducted to analyse the differences of the AUC between the repeated measures. Differences that were not statistically significant indicated that the protocol is reproducible as it did not produce different results.

Cold Exposure, Resting Energy Expenditure, and Temperature Change

Data to compare REE_{baseline} and $REE_{\text{cold exposed}}$ were expressed as mean + SEM. Data for REE and temperature change were expressed as absolute values. A paired parametric t-test conducted to compare the change in REE following cold exposure. Linear regression analysis was performed for temperature change and REE change. Temperature change was the independent variable while the REE_{baseline} and the difference of $REE_{\text{cold exposed}} - REE_{\text{baseline}}$ were the dependent variables.

2.6 Study Overview

The four segments of this pilot study are:

1. Preliminary testing to establish an IRT protocol to estimate BAT volume and interpret the collected data.
2. Optimisation of the protocol by comparing two different sites of cold water exposure such as the arm or both feet.
3. Assessment of the reproducibility of the established protocol by repeated measures on the same individuals across different days.
4. The effect of cold exposure on resting energy expenditure, as well as its relationship with temperature change of the SCV region measured using IRT imaging.

CHAPTER 3. RESULTS

3.1 Preliminary Testing

3.1.1 Assessment of Infrared Thermography Camera Equipment

To test the ability of the thermal camera to track changes in temperature over time, we measured a glass of hot water cooling over time under room temperature. Figure 3.1 shows that temperature of the water in the glass cooled over time in a consistent, linear fashion. The mean and maximum temperature of the water demonstrated consistency during its cooling, while the background temperature of the wall remained stable throughout the experiment. These findings indicated the ability of the equipment to accurately track the changes in temperature.

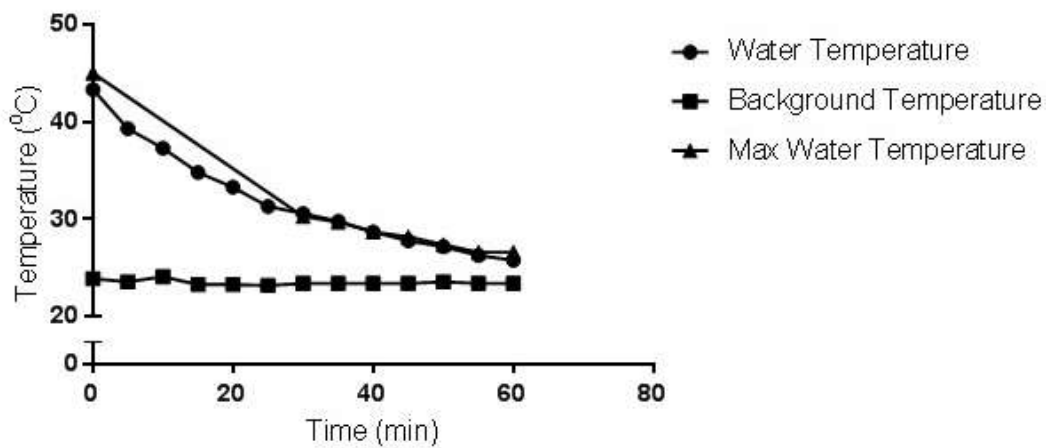


Figure 3.1 Hot water in glass cooling at room temperature. In this experiment, a glass of hot water was placed in front of the thermal camera at a distance of 1 metre. An image was captured every 5 minutes for 60 minutes to track the rate of temperature cooling. The maximum and mean temperature of the water in the glass, as well as the background temperature were analysed using the FLIR Tools software.

3.1.2 Threshold Analysis

The aim of this experiment was to assess the feasibility of estimating BAT with the threshold analysis technique. Using this technique, we were able to detect an increase in the number of pixels which met the threshold temperature when the participants were exposed to the cold stimulus (Figure 3.2A). Similarly, the mean and median temperature of pixels above the threshold temperature also increased with the number of pixels that exceeded the predetermined threshold (Figure 3.2B). This finding indicated that the increased temperature is associated with the transfer of heat to surrounding tissues. This experiment was repeated three times with one other participant which all demonstrated similar results (data not shown).

However, the area above the threshold temperature appeared to experience increases prior to the introduction of the cold exposure, thus inferred a necessity to assess the variability of the temperature at baseline as well as the time needed for the temperature to stabilise. The spike in temperature prior to the cold stimulus may also indicate some degree of anticipation effect and would be minimised if the participant were blinded to the timing of the exposure. Therefore, we next tested the stability of the threshold measurement at baseline (prior to cold stimuli). To do so, we needed to capture the images of the participant at rest, over a period of time. This data will then be analysed to assess the behaviour of the SCV skin temperature following a change in the environment. In addition, for the subsequent experiments, the participants were not verbally cued prior to the cold exposure to minimise any potential confounding effects from anticipation to cold stress.

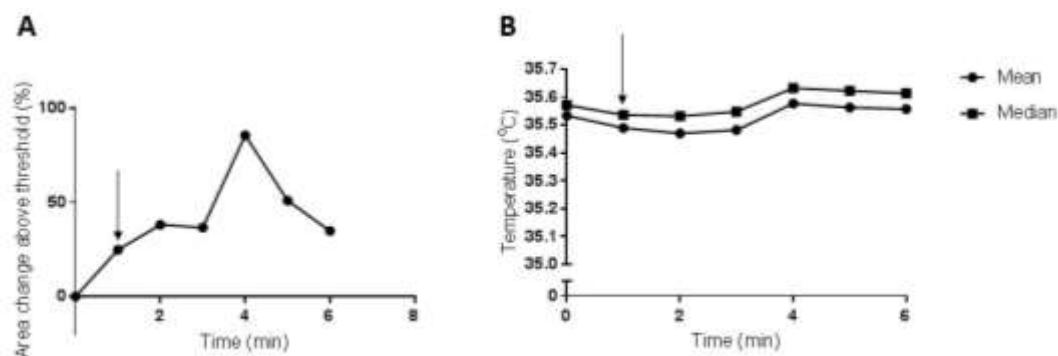


Figure 3.2 Threshold analysis of temperature changes in SCV region upon cold exposure for one experiment. Subfigure A show the temperature change as the number of pixels above the predefined threshold temperature, while subfigure B show the average temperature change of the pixels above the threshold. Four experiments were analysed using the threshold analysis technique. Data were collected from two participants, one of whom were studied three times. In these experiments, the participants were 90 minutes post-prandial. During one experiment, the participant had caffeine consumption with their last meal. The participants were seated in front of the camera positioned 1 metre away and the room temperature was thermoneutral at 23-24°C. The participant was instructed to remain in their testing position for 5 minutes prior to the measurement. Images were taken at 1 minute intervals. The cold stimulus was the submersion of a part of a limb in cold water maintained at 16°C. The point of cold exposure introduction is indicated by the arrows on the figures.

3.1.3 Stabilisation of Body Temperature

Duration the testing for SCV temperature change, the participant was subjected to a change in environment, different clothing and physical movement was limited. These changes would consequentially result in changes in heat production and therefore body temperature detected in the SCV region. In this experiment, the variability in SCV region temperature from the start of being in the testing condition was assessed. Figure 3.3 A and B suggest that SCV temperature began to show stabilisation by the first 10 minutes. This finding was consistent across the different threshold values used. The experiment was repeated for 5 participants, all of which demonstrated similar results. To

minimise temperature fluctuations from the introduction of the new environment, it appeared that stabilisation period of 10 minutes was likely to suffice for the equilibration of body temperature.

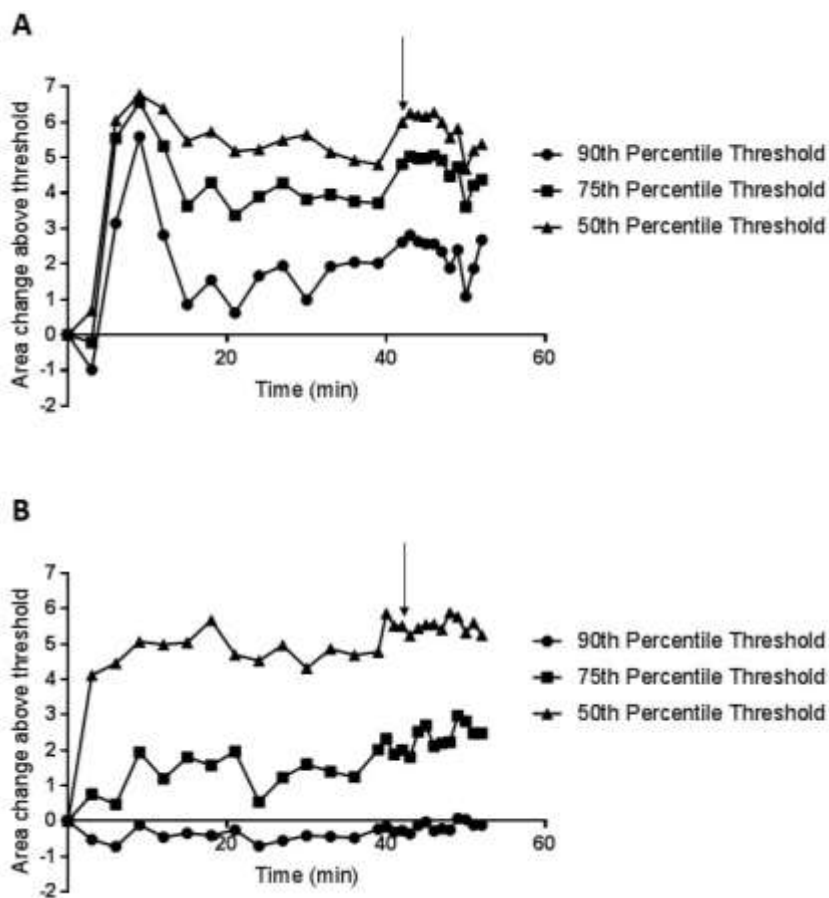


Figure 3.3 Stabilisation of SCV region temperature in two participants, A and B. The change in the number of pixels above the threshold temperature was analysed for three different threshold cutoff points, 90th, 75th and 50th percentile. Upon changing into the singlet, participants were required to be seated in the position of testing to be recorded immediately. Images were captured every 3 minutes at baseline for 42 minutes prior to the introduction of the cold stimulus for a further 10 minutes. Images were captured every minute during the cold exposure.

3.1.4 Contraindications for Threshold Analysis

A limitation with the threshold analysis was that it was unable to measure the temperature of the control region. A negative control region that is unaffected by BAT thermogenesis was important as it could then account for the general changes in skin temperature. This net difference between the control temperature and SCV skin temperature would best represent BAT thermogenesis. The limitation of the threshold analysis technique was that it used pixels that exceeded the defined threshold temperature to account for the changes in temperature. However, in the case where all of the pixels exhibit decreases in temperatures below the threshold, no pixels would be counted and the change would not be quantified. This is illustrated in Figure 3.4 where the maximum decrease in temperature able to be

quantified would be -100%. Therefore, an alternative technique was needed to account for data which presented with this problem.

Furthermore, the threshold analysis appeared to show high variability which made the changes in temperature difficult to quantify. While an overall increasing trend in temperature under cold exposure was observed, the changes in temperature and each data point did not demonstrate consistency (Figure 3.4). This limitation provided further contraindications for the use of the threshold analysis technique in the quantification of SCV temperature change under cold exposure.

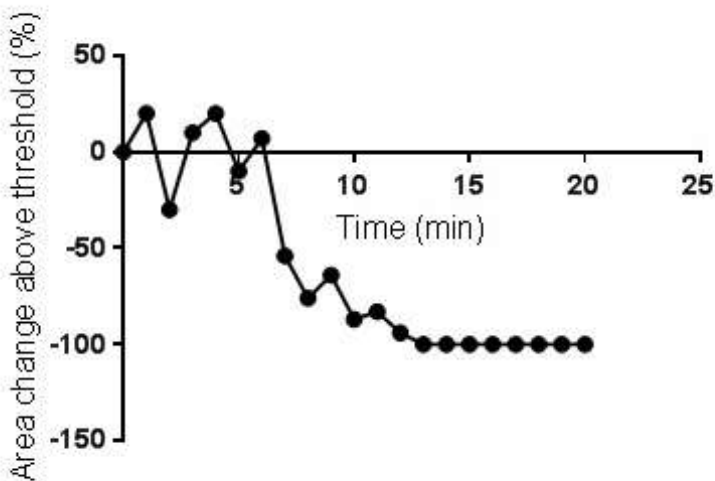


Figure 3.4 Illustration of control temperature decreasing below the defined threshold.

3.1.5 ResearcherPro Analysis

As the ThermoCAM ResearcherPro software directly measures the mean temperature of a selected area, it was able to measure the changes in the control region. In this experiment, the ResearcherPro analysis technique was applied to a series of experiments to compare with the results from the threshold analysis technique.

The SCV fossae were divided into the left and right side to assess whether a difference in temperature change between the two sides. As observed in Figures 3.5 A and B, both left and right SCV skin temperatures increased following the introduction of cold exposure. The temperature changes between the left and right sides showed similar trends. Therefore, the means of the two sides were taken to compare with the threshold analysis.

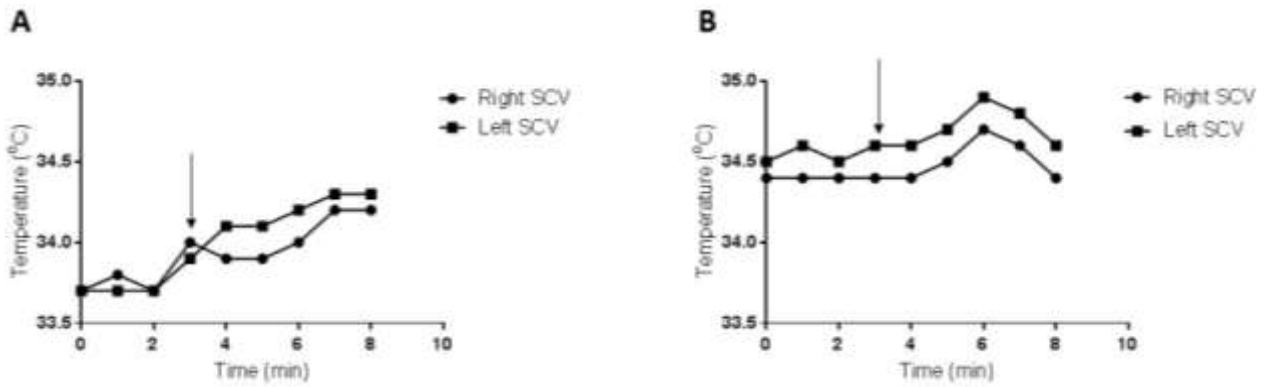


Figure 3.5 Change in temperature of the left and right SCV fossae in two participants, A and B. Images were analysed using the ThermoCAM Researcher Pro 2.1 software package. The cold stimulus was introduced after the third minute as indicated by the arrows. Both participants demonstrated similar increases in mean SCV temperature of both left and right sides following cold exposure.

As seen in Figure 3.6, the ResearcherPro analysis demonstrated comparable trends with the threshold analysis under cold exposure. Moreover, the ResearcherPro analysis was able to provide results for the absolute changes in temperature, allowing us to compare the data in degrees Celsius. Due to the observed similarities between the two methods, along with the ability to interpret the control region measurements and express results in units of temperature, the ResearcherPro technique was selected as the preferred candidate for the analysis.

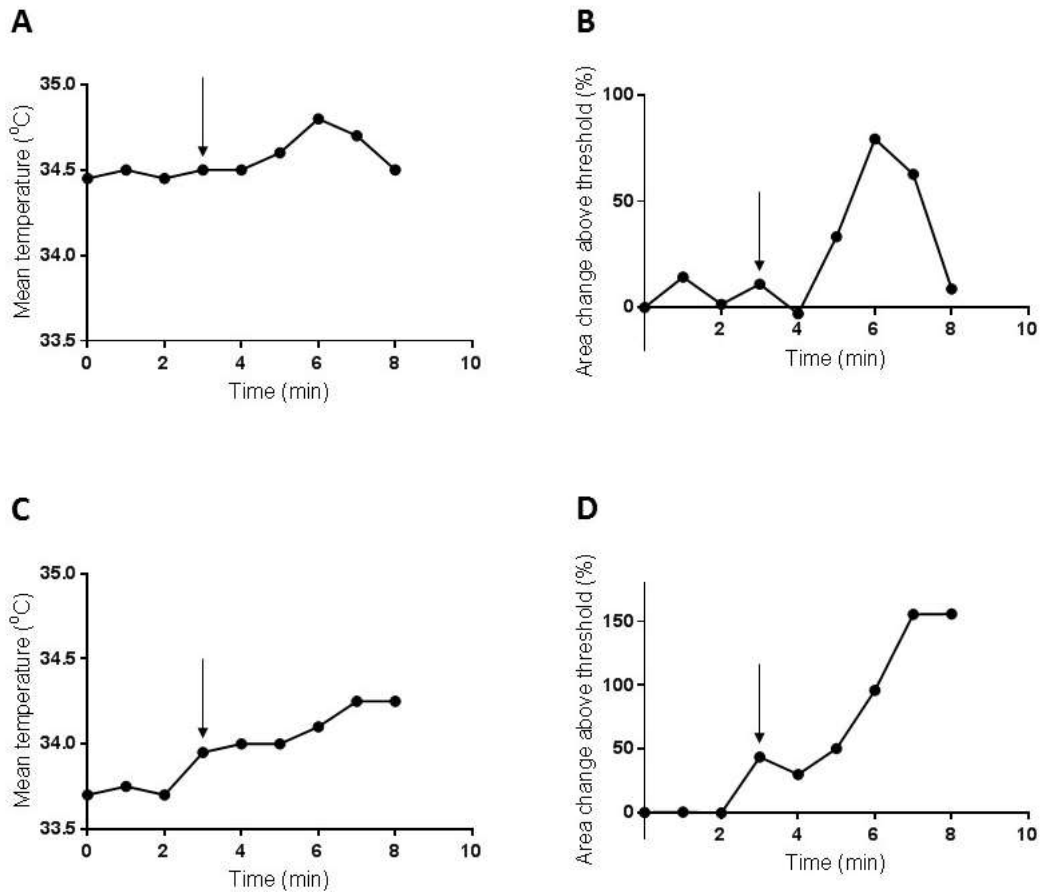


Figure 3.6 Temperature changes of the SCV region as measured by ResearcherPro analysis (subfigures A and C) and threshold analysis (subfigures B and D). Subfigures A and B, show the SCV temperature change for experiment 1, while C and D show the SCV temperature change for experiment 2. Two participants were measured, one of whom was studied three times. Images were captured every minute and analysed by both ResearchPro analysis and threshold analysis techniques. The point of cold exposure introduction is indicated by an arrow.

3.1.6 ImageJ Analysis

While the ResearcherPro technique showed favourability as the method of analysis, only a trial version of the software programme was available. As we were unable to purchase the software, another programme with the capacity of measuring the mean temperature of an area was sought for as substitution. ImageJ was a software programme selected to substitute the function of the ResearcherPro analysis method. Its advantages included its free cost and convenience of use. However, as ImageJ calculates data based on the brightness of the colour scale on the image, the values do not make for meaningful comparisons when temperature is the parameter of interest. Thus, a calibration experiment was conducted to determine the relationship between the ImageJ brightness value and temperature. A linear relationship between brightness and temperature was demonstrated as seen in Figure 3.7. This equation was then applied to all ImageJ data to determine the temperature in degrees Celsius.

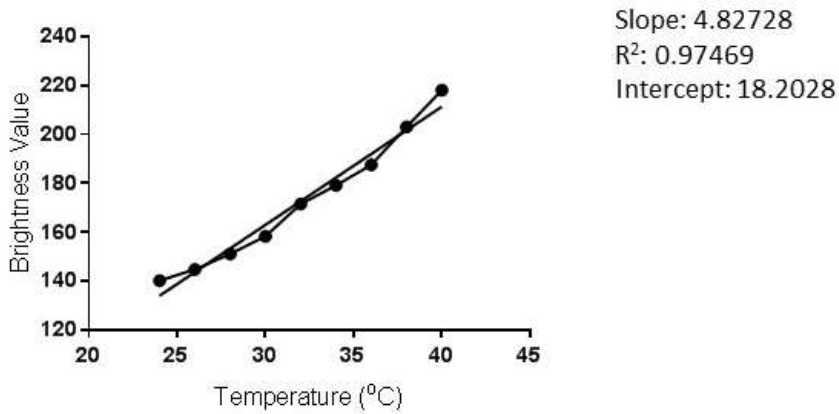


Figure 3.7 Calibration curve of ImageJ brightness and temperature. A glass of hot water was the object of interest and an image was captured for every 2°C drop as determined by a digital thermometer. The images were analysed using ImageJ and the values were plotted against the temperatures recorded. The figure shows a positive linear relationship between brightness and temperature.

The temperature measurements calculated through the ImageJ analysis was then compared with that of the ResearcherPro analysis to assess the accuracy of our calibration. The same experiment was analysed using both techniques and compared with one another. Figure 3.8 shows that the trend of the temperatures are comparable between the two analysis techniques, however, the absolute values differed. The ResearcherPro analysis demonstrated the same trend as the ImageJ analysis but at a higher temperature range. However, as relative temperature change over time was our measurement of interest, the absolute temperatures of the SCV region were less important. The changes relative to the temperature at 0 minutes for each analysis technique demonstrated concordant values, indicating that the ImageJ analysis along with our calibration was able to accurately measure the changes in temperature in degrees Celsius. As these images were analysed separately, there were discrepancies between the area measured and would therefore result in slight variations as seen in the figure. As we were now able to successfully analyse the temperature of any given area using the ImageJ analysis technique, the experimental protocol was able to now be further optimised.

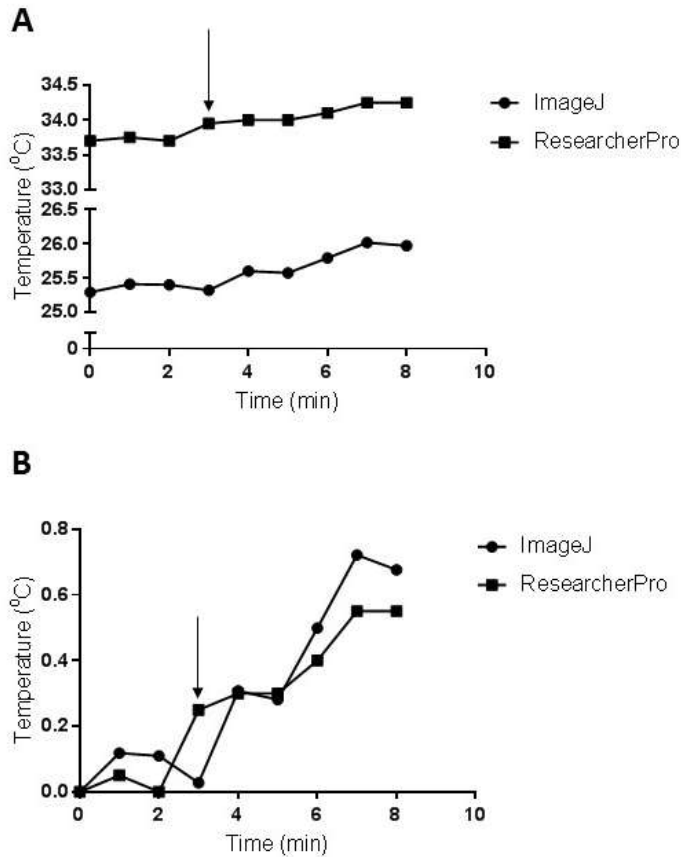


Figure 3.8 Comparison of ImageJ calibration with ResearcherPro analysis. In this experiment, an IRT image was captured every minute and the cold stimulus was introduced at 3 minutes. The SCV fossae was analysed with both the ImageJ technique and ResearcherPro technique. Subfigure A show that absolute temperature measurements demonstrated the same trend but was at different temperature ranges. When the temperature change relative to the first time point was compared, subfigure B show that these values were consistent.

3.2 Optimisation of Protocol

3.2.1 Water Temperature

The temperature of the water was tested to assess whether there was a difference in the inducibility of BAT activity. We tested the water temperatures of 16°C and 20°C, while warm water at 35°C was used as a negative control. Figure 3.9 shows that the cold water temperature of 16°C resulted in an increase in SCV temperature than the 20°C and 35°C water temperatures. This may suggest that the greater the cold stress, the greater its capacity to induce BAT thermogenesis.

During the experiment, the participant reported that the 16°C water temperature was tolerable, not uncomfortable, and was not too cold to cause shivering. This suggested it was appropriate to use the colder temperature of 16°C which conferred greater cold stress to induce BAT activity. Furthermore, a likely explanation as to why the 20°C water temperature did not induce an observable SCV temperature change would be that the hand alone may not provide enough surface area to conduct the cold stimulus.

Therefore, a larger surface area in contact with the cold stimulus, such as the arm or feet and legs may be necessary to achieve a greater BAT response. The combination of the water temperature 16°C with the exposure of a greater surface area such as the arm was decided as the method of delivering cold stimulus.

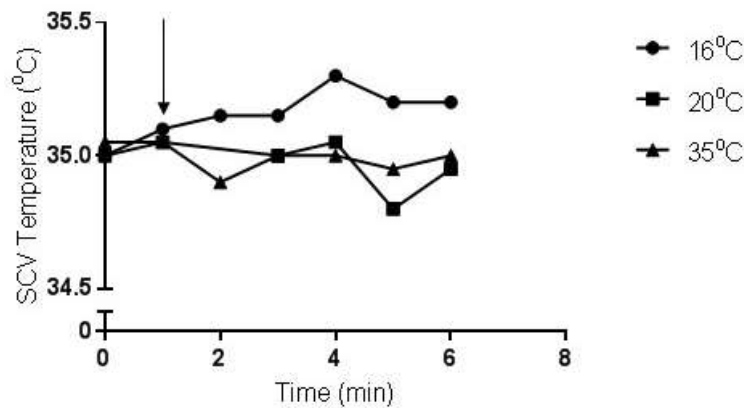


Figure 3.9 SCV temperature change under different water temperatures exposures at 16°C, 20°C, 35°C. The ResearcherPro analysis technique was used to measure the temperature change of the SCV fossae as this experiment was conducted prior to the expiry of the Researcher Pro software package trial. In this experiment, the hand alone was submerged in the water after the image taken at 1 minute.

3.2.2 Room Temperature

The differences in the ambient room temperature was of interest as this may augment the BAT-stimulating capacity of the cold water exposure. Even at thermoneutrality, the response to the cold stimulus may be blunted at the warmer end of this temperature range, while the same response may be enhanced under cooler temperatures of this range. Currently, there is limited evidence on BAT behaviour at the range of temperature that is considered thermoneutral. Unfortunately, we were unable to change the temperature of the room evenly as we did not have control over the air conditioning system and a portable heater was ineffective. Therefore, the effects of different “thermoneutral” temperatures on the cold water stimulation of BAT activity remains from a hypothetical viewpoint. The assumption was made that the thermoneutral temperature range of 21-24°C did not have an effect on the BAT response to cold stimulus.

3.2.3 Measurement Distance

The distance between the thermal camera and the participant directly affects how many data points are captured as each pixel corresponds to a temperature reading. The closer the distance, the more the data points on the participant was captured and vice versa. For this reason, we explored whether there was a functional difference between two different distances, at 0.7 and 1 meter apart. The results demonstrated similar trends between the two distances as seen in Figure 3.10.

The optimal distance of the camera to the participant was therefore set at 1 meter to accommodate for the experimental procedure, as it provided adequate resolution quality while still being able to capture the regions of interest of larger participants. Obtaining thermal images at closer distances resulted in the images being unable to be analysed as they were overly magnified and cropped the regions of interest. On the contrary, obtaining thermal images at farther distances lowered the resolution of the regions of interest, rendering them difficult to analyse with accuracy.

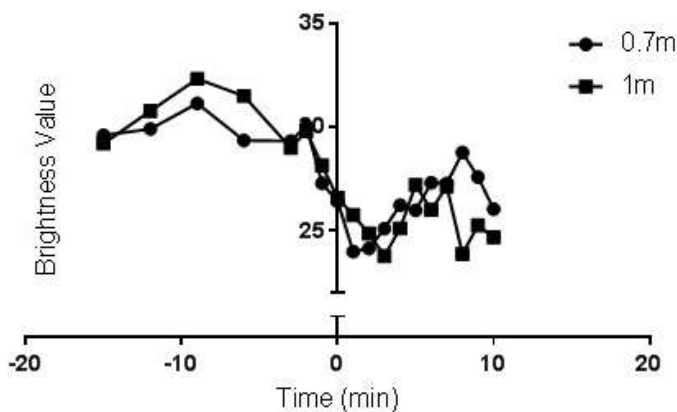


Figure 3.10 Difference in measurement readings between distances, 0.7 and 1 meter. In this experiment, an image at each distance was captured at each time point. Consistency in the distances during its continuous changing was achieved by placing tape on the floor where the tripod would stand at either distances. The tripod was then moved to these positions at every time point to capture the images. In the ImageJ analysis of the images, a fixed oval shaped area tool was used to include the entire SCV region from the outlines of the trapezium to the clavicles. This experiment was repeated with three other participants which all demonstrated similar results (data not shown).

3.2.4 Variability of Temperature at Baseline

Due to the skin temperature variability between individuals, each participant acted as their own control in order to compare the conditions of pre- and post-stimulus. For the participants to act as their own control, the temperature changes were expressed as a fold change to standardise the participants. To determine which point or points were optimal during baseline to standardise to the variability in baseline temperature was assessed. Five participants were measured under this protocol in this experiment and all participants demonstrated similar results. The results for two participant are shown in Figure 3.11 A and B. While the baseline temperatures demonstrated variability as expected, cold exposure resulted in significant elevations in SCV temperature which rendered the baseline variability to appear relatively insignificant. Therefore, we were able to group the data into four time points for the comparison between the conditions expressed as a fold change.

The four time points were grouped as:

1. 5-10 minutes pre-stimulus
2. 0-5 minutes pre-stimulus
3. 0-5 minutes post-stimulus
4. 5-10 minutes post-stimulus

The first time point was used as the control for the subsequent time points to be compared against. The second time point was to ensure that baseline temperatures remained consistent. The third and fourth time points were to demonstrate the rate and capacity of the temperature change between the SCV and control regions.

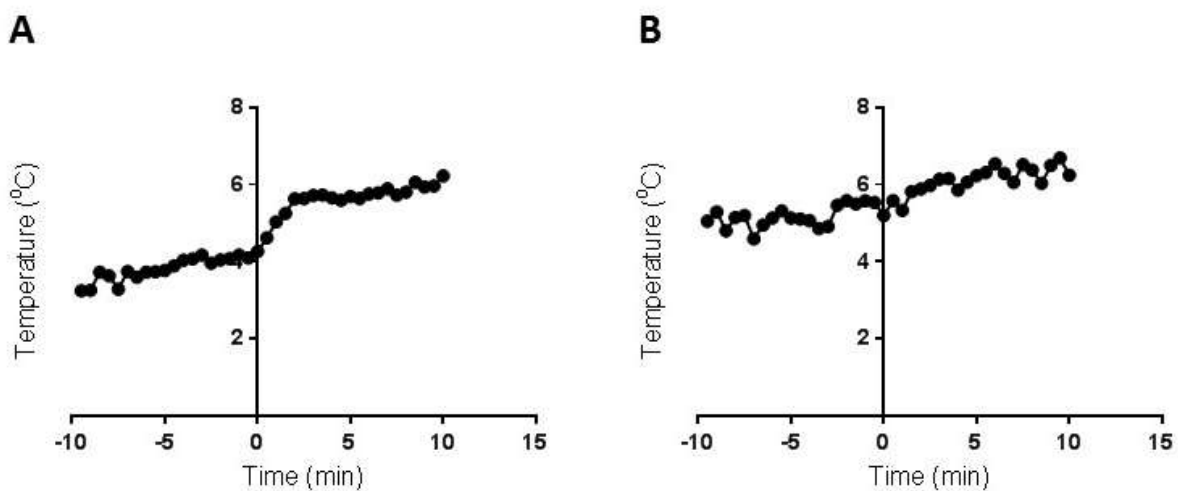


Figure 3.11 Variability of SCV temperature under baseline and cold exposed conditions. Participants were in the testing position 10 minutes prior to the recording, where images were captured every 30 seconds for a further 20 minutes. The point of cold exposure introduction is indicated at 0 minutes. The images were analysed using ImageJ and the temperature change was the net difference of the SCV and control region. The values were expressed as degrees Celsius.

3.2.5 Defining the Measurement Area of the Region of Interest

While it is known that the region of interest of BAT is the SCV region, the shape of the area tool needed to be defined. The aim was to explore the superiority of BAT activity detection of two methods: a fixed oval shaped area to measure the region of interest and the surrounding skin; or a fixed triangle shaped area to measure strictly the region outlined by the trapezius and clavicle on one side of the body (SCV fossa). The left side was chosen as it has been previously demonstrated that the left side may exhibit greater temperature change than the right side (8). These two shapes determining the regions of interest can be seen in Figure 3.12 A and B.

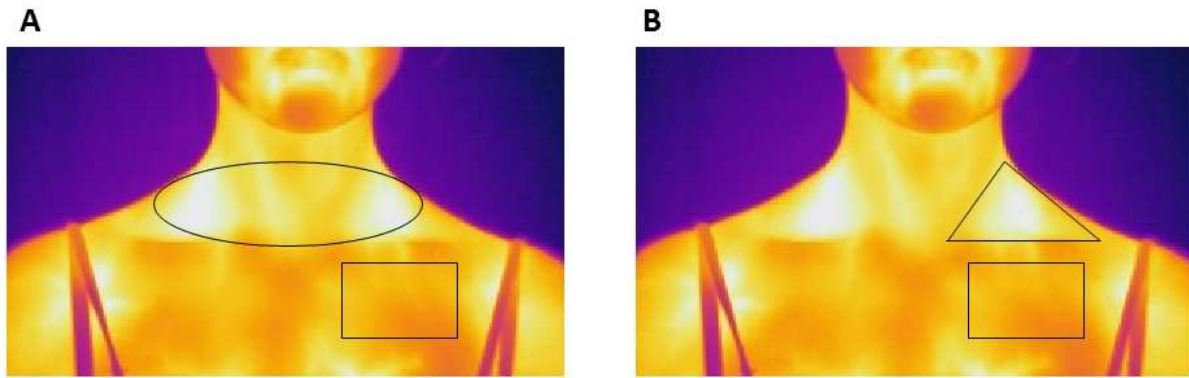


Figure 3.12 Illustration of the selected area of oval (A) and triangle (B) shapes for analysis, along with the rectangular area displayed in both subfigures A and B as the control region. The oval shape was defined by the sternal end of the clavicles and the most lateral end of the trapezius muscle outline, while not including the background temperatures or regions above the most superior end of the SCV fossae. The triangle shape was defined by the left SCV fossae. The control (rectangle), was defined by the left mediastinal region. It was important for the SCV and control regions to not overlap to avoid any duplication of data.

The oval shaped area demonstrated a difference between the baseline temperature and temperature at both time periods under cold exposure, while the triangle shape remained relatively stable at all time points (Figure 3.13). Therefore, the oval shaped was selected for the area of measurement.

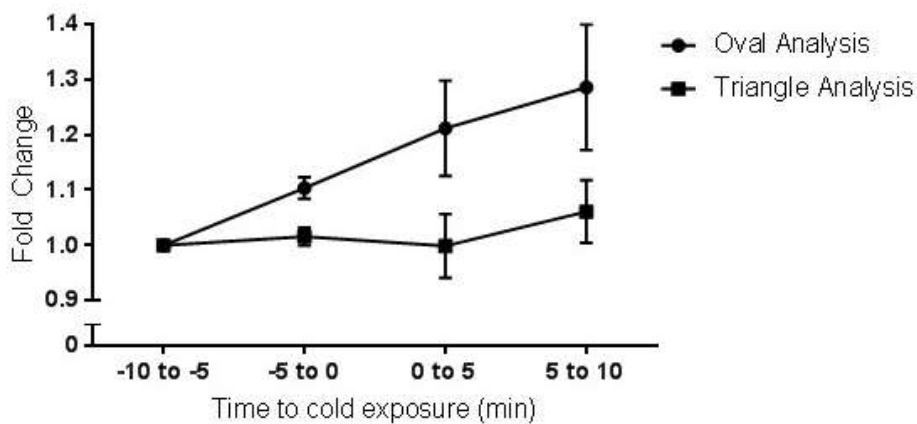


Figure 3.13 Differences in temperature fold change between oval shaped area and triangle shaped area (n=5, data are expressed as mean +/- SEM). Using the base protocol described above, the experiment was repeated for five participants where the cold stimulus was exposed to their right arm. The images captured every 30 seconds for 10 minutes prior and 10 minutes after the introduction of the cold exposure. The images were then analysed using the two defined areas, oval or triangle, as described above.

3.2.6 Exposure Location of Cold Stimulus: Arm versus Feet

Data was collected for 17 participants. Results from the Two-way ANOVA showed that there were statistically significant differences in temperature between the different time points relative to cold exposure in both groups ($p < 0.001$). There was no interaction observed ($p = 0.19$). The mean fold change of the skin temperature at the four different time points for each group are shown in Figure 3.14. There were no statistically significant differences between the arm exposed group and the feet exposed group ($p = 0.49$).

As the temperature change between time points were statistically significant, Sidak's multiple comparisons test was used to test which of these points were different. For the arm exposed group, the mean fold change from 5-10 minutes pre-stimulus (baseline) was the greatest at 5-10 minutes post-stimulus ($M = 1.22 \pm \text{SEM } 0.04$), than that at 0-5 minutes pre-stimulus ($M = 1.06$, $\text{SEM} = 0.01$). The mean difference of 16.5% between these two time points was statistically significant (95% CI: 11% to 22%; $p < 0.0001$). For the feet exposed group, the mean fold change from baseline was greater at 5-10 minutes post-stimulus ($M = 1.16$, $\text{SEM} = 0.05$), than that at 0-5 minutes pre-stimulus ($M = 1.05$, $\text{SEM} = 0.02$). The mean difference of 10.6% between these two time points was statistically significant (95% CI: 5% to 16%; $p < 0.0001$).

The different sites of cold exposure was of interest due to the differences in the surface area of each location. Cold water submersion of the feet up to the calves would expose more surface area than an arm up to the elbow, thus having both feet in the cold water may confer greater cold stress than just the arm alone and perhaps better stimulate BAT activity. As a significant effect of cold exposure on skin temperature change is observed in both groups, with no difference between the groups, the arm was selected as the preferred site of cold exposure. The arm was selected primarily for its convenience over having both feet submerged in cold water and that participants were able to undergo cold exposure without moving the body.

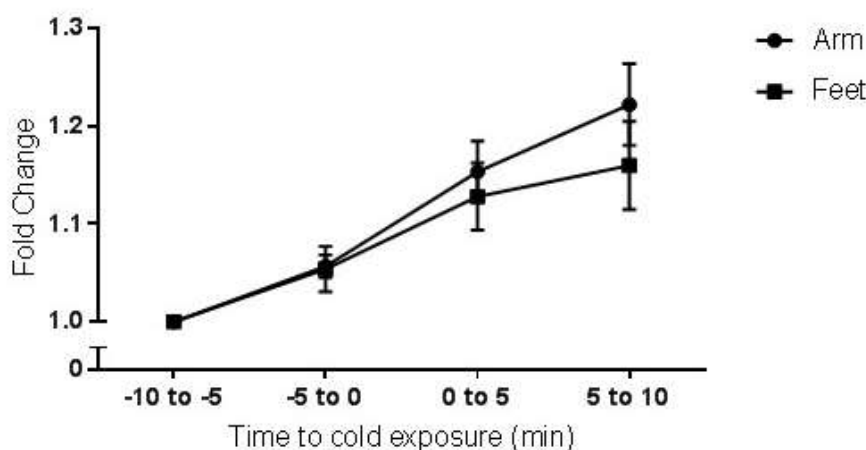


Figure 3.14 Fold change of skin temperature during cold exposure for both exposure location groups ($n = 17$, data are expressed as mean \pm SEM). The first time point was the baseline used as the control for the subsequent time points to calculate the fold change. The draft protocol

was repeated to compare the effects of cold exposure to either the arm or both feet on temperature change.

Table 3.2 Sidak’s multiple comparison test for temperature change

Group	Times (relative to stimulus)	Mean Difference	Standard Error of Difference	Significance	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Arm	0-5min Pre vs 0-5min Post	-0.09653***	0.02325	0.0003	-0.1535	-0.03951
	0-5min Pre vs 5-10min Post	-0.1651****	0.02325	<0.0001	-0.2221	-0.108
	0-5min Post vs 5-10min Post	-0.06854*	0.02325	0.0133	-0.1255	-0.01152
Feet	0-5min Pre vs 0-5min Post	-0.07396**	0.02325	0.0068	-0.131	-0.01695
	0-5min Pre vs 5-10min Post	-0.1055****	0.02325	<0.0001	-0.1626	-0.04853
	0-5min Post vs 5-10min Post	-0.03158	0.02325	0.4472	-0.08859	0.02544

Means expressed as a fold change from the baseline time point, 5-10 minutes pre-stimulus.

*Footnotes: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*

3.3 Reproducibility of Protocol

All of the participants were recalled for repeated testing for cold exposure to the arm. Two more experiments were conducted to assess whether the results were reproducible. Data was collected for 11 of these participants. The AUC was calculated to quantify the total rate of change in SCV temperature following cold exposure. The area starting from timepoint 0-5 minutes pre-stimulus to the timepoints at 0-5 and 5-10 minutes post-stimulus (Figure 3.15). The AUC was calculated for all three repeated tests of the arm in water protocol for every participant.

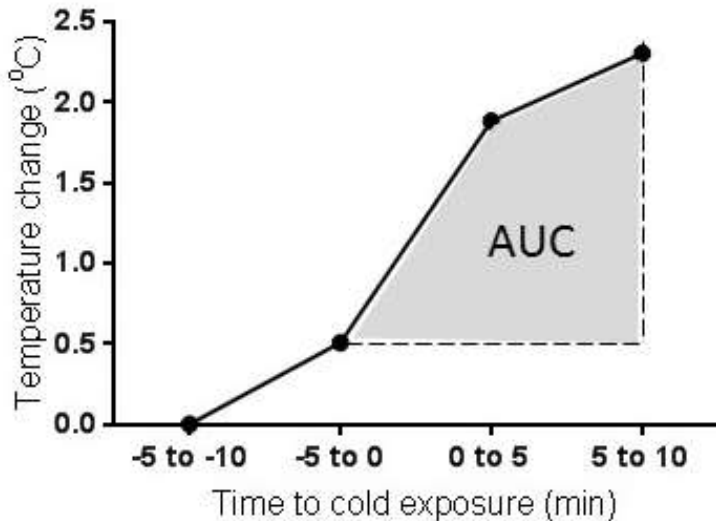


Figure 3.15 Illustration of the area taken to calculate the AUC.

A subject-matched, one-way ANOVA was conducted for the mean AUC of each repeated tests. As seen in Figure 3.16, results from the One-way ANOVA showed that there were no statistically significant differences in the rate of temperature change between the three repeated measurements ($p = 0.35$), indicating that these results were reproducible. The temperature change results for most of the participants appear to be fairly consistent between the repeated tests, as seen in Figure 3.17 subfigures A-K. While much of the variability is seen in timepoints 0-5 minutes pre- and post-stimulus, the final timepoint of 5-10 minutes post-stimulus are less variable.

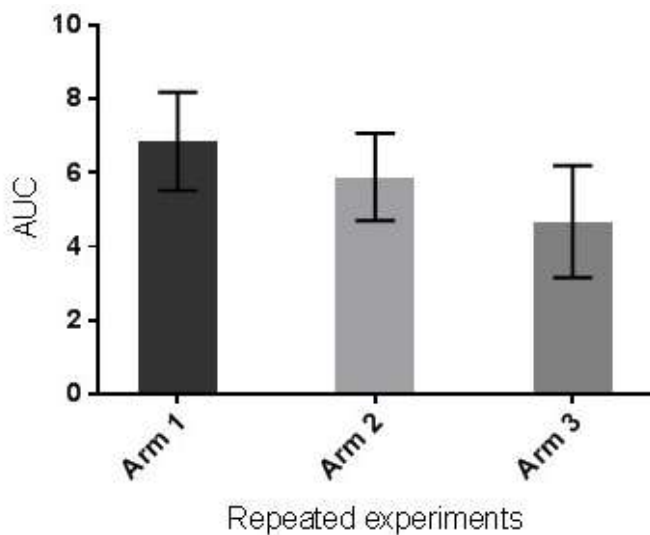
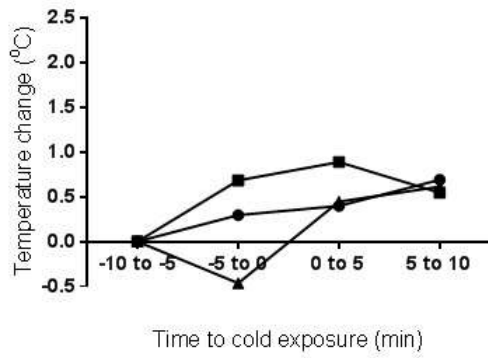
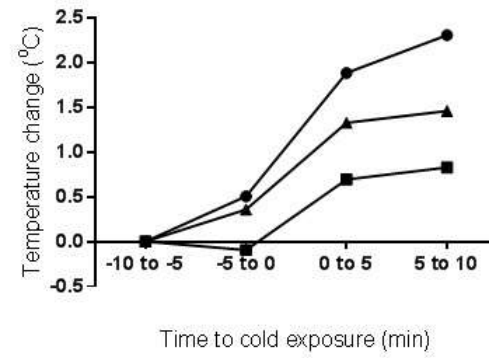
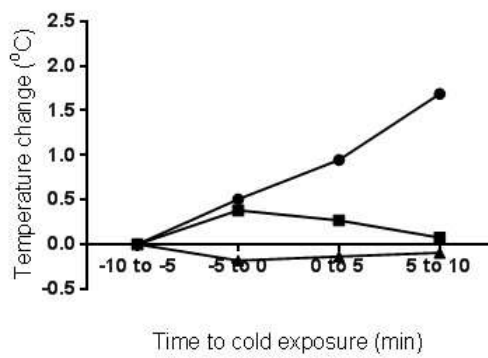
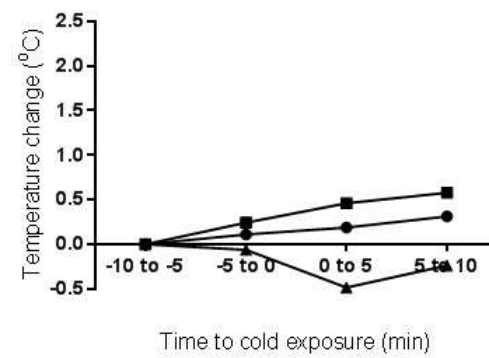
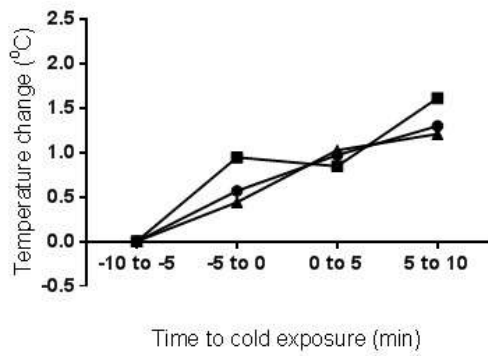
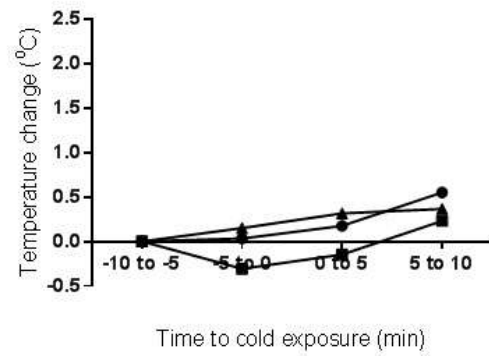
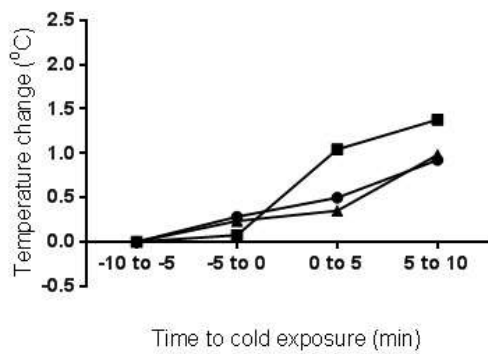
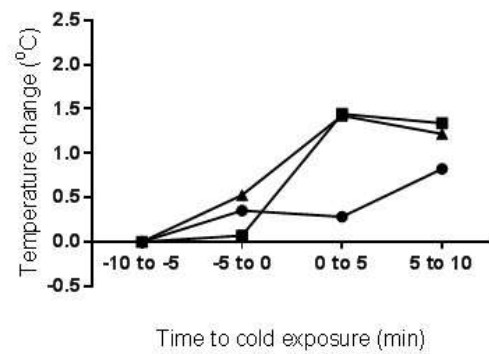


Figure 3.16 AUC of temperature change for repeated experiments (n = 11, data are expressed as mean +/- SEM). The AUC of temperature change for all repeated tests of all participants were calculated and grouped by the test number. The pooled data show that there is no difference between the tests.

A**B****C****D****E****F****G****H**

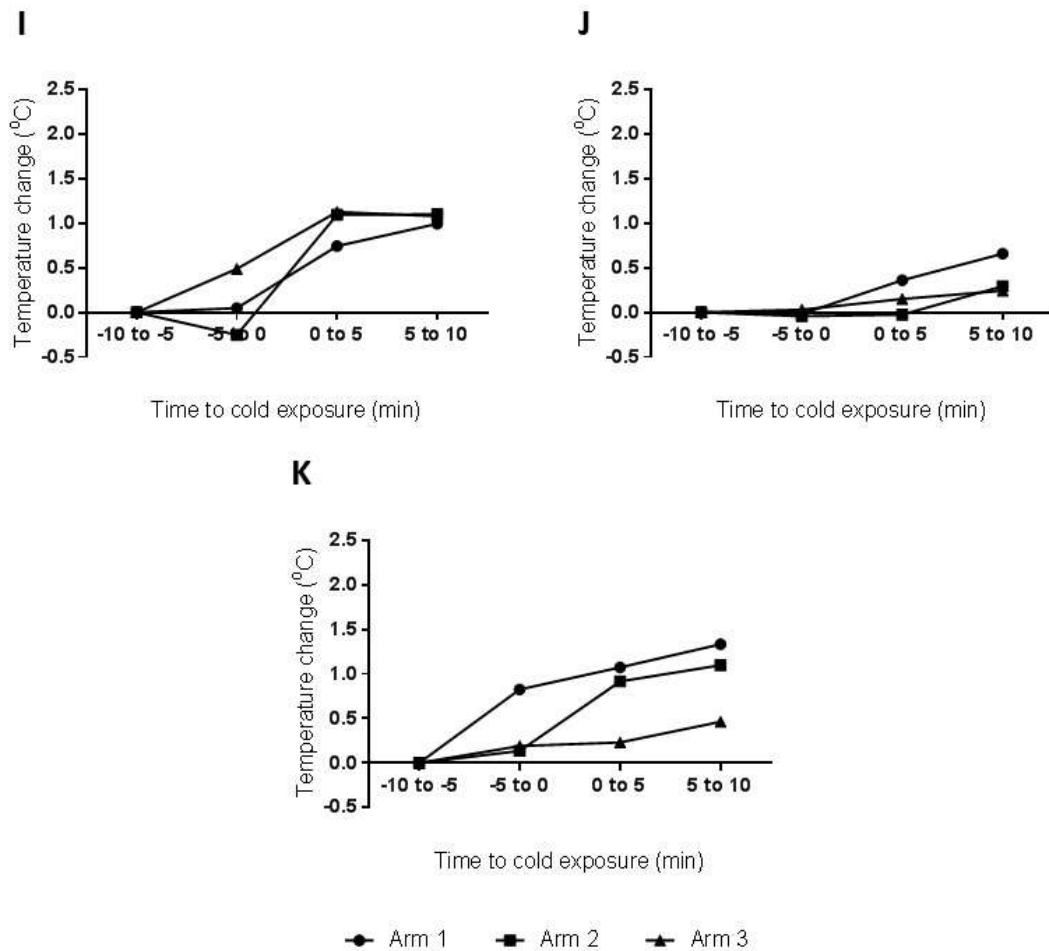


Figure 3.17 Mean temperature change of each participant (subfigures A-K) during cold exposure in repeated experiments (n = 11). Data is expressed as a mean temperature change in degrees Celsius from the first time point.

A subject-matched one-way ANOVA was also carried out to compare the absolute temperature change between the three repeated tests. Similarly, there was no difference between the repeated tests ($p = 0.13$). However, there was an absolute temperature difference observed between individuals ($p < 0.05$). The results of the one-way ANOVA can be seen in Figure 3.18. The absolute temperature change for the repeated tests of each individual can be seen in Figure 3.19.

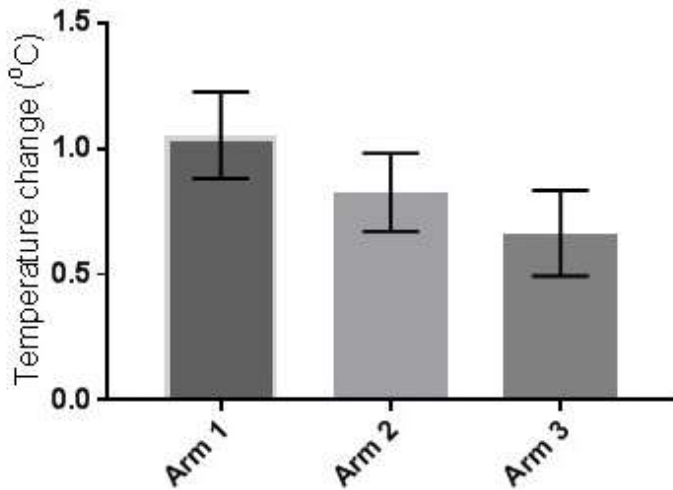


Figure 3.18 Mean temperature change of repeated experiments (n=11, data are expressed as mean +/- SEM). The temperature change was measured by the difference between the timepoint of 0-5 minutes pre-stimulus, and the final timepoint of 5-10 minutes post-stimulus.

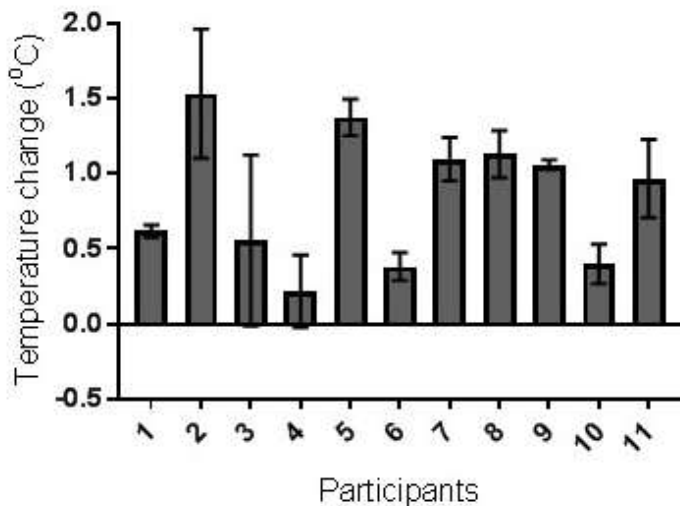


Figure 3.19 Mean temperature change of repeated experiments (n = 11, data are expressed as the mean +/- SEM). The temperature change was measured by the difference between the timepoint of 0-5 minutes pre-stimulus, and the final timepoint of 5-10 minutes post-stimulus.

3.4 Resting Energy Expenditure

3.4.1 Measuring Cold-Exposed Resting Energy Expenditure

When measuring REE through indirect calorimetry, stabilisation of the participants' breathing was necessary. The breathing pattern of the participant changed with mental arousal, resulting in the increase of energy expenditure estimation. This was seen as a spike in the participants' energy expenditure in a response of shock to the introduction of cold exposure. This spike as related to the participants' response to a new condition is seen in Figure 3.20 where at the point of cold exposure,

energy expenditure rises significantly. Therefore, there was a need for the participant to relax following the introduction of cold exposure to allow for the stabilisation of breathing before comparing the REE. The lowest and most stable 5 minutes of both conditions were taken based on the assumption that during this periods participants are most relaxed and best represent energy expenditure at rest.

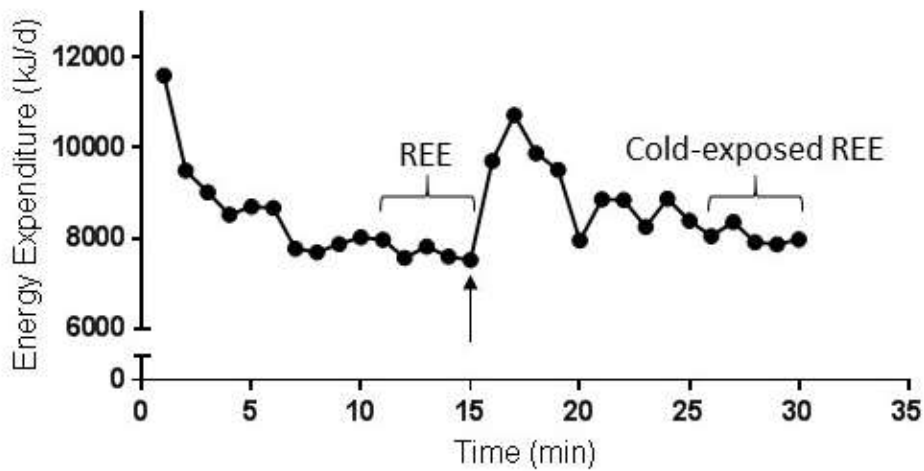


Figure 3.20 Representative figure for the energy expenditure over time before and after cold exposure. Energy expenditure rapidly spiked when the cold exposure was introduced, before stabilising at a higher range than prior to the cold exposure. Energy expenditure also increased at the beginning of the experiment due to the stabilisation in both the participant’s breathing and the gases in the canopy becoming representative of the participant’s gas exchange. The point of cold stimulus introduction is indicated by an arrow on the figure.

3.4.2 Cold-Exposed Differences in Resting Energy Expenditure

Data was collected from 19 participants. As seen in Figure 3.21, the mean REE increased from 7337 +/- 293.5kJ at baseline to 9569 +/- 283.2kJ following cold stimuli ($p < 0.05$).

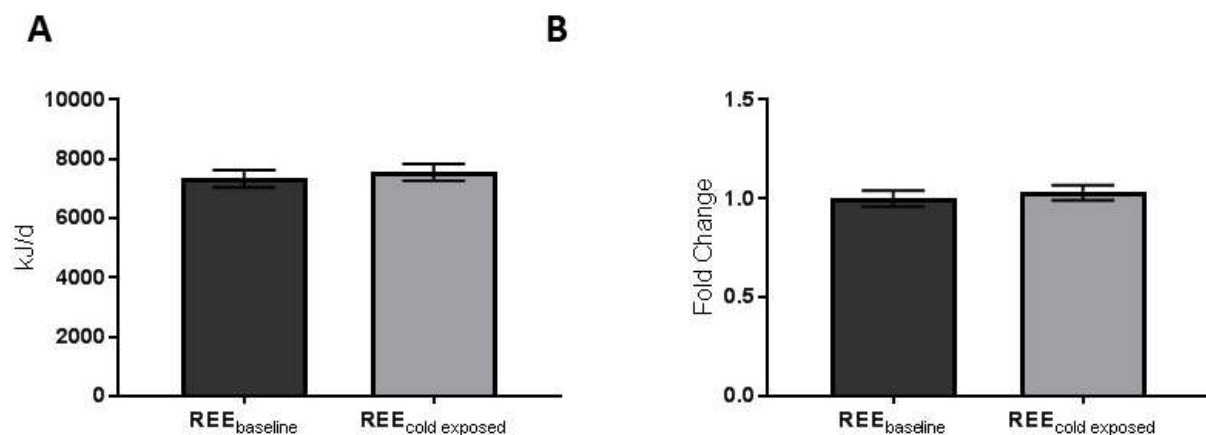


Figure 3.21 Resting energy expenditure at baseline versus during cold exposure ($n = 17$, data expressed as mean +/- SEM). Subfigure A shows the absolute mean difference in REE between the conditions, while subfigure B shows the fold change of REE_{cold exposed} from REE_{baseline}.

3.4.3 Linear Regression of Temperature Change and Resting Energy Expenditure

Data from both REE and IRT experiments were available for 16 participants. As seen in Figure 3.22, on average, the mean REE was 104kJ/kgBW/d (SEM = 3.713kJ/kgBW/d) and the mean temperature change was 0.6°C (SEM = 0.1°C). A linear regression was calculated to predict REE based on temperature change but was statistically insignificant ($F(1,14) = 0.367$, $p = 0.55$), with an R^2 of 0.026. This finding indicated that we were unable to demonstrate any relationship between temperature change and REE and therefore we could not predict REE using temperature change.

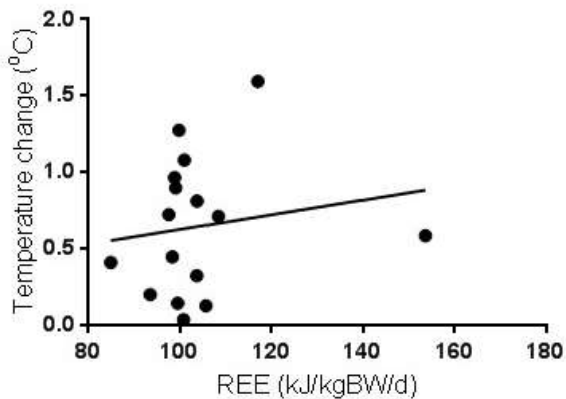


Figure 3.22 Relationship between temperature change and resting energy expenditure (n = 16, data expressed as means of temperature change and REE). The temperature change was the difference between the last time point of the cold exposure (5-10 minutes post-stimulus) and the time point immediately before the cold exposure (0-5 minutes pre-stimulus). Results from the arm-in-water experiments were used for the temperature change data; for those who underwent the repeated tests, the mean of the three tests were used. The REE was taken using the lowest and most stable 5 minutes without cold exposure and adjusted for body weight.

The mean cold-exposed REE was 2.656kJ/kgBW/d (SEM = 1.153kJ/kgBW/d), as seen in Figure 3.23. A linear regression was also calculated to predict cold-exposed REE changes based on temperature change. The regression was also found to be statistically insignificant ($F(1,14) = 0.062$, $p = 0.81$), with an R^2 of 0.004. Similarly, the absence of a relationship indicated that we could not predict REE change using temperature change.

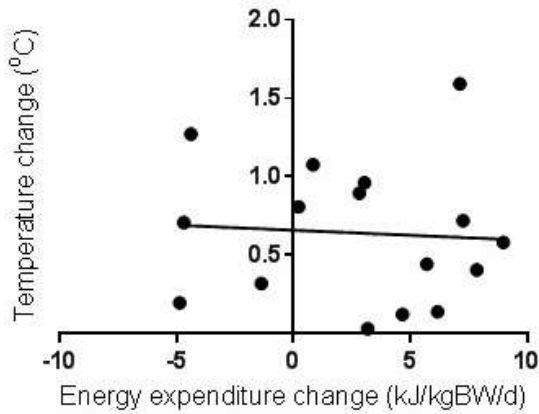


Figure 3.23 Relationship between temperature change and energy expenditure change following cold exposure (n = 16, data expressed as means of temperature change and REE change under cold exposure). The temperature change was the difference between the last time point of the cold exposure (5-10 minutes post-stimulus) and the time point immediately before the cold exposure (0-5 minutes pre-stimulus). Results from the arm-in-water experiments were used for the temperature change data; for those who underwent the repeated tests, the mean of the three tests were used. The change under cold exposure was taken using the difference between $REE_{\text{cold exposed}} - REE_{\text{baseline}}$. The REE of both conditions were taken using lowest and most stable 5 minutes before and following cold exposure.

CHAPTER 4. DISCUSSION

The use of IRT imaging has emerged as a promising method for estimating BAT volume. Current research evidence supports its use through its feasibility and practicality. However, the novel use of IRT remains as a relatively new field with only a few studies having explore different potential protocols. We hypothesised that as BAT has a thermogenic function which may be induced by exposure to a cold stress, the temperature change of the overlying skin may yield a reflection of BAT volume. We also hypothesised that as BAT thermogenesis occurs through the dissipation of energy as heat, inducing its activity using the same cold exposure technique may therefore increase the energy expenditure of an individual at rest. Furthermore, this increase in energy expenditure may be associated with the SCV temperature change as both of these changes are a function of BAT activity. As BMI is related to BAT activity, the relationship between BAT activity and REE under thermoneutral conditions of daily living was also of interest.

To answer these questions, along with future questions in human BAT research, a cost-effective, low-burden, and non-invasive method was necessary. In our study, we optimised an IRT imaging protocol to measure the overlying skin temperature change associated with BAT thermogenesis in the SCV regions. We compared this temperature change with energy expenditure both at thermoneutrality and under cold stress. We found that our established protocol was capable of measuring temperature change of the skin overlying SCV BAT depots which may reflect BAT volume. The findings presented in this thesis therefore have practical implications on future BAT research.

4.1 Part 1 – Preliminary Testing: Drafting the Protocol

The present study was the first project using IRT equipment in our laboratory. For this reason, preliminary testing included ensuring that the equipment were accurate and exploring optimal standard operating procedures to best fit our purpose. We first tested for the accuracy of the FLIR e60 thermal imaging camera by taking temperature measurements of a glass of hot water cooling at room temperature. The results showed a consistent decreasing trend which was sufficient to suggest appropriate function of the equipment. While it was straightforward to measure an inanimate object which does not move, the processing of data on human subjects needed refining as participants tended to move slightly throughout the experiment. Determining the technique for interpreting radiometric data consistently in participants was a priority.

Previous research have used software programmes such as FLIR ThermaCam QuickReport 1.2 proprietary software (Wilsonville OR, USA, FLIR Systems) (11), FLIR Researcher Pro 2.10 (10), FLIR Research IR Professional Analysing Software (Wilsonville OR, USA, FLIR Systems) version 1.2 (8) and version 3.3 (7). All of these software packages are able to interpret and express radiometric data as units of temperature. The most recent method of interpreting IRT data in research is the use of video imaging with a seeded region growing technique which uses an algorithm to detect a central region of hot spot (7). However, the study identified that this technique was highly time-consuming as each data point of 5 seconds contained 150 frames, all of which had to be processed manually. Another method IRT processing is the use of the hottest 25% of the region of interest, including the SCV fossae and

sternocleidomastoid muscles, to determine a change in the average temperature (10). Similarly, the upper 10th percentile of the entire neck and upper thorax region can also be used to compare temperature change of the skin overlying SCV BAT depots (11). The temperature change can also be quantified by visually placing a measurement area tool of a fixed size onto the region of interest of each image (8). It is also important to note that Jang et al. (8) highlighted the necessity for a control region to account for general changes in skin temperature that may otherwise confound results. In our study, the video imaging seeded region growing technique was forgone due to the time-consuming process as well as that we were unable to identify a suitable software programme that were compatible with our intended of the video imaging data. In our preliminary studies, we explored the other methods of data interpretation and selected the one that was most appropriate to further optimise. The exploration of these methods are detailed below.

To allow for the assessment of different image processing techniques, a protocol to induce BAT activity was required. It is suggested that the rate of BAT detectability by PET/CT in a thermoneutral environment is about 5-10% and increases to over 90% upon cold stimulation (80). Indeed BAT activity is significantly related to the level of cold-induced thermogenesis (171), and therefore, its cold stimulated FDG uptake would better represent BAT volume than under basal conditions. Furthermore, the use of IRT in the detection of cold-induced skin temperature change of SCV BAT depots is validated with PET/CT imaging of the cold-induced FDG uptake in BAT, showing that IRT imaging is able to reflect BAT volume (8). The SCV area in which skin temperature is elevated in IRT images is also consistent with location of BAT identified using FDG-PET/CT imaging (11). As seen in previous studies, the effect of cold exposure on SCV temperature demonstrates a measurable change by IRT imaging only under a cold environment at 19-20°C, but not warm environments at 25-26°C (11). These findings indicated that the use of IRT to measure skin temperature change of BAT regions in our study was appropriate. A cold induction protocol similar to that adapted from Symonds et al. (11) was used. This protocol consisted of the submersion of the hand in water maintained at 16-18°C for 5 minutes as BAT thermogenesis can be observed within that time span (11). Although, the protocol described in Symonds et al. was not validated with PET/CT imaging, it did demonstrate the already known relationship between age and BAT activity (169).

We used the threshold analysis, which compares the number of pixels meeting a predefined temperature threshold in the images over time, in a series of experiments using this cold exposure protocol. The threshold analysis was similar to the method used in the study by Symonds, et al. (11) which also took the hottest 10th percentile of pixels in the neck and upper thorax region. An increase in the area of the threshold temperature was observed with the introduction of cold exposure. However, it was identified that this temperature had started to trend upwards prior to the cold exposure. This was speculated to be an effect of the rapid loss of heat following the participant changing into a singlet from warmer clothing. Therefore, it was necessary to consider the fluctuations in SCV temperature from the moment the participant was in the seated experimental position. We were unable to find any reports of the time needed for body temperature to stabilise in previous research. Our assessment of this showed that threshold temperatures started to stabilise after 10 minutes in the seated experimental position.

However, from our pooled data of the protocol optimisation and reproducibility assessment, it appears that the second pre-stimulus time point was consistently higher than the first pre-stimulus time point. This finding shows a trend of minor temperature increases during the pre-stimulus state, suggesting that perhaps a 10-minute rest period was almost but not enough to stabilise the body temperature. Therefore, it is important for future experiments following this method to re-evaluate the time needed for the stabilisation of body temperature to ensure that adequate time is allowed for more accurate measurements.

The threshold analysis also presented with an issue in some of the experiments, which showed a rapid transient decrease in the SCV temperature immediately after cold exposure. This was concordant with the general cooling of the body surface as described in previous research (8). However, in the study by Jang et al. (8) the general cooling of the skin was measured at 60 and 120 minutes in a cold, air-conditioned room. However, the transient decrease in SCV temperature seen in our experiments occurred at the first 1-2 data points post-stimulus, which was only over the first 2 minutes. This observed effect may suggest that it was just be a result of measurement errors or the inherent fluctuations in skin temperature. However, this highlighted the need for a negative control to account for such confounding errors. The mediastinal region, an area not known to demonstrate BAT activity was selected as a negative control for every image processed in our study. The need for a negative control and well as the use of the mediastinal region as the control region was also reported in the study by Jang et al. (8). However, our threshold analysis was unable to successfully quantify the temperature of the negative control as it was unable to quantify temperatures below the predefined threshold. Indeed, the difference between SCV and chest skin temperature is suggested to reflect BAT volume better than the SCV temperature alone (8). For this reason, the ImageJ analysis, which uses a fixed shape to quantify the mean values of an area on the images was selected to be optimised. Furthermore, the advantages of ImageJ include its free cost to download, accessibility with the use of a computer, and is easy to use.

4.2 Part 2 – Optimisation of Protocol

4.2.1 Water temperature

The temperature of the cold water stimulus was of interest as each temperature may confer a different level of cold stress. In previous IRT imaging studies, a water temperature of 18-20°C was exposed to the hands or feet and yielded measurable changes in SCV skin temperature (7, 10, 11). As we hypothesised that a greater cold stress may confer greater inducibility of BAT activity, a water temperature of 16°C was compared with that at 20°C. In our study, we were able to demonstrate a measureable change in SCV skin temperature with a cold stimulus temperature of 16°C but not at 20°C. As this test was only analysed for one participant, we are unable to make an inference to suggest that the 20°C water temperature is insufficient to induce temperature change. However, during the experimentation of submerging the hand in water, participants reported no shivering or major discomfort at 16°C. It is also likely that the hand alone did not provide enough contact with the cold stimulus under the confounding effects of our testing conditions such as clothing of the participant, ambient room temperature, and air circulation. Therefore, it appears pragmatic to use the greatest tolerable cold

stress, which includes a colder temperature along with exposing greater skin contact with the stimulus, to assume maximal inducibility of BAT activity.

4.2.2 Room Temperature

The term “thermoneutrality” in describing the range of temperatures not known to induce a thermoregulatory response in BAT research is relatively arbitrary. In previous studies, ambient room temperatures regarded as thermoneutral showed both a positive relationship and no relationship between BAT and REE (80, 82, 83, 120). These findings in literature suggest that even temperatures regarded as thermoneutral may still confer a mild cold stress, in turn resulting in the basal activation of BAT. In IRT imaging studies, ambient room temperatures ranging from 19-24°C demonstrated an inducible effect of BAT activity under cold stress (7, 8, 10, 11), thus indicating that basal activity is not reflective of its total capacity and that change maintains capable of being measured. However, the absolute skin temperature of the SCV region at baseline cannot be compared between studies, as different methods of analysis were used. Therefore the level basal stimulation at the different thermoneutral temperatures remains unknown. This highlights the need to address the influence of different thermoneutral temperatures on basal BAT stimulation. However, we were unable to successfully manipulate room temperature due to the combination of having inadequate air-conditioning equipment, as well as the lack of control over the central air-conditioning system of the building complex. For this reason, the basal stimulation of BAT was assumed to be minimal or at least constant between the individuals.

4.2.3 Measurement Distance

We found that the distance between the camera and the participant did not influence the results of skin temperature change in the SCV region. Therefore, the distance of 1 metre was chosen as the standard measuring distance for practicality, as it was able to capture the regions of interest of larger participants. In retrospect, the distance between participant and camera did not have much significance in the measurement of temperature change. As each participant acts as their own control and our method of analysis uses the mean of a defined area, producing consistent images of participants is thus more important in the quantification of temperature change. Therefore, adjusting the distance for each participant to capture the greatest resolution of the regions of interest in future studies may be more beneficial than using a predetermined distance.

4.2.4 Variability of Temperature at Baseline

During our experiment, images were taken every 30 seconds for 20 minutes to capture the rate of change in temperature. The individual data points at these times showed significant variability, suggesting the need to reduce the noise of the data. In the study by Symonds et al. (11), images were taken every minute from -2 to 6 minutes relative to the time of cold exposure. However, there was not much variability between the data points as they demonstrated a consistent increasing trend. The discordance in the variability of time points between our findings and that of the study by Symonds et al (11) may be attributed to the different analysis techniques used. Previous studies have also used time points that were spaced further apart, ranging from 5 minutes to 60 minutes (7, 8, 10). It is likely

that by allowing for a longer time between data points, the magnitude of the induced BAT activity would override the variability otherwise seen in data points closer in time. By plotting the individual time points in our data set, there was a consistent increasing trend following cold exposure, despite the variability between the 30 second time points. To adjust for the variability between each data point, the timeframe of the experiment was divided into four groups to compare the mean values at each of these time points. However, the slight increasing trend of temperature pre-stimulus resulted in the second baseline time point being greater than the first. Although this was the case, the relatively significant increases in temperature following cold exposure indicated that it was still appropriate to use the data from the second time point. Furthermore, the increasing trend of temperature at baseline appears consistent with findings from previous studies, where the ambient room temperature regarded as thermoneutral resulted in BAT-associated increases in energy expenditure (80). Therefore, this upward trending temperature at baseline suggests that the SCV temperature was not completely stabilised, highlighting the need to re-evaluate the stabilisation time required for future studies.

4.2.5 Determining the Measurement Area of the Regions of Interest

In our study, we highlighted that when defining the region of interest of the SCV region, it was important to include more than just the SCV fossae alone. While previous studies have defined this region by using the SCV fossae with the sternocleidomastoid muscle (10) or simply an area within the SCV fossae (8), our study found that we were unable to demonstrate an increase in temperature following cold exposure by measuring this region alone, even with adjustment of a control region. In our measurement of the SCV fossae, the left side was used as it was previously reported to demonstrate a higher temperature than the right side (8). Our comparisons of the two sides of SCV fossae also supported this notion, with the left SCV fossa displaying a higher temperature. Although, this difference is suggested to be unrelated to BAT or the cooling method, but rather the anatomical positioning of the capacious left branchiocephalic vein (8). We demonstrated that by sampling an area which included SCV fossae along with the neighbouring cervical SCV region between the two sides, a SCV temperature change could be then be detected. This may be due to the quantifiable transfer of heat to the neighbouring cervical regions, as well as the contribution from BAT depots scattered in the neck regions (169, 176). Furthermore, as the overlying skin temperature increases as a result of BAT, measuring the spread of this increase in temperature may be more representative of the magnitude of BAT activity compared with the area of skin directly overlying BAT depots.

4.2.6 Exposure location of cold stimulus: Arm versus Feet

The present study showed that an increase in the skin temperature overlying SCV BAT depots can be induced by submersion of a limb in cold water maintained at 16°C and the measurable thermogenic activity may reflect BAT volume. This finding is concordant with previous studies that have also used the limb-in-water method as cold stimulation (7, 10, 11). In the existing literature, other forms of non-invasive cold stimuli have been utilised to estimate BAT volume, including air-conditioning control of room temperatures (8, 9, 80, 120), drinking a glass of ice cold water (197), intermittently resting a leg on an ice block (82) or a liquid-controlled tube suit (83). We explored the limb-in-water technique as it

was the most practical method of inducing cold activation of BAT thermogenesis. Furthermore, this method was the most flexible as it can be done in almost all settings and without requiring accessibility to certain equipment such as an air-conditioning unit or liquid tube suits.

The hands and feet also have other advantages as the chosen locations of cold exposure. The surface area to mass ratio for the hand and feet are respectively, 4-5 times and 2.5-3 times greater than the rest of the body (198), thus providing an easily accessible yet sufficient means of delivering cold stress. While the initial response of cold exposure is strong vasoconstriction (199), in the case where core temperature is normal or raised, insufficient tissue perfusion and heat loss impairs the contractility of vascular smooth muscles (198). The resulting response is vasodilation which is proposed as a defence mechanism against tissue damage from cold injury (198). Due to this physiological response, these areas provide an effective means of heat exchange with the environment. Furthermore, thermal sensations are recognised in the somatosensory cortex, where a large volume receives feedback from the hands, feet, and toes (198).

Our study found that both of the cold exposure locations, the right arm or both feet, were equally capable of inducing a significant change in temperature. Between the location groups, no difference in temperature change were found. It is likely that the greater surface area exposed for the feet did not elicit a greater response because the surface area of the exposed arm was already sufficient for the maximal stimulation of BAT. Although, while the differences between arm and feet were not statistically significant, the arm group showed a slightly higher mean than the feet group. This may be explained by the anatomical and physiological differences between the hands and the feet. Under thermoneutral conditions, blood flow to the hand is 3-4 times greater than the foot and is around twice greater once adjusted for the skin surface area (198, 200). This difference in blood flow may result in differences in the heat lost between the two limbs during the first moments of cold exposure. In addition, the hand also provides slightly more powerful sensory feedback to the somatosensory cortex than the feet and toes (198). The combination of these differences may explain some of the differences in BAT response, however, this is only maintained from a theoretical viewpoint.

Interestingly, when comparing the two post-stimulus time points within the groups, there was a difference in the arm group but not the feet group. However, as discussed above, no significant differences in the temperature of the final time point were observed between the arm and feet groups. As the final point of the two groups were similar, a plausible explanation is that the arm group may have taken longer for BAT to reach the same level of activity. Which would be concordant with the larger area of the feet group.

4.3 Part 3 – Reproducibility of the Protocol

This study showed that the proposed protocol for estimating BAT volume was reproducible as it yielded consistent results through the repeated tests by showing no differences between them. When looking at the individual experiments for each participant, most participants show consistent temperature changes across each testing while some show more variable results. This finding is similar to some of the discordance found between FDG-PET/CT scans of BAT in previous research. Jang et al., (8)

reported that of 12 participants that were studied twice, only 10 showed consistency in PET/CT scans. These results were considered discordant based on whether the uptake met the criteria of being BAT-positive or BAT-negative. Although, it is worthy to note that for the repeated scans that were concordant in being BAT-positive or –negative, the differences in FDG uptake may still vary within its classification. Therefore, slight variations between tests may be inevitable as it is BAT activity, rather than BAT mass that is being measured. In our study, the inconsistencies of temperature change mainly came from the middle time points. However, this did not result in significantly different absolute temperature changes at the final time points. Individual results also show observable trends in support of this finding. These observations suggest that the variability may lie within the rate of thermogenesis, rather than its absolute capacity. Therefore, in order to accurately measure the capacity of thermogenesis with consistency, emphasis must be placed on ensuring that BAT activity is sufficiently stimulated.

While there were no statistically significant differences between the three repeated tests, the AUC of SCV temperature appear to be highest in the first set of experiments, followed by the second, and third. The variability of this appear to show similar trends with the outdoor temperatures at the time. It is known that BAT activity is inversely correlated the outdoor ambient temperature at the time of testing as detected by heat production and FDG uptake, demonstrating higher activity in the winter months than the summer months (100, 101, 169). Similarly, the regression of recruited BAT can also occur as the ambient temperature gets warmer, as detected by FDG-PET/CT imaging (172). In our study, the first set of repeated tests were conducted in July, while the second and third tests were conducted in late August and early September. The visually observed decline in mean AUC through these repeated tests appear in concordance with the outdoor temperatures of Auckland through these months, where July was the coldest, followed by August, then September (201). These trends in the increase of SCV temperature, albeit not significant, appear to reflect the plasticity of recruited BAT as the climate temperature rises.

The estimation of BAT with IRT imaging has previously been assessed by benchmarking with PET/CT under cold stimulation. It is suggested that a BAT-positive PET/CT scan can be predicted by a left SCV fossa temperature change of over 0.9°C, with a sensitivity of 77% and a specificity of 78% (8). While for the right SCV fossa temperature, the same change conferred with a sensitivity and specificity of 59% and 89%, respectively (8). We found a difference in the absolute temperature changes between participants, indicating that there are differences in BAT activity that may be classifiable into BAT-positive and –negative groups. In our study, the temperature of the SCV region taken included the neck region between the SCV fossae, meaning that this temperature would be lower than that taken solely from the SCV fossae. Nonetheless, in the present study, the means of the repeated tests of each participant can visually be classified as having either a strong or weak BAT response based on the 0.9°C temperature change criteria. Upon the categorisation of BAT-positive and BAT-negative using the 0.9°C cut-off point, the BAT-positive group showed significantly greater increases in temperature than the BAT-negative group. The temperature of the BAT-positive group increased by 0.97 to 1.42°C, while the BAT negative group increased by 0.24 to 0.63°C. There may be several factors influencing the variability in BAT responses between our participants. As well as the outdoor ambient temperature,

as discussed earlier in this manuscript, the recruitment and regression of BAT may also be influenced by other factors. These factors include BMI, ethnicity, age, beta-blocker use (100, 102, 169, 202). Although, the pertinent medication of beta-blockers was in our exclusion criteria, and our participants were mostly university students that were similar in age, therefore these two factors would not contribute to the variable BAT responses. A likely factor may be the activity level of the participant, as emerging evidence suggest that human BAT may be activated and recruited with exercise (119). Evidence from mice studies also suggest a potential role of nutrition in BAT recruitment, where dietary restriction in conjunction with exercise induces gene expression changes in BAT (203). However, as these factors were either unknown or not analysed, they can only be maintained as hypothetical contributors to the variability in the level of BAT responses in our study.

While the thermogenic responses vary between individuals, all individuals demonstrated an increase in the SCV temperature of $>0.5^{\circ}\text{C}$ in at least one of the repeat tests. It is suggested that there is high prevalence of SCV BAT in adult humans regardless of PET/CT imaging status, as supported by histological and molecular evidence (204). While there may be up to 15- to 20-fold more BAT in BAT-positive individuals than BAT-negative, the almost universal presence of BAT is indicated by signature transcripts and UCP1 immunoreactivity (204). This may explain the increased detectability of BAT under cold exposure (80). When looking at the individual data of the repeated tests from our study, only two tests showed a decrease in the temperature change, both of which were from the BAT-negative group. By arbitrarily using an increase greater than 0.2°C to benchmark the presence of the thermogenic activity of BAT, up to 12 of the 15 repeated tests between 5 BAT-negative participants demonstrated a temperature change to this extent. Therefore it appears that our findings support the notion that BAT function is indicated in almost all adult humans.

4.4 Part 4 – Resting Energy Expenditure

As previously discussed, the thermogenic function of BAT occurs through the dissipation of energy as heat, thereby contributing to energy expenditure. Marked elevations in energy expenditure should therefore be observed when participants are exposed to a cold stress. This study found that cold exposure by the submersion of the feet in cold water was able to induce a significant increase in the REE in the absence of shivering, therefore indicating the likely presence of non-shivering mechanisms of thermogenesis. During the experiment, once the participants' breathing had stabilised after the introduction of the cold stimulus, REE remained elevated. This is expected as cold exposure would induce a thermoregulatory response to maintain core body temperature, such as the non-shivering thermogenesis of BAT through energy dissipation (114), thereby increasing energy expenditure to do so (205). This finding is similar to that previously demonstrated in research, where cold exposure increased the REE of participants either using a climate chamber, cold water limb submersion, or by a liquid-conditioned tube suit at 18°C (80-83). Furthermore, the absence of shivering in the presence of elevated REE under cold exposure indicates the non-shivering thermogenic function of BAT metabolism (82, 83). Our study also found that cold-induced changes in REE demonstrated high variability between individuals, similar to that reported previously (80, 205). These differences may be attributable to the factors that also affected the variable levels of BAT responses seen in our IRT imaging data.

In our study, we did not find a significant relationship between BAT activity and REE at thermoneutral environments. This was not unexpected as for basal REE in the absence of cold stress, some studies suggest no relationship with BAT volume (82, 83, 120), while others suggest a positive correlation (80). This discrepancy seen in the current literature may relate to the activity of BAT under the experimental conditions in these studies, such as the ambient room temperature as the detectability of BAT increases dramatically under cold stimulus (80). Indeed, in the study which demonstrated a relationship between BAT activity and basal REE, REE was measured under an ambient room temperature of 22°C which was regarded as thermoneutral (80). In the studies that did not demonstrate such relationship, REE were measured under warm conditions of 25°C in one study (83) and 27°C in the others (82, 120). This finding highlights that even an ambient temperature of 22°C that is often regarded as thermoneutral poses a minor cold stress which may result in basal BAT activity. However, it also suggests that this temperature of 22°C is thermoneutral enough to allow for further BAT stimulation. As the presence of BAT is present in almost all individuals (204), it is likely that we did not observe a significant relationship due to the inactivated state of BAT at warmer ambient room temperatures. This appears to be the case as our REE experiments were conducted in an ambient room temperature of 25°C and above.

On the other hand, it is widely suggested that the cold-induced elevation in REE is positively correlated with BAT volume (80-83). In our study, we found an overall increase in the skin temperature overlying the SCV region, as well as an overall increase in energy expenditure following cold exposure. However, contrary to our expectations, we also did not find a significant relationship between BAT activity and the cold exposed elevation in REE of individuals. There may be several reasons as to why this was not observed in our study. While we adjusted REE for differences in body weight, the adiposity of the individuals were not accounted for. As REE is largely predicted by fat-free mass (65, 72), it was thought that adjusting for body weight alone may not accurately represent the cold exposed changes in REE in a group of participants with high individual variability in body fat percentage. We adjusted for adiposity using BMI as BMI is inversely associated with BAT activity (10, 100). This inverse relationship would theoretically mean that the higher the adiposity of an individual, the lower the BAT activity and consequently a lower REE under cold exposure. However, we did not find a relationship between BMI and SCV temperature change ($p = 0.51$) or cold exposed elevation of REE ($p = 0.13$). Therefore, it is unlikely for BMI to be responsible for the absence of a relationship in our data. However, in retrospect, as the cold exposed elevation in REE is assumed to be a function of BAT oxidative metabolism, it appears unnecessary to adjust for the body weight to quantify BAT activity. Despite this, after un-adjusting for body weight, the relationship between temperature change and REE change remained insignificant ($p = 0.76$). Furthermore, a likely explanation as to why we did not see the expected correlations between IRT imaging of SCV temperature change and cold-induced elevations in energy expenditure is the timing of the experiments. For practicality reasons, our study design included the measurement of energy expenditure within the hour after the IRT imaging, where the participant was already exposed to the cold stress. Research evidence suggests that in lean individuals, the cold-induced increases in energy expenditure remain elevated during an hour of rewarming (206). As the majority of our participants in this experiment were classified as being lean using BMI data, this effect

from the experimental timing may be the primary reason for the absence of a relationship in our linear regression analysis.

An interesting speculation from our REE data is that some participants experienced a decrease in energy expenditure after the introduction of cold exposure. This finding appears contradictory to the body's thermoregulatory response as maintaining core body temperature would increase the energy expenditure through thermogenesis. An explanation which could explain this finding is that some young adults (along with most elderly adults) show a decrease in non-shivering thermogenesis to mild cold exposure, attributable to a decrease in cellular metabolism of the cooled tissues (205). While this may account for the observed decreases in energy expenditure following cold exposure, measurement errors may also contribute to this finding, as well as affecting the accuracy of each data set in general. These measurement errors include changes to the participants' breathing, such as falling asleep, or becoming more calm and relaxed through the progression of the measurement (67). This is further discussed in the limitations.

4.5 Limitations

There were several limitations within this study that may have impacted on our findings. Firstly, it should be acknowledged that our sample size was small for each experiment ($n = 17$ for optimisation, $n = 11$ for reproducibility, $n = 16$ for REE). While there were no significant outliers, our findings were subjected to the bias of a small sample size. Type II errors for our linear regression may have also occurred as the preferred sample size for a regression analysis is over 20 subjects per variable.

A limitation with the use of the IRT camera was the need for manual focus adjustments as this was not automated. The focus of the image depicted its resolution and quality of the measurements. The manual adjustment therefore led to slight differences in focus between participants, and to a small extent, between the images of individuals due to body movements. However, as our parameter of interest was temperature change in the same individual, the differences in focus between participants were less important for the sole purpose of demonstrating consistency. Nonetheless, this limitation becomes more significant when comparing absolute temperature differences between tests. As the focus requires user adjustment, we therefore have to assume that the focus is adequately adjusted at the beginning of each testing and ensure that it is not altered during the experiment.

The primary limitation with IRT is that the movements of the individuals between each image taken would affect its consistency as discussed earlier on in this manuscript. These movements affected the consistency of the images in different ways: 1) although probably insignificant, slight movements towards or away from the camera would therefore affect the resolution to some degree, 2) movements of the torso in the seated position would change the plane of the SCV region and therefore the area captured on the image, 3) movements of the head may cover the neck area sampled as part of the SCV region, such as the drop of the chin covering the neck, and 4) movements of either shoulder would change the anterior outline of the trapezius which defined the sampled area during analysis. While all participants were instructed to choose a comfortable position which they were able to maintain for 20 minutes, slight movements were inevitable, despite the ongoing correction of these movements during

testing. Therefore, the researcher should be vigilant in correcting the ongoing movements of the participant.

The temperature of the clinical rooms used for IRT imaging was also a limitation as they fluctuated between 21-24°C depending on the time of day. This parameter was a limitation as we were unable to control the centrally controlled air conditioning. While this temperature range was considered as thermoneutrality, the warmth of this range may have implications on the basal activity of BAT (80), and its inducibility by the cold stimulus. The higher the basal activity of BAT, the closer it is to its maximal activity, therefore underestimating the thermogenic capacity. It is also plausible that when ambient temperature was higher, the BAT took longer to exhibit the same response induced from the cold stimulus or that BAT had a less sensitised response to the cold stimulus, and vice versa. Although, the rate of BAT activity under cold stimulus under different ambient room temperatures has not been studied, therefore, this mechanism remains as a potential hypothesis. Furthermore, the warmth relative to the participant may also be affected by other factors, such as the insulating capacity of their pants on different testing days. To minimise this limitations, a full set of standardised clothing for the experiments should be provided and the room temperature recorded.

Another limitation of the study was its long duration which spanned from April to September. The first set of the repeated experiments were conducted in July and the subsequent sets were conducted in late August and early September. The implication of this was the change in season and subsequent cold exposure experience by the participants in their daily lives. With August being the last month of winter, it is possible that participants experienced more cold exposure during this time. As recent studies suggest evidence for the cold-acclimated recruitment of BAT (120, 171, 197), this month-long delay between the first and subsequent repeated tests may expose participants to more cold stress and augment the magnitude of the activity being measured. Indeed, the seasonal variation in both non-shivering thermogenesis and cold-activated BAT suggests the plasticity of BAT to the changing climate (100, 101). Similarly, during this period participants may also have worn more clothing and perhaps stayed warmer, thus resulting in any possible regression of BAT recruitment on the basis that BAT activity experiences high plasticity. While it is not always feasible to do so, repeated measurements should be conducted during a shorter period of time to avoid such changes in BAT.

For practicality reasons, the REE experiment for a majority of the participants were conducted after IRT imaging experiment. The time between these consecutive experiments was about 20 minutes apart. While in our preliminary experiments, we demonstrated that body temperature started to stabilise at around 10 minutes, this was under thermoneutral conditions with a mild cold stress which was heat loss from their clothing upon changing into a singlet. The stabilisation of BAT activity following prolonged cold exposure used in our IRT imaging experiment was not assessed. However, as there is evidence suggesting the continued elevation in energy expenditure for 60 minutes following cold exposure (206), measurements requiring cold stimuli should be conducted on separate days until there is further research to recommend an appropriate wash out period.

Lastly, the accuracy of REE measurements require the participant to be rested, relaxed, including the mental and emotional state as well as familiarity with the testing procedure (67). The major barrier to this requirement is the continuous stimulus of the cold water exposure, whereby participants must keep a calm mental state and remain unaroused through the duration of cold stimulus. As we were unable to assess the difference in arousal levels between the pre- and post-stimulus states, its likely contribution to our data remains hypothetical. To minimise this, a practise measurement may be conducted with the participant prior to the actual experiment, although this may be time-consuming.

4.6 Conclusions

Recent findings in research show promising evidence for human BAT as an anti-obesity target, resulting in its increasing popularity in research over the last decade. Its growing interest has led to the development of different methods in its measurement in human subjects, as the current gold standard using FDG-PET/CT scan is costly and invasive. Of these methods, IRT imaging is a non-invasive technique which shows potential for measuring BAT volume. Therefore it is important that future research is dedicated to optimising and validating the use of IRT imaging as well as understanding the implications of BAT on energy balance and obesity in humans. The IRT protocol developed in our study was practical, low-cost, low-burden, and demonstrated accuracy and reproducibility. Our protocol was able to induce and detect an increase in the temperature change of skin overlying the SCV BAT depots as well as that its accuracy and consistency is demonstrated by its reproducibility. In consideration of all these aspects, overall the use of our IRT imaging protocol appears appropriate for estimating BAT in human subjects. However, we were unable to demonstrate a relationship between BAT activity and energy expenditure using this protocol, which was likely to be confounding error due a carryover effect from the IRT imaging experiment, resulting in the continued elevation of energy expenditure.

4.7 Future Directions in Research

An identified barrier in our study, as well as other studies that explored IRT imaging protocols, methods of analyses were heavily user dependent. This subjects the results to human error as well as having the potential to be a time-consuming process. Future research involving computer algorithms that minimise user dependence is required to allow for greater reproducibility and larger sample sizes. The ambient temperature range regarded as being thermoneutral to human BAT activity will also need further research, as basal BAT stimulation observed in some of these temperatures may confound findings in future studies. Larger studies to validate the use of IRT with the current gold standard of FDG-PET/CT imaging, as well as the non-shivering thermogenesis-related increases in energy expenditure are required. Future research in these areas are integral to provide further understanding in the role of human BAT in energy metabolism in order to exploit BAT as an anti-obesity target.

APPENDICES

Appendix A: Recruitment Poster



Department of Molecular Medicine & Pathology
Faculty of Medical and Health Sciences
+64 (0) 9 923 6733
The University of Auckland
Private Bag 92019
Auckland, New Zealand

How much **brown fat** do you have?

Healthy volunteers wanted

Dr Troy Merry (Supervisor) and Henry Lin (Masters Student) are looking for people aged 12-14, 18-25, and 50+ years to participate in a study for brown fat research. In this study, we aim to develop a technique for measuring brown fat. The experiment will take approximately take 2 hours on 1-4 separate occasions at The University of Auckland Grafton Campus.

Benefits of participation:

- Measurement of your **brown fat**
- Measurement of your **energy metabolism**
- Measurement of your **body composition** (muscle mass, body fat, bone health)
- Contribute to health research



If you would like to find out more about what brown fat is, how fast your metabolism is and the muscle and fat percentage of your body, please email Henry Lin at:

Email: plin594@aucklanduni.ac.nz



APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 21ST March 2017 FOR (3) YEARS REFERENCE NUMBER 018504

Establishing a Non-invasive Technique for Measuring Brown Adipose Tissue Volume, Part I: Developing a Protocol

PARTICIPANT INFORMATION SHEET

Name of researcher: Henry Lin

Name of supervisor: Troy Merry

Researcher Introduction

I am Henry and I am a student studying towards a Master of Health Science at the University of Auckland. My supervisor is Troy Merry, who is a researcher at the University of Auckland, Department of Molecular Medicine and Pathology.

We invite you to participate in a pilot study aimed at measuring brown fat. Your participation in the research is entirely voluntary (your choice). If you are interested and agree to take part, you may contact the investigators to withdraw from the study at any time, without having to give reason. You can also request that any data you provide for this research be withdrawn prior to the publication of any results.

1. Background

Brown fat is a type of fat which may have beneficial effects on reducing obesity and its related diseases. The main function of brown fat is heat production for the body when it is cold. Like many bodily processes, brown fat requires energy to produce heat, thereby contributing to total energy expenditure. As changes in body weight are a formula of energy in versus energy out, the more energy used by heating the body the easier it will be to lose weight.

2. What is the aim of the research?

The aim of the research is to establish an accurate, non-invasive technique for measuring brown fat.

3. Eligibility

You can take part if you are a healthy male or female between 18-35 years of age.

4. What happens if I decide to take part?

You will be required to visit the University of Auckland (Grafton campus) on 85 Park Road, Grafton for one to four occasions and each visit will last 0.5-2h. We will discuss this participant information sheet with you and if you wish to participate you will be asked to sign an informed consent form. You will be asked some questions about your health, height, and weight and wear a singlet before proceeding with the study. You will then be required to submerge parts of your arm/leg (to your elbow/knee) in a water bath at temperatures ranging 16-20°C for 1-30 minutes each bath. The cooling is necessary to stimulate the brown fat activity that we will be measuring. If you start to feel uncomfortable you will be free to remove your limb from the water. During these experiments, we will record the change in your body temperature on the neck region using a thermal camera and thermistors that will be attached to your neck with tape.

Note: A singlet is defined as a sleeveless shirt which exposes the arms, neck and the collarbone region near the neck. Both male and female participants are required to wear a singlet for more accurate measurements.

5. Who designed the study?

The trial was designed by research staff and students at the University of Auckland.

6. The risks and benefits of the research

There is little risk of experiencing an adverse event or injury through participating in this research. Participants may experience some minor discomfort associated with the cooling of limbs using the water bath. In the very unlikely event that there is any major adverse events, the Auckland City Hospital and emergency department is located within a couple of minutes of walk from the University of Auckland Grafton Campus.

The benefits include having your body heat production measured and having the opportunity to contribute to health research through participation.

In the unlikely event of an injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation, and Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Injury Prevention, Rehabilitation, and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator. You are also

advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.

7. Confidentiality

Research files, data, and all other information that you provide will remain strictly confidential. The data will be used for scientific publications and presentations. No material that could personally identify you will be used in any reports on this research. All computer records will be password protected. Upon completion of the research your records will be stored for 6 years in a secure place, before being destroyed by the principle investigator or co-investigators. If this is not possible for any reason the head of the principle investigators department or otherwise designated research will take responsibility for this process. A copy of your results will be given to you upon completion of the research at your request.

Thank you for your consideration in participating in this study.

For more information, please contact:

Henry Lin, Student Dietitian, The University of Auckland, 85 Park Rd, Grafton. Email: plin594@aucklanduni.ac.nz

Troy Merry, Supervisor, Department of Molecular Medicine and Pathology, The University of Auckland, New Zealand.

Telephone: 09 9239008

Email: t.merry@auckland.ac.nz

The investigators of this research are:

CONTACT DETAILS AND APPROVAL

Student Researcher	Supervisor	Head of Department
Henry Lin The University of Auckland plin594@aucklanduni.ac.nz	Troy Merry Department of Molecular Medicine and Pathology t.merry@auckland.ac.nz 09 9239008	Professor Peter Browett Head of Department of Molecular Medicine and Pathology p.browett@auckland.ac.nz +64 (0) 9 923 6281

For any queries regarding ethical concerns you may contact the Chair, The University of Auckland Human Participants Ethics Committee, The University of Auckland, Research Office. Private Bag 92019, Auckland 1142. Telephone 09 373-7599 ext. 83711. Email: ro-ethics@auckland.ac.nz

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 21st March 2017 FOR (3) YEARS REFERENCE NUMBER 018504

Establishing a Non-invasive Technique for Measuring Brown Adipose Tissue Volume, Part II: Validating the Protocol

PARTICIPANT INFORMATION SHEET

Name of researcher: Henry Lin

Name of supervisor: Troy Merry

Researcher Introduction

I am Henry and I am a student studying towards a Master of Health Science at the University of Auckland. My supervisor is Troy Merry, who is a researcher at the University of Auckland, Department of Molecular Medicine and Pathology.

We invite you to participate in a pilot study aimed at measuring brown fat. Your participation in the research is entirely voluntary (your choice). If you agree to take part, you may contact the investigators to withdraw from the study at any time, without having to give reason. You can also request that any data you provide for this research be withdrawn prior to the publication of any results.

1. Background

Brown fat is a type of fat which may have beneficial effects on reducing obesity and its related diseases. The main function of brown fat is heat production for the body when it is cold. Like many bodily processes, brown fat requires energy to produce heat, thereby contributing to total energy expenditure. As changes in body weight are a formula of energy in versus energy out, the more energy used by heating the body the easier it will be to lose weight.

2. What is the aim of the research?

The aim of the research is to establish an accurate, non-invasive technique for measuring brown fat.

3. Eligibility

You can take part if you are a healthy male or female in the following age groups: 18-25, and 50+ years. You must be willing to visit the University of Auckland (Grafton campus) and have parts of your arm or leg submerged in water baths, have breath sampled through a mouth

piece and have your body composition measured by a scanner. The duration of the visit may take up to 2 hr.

4. What happens if I decide to take part?

You will be required to visit the University of Auckland (Grafton campus) at 85 Park Road, Grafton for one occasion in the morning. You will be requested to come to the laboratory fasted, only consuming water after 10 pm the night before. We will discuss this participant information sheet with you and if you wish to participate you will be asked to sign an informed consent form. You will be asked some questions about your health, height, and weight and wear a singlet before proceeding with the study. You will then be required to submerge parts of your arm/leg (to your elbow/knee) to a water bath of a specific temperature between 16-20°C for up to 30 minutes. The cooling is necessary to stimulate the brown fat activity that we will be measuring. If you start to feel uncomfortable, you will be free to remove your limb from the water. During this experiment, your body temperature around your neck region will be measured with a thermal camera and with thermistors taped to your neck. You will also be required to breathe through a mouthpiece which will measure your energy expenditure. After this test you will be able to eat. This will be the first measure of the day. Lastly, you will also be required to undergo a DXA scan, which will give you information about your bone, fat, and muscle mass.

Note: A singlet is defined as a sleeveless shirt that exposes the arms, neck and the collarbone region near the neck. Both male and female participants are required to wear a singlet for more accurate measurements.

5. Who designed the study?

The trial was designed by research staff and students at the University of Auckland.

6. The risks and benefits of the research

There is little risk of experiencing an adverse event or injury through participating in this research. Participants may experience some minor discomfort associated with the cooling of limbs using the water bath or breathing through a mouth piece to measure respiratory gases. The DXA scan will expose participants to a small amount of radiation, but this is no more than the background radiation one would be exposed to on a daily basis. In the very unlikely event that there is any major adverse events, the Auckland City Hospital and emergency department is located within a couple of minutes of walk from the University of Auckland Grafton Campus.

The benefits include having your body heat production, fat-free mass, and bone mineral density measured as well as having the opportunity to contribute to health research through participation. Fat-free mass is an indication of one's adiposity, giving a more meaningful comparison of how much body fat an individual carries. Bone mineral density reflects the

structural integrity of your bones and may provide insight on the risk of developing osteoporosis.

It is possible that DXA scans can identify conditions as bone abnormalities, biliary and urinary stones, vascular and other soft tissue calcifications, and abnormal anatomical structures. However, as scans will not be reviewed in any diagnostic matter by medically qualified persons, it is very unlikely any of these conditions will be uncovered in you.

In the unlikely event of an injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation, and Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Injury Prevention, Rehabilitation, and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator. You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.

7. Confidentiality

Research files, data, and all other information that you provide will remain strictly confidential. The data will be used for scientific publications and presentations. No material that could personally identify you will be used in any reports on this research. All computer records will be password protected. Upon completion of the research your records will be stored for 6 years in a secure place, before being destroyed by the principle investigator or co-investigators. If this is not possible for any reason the head of the principle investigators department or otherwise designated research will take responsibility for this process. A copy of your results will be given to you upon completion of the research at your request.

Thank you for your consideration in participating in this study.

For more information, please contact the investigators of this research:

CONTACT DETAILS AND APPROVAL

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Henry Lin The University of Auckland plin594@aucklanduni.ac.nz	Troy Merry Department of Molecular Medicine and Pathology t.merry@auckland.ac.nz +64 (0) 9 923 9008	Professor Peter Browett Head of Department of Molecular Medicine and Pathology p.browett@auckland.ac.nz +64 (0) 9 923 6281

For any queries regarding ethical concerns you may contact the Chair, The University of Auckland Human Participants Ethics Committee, The University of Auckland, Research Office. Private Bag 92019, Auckland 1142. Telephone 09 373-7599 ext. 83711. Email: ro-ethics@auckland.ac.nz

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 21st March 2017 FOR (3) YEARS REFERENCE NUMBER 018504

PARTICIPANT CONSENT FORM

Title: Establishing a non-invasive technique for measuring brown adipose tissue volume

Investigators:

Troy Merry (supervisor), Department of Molecular Medicine and Pathology, The University of Auckland

Henry Lin, Student Dietitian, The University of Auckland

I have read the Participant Information Sheet; have understood the nature of the research. I have had the opportunity to ask questions and have them answered to my satisfaction.

- I agree to take part in this research
- I understand that I am free to withdraw participation at any time
- I understand the DXA scan will expose me to a small amount of radiation that is equivalent to the amount radiation I would be normally be exposed to on a regular day
- I do/do not wish to receive a summary of the findings (please strike out which does not apply)
- I understand that all the data collected for future analysis will be stored securely for 6 years following publication before being destroyed.

Name _____

Signature _____ Date _____

Email (only provide if you would like a copy of the research outcomes): _____

APPROVED BY THE UNIVERSITY OF AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 21st March 2017 FOR (3) YEARS REFERENCE NUMBER 018504

Establishing a Non-invasive Technique for Measuring Brown Adipose Tissue Volume

HEALTH SCREENING QUESTIONNAIRE

Participant Information

Name _____

Sex: M / F (please circle) Date of Birth (dd/mm/yy): _____

Anthropometry

Height: _____ cm

Weight: _____ kg

Medical History

1. Do you have a history of diabetes? YES / NO

2. Do you have a history of high blood pressure? YES / NO

3. Are you a smoker? YES / NO

4. Do you have a history of cardiovascular disease? YES / NO

5. Do you have any other medical conditions? YES / NO

If yes, please specify: _____

6. Are you currently taking any medications? YES / NO

If yes, please specify: _____

7. Please list any family history you are aware of:

Fitness Level

1. How many days did you exercise on the week of your testing for at least 30 minutes? (*Exercise includes any structured recreational physical activities that elevates your heart rate)

0-1

2-3

4-5

6+

2. Please list the type of activity you did last week, as well as how long you did the activity.

3. How INTENSE would you rate your previous workouts in the month of your testing? In other words, how HARD was your body usually working?

- very intense worked hard an average workout a light workout

4. Are the previous questions representative of your typical workouts over the past 6 months?

- Yes No

- If no, why is this not a typical example of your workout history?

5. Please list the activities/athletics you have participated in over the previous 5 years. Indicate what level you participated in each event (National, Atlantic Region, Provincial, or Recreational.)

ACTIVITY	LEVEL	YEAR

Parvo True One Metabolic Cart: Setup and calibration

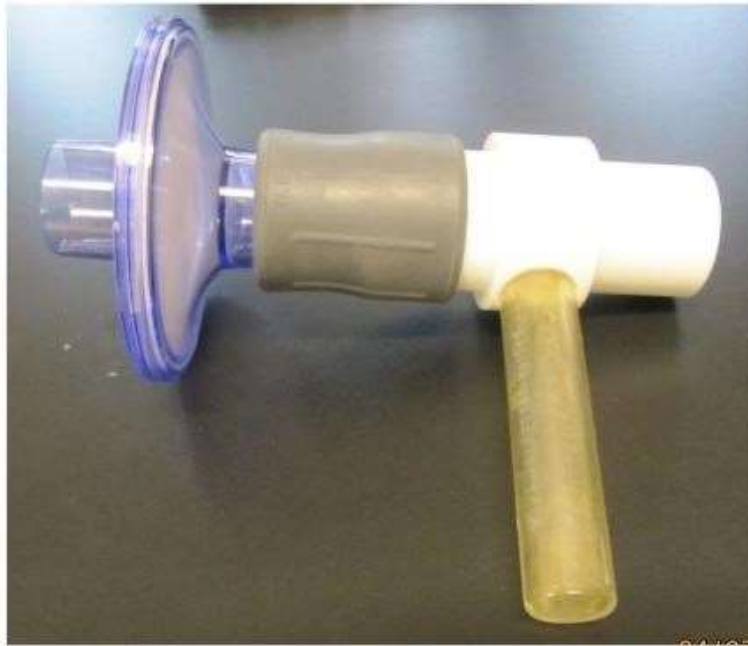
1. **AT LEAST 45 MINUTES** prior to your participant arriving turn on:
 - Main power switch (green switch) on the back of the unit
 - Pump/Heater switch on the front of the unit (circle with dot in the middle is the On position)
 - Computer and True one software



2. Turn on the device used for testing.

3. Set up metabolic cart for testing:

Connect the purple filter to the T-connector using the grey connector. Gently push the grey connector on to the T-connector. Then gently push the purple filter into the grey connector. Ensure the tapered side of the purple filter is the side being attached.



Attach this assembly to the pneumotach. Again all that is needed is slight pressure to make the following attachment. Ensure that the mixing chamber (tinted rectangular box) does not fall to the floor. Grab the white connector (not the round oven) of the pneumotach and gently push the assembly (purple filter end) together to make the connection.



The hose can be attached to the T-connector assembly.



The hose can be attached to the reticulated arm using Velcro straps. The black device (with the orange dot) is the heart rate monitor receiver (the chest strap on the subject is the transmitter). The result will be something like this. The weak point is the hose mating with the T-connector. Ensure a good connection. If this separates during testing, continue testing and re-attach as quickly as possible



4. Calibration: Should be done 30 minutes after turning metabolic cart on.

The smaller grey connector, that is smooth on the inside with a ridge halfway down the middle, is used to interface the calibration mouthpiece and the 3 litre syringe. Attach this to the syringe.



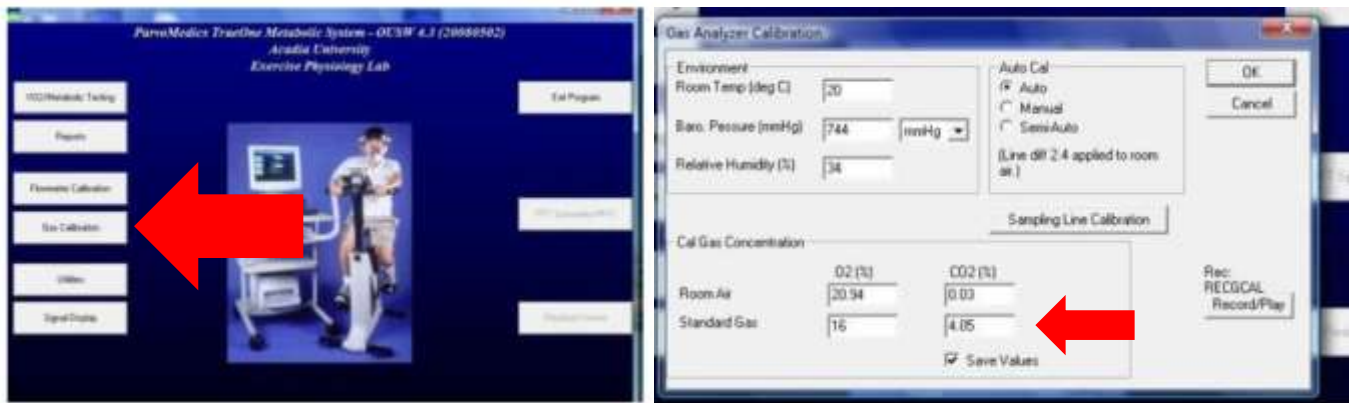
Attach the two way valve to the calibration syringe. Ensure you are on the output valve when making this connection.



Gas Calibration

On the home screen click on gas calibration and follow the instructions. Temperature, humidity and barometric pressure is found on the weather station next to the computer.

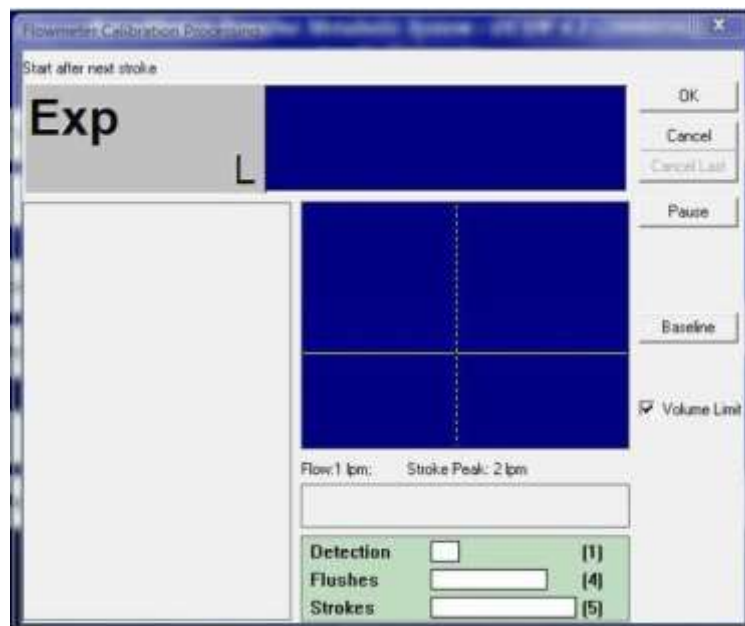
On the calibration gas bottle there is the percentage on carbon dioxide and oxygen. Check this is the same as "standard gas" when entering environmental conditions. Only turn the calibration gas as far as it will allow, do not force it further



Turn off calibration gas. Hit “save” if the difference is less than 5%- if greater cancel calibration and start again.

Flow Calibration

Open flowmeter calibration. Again, enter in the weather information and click “sample baseline.”



Detection stroke

- one input stroke indicates air is flowing into the system (mouthpiece and all assemblies are properly configured).

*Notice the Flow indicator will show the real time input velocity, Stroke Peak with show the peak in real time and in the blank blue field beside the Cancel button, the volume of the syringe will be displayed at the end of any stroke. This value should be around 3 litres. If it reads high – 9, 12, 15 litres etc. The pneumotach has a moisture issue (if testing multiple subjects over a short period of time) and must be allowed to dry. You can use the syringe setup to do the Flowmeter calibration, but don't open the software. You are just physically driving air across the pneumotach, through the mixing chamber and into the system

Flush Stroke

- Four input strokes to push fresh air into the system
- The pneumotach can sense 800 lpm. Of the five calibration strokes input next, two must be less than 80 lpm. The number will fluctuate beside the Flow numerical indicator (to the left of Stroke Peak)

For Exercise Stroke

Velocity is very very slow, very slow, slow, fast, faster.

Stroke 1 → approximately 50 -80 lpm (very, very slow)

Stroke 2 → approximately 100+ lpm (very slow)

Stroke 3 → approximately 200+ lpm (slow)

Stroke 4 → approximately 300+ lpm (fast)

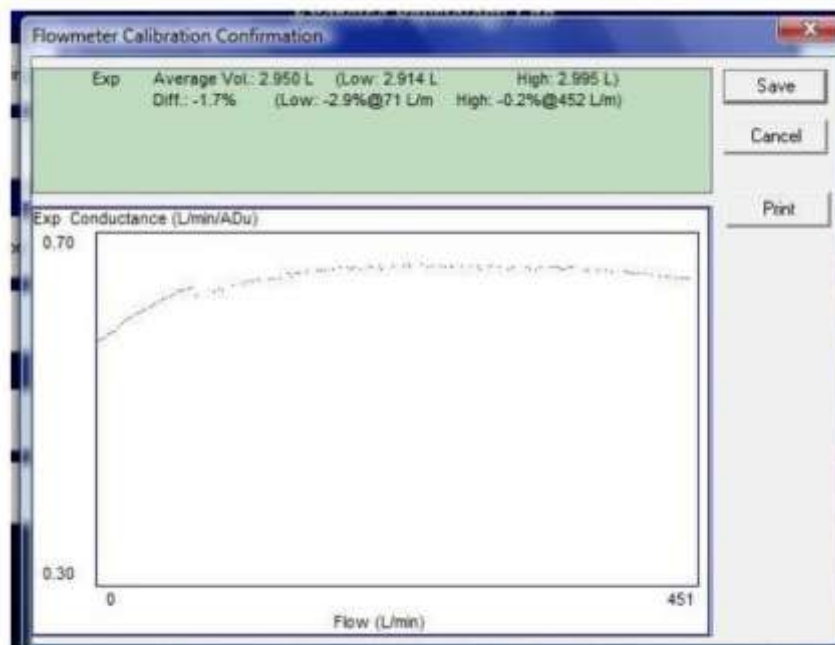
Stroke 5 → approximately 400+ lpm (faster)

For Resting Metabolic Rate Stroke

Velocity is very slow.

Stroke 1-5 → 40-50 lpm

The result is something like this curve:



Hit "save" if the difference is less than 5%- if greater cancel calibration and start again.

Testing Participants: Resting Metabolic Rate

1. On the home screen on the metabolic cart click "VO2 metabolic testing." If the participant has done a VO2 on that machine before click "patient look up" and search using their last name. Otherwise enter their details.



The screenshot shows the 'Patient/Test Info' window of the metabolic cart software. The window is titled 'Acacia University' and 'Respiratory Physiology Lab'. It contains the following fields and options:

- Patient Information:** Last Name (ROBINSON), First Name (KAYLA), Med Rec # (0), Age (20 yr), Sex (Female), Height (156 cm), Weight (125 lb).
- Test Protocol/Environment:** Doctor (), Tech (Samantha Robinson), Test Degree (Manual), Test Date / Time (2014/4/28), Exercise Device (Treadmill), Heart Rate/ECG (Active).
- Gas Calibration:** Insp. Temp. (20 deg C), Insp. Rel. Humid. (34 %), Insp. O2 (20.94 %), Base Pressure (744 mmHg), Calc. Humidity (), Insp. CO2 (0.03 %), Selected Flowmeter (00000 L/min).
- Base Values:** Base O2 and CO2: Same as Insp. O2 and CO2.

Buttons for 'OK', 'Cancel', and 'Rec: RECIP Records/Plat' are visible.

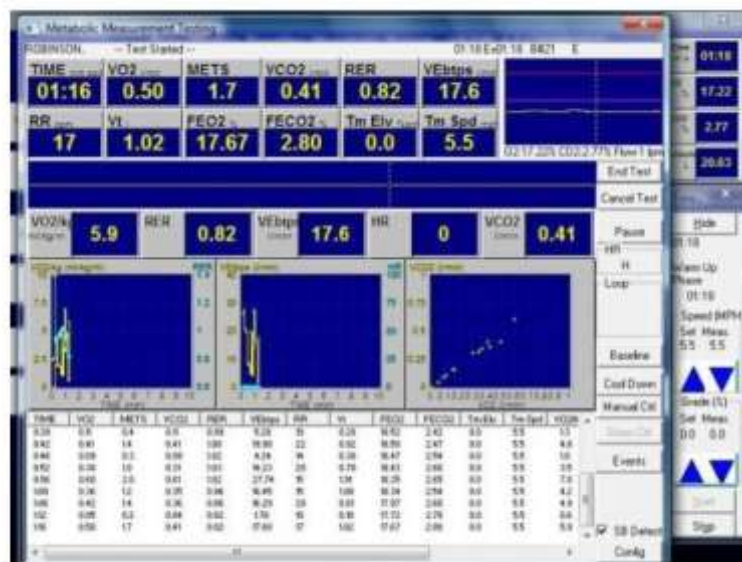
2. Lie participant down on bed and place canopy/ hood over their head. Ensure that where the testing tube meets the hood is centred and above their chest/ between their clavicles.



3. Tightly wrap the plastic sheet under the pillow, bed, and participant's arms. This will form a seal and stop air leaking. This only has to go as far as the participant's sternum.
4. Attach the gas sampling tube to the hood, as seen in photo above.
5. On the participant details screen click "OK" to begin test. You will see the ready to start testing screen appear. Adjust the dilution pump (under weather station) so CO2 is between 0.8-1.2% (body mass/3).

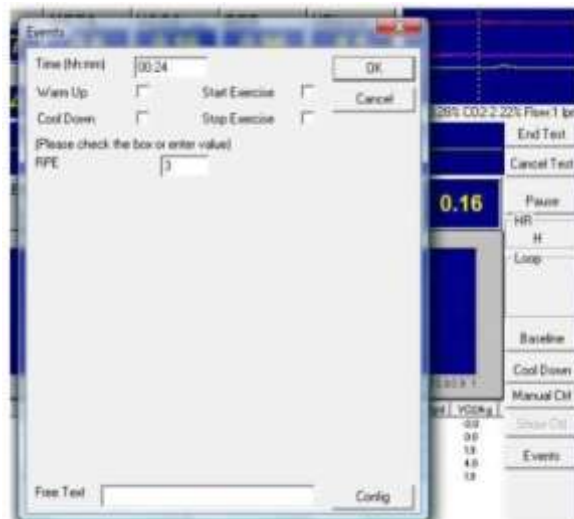


- There will be a delay of approximately 15 – 20 seconds before the Metabolic Measurement Testing window updates with data. The white space below will be filled with data every 60 seconds (this can be changed). Every 5 minutes there will be re-sampling and configuration of room air.



** During testing if condensation starts to form inside the hood, adjust the flow rate.

- The Events button to the right can be used to enter RPE values, blood pressure, etc. A pop-up window will appear. Simply enter the RPE value to the empty data field in the pop-up. This new event will be added to the data lines in the white space below.



8. At the end of testing click “End test” this will take you to a summary page. All tests are saved onto Parvo True one for later access but to be safe export data to excel and then save onto hard drive or memory stick.

Clean Up

9. Remove the hood and allow the participant to get up.
10. Spray the inside of the hood with disinfectant and wipe clean.
11. Gently fold the plastic sheet into the hood. This will prevent the sheet from tearing.

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