

The assessment of environmental stimulation of brown adipose tissue thermogenesis in adult humans using infrared thermography

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Abstract

Introduction: Obesity increases the risk of non-communicable diseases (NCDs) including cardiovascular (stroke, myocardial infarction, and hypertension), and metabolic (fatty liver and Type 2 diabetes mellitus) disease. The prevalence of obesity and related NCDs continues to grow despite the increased action of global governments. Promotion of exercise and healthy eating as prevention strategies remains challenging to implement so additional actions are required. Brown adipose tissue (BAT) is a potential target, due to its unique role in energy homeostasis. Activation of BAT promotes heat production and lipid and glucose metabolism, which could aid weight loss and promote health. The most prominent means of triggering BAT thermogenesis is through mild cold exposure which stimulates activation of the sympathetic nervous system (SNS). The same effect can be achieved through nutritional interventions, but this is less well explored. One potential nutrient is caffeine which stimulates the SNS and thus could activate BAT. There is a genetic component relating to the effectiveness of BAT and overall thermogenesis. Specifically, the thermoreceptor TRPM8 which detects mild cold exposure has been shown to aid in thermogenesis and may play a role in BAT thermogenesis. To investigate these potential outcomes multiple studies were conducted using infrared thermography to detect changes within the supraclavicular BAT depot as a result of caffeine supplementation or cold exposure. Genetic analysis was also conducted to investigate the link between thermogenesis related single nucleotide polymorphisms of TRPM8 (rs10166942) and the response to cold.

Methods: Non-invasive infrared thermography (IRT: thermal imaging) was used to measure the change in the temperature of the supraclavicular (SCV) region which overlays the most prominent BAT depot. An initial nutritional study used coffee, and this was followed by a

double-blind placebo trial to assess the impact of caffeine for which I hypothesised that 200mg of caffeine will promote BAT thermogenesis and increase supraclavicular temperature.

For the study investigating the impact of drinking a caffeinated beverage (Nescafe 100g sachet ~89mg caffeine) on the acute response of supraclavicular BAT participants underwent IRT to assess their baseline temperature followed by further imaging after consumption of the randomly allocated beverage (coffee or water).

For the study investigating the impact of caffeine as a supplement (Bulk Powders PureSeries 200mg caffeine tablet) vs a placebo (lactose 200mg tablet). This study was a double-blind crossover design. Participants were randomly allocated a single intervention per study visit. Prior to imaging participants underwent photoplethysmography (PPG) analysis to assess sympathetic nervous system activity followed by baseline SCV temperature assessment by IRT. Participants where then given their randomly allocated intervention and after an ingestion period (30mins) were assessed further, first by PPG and then a second session of IRT of the SCV region with co-locates BAT. Participants were then invited back to complete the assessment with their second randomly allocated intervention allowing results to be compared.

For the study investigating single nucleotide polymorphisms, assessment of BAT activity was conducted prior to the collection of saliva samples in collaboration with Nottingham University Hospitals NHS Trust, NIHR Nottingham Digestive Diseases Biomedical Research Unit (NDDBRU) and the NIHR Biomedical Research Centre, School of Medicine, Nottingham. BAT thermogenic response Participants were assessed initially at baseline prior to undergoing forearm cooling to illicit a thermogenic response. Participants where then assessed again

using IRT to assess their response to the cooling stimulus. This thermal data and participant response to cooling was then compared to participant genetic data from saliva samples and obtained using real-time PCR investigating the thermogenesis related single nucleotide polymorphism of TRPM8 (rs10166942).

Results: In the study involving the consumption of the caffeinated beverage, results showed consumption of caffeine significantly increased the temperature of the supraclavicular region co-locating with BAT. This effect was seen as an increase of TSCV and TRel (TSCV before intervention: 33.81 ± 0.45 °C, TSCV after intervention 34.187 ±0.59 °C, $P > 0.05$, TRel 0.4067 ±0.28 °C $p < 0.001$) an effect not seen with the consumption of water alone.

Results from the caffeine supplement 200mg caffeine vs placebo lactose 200mg tablet study showed that the 200mg caffeine intervention had no significant impact of the temperature of the supraclavicular region (TSCV for caffeine intervention baseline: 34.43 ± 0.22°C, TSCV after caffeine intervention 34.51 ± 0.25°C $P = 0.24$). This effect was also not present within the lactose placebo (200mg) sample (TSCV for lactose intervention baseline: 34.45 ± 0.43°C, TSCV after lactose intervention 34.48 ± 0.57°C $P = 0.68$)

Analysis of the genetic data demonstrated that the T Allele frequency among Caucasians and South Asians was 0.8 and 0.48 respectively and sits in accordance with expected values for the allele frequency within these demographic populations. The presence of the T Allele was strongly associated with increased BAT activity (ΔTrel) in our sample set (0.49 +/- 0.22 vs 0.33 +/-0.21 $p = 0.006$).

Conclusions: This thesis has demonstrated that the supraclavicular region which co-locates a major adult BAT depot can show a demonstrable thermogenic response to the ingestion of coffee (Nescafe 100g sachet ~89mg caffeine) but not after ingesting a caffeine supplement

(Bulk Powders PureSeries 200mg caffeine tablet). It also demonstrated that there may be a significant genetic impact of the thermogenic cold response system, specifically the response to mild cold stimuli in relation to the presence of the T allele (rs10166942) of the TRPM8 gene.

1.Introduction

1.1 Obesity Incidence – A global Concern

Obesity – defined as a body mass index (BMI) greater than 30kg/m^2 in adults has seen increased incidence throughout the world in the last 30 years (Dietz, 2011). The year 2005 saw the World Health Organisation (WHO) categorise global obesity and overweight as a "pandemic" with significant and increasing health risk in most of the developing and developed nations of the world (WHO, 2018).

Obesity is also a major risk factor for noncommunicable diseases (NCDs). Obesity dramatically increases one's risk to a broad scope of NCDs including cardiovascular (stroke, myocardial infarction, and hypertension), metabolic (fatty liver and type 2 diabetes mellitus) neurodegenerative (such as Alzheimer's) and mental health (depression) disease. And excessive fat accumulation can have a profound negative impact on the musculoskeletal system and proliferate certain cancers (Bray et al., 2017). NCDs now represent a vast and growing number of deaths worldwide and are the leading cause of premature mortality (Organization., 2017). More recently obesity has been labelled a chronic progressive disease (Bray et al., 2017) in a growing trend by a range of health organisations across the world and not merely a risk factor for NCDs. Since 2010 the WHO has made reducing and controlling the global obesity crisis a focus to reduce NCDs and called to action global political leaders across the UN in a bid to further educate the global populace on the increased dangers of unhealthy diet and a sedentary lifestyle (Organization., 2017).

1.1.2 Obesity Global Prevalence and epidemiology

Obesity is now prevalent throughout the global populace in both adults and children (Kaplan, 2011; ProQuest, 2002) with recent estimates indicating a prevalence of overweight (BMI >25kg/m^2) in 1.46 billion (Kaplan, 2011) and obesity in over 600 million adults (Verma & Hussain, 2017) and 170 million children (<18 years old) globally classified as overweight or obese (Lobstein et al., 2004).

Obesity prevalence and related NCDs are continuing to grow despite the increased action of global governments. Some regions have seen continued acceleration of obesity growth rate within this period when compared to others. Specifically, southeast Asia, south Asia, and many parts of south America have all seen dramatic increase in obesity and related NCDs over the course of the decade (Kelly et al., 2008). Studies have also revealed large discrepancies between adult obesity incidence based on global location with over 30% of the population of the US now considered obese, whilst just ~5% of adults in Japan and South Korea have a BMI of >30kg/m^2 (Emily et al., 2019). This dramatic shift in incidence rates between global populace does offer potential insight into the most prominent and potentially targetable aspects of weight gain and subsequent body fat accumulation.

1.1.3 The Socio-economic Burden of Obesity

The origin of the obesity epidemic can be traced back to the 1970's and 80's in countries with high net wealth seeing a surge in obesity followed by middle and low income countries towards the turn of the 21st century (Sassi et al., 2009). This trend in prevalence has however started to alter once again. Higher net worth individuals are now starting to demonstrate an inverse correlation with obesity whilst those in lower socioeconomic groups are most at risk

(Levine, 2011). Changing sociocultural norms demonstrate how increased wealth allows an individual better access to education and higher quality fresh produce. Whilst those in the poorest areas see an influx of readily available, cheaper but often unhealthy fast food and a lack of access to STEM (science, technology, English and maths) education which can subsequently impact a general understanding of nutrition and diet and their impact on health. This trend is also demonstrated with a steep rise in prevalence in developing countries. This surge demonstrates a pattern of economic influence on obesity within an economically developing populace with new wealth leading to higher rates of incidence (Hruby & Hu, 2015). The influence of western culture and lifestyle have started to shape both the quality and quantity of diet and adoption of a more sedentary lifestyle have compounded the effects of poor dietary habits resulting in an overall increase in overweight and obesity (Ng et al., 2014).

1.1.4 Known Causes of Obesity and the 'Metabolic Syndrome'

The consensus for the fundamental cause of weight gain relies on the premise that an energy imbalance i.e. more calories consumed than expanded will result in increased adiposity (Blüher, 2019). As such current legislation relies on the promotion of consuming a healthy diet whilst maintaining consistent levels of exercise. Historically however, this has not been a successful method for the management of weight over a longer period of time and increasing levels of overweight and obesity support this (Montesi et al., 2016). Intra-population rates of obesity are also highly inconsistent between subsets of those with similar lifestyles. Obesity is therefore far more complicated than a mere abundance of caloric intake and lack of energy expenditure. In fact, it is currently considered a multifaceted syndrome reliant on several

components including genetic factors, physiological mediators and one's external environment (Hruby & Hu, 2015).

Obesity is strongly associated with a range of over 45 comorbidities and atherogenic disorders which ultimately lead to the development of the metabolic syndrome (Verma & Hussain, 2017). Type 2 Diabetes mellitus (T2DM) is one such comorbidity revealed through a range of epidemiological studies to share close incidence and rate of escalation with obesity (Deepa et al., 2006).

DM is a chronic disorder, known to impact protein, carbohydrate and fat metabolism (Al-Goblan et al., 2014). DM is caused by an autoimmune response acting against insulin and β-Langerhans islet cells of the pancreas or a deficiency within β-cells to secrete sufficient insulin (Al-Goblan et al., 2014) classified as Type 1 (T1DM) or Type 2 (T2DM) respectively. Whilst T1DM pathophysiology is largely due to an autoimmune response, leading to β-cell loss and eventual hyperglycaemia (Atkinson, 2012). T2DM has an alternate pathophysiology which sees a combination of low insulin production from the pancreatic β-cells alongside a continued increase in peripheral insulin resistance resulting in an accumulation of plasma glucose (Gesta et al., 2007).

Alongside obesity, T2DM has become a global health care concern, that is projected to reach pandemic levels of incidence by the year 2030 (Huang et al., 2017). There are currently an estimated 387 million people globally living with diabetes and a further 316 million with diagnosed impaired glucose tolerance who are at high risk of developing the disease (Huang et al., 2017). Furthermore, diabetes and specifically T2DM (which accounts for ~90% of diabetes incidence) diagnoses' are set to rise to 592 million people by the year 2035 (Huang et al., 2017), a prevalence tied closely to increased obesity (Mraz & Haluzik, 2014).

The increasing incidence of T2DM is closely related to an increase in body weight due to an accumulation of excess adipose tissue (Hossain et al., 2007). It is estimated that overweight induced T2DM occurs in ~90% of T2DM cases (Hossain et al., 2007) demonstrating strong evidence for the close tie between increased adiposity and insulin resistance. Whilst the accumulation of white adipose tissue (WAT) has been linked closely to the development of obesity and obesity related disorders including T2DM (Verma & Hussain, 2017) it is with the recent rediscovery of activated brown adipose tissue (BAT) in adults with its thermogenic capacity to utilise fatty acids and glucose to dissipate energy as heat (Nedergaard et al., 2007) which has become a novel potential target against obesity and T2DM.

1.1.5 Early life nutrition, developmental programming and the impact of diet on development of the metabolic syndrome in adulthood

Continued evidence demonstrates strong links between the intrauterine milieu and the eventual health and development of the fetus into adulthood (Ismail-Beigi et al., 2006). Whilst diet, environmental factors and genetics are most often discussed with relation to the development of obesity and the metabolic syndrome (Blüher, 2019), the concept of fetal programming and the strong influence of the mother and their metabolic state is demonstrably shown to affect the long-term health of the child and likelihood of adulthood obesity and development of T2DM (Vickers et al., 2000). The concept of metabolic programming of the young is no longer a phenomenon observed solely in animal models (although these studies have proven useful in our understanding (see (Vickers et al., 2000)). With the rapid rise in global incidence of obesity, alongside obesity related comorbidities, strong epidemiological evidence has shown that fetal undernutrition during gestation can

impact a range of metabolic and physiological factors leading to increased risk of the development of childhood obesity and T2DM (Barker, 1995).

1.2 Adipose tissue

Adipose tissue plays a major role in a range of physiological processes, taking place throughout the life course. From providing a source of heat by thermoregulation, maintaining and orchestrating metabolic control, to the release of a range of hormones responsible for regulating reproduction and satiety. Adipose tissue is unique in its ability to continually develop over time based on the physiological state of the animal and is most commonly found in two forms; visceral adipose tissue, which forms around the vital organs and subcutaneous which resides beneath the skin (Ali et al., 2013).

Adipose tissue in all its forms provides an intricate and crucial role in the maintenance of homeostasis; originating in the earliest stages of neonatal development and continuing throughout life (Ali et al., 2013).

1.2.1 Evolutionary Origins of Adipose Tissue Storage and Regulation

The primary role for adipose tissue is that of a fat store utilised as an energy source in times of nutritional scarcity. Thus, there is an evolutionary benefit of being able to retain excess calorific in-take during times of hardship. This is seen throughout nature as both vertebrates and invertebrates possess the ability to utilise energy consumed through feeding as a source of both immediate and prolonged energy. However, the complex role of deposited adipose tissue extends beyond energy storage. Adipose tissue is the body's largest endocrine organ, responsible for a range of metabolic functions, including the control and release of lipid, adipokines and cytokines and the management and regulation of a range of physiological

actions including satiety, insulin sensitivity, glucose homeostasis and thermoregulation (Gesta et al., 2008).

Adipocytes originate within the mesoderm which are formed during embryogenesis (Arner et al., 2011). Each form of adipose tissue white adipose tissue (WAT) and BAT demonstrates a unique cell lineage. The time at which they first emerge varies between adipose subtypes. In humans, for example, BAT emerges first, followed later in mid gestation by WAT (M. E. Symonds, M. Pope, et al., 2012). The intricacies of adipose tissues in all forms (WAT and BAT) stem from a selective composition of cell types and deposited areas which give rise to multiple forms of tissue with varying physiological roles. The basic composition of these tissues include adipocytes (the fat storing cell), adipose stem cells (the adipocyte precursor), alongside neuronal, mural and endothelial cells each aiding in the endocrine and energy storing function of the cell type (Smorlesi et al., 2012). Adipose tissue is located throughout the body, including depots within the interscapular, inguinal, perigonadal, mesenteric and retroperitoneal depots.

1.2.2 White and Brown Adipose Tissue: Physiological Role and Developmental Origin

Mammals possess two physiologically distinct forms of adipose tissue WAT and BAT that are histologically different. Each are deposited in different depots in the body that reflect the bodies physiological and metabolic demands (Tchkonia et al., 2007). WAT provides a general long-term energy storage and endocrine function (Badoud et al., 2015). BAT however, plays a crucial role during the early stages of development and early postnatal period where it acts as a source of non-shivering thermogenesis (NST), due to the presence of uncoupling protein one (UCP1) (*see 1.4*) but remains active into adulthood (Nedergaard et al., 2007). More

recently a third unique type of adipocyte containing characteristics of both white and brown adipocytes has been categorised as 'brite' (brown in white) or beige adipocytes (Min et al., 2016). A detailed list of the histological differences between BAT and WAT can be seen in Table 1.

1.2.3 Adipogenesis

Adipocytes originate as mesenchymal stem cells (MSC) followed by committed precursor cells known as 'preadipocytes' and later differentiation occurs resulting in mature adipocytes (Ali et al., 2013). The specific mode of action committing pluripotent mesenchymal stems cells to their adipose lineage is not fully understood in humans, however bone morphogenetic (BMP) signalling is believed to play a role. Specifically, BMP2 and BMP4 have been shown to predispose stem cells to preadipocytes (Nam et al., 2015).

	White	Brown
Anatomical Location	found dispersed throughout body, with major visceral depots within the perirenal areas, omentum and intestines and subcutaneously surrounding the abdomen and thighs as well as interspersed within a range of other areas	(at birth); perirenal, cervical, axillary and periadrenal regions, with discrete depots surrounding the jugular vein and carotid artery
Lipid Component	Uniocular	Multiocular
Mitochondrial Concentration	Low levels of mitochondria	High levels of mitochondria
Vascularisation	Low levels of vasuclarisation	High levels of vascularisation
Thermogenic Capacity	Non-thermogenic	Thermogenic
Characterising Protein	Leptin	UCP1
Functional Role	Energy storage, insulation and endocrine functionality	Able to produce heat via UCP1 for non-shivering thermogenesis

Table 1. Demonstrates the histological changes seen in white adipocytes and brown adipocytes, based on their physiological function and depot location.

1.2.4 Differentiation of Adipocyte Precursor to Adipocytes

Precursor adipocytes undergo the process of terminal differentiation leading to the production of adipocytes. A range of transcription factors mediate this process. However, primary differentiation is completed through the activation of C/EBP-β and C/EBP-δ, which lead to the expression of PPAR-ϒ and C/EBP-α (Ali et al., 2013). Other factors which play a key role in differentiation include Kruppel-like factors and cyclic AMP response element-binding protein (CREB) lead to the proportion of preadipocytes to adipocytes (Huang et al., 2009). Adipogenesis is also subject to growth factor and hormonal control within the extranuclear environment (Lee, 2011). Thyroid hormones have been shown to induce adipogenesis by thyroid receptor alpha 1 action and insulin-like growth factor (IGF-1) and insulin can cause adipogenesis through pathways such as the phosphorylation of CREB (Lee, 2011).

1.2.5 Brown and White Adipose Tissue Adipogenesis Pathways

White and Brown adipocytes share multiple transcriptional cascades which lead to their initial differentiation such as PPARϒ and C/EBP (Ali et al., 2013). Morphological differences between the two adipose tissue subtypes would warrant individualised transcription pathways. As such Brown and white adipocyte adipogenesis occurs as a result of two distinct pathways, each with a unique set of transcription factors (Kajimura et al., 2010).

Brown adipocyte adipogenesis in controlled by a range of factors which include PR (PRD1-BF1-RIZ1 homologous) – domain containing protein (PRDM16), BMP 7, perioxisome proliferator-activated receptor ϒ (PPARϒ) and PPARϒ co-activator (PGC) 1α (Ali et al., 2013;

Nam et al., 2015; Schulz & Tseng, 2009). These adipocytes originate from the dermomyotomal precursors that demonstrate positive demonstration of the myogenic factor 5 (Myf5) gene which has also been shown to be important within the development of myocytes (Carobbio et al., 2013). The PRDM16 transcription is a key component of brown adipocyte differentiation, owing to an action which increases the transcriptional activity of PGC1α, which plays a strong role in the regulation of oxidative metabolism (Kajimura et al., 2010)

White adipocytes however, owe their origin to another range of genes and transcription factors which include; BMP4, homobox (Hox) C9, adipose differentiation related protein (ADRP) and BMP4 (Ghaben & Scherer, 2019).

1.3 White Adipose Tissue

1.3.1 White Adipose Tissue Structure and Physiology

WAT provides a range of metabolic functions, both as an energy source and an endocrine organ (Berry et al., 2013). Histologically, WAT is subdivided into two forms based on gross anatomical location, subcutaneous (found beneath the skin) and visceral (found close to and surrounding many internal organs). These WAT subtypes can be distinguished by several specific features including the developmental timing, their histology and function (Saely et al., 2012).

Subcutaneous WAT can be distinguished by its heterogeneous mix of both mature unilocular adipocytes, interspersed with smaller multilocular adipocytes, whilst Visceral adipose tissue has a much more uniform histological appearance of mainly larger unilocular adipocytes (Tchkonia et al., 2007).

The lipid contained within each cell consists of triglycerides which can be stored and utilised as an energy source when required. Due to the large lipid component of each adipocyte, the mitochondria contained within each cell are elongated and can vary in number (Cinti, 2002). It has been noted that both visceral and subcutaneous WAT depots are able to respond differently to a range of external factors based on the stimulus itself and the depots location. For example in mice and humans, oestrogen has been shown to enlarge the size of subcutaneous depots were as visceral depots are more responsive to glucocorticoids (Shi & Clegg, 2009).

Gene expression profiling of both visceral and subcutaneous adipose tissues have demonstrated each has distinct molecular phenotypes (Gesta et al., 2007) and overall adipocyte size (Jernås et al., 2006). Endocrine function also differs, between WAT subtypes. For example, visceral WAT found in the omentum express greater amounts of the insulin receptor 11β hydroxysteroid dehydrogenase (11β HSD) and interleukin 6 (IL6), whilst subcutaneous fat has more leptin and glycogen synthase (Gesta et al., 2007). These variations in gene expression between depots and tissue type are particularly important during early development of the tissue (Budge et al., 2005).

1.3.2 White Adipose Tissue Endocrinology

1.3.3 Leptin

Leptin is a form of anorexigenic adipocytokine which comprises 167 amino acid residues, is commonly referred to as the 'fullness hormone' and is a product of the ob gene which is made almost exclusively expressed by WAT (Warchol et al., 2014a). Leptin is secreted in small quantities by the placenta, gonads, stomach, pituitary gland, vascular endothelium, BAT

and skeletal muscle (Zhang & Scarpace, 2006). Leptin displays pleiotropic roles as the leptin receptor (Ob-R) has multiple isoforms which are present in various organs in the body including the hypothalamus (Bjorbaek & Kahn, 2004). One of its main actions is the regulation of energy homeostasis through its effect on the hypothalamus (Dessi et al., 2013; Warchol et al., 2014a). Leptin reaches the hypothalamus through the circulation and binds to the Ob-R receptor of the POMC/CART and NPY/AgRP neurons around the Arc nucleus (Horvath, 2005). This activates a cascade of intracellular signalling pathways involving the janus activating kinase - signal transducer and activator of transcription 3 (JAK-STAT3) (Bjorbaek & Kahn, 2004). The resulting activated STAT 3 then dimerises and translocates into the nucleus to modulate the expression of target genes such as increasing the expression α-MSH from POMC/CART neurons and suppressing the expression of NPY and AgRP from NPY/AgRP neurons (Breton, 2013; Wattez et al., 2013). α-MSH then binds to the MC4R receptor, especially around the PVN, activating anorexigenic secondary neurons to send a signal along the nucleus of solitary tract to suppress appetite and reduce lipogenesis, thereby controlling body weight (Dessi et al., 2013; Warchol et al., 2014a). Besides that, the previously described activated STAT3 also modulates the expression of suppressors of cytokine signalling 3 (SOCS3). SOCS3 inhibits the leptin-induced tyrosine phosphorylation of the JAK-STAT3 pathway (Wattez et al., 2013). This is postulated to be a potential mechanism for neuronal leptin resistance which is associated with obesity in adults.

Leptin also shares a common pathway with insulin in the hypothalamus at the level of PIP3 allowing cross-interaction between insulin and leptin (Breton, 2013; Wattez et al., 2013). Leptin and insulin hormones release ultimately lead to a reduced expression of orexigenic neuropeptide while an increased expression of anorexigenic neuropeptides. It plays a neurotrophic role in the hypothalamus as well. This is crucial in the plasticity or programming

of the appetite regulatory pathway in the hypothalamus. It has a trophic effect on the hypothalamic neural progenitor cells (Desai et al., 2011) and promotes neuronal outgrowth from Arc to PVN during the early postnatal period (Bouret et al., 2004). Leptin also affects growth by its interaction with the somatotroph which is also known as the growth hormone. It inhibits the secretion of somatostatin in the hypothalamus besides having a direct effect on the somatotroph cells in the pituitary gland (Horvath, 2005).

Leptin may also play a role in fetal adipose tissue formation, as its presence is noted in the fetus between 6 to 10 weeks of gestation, coinciding with the beginning of lipogenesis, and promotes the differentiation of pre-adipocytes and hence directly activating adipogenesis (Warchol et al., 2014a).

1.3.4 Adiponectin

Adiponectin is an adipocyte secreted protein, that controls lipid and glucose metabolism within insulin sensitive tissues and is negatively correlated with adult body weight (Poulos et al., 2010). It is a 30kDa protein which circulates in relatively high concentrations, compared to leptin, with a primary structure composed of a collagen-like tail, N-terminal signal sequence, a variable domain region and C-terminal globular head which resembles that of TNF-α (Poulos et al., 2010). Adiponectin receptors mediate the phosphorylation of AMPK (AMP-activated protein kinase) and are composed of seven transmembrane domains and found in two forms, ApidoR1 and AdipoR2 (Wagner de Jesus, 2014). AdipoR1 receptors show a high affinity for binding the globular form of adiponectin, with low affinity for full length adiponectin. AdipoR2 receptors are found in high abundance within the liver and demonstrate a high affinity for binding both forms of adiponectin (full length and globular) (Fasshauer & Blüher, 2015).

Adiponectin bound receptors mediate the oxidation of fatty acids and supress gluconeogenesis through activation of AMPK, insulin receptor phosphorylation and the modulation of nuclear factor κB (NF-κB) (Fasshauer & Blüher, 2015).

Due to the importance of maternal gluconeogenesis and the concentrations of insulin and glucose affecting fetal growth and development, circulating levels of maternal adiponectin impact fetal development (Luo et al., 2013). Adiponectin within the fetus is produced through endocrine tissues including adipocytes as well as a range of fetal vascular tissues. It has been demonstrated to be highly abundant within fetal plasma and closely related to lower insulin concentration and increased insulin sensitivity (Pinar et al., 2008). Adiponectin therefore demonstrates a clear role in multiple areas of neonatal development and could contribute to insulin sensitivity (Hansen-Pupp et al., 2015).

1.4 Brown Adipose Tissue

BAT is a crucial heat generating organ present in all mammals which serves an important evolutionary benefit (Cannon & Nedergaard, 2004). The evolutionary development of BAT alongside the BAT specific protein uncoupling protein 1 (UCP1) represent a key stage in mammalian development offering an evolutionary advantage against cold climate survival and the extremes of the extra uterine environment (Symonds, 2013).

Originally described in 1551 by the naturalist Conrad Gessner, BAT has since been noted in all mammals (Cannon & Nedergaard, 2004). The thermogenic characteristics of BAT however, are a more recent discovery (Smith, 1964) including the involvement and role which UCP1 plays in adaptive thermogenesis (Heaton et al., 1978). Even more recent is the notion that BAT remains active throughout the life course and long into adulthood which was once not believed the be the case (Nedergaard et al., 2007). The metabolic inefficiency of UCP1, and

BAT utilising free fatty acid (FFA) and glucose stores make BAT a potential therapeutic target in our ongoing fight against obesity. This realisation has prompted a resurgence in BAT research.

1.4.1 Brown Adipose Tissue Structure and Physiology

Brown adipocytes share a similar stem cell origin with the myocyte, both carrying the myogenic factor 5 (Myf5) marker (Ravussin & Galgani, 2011). Both cell types are also regulated by the Zn-finger transcriptional regulator PRD1-BF1-RIZ1 homologous domain which contains the PRDM16, enforcing the cell fate, switching between myoblast and BAT cell lines (Seale et al., 2007). Once mature, both cell types demonstrate abundant similarities in the form of; a large core sympathetic innervation, an abundance of mitochondria and an ability to facilitate thermogenesis (Ravussin & Galgani, 2011), that in many large mammals is initiated around the time of birth following cold exposure to the extra-uterine environment (M. Symonds et al., 2012). BAT cells are unique in their composition, containing high numbers of mitochondria and a unique thermogenic profile in the form of the protein UCP1. UCP1 is a 32Kda protein housed within the inner mitochondrial membrane which allows the cell to utilise stored fatty acids to generate heat by a process of energy uncoupling within the mitochondria by alternating the proton concentration gradient generating heat rather than adenosine triphosphate (ATP), at a maximal rate of 300watts/kg, much higher than the standard 1watts/kg in most common tissues (Symonds, 2013). This heat generating capacity plays a crucial role in thermogenesis and the maintenance and control of the core body temperature.

Brown adipocytes can be found in a number of anatomical locations which include (at birth); perirenal, cervical, axillary and periadrenal regions, with discrete depots surrounding the

jugular vein and carotid artery, and can also be directly impacted by endocrine and environmental changes (Cannon & Nedergaard, 2004). Unlike WAT, BAT is highly abundant during early life and is usually reduced during early postnatal development, as the body becomes more adapted to controlling temperature through means such as shivering thermogenesis (Symonds, 2013). Whilst BAT was not considered to be found in significant amounts in adults, recent imaging studies utilising positron emission tomography with computed tomography (PET/CT) (Lee et al., 2011) and thermal imaging (Law, Morris, et al., 2018) have demonstrated active BAT within the supraclavicular region of adults, which may impact metabolism and energy expenditure.

1.4.2 Neonatal and Fetal Adipose Tissue Development

Neonatal and fetal fat deposition is determined in part by the overall glucose environment, with higher maternal glucose availability resulting in an increase in adipose tissue deposition (M. Symonds et al., 2012). In human development the longer gestation period results in larger amounts of WAT deposition during mid-gestation and of subcutaneous BAT deposition during the late gestation period (M. Symonds et al., 2012). Adipose tissue deposition within the human fetus begins around 14 weeks gestation within the head and neck, followed by the trunk and limbs (Poissonnet C.M. A.R. Burdi, 1983). Within the hypoxic and hypoglycaemic uterine environment, fetal temperature is reliant on maternal regulation and usually remains at a steady state. It is therefore unsurprising that due to the higher metabolic demands of lipid deposition, when compared to carbohydrate or protein, the amount of fat deposited is low during fetal development. Fat deposition increases postnatally as available oxygen and glucose is much higher and lipid supply increases (Symonds et al., 2003).

During fetal development in humans and sheep, adipose tissue is present from mid-gestation. It continues to develop throughout the remainder of pregnancy with the highest rates of deposition occurring towards term. This coincides with the increased demand for heat generation following exposure to the extra uterine environment (Clarke et al., 1997). The longer gestation of precocial mammals such as humans and sheep, allows for the complete maturation of the hypothalamic pituitary axis prior to birth. The development of this system prior to birth allows for the autonomic control of none shivering thermogenesis under extra uterine conditions (Clarke et al., 1997). In humans, subcutaneous adipose tissue depots represent the primary stage of adipose tissue development predating the deposition of visceral depots and account for the highest proportion of adipose tissue in humans at birth. This is also true throughout the life course, as adipose tissue is continually developed and deposited based on metabolic and physiological demands (Kiess et al., 2008).

Adipose tissues, are in a continual state of growth and development based on nutritional availability (Arner et al., 2011). Based on early anatomical investigation (Poissonnet C.M. A.R. Burdi, 1983) prenatal adipose tissue development can be divided into morphological developmental phases:

Phase one can be seen prior to 14 weeks gestation wherein, adipocyte precursor cells consist of loose connective tissue.

Phase two demonstrates the earliest signs of adipogensis. During this phase mesenchymal cells begin to aggregate into a dense mass. This stage also coincides with the proliferation of early stage vessels and ends the undifferentiated cell phase (Poissonnet C.M. A.R. Burdi, 1983).

Development of adipocyte vasculature begins with a network of capillaries stemming from these vessels forming a rich network of capillaries, which the mesenchymal cells differentiate into preadipocytes. These mesenchymal cells organised within a glomerulus represent phase 3 of the developmental process and at this stage do not contain the lipid droplet characteristic of the adipocyte.

As the vascular network continues to proliferate and increase in density, development of early adipocytes occurs within the mesenchymal lobules, as lipid vacuoles form within the cytoplasm concluding phase 4.

During the final stage of adipocyte development fat lobules are surrounded by perilobular mesenchyme, which condenses at a high rate and eventually thickens to form interlobular septa (Poissonnet C.M. A.R. Burdi, 1983).

Fetal adipose tissue development is highly reliant on a well-established vascularisation of the surrounding cells. This allows for the secretion and mobilisation of a range of endocrine factors, including a range of cytokines (Stephenson et al., 2001). With the expansion of fat mass towards the end of the gestation period, there is a marked increase in the number of hormone receptors. This alongside the development of the sympathetic nervous system result in increased abundance of UCP1 at the time of birth, ensuring thermoregulation in the extra uterine environment (Warchol et al., 2014b).

1.5 Uncoupling Protein 1

UCP1 is critical for BAT thermogenesis. The protein which resides on the inner mitochondrial membrane within brown adipocytes , is unique to BAT and mediates non-shivering thermogenesis through a process of metabolic inefficiency, that results in the production of heat (Nedergaard et al., 2001). UCP1 activation leads to heat production through a process which disrupts the conventional action of the electron transport chain. Oxidation of substrates coupled to adenosine triphosphate (ATP) generation caused by the movement of electrons within the mitochondrial matrix leads to a proton electrochemical gradient which is dispelled when hydrogen ions (H^+) move back into the inner mitochondrial membrane. This movement of H^+ ions leads to the rotation of the ATP synthase complex causing the generation of ATP from adenosine diphosphate (ADP) (Mitchell, 1961). UCP1 then acts on this process by disrupting the chain, causing the protons to pass from the membrane to the mitochondrial matrix through different means, which results in a dissipation of the proton motive force and instead causing energy to be released as heat (Nedergaard et al., 2001).

1.5.1 UCP1 Activation

UCP1 is inactive within its resting state requiring activation to impact the process of ATP generation. The process which leads to the activation of UCP1 within the inner mitochondrial membrane is not well understood, however current theory demonstrates the presence of free fatty acids being a key driver of non-shivering thermogenesis (Cannon & Nedergaard, 2004). An increase in the concentration of intracellular cAMP through the activation of β-adrenoreceptor activation, is one means by which UCP1 is regulated (Nedergaard et al., 2001).

1.5.2 Activation of BAT – The Thermogenic Pathway

The primary role of BAT is in NST resulting in heat generation (Bahler et al., 2015). This thermogenic process can be induced through the action of cold exposure resulting in sympathetic stimulation (Bartness et al., 2010). The process is heavily mediated, originating in the hypothalamus and follows the autonomic nervous system (ANS) until it meets a dense network of sympathetic fibres connecting to BAT depots (Bartness et al., 2010).

1.6 Thermogenesis

1.6.1 The Hypothalamus and Central Nervous System – Role in Thermogenesis

Energy homeostasis is controlled through the action of the central nervous system (CNS), with the majority of sensory signals reaching the hypothalamus where they are then converted into a response which will regulate energy expenditure (EE) and nutritional intake (Contreras et al., 2017).

The hypothalamus is composed of interconnected nuclei, made up of a distribution of neurons. Energy balance Is regulated by the ANS, whilst the thermogenic response induced in BAT is regulated through the activation of the sympathetic nervous system (SNS) (Contreras et al., 2016). The regulation of the ANS is tightly controlled within the hypothalamus through signalling by a complex network of neuronal populations within six distinct regions (Contreras et al., 2017).

Temperature changes are sensed through the preoptic area (POA). This region can receive sensory temperature signals through temperature sensitive neurons and in turn regulate the core body temperature (Boulant, 2000). Thermosensitive areas around the body are able to

signal to the POA which is connected to the ventromedial nucleus of the hypothalamus (VMH) when cold induced, which can trigger the thermogenic mechanism of BAT (Nakamura & Morrison, 2011). The VMH has been shown to play a key role in this process, as rats who develop obesity induced lesions of the VMH, are unable to instigate cold induced BAT thermogenesis (Hogan et al., 1982). The POA is also instigated in times of illness, when it induces the febrile response seen in fever, a state of hyperthermia required to defend against invading pathogens (Steiner et al., 2002). The febrile response can induce BAT thermogenesis through the action of prostaglandins (PG) being released into the vasculature and peripheral tissues. PG eventually make their way to the POA where the EP3 subtype receptors for PG, connected to the dorsomedial nucleus of the hypothalamus (DMH) and rostral raphe pallidus (rRPA) of the brainstem utilise cAMP to induce BAT activation (Steiner et al., 2002).

The proximity of the arcuate nucleus of the hypothalamus (ARC) to the median eminence (ME) and permeable region of the blood brain barrier (BBB) allows for the transfer of peripheral signals to the brain allowing for neuronal detection of circulating temperature mediated factors (Lage et al., 2016). The ARC contains a range of neuronal populations including those expressing orexigenic neuropeptides promoting appetite; neuropeptide Y (NPY) agouti-related peptide (AgRP), alongside anorexigenic neuropeptides such as cocaine and amphetamine regulated transcript (CART) and pro-opiomelanocortin (POMC) (shown to increase BAT thermogenesis through SNS activation (Butler & Cone, 2002)) which promote satiety (Contreras et al., 2017). The ARC also plays a key role in the maintenance of energy expenditure and sympathetic activation of BAT through the action of leptin, which is able to induce an action potential allowing for the firing of nerves regulating BAT activity (Contreras et al., 2016). Whilst the neuronal make-up of the ARC is involved in the regulation of

thermogenesis, the molecular mechanisms involved remain unclear. Although a stress induced response on the endoplasmic reticulum (ER) has been noted to increase energy expenditure and BAT thermogenesis in times of low ER stress through ER-stress mitochondrial signalling (Kwon et al., 2016). Whilst high amounts of ER stress in POMC neurons can reduce BAT thermogenesis (Schneeberger et al., 2013).

The DMH is also instigated in the thermogenic response in BAT through sympathetic transmission (Contreras et al., 2016). During the febrile response DMH glutamatergic neurons are able to activate sympathetic fibres to BAT through rRPa, a process requiring two groups of neurons (Contreras et al., 2017). GABAergic neurons of the DMH initially inhibit the glutamatergic neurons. However, when under prostaglandin (PG)E_2 activation in the POA, a second set of GABAergic neurons within this region are able to inhibit the DMH GABAergic neurons supressing the DMH activated response (Morrison et al., 2014).

NPY expressing neurons are also present within the DMH that have been shown to regulate sympathetic innervation in BAT resulting in thermogenesis. These NPY expressing neurons are affected by disruption, causing an increase in BAT thermogenesis (Monge-Roffarello et al., 2014). Leptin receptor expression (LepR) within DMH neurons has also been instigated in the thermogenic process and the maintenance of healthy weight. One study in mice, demonstrated the importance of LepR expression in the process of sympathetic innervation of BAT, in which lower expression of LepR within the DMH resulted in a decrease in BAT thermogenesis and contributed to weight gain (Rezai-Zadeh et al., 2014).

The VMH has also been shown to play an important role in BAT activated thermogenesis, through the action of coordinating peripheral signals into a thermogenic response (Contreras et al., 2016). Anatomically the VMH shares a close link to the brown fat pad through the

inferior olive and rRPa of the brainstem (Bartness et al., 2010). This resulting in the VMH being shown to both inhibit BAT thermogenesis, through the action of GABA agonists (Amir & Schiavetto, 1990) and activate thermogenesis by neuropeptides such as the catecholamine noradrenaline (Hugie et al., 1992). Peripheral signals such as thyroid hormones, leptin and drugs such as nicotine have been shown to activate BAT through a process mediated by the VMH (Miguel et al., 2010). This shared mechanism utilises the inhibition of AMP-activated protein kinase (AMPK) resulting in the activation of BAT sympathetic fibres which innervate the tissue (Mamoru et al., 2013).

The lateral hypothalamic area (LHA) plays a small yet important role in maintenance of thermogenesis through the action of orexigenic neuropeptides; orexins A and B (OX) which are heavily expressed within the LHA and can induce thermogenesis in BAT (Hara et al., 2001). OXs have also been demonstrated to constitute a key determinant of BAT development and differentiation making the LHA a key component of not only BAT activation but of brown adipogenesis (Sellayah et al., 2011).

Finally, the paraventricular nucleus of the hypothalamus (PVH) plays a role in sympathetic activation of BAT through the regulation of energy homeostasis (Contreras et al., 2016). The PVH shares an anatomical connection to BAT, and multiple studies have demonstrated a thermogenic effect of BAT when this region is administered with nervous system stimulating substances, including corticotrophin releasing hormone and leptin (Jiang et al., 2015; Lefeuvre et al., 1987).

The combined effect of nutritional and hormonal signals to the various regions of the hypothalamus, culminate in the transmission of this information through efferent fibres to the brainstem (Cohen & Spiegelman, 2015).

The brainstem has been shown to utilise an inhibitory pathway in the activation of BAT thermogenesis, through the presence of rRPa neurons, which receive inhibitory inputs from warm sensitive GABAergic POA neurons. Subsequently when these rRPa neurons are not inhibited, increased sympathetic activity results in BAT activation and thermogenesis (Morrison et al., 2014). rRPAa neurons of the brainstem play a key role in the integration of peripheral efferent signals from multiple regions of the hypothalamus. These neurons ultimately aid in the coordination of thermogenesis and core temperature through sympathetic output to BAT tissues (Contreras et al., 2016) which have been demonstrated to innervate BAT through the use of viral tracers (Nguyen et al., 2014).

1.6.2 The Autonomic Nervous System Function in BAT Thermogenesis

Efferent nervous signals from the hypothalamus and brainstem are eventually orchestrated through the ANS. This tightly controlled system is responsible for the maintenance of homeostasis and energy balance of the bodies major organs which are highly innervated. The ANS is subdivided into two subsystems which work antagonistically to illicit and control the nervous response; the SNS and the parasympathetic nervous system (PSNS) (Bahler et al., 2015). The SNS and PSNS serve two distinct functions, whereby the SNS is responsible for instigating the fight or flight response, increasing overall nervous system activity, inducing a heightened state of alertness. Whilst the PSNS works to control the SNS to maintain a sense of calm and mediate the rest and digest response (Contreras et al., 2017).

1.6.3 Adrenergic Signalling in BAT

BAT relies on the presence of UCP1 to elicit a thermogenic response in the form of dissipating energy as heat through uncoupled mitochondrial fatty acid oxidation (Cohen & Spiegelman, 2015). Brown adipocytes are specially adapted to play this role through a unique histological composition, containing high concentrations of mitochondria (when compared to WAT) and multilocular lipid droplets (rather than single lipid droplets seen in WAT) (Saely et al., 2012). It is a highly innervated and vascularised tissue with a high abundance of β, α_1 and α_2 adrenoreceptors with β_3-adrenoreceptors (β_3-AR) being the most significant signalling pathway (Cannon & Nedergaard, 2004). B_1 adrenoreceptors are expressed within brown adipocytes and are most notably coupled to the production of cAMP rather than any signalling processes (Bronnikov et al., 1999). Whilst β_2 are not actively expressed within brown adipocytes at all, but rather can be found within the tissue particularly localised to the vasculature, where they act as binding sites in membrane preparations of BAT (Cannon & Nedergaard, 2004).

BAT thermogenesis is triggered upon the arrival of the sympathetic signals (from peripheral thermoreceptors) from the hypothalamus to the nerve terminal whereby noradrenaline (NA) binds to the β_3-AR on the postsynaptic terminal triggering the thermogenic response in BAT (Cannon & Nedergaard, 2004). Noradrenaline is responsible for inducing thermogenesis by BAT activation and controlling brown adipocyte proliferation (Cannon & Nedergaard, 2004). This has been demonstrated in a range of studies which have administered adrenergic agonists leading to BAT activation (Bartness et al., 2010). The adrenergic controlled signalling in BAT is therefore fundamental to the overall function of the cell type.

Thermogenesis is instigated when nerve fibres innervating BAT release NA which in turn bind the β_3-AR found within the brown adipocytes (Bartness et al., 2010). Upon binding of NA to β_3-AR, adenylate cyclase (AC) is activated through the action of the stimulatory G-protein coupled to the β_3-AR. This AC activation catalyses the conversion of ATP to cAMP, which in turn activates the protein kinase A (PKA) (Contreras et al., 2016). PKA plays an important role in the downstream activation of brown adipocytes. Initially PKA activates the extracellular signal regulated kinases (ERK) 1 and 2 and p38 mitogen-activated protein kinase (MAPK) which result in the transcription of UCP1 (Contreras et al., 2017). Importantly, PKA also initiates the activation of lipolysis within the brown adipocytes. The lipases, hormone sensitive lipase, adipose tissue triglyceride lipase, and monoacylglycerol lipase are released allowing the hydrolysation of triacylglycerol, diacylglycerol and monoacylglycerol into free fatty acids (FFA) and glycerol ready for uptake into the mitochondria (Cohen & Spiegelman, 2015). This uptake of FFA utilises carnitine palmitoyltransferase 1a whereby FFAs reaching the mitochondria then undergo oxidation through a process of β-oxidation. This process sees NAD^+ and FAD reduced to form NADH and $FADH_2$ where they are utilised to power the electron transport chain. The process of oxidation and action of UCP1 then uncouples these protons from the electron transport chain dissipating this excess energy as heat. The continued initiation of the sympathetic drive towards BAT utilises this process to induce thermogenesis (Cohen & Spiegelman, 2015).

Neuronal control of thermogenesis through activation of BAT is of crucial importance in our ability to maintain a healthy homeostatic environment. It is therefore apparent that any dysregulation within the ability of BAT or the nervous system to maintain adequate control of the homeostatic environment could lead to an increased risk in development of metabolic

disease and obesity. Coincidentally, this also makes BAT and specifically the sympathetic innervation of BAT a potentially important target against obesity and T2DM.

1.7 BAT as a therapeutic target in Obesity

BAT has historically been regarded as an organ specific to early development with rapid involution in early childhood and little functional role in adulthood, 2007 saw the culmination of studies in the field of radiology demonstrate what was believed to be BAT in adults through the use of positron emission tomography and computed tomography (PET/CT) (Nedergaard et al., 2007). PET utilises radiotracers such as ^{18}F-fluorodeoxyglucose (^{18}F-FDG) to measure the metabolic activity of tissues, CT is then able to capture high resolution images of the targeted anatomical region (Stephens et al., 2011). Overlaying the two techniques allows for both structural and functional information and is a favoured technique in cancer diagnosis, as developing metastases are highly metabolically active (Schoder et al., 2004). Radiologists where originally sceptical of the view that metabolically active fat seen through PET/CT in adults was BAT. In 2009 a collective of research groups working independently using PET/CT were able to demonstrate active BAT in adult humans (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

The BAT discovered in adults was predominantly shown within the major depot of the cervical-supraclavicular region, and post PET/CT histology demonstrated UCP1 and β_3-AR expression within the tissues (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). Increasing evidence that BAT was not only present in adults but remained active well past the stage at which was originally considered possible.

Expanding on the notion that BAT remains active in adults one group retrospectively assessed 2000 individuals for the presence of BAT and showed not only widespread evidence of BAT in

adults, but also the correlation between BAT activation, ambient temperature, medication use (such as beta-blocker) and BMI (Cypess et al., 2009), demonstrating the potential connection between sympathetic nervous system stimulation and BAT activation. This notion of BMI impacting on BAT prevalence was further confirmed in similar studies demonstrating the connection between excess adiposity leading to reduced BAT (Saito et al., 2009; Zingaretti et al., 2009). Whether this connection is causative or as a result of overweight is as of yet confirmed.

Considering that BAT has a high capacity to dissipate energy as heat through the use of FFA and glucose, this makes BAT a relevant target for anti-obesity and T2DM therapy. Pharmacological treatments for obesity are severely lacking. Currently these focus on reducing energy intake (Kaplan, 2005) rather than utilising the body's natural energy producing processes (such as thermogenesis), and none have demonstrated long term efficacy (Rodgers et al., 2012). This opens an avenue for the use of BAT thermogenesis as a potential therapeutic target. Current theoretical approaches to the use of BAT as an obesity intervention focus not only on inducing BAT thermogenesis but also increasing BAT mass through the use of pharmacological agents and growth factors. The efficacy of inducing thermogenesis from a pharmacological intervention has already been demonstrated to be a possibility, in the form of the drug dinitrophenol. This nonselective uncoupler of mitochondrial oxidation has been shown to be an effective way of inducing weight loss, however a host of side effects associated with the drug prevent its use in humans (Grundlingh et al., 2011).

Another popular pharmaceutical target for BAT related interventions have focused on the activation of the central nervous system, known to be the key stimulating component of BAT thermogenesis (Cohen & Spiegelman, 2015). One such approach was the use of the alkaloid

ephedrine. This drug stimulates the nervous system leading to vasoconstriction and bronchodilation (Pipe, 2004) and stimulates moderate short term weight loss, utilising in part BAT thermogenesis as demonstrated in PET/CT studies (S. Baba et al., 2007). More recently, the β_3-AR agonist mirabegron, currently used in the treatment of overactive bladder, was demonstrated to have efficacy in inducing BAT activity in a human study (Cypess et al., 2015). The study utilised PET/CT on 12 healthy male participants who previously demonstrated BAT prevalence through cold exposure and showed a significant increase in both BAT activation and resting metabolic rate in these subjects, demonstrating the potential for use of β_3-AR agonists as a target in the activation of BAT.

These studies and the close association to pharmacological nervous system stimulation has been demonstrated as a potential target in our efforts to induce BAT thermogenesis through the use of pharmaceuticals which may play a key role in aiding in weight loss strategies.

1.8 Caffeine as a target in the activation of BAT

Caffeine is a stimulant which may act on the nervous system and BAT in a way which could promote and potentially sustain thermogenesis. It is the world's most popular drug, consumed by 80% of the adult population (Harland, 2000). As a naturally occurring alkaloid, caffeine is found in a range of leaves, beans and fruits in varying quantities, with the most common sources being the kola nut, guarana berry and cacao bean (Harland, 2000). Caffeine is absorbed rapidly by the gastrointestinal tract and metabolised within the liver into three metabolites within 15-45 minutes of consumption: paraxanthine (the major metabolite), theophylline and theobromine (Heckman et al., 2010). It is a lipid soluble compound and can alter neural function by free movement across the blood brain barrier (Goldstein et al., 2010).

1.8.1 Caffeine and the Sympathetic Nervous System

Due to its ability to freely permeate the blood brain barrier and interact with neural function, caffeine is able to induce activity of the sympathetic nervous system (SNS). The action of methylxanthines such as caffeine can stimulate the SNS through the inhibition of phosphor-diesterase (PDE). The resulting intracellular signal ultimately leads to nervous activation, increased lipolysis and heat generation is dependent on the availability of cAMP (Heckman et al., 2010). Under normal circumstances, the action of cAMP within the nerve terminal is short lived due to the rapid degradation by PDE. However, methylxanthines such as caffeine can inhibit PDE resulting in sustained cAMP presence. Caffeine has a dual action on sympathetic activation. Alongside inhibition of PDE, caffeine also antagonises the effect of adenosine in the presynaptic nerve terminal by reversing the adenosine-mediated inhibition of

catecholamines, leading to sustained BAT activation by adrenoreceptors β_1, β_2 and β_3 (Dulloo, 2011).

The use of caffeine is widespread, found in a range of drinks, foods and pharmaceuticals. There is an increasing array of evidence stipulating the thermogenic effect of caffeine, and the detrimental effect in which dysregulation of thermogenesis can have in the development of obesity. Caffeine could be a valuable research target within this field, potentially elucidating not only itself as a consumer safe thermogenic drug, but also in our continued research into the differences seen in those who are overweight and obese and those who are not. Caffeine has been studied extensively when relating to energy expenditure and multiple studies have demonstrated the benefits of caffeine in this regard (S. Baba et al., 2007; Westerterp-Plantenga, 2010). It has also seen some use in BAT research, especially in rodents where intravenous injection of caffeine has been shown to increase expression of BAT specific proteins including UCP1 (Yoshioka et al., 2003). However, there is a significant lack of research and evidence for the effect of caffeine in adults, especially when related to the activation of BAT. This study aims to assess the acute thermogenic effect of caffeine in BAT using a novel BAT analysis technique i.e. thermal imaging.

1.8.2 Caffeine, energy balance and thermogenesis

Physiological effects of caffeine include enhanced cognition and physical performance, as well as being shown to impact resting energy expenditure (Glade, 2010). The beneficial impact of caffeine on metabolic syndrome related conditions has been documented in laboratory controlled conditions, especially in studies relating to rodents. For example, caffeine consumption in lab studies has been shown to reduce weight gain in rats fed a diet

high in fat (Conde et al., 2012). As well as decreasing insulin and plasma glucose concentrations when subject to caffeinated drinking water (0.1%) upon consumption of high fat and high sucrose diets (Conde et al., 2012). The impact on humans however is much less apparent owing to the difficulty in simulating laboratory based conditions on human participants. Caffeine from drinking coffee however, has been classically shown to impact a range of physiological factors positively in both obese and non-obese participants, especially those relating to lipolysis, insulin secretion and thermogenesis (Astrup et al., 1991; Bracco et al., 1995; Hursel et al., 2011) and caffeine consumed in the form of beverages, has been linked to increased energy expenditure equivalent to a 4% energy expenditure increase (from consumption of 150mg) (Dulloo et al., 1999), all of which would aid in the overall maintenance of energy balance.

1.8.3 The receptor non-specific nature of caffeine and deficiencies of caffeine as a pharmacological intervention

A more controlled energy balance profile however does not necessarily aid in weight loss. Coffee consumption itself has not been shown to demonstrate a weight loss effect in humans (Van Schaik et al., 2021). Likely owing to the fact that continued coffee drinking and therefore caffeine exposure can lead to a dulled catecholamine response and reduced lipolysis over time (Dekker et al., 2007). Caffeine is also adrenoreceptor non-specific and as such may negatively impact other areas of physiology.

Caffeine administration especially at higher doses can have significant side effects on the cardiovascular system and arterial pressure for example (Turnbull et al., 2017) which would make it a potential unsuitable target for metabolic control, especially in those most vulnerable. Caffeine consumption has also been linked to higher blood pressure, increasing

an individuals risk of hypertension (Noordzij et al., 2005), however multiple epidemiological studies have demonstrated that dietary caffeine consumption does not increase the risk of developing hypertension (Geleijnse, 2008; Savoca et al., 2004).

Whilst caffeine's physiological impacts have been well documented, its mechanisms stemming from sympathetic control are less well known. It is believed that in humans caffeine has a centralised control (Ferre, 2008), and therefore any thermogenic impact must also be initiated in this way. In rodents, studies have demonstrated the impact of low doses of caffeine on the orexinergic neurons of the hypothalamus (Murphy et al., 2003). Regions which have been closely linked to the regulation of sympathetic innervation of interscapular BAT, leading to BAT thermogenesis (Tupone et al., 2011). An increase in cAMP could be one such mechanism.

1.8.4 The impact of caffeine on the sympathetic nervous system and BAT thermogenesis

Caffeine is a non-selective phosphodiesterase inhibitor (Harland, 2000). Phosphodiesterase plays a role in the conversion of cAMP to adenosine monophosphate, as such caffeine consumption will lead to an increase in release of intracellular cAMP (Boswell-Smith et al., 2006) and may interact with UCP1 by increasing BAT UCP1 activity (Fukano et al., 2016). Caffeine also acts on the β-3 adreno receptors which innervate BAT (Yoneshiro et al., 2017). Activation of these G-coupled receptors results in subsequent cAMP production, which leads to the activation of protein kinase A, which itself has been shown to stimulate lipolysis through the release of free fatty acids and in turn activating UCP1 (Cannon & Nedergaard, 2004).

Caffeine is also largely dependent on the antagonism of the adenosine receptor A_{1A}, which

leads to the release of orexinergic neurons from inhibition (Murphy et al., 2003). As such it is proposed that this is one mechanism of action for caffeine's effect on BAT thermogenesis, through antagonism of A_{1A}. A mechanism that has been linked to increased oxygen consumption and BAT thermogenesis in rats (Wu et al., 2017). Adenosine signalling is transmitted through the A_1 and A_3 adrenoreceptors (Ferre, 2008) through the G_i/G_o family or through A_{2A} and A_{2B} via the G_s family of G proteins (Rivera-Oliver & Díaz-Ríos, 2014). The G family of proteins specially modulate the cAMP available through downstream signalling; G_s stimulating the release of adenylate cyclase, and G_i/G_o inhibiting the release of adenylate cyclase (Rivera-Oliver & Díaz-Ríos, 2014), thereby increasing or lowering available cAMP acting on the A1 or A3 receptors. When it comes to obesity, adenosine has been shown to be protective of obesity in rats fed high fat diets (Cully, 2014), whilst lipolysis of WAT has been shown to be inhibited through the activation of A_{1A} receptors (Johansson et al., 2008) and increases in cAMP promote further lipolysis therefore promoting usage of stored energy (Arner et al., 2011).

For BAT thermogenesis, adenosine A_{2A} and A_{2B} receptor agonists have been shown to increase both lipolysis and BAT thermogenesis, alongside promotion of A_{2A} receptor expression in cold-exposed mice, an effect also seen with exposure to increased levels of intracellular cAMP (Gnad et al., 2014), a known effect of caffeine consumption. Overall then it is clear that caffeine's impact as a non-specific adenosine receptor antagonist could act on multiple fronts relating to lipolysis and thermogenesis, and therefore aid in the promotion of energy balance through this action.

Caffeine consumption has also shown to be beneficial in the central control of insulin resistance by enhancing insulin sensitivity and signalling in the solitary tract of rodent models (Yeh et al., 2014). The solitary tract has been linked closely with the control of blood pressure

through nitric oxide production via the A_{2A} receptor signalling pathway (Ho et al., 2008). Consequently, the solitary tract has shown to be one means by which BAT thermogenesis is centrally controlled, as inhibition of these pathways lead to diminished BAT thermogenesis through the activation of A_{1A} receptors and subsequent lowering of cAMP (Cao et al., 2010). Having demonstrated that caffeine is able to antagonise these receptors it is possible to suggest that dietary caffeine consumption may be beneficial in maintaining this balance over time.

The continued findings linking the sympathetic control of BAT and the interplay between cAMP, A receptors, β-3 adreno receptors and the adenosine signalling molecule have all been shown to impact BAT thermogenesis and energy metabolism (Van Schaik et al., 2021) however, this phenomenon has not yet been investigated using non-invasive techniques.

1.9 Methods of Detecting BAT Activity

Due to the recent resurgence in interest in the assessment of BAT both in adults (El Hadi et al., 2016) and children (Robinson et al., 2014) it is important to consider the range of current methodologies available. As previously discussed brown adipocytes have a specific morphology which makes them distinct from white adipocytes (See table 1 p 18). Functional BAT in adults was detected using 2-deoxy-2-[^{18}F]fluoroglucose (^{18}F-FDG)-PET/CT) (Nedergaard et al., 2007) and subsequently confirmed using a range of methods including biopsies and molecular analysis (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009). In order to continue to research the potential of BAT it is important that non-invasive means of assessment are utilised that will allow large scale trials in both adults and children, these methods should ideally be non-ionising, price efficient and consistently reproducible on a scale that makes them practical for volunteer studies. Current methods offer a range of these

features but as of yet a single non-invasive method has not been established as the 'gold standard' for BAT detection.

1.9.1 Positron emission tomography and Computed tomography

Currently, PET is the most commonly utilised method for the assessment of BAT. This is generally a mixed methods approach that uses the ^{18}F-FDG tracer alongside a PET/CT scan to create an image of increased metabolic activity. The method requires the injection of a positron emitting radiotracer with a short half-life, which binds to tissues undergoing high metabolic demand. The radioactive decay of the tracer results in the release of positrons, which collide with electrons resulting in the release of a neutrino and photons. The PET scanner itself is able to detect the movement of these photons and their current position, which in turn can be quantified alongside their accumulation within specific regions (Chondronikola et al., 2018). PET data can be acquired in two formats; static and dynamic. The static format allows for the acquisition of an image which covers the location of the radiolabelled tracer across a single time point. Dynamic acquisition however, a more modern approach allows users to assess radiotracer uptake across a time series which enables the assessment of the specific uptake rate and position of the radiotracer label over a specific period of time (Chondronikola et al., 2018), which makes this method specifically useful for the assessment of BAT activity over a time course and could provide a useful comparison in a mixed methods approach alongside IRT or MRI.

There are currently a range of possible radiotracers being utilised across the field of BAT research. ^{18}F-FDG is currently the most established tracer used in studies of this type. The mechanism of action for this tracer relies on BAT to uptake the tracer alongside plasma glucose. Once the tracer has entered the cell it becomes phosphorylated by hexokinase with

further breakdown being very slow and therefore detectable by the CT scan (Chondronikola et al., 2018). Recently, the assessment of BAT using ^{18}F-FDG has been standardised by the BARCIST 1.0 (Chen et al., 2016). This standardisation will have a profound impact on the fields ability to coordinate assessment of BAT via this method as prior to this there has been a significant lack of standardisation across groups and should make interexperimental quantitative assessment more feasible. Another method of BAT assessment is by utilising the uptake of glucose as a proxy for BAT activity, which dynamic methods have enabled. Glucose uptake has been closely linked to possible BAT activity (Chondronikola et al., 2014; Jacene et al., 2011; Law et al., 2021).

Computed tomography (CT) makes use of x-rays and tomographic image overlay to visualise internal physiology. When imaging BAT CT can target the unique morphology of BAT focusing on low radio density (vs water) but a higher density than lung tissue (Chen et al., 2016). Importantly BAT is distinguished from WAT via changes in radiodensity, with studies demonstrating that WAT has a higher radiodensity that BAT (Shingo Baba et al., 2007).

1.9.2 Limitations of 18F-FDG-PET/CT

^{18}F-FDG-PET/CT has undoubtably improved our understanding of BAT within adults and without the use of this technique the assessment of BAT within adults and related research would be much less robust. There are a range of limitations with this methodology however that must be considered. First and foremost, the ethical concerns relating to the utilisation of ionising forms of radiation make this technique unsuitable for multiple repeat assessments or for the use in those deemed vulnerable or in children. The technique also requires a high degree of specialist equipment and trained users which, making this type of work inaccessible to those without high levels of funding and very specific subject knowledge and know how.

1.9.3 Magnetic resonance Imaging

Owing to the complex morphology of adipose tissue, BAT has been a target of investigation through the use of magnetic resonance imaging (MRI). MRI works on the principles of spin which occurs when samples with high levels of nuclei are subjected to a magnetic field, spin can be altered using radio frequency which produce a detectable signal unique to the chemical composition of the tissue being assessed (Chondronikola et al., 2014). Samples which have an abundance of nuclei have a non zero spin which can be detected using MR (Chondronikola et al., 2018). ^{1}H spins are particularly abundant within water and lipids. Bulk longitudinal (T_1) and transverse (T_2) relaxation time constants are used in MR studies to target structural differences in water/fat ratio, lipid droplet size and mitochondrial density between BAT and WAT. (Romu et al., 2015; Sbarbati et al., 1997). Significant work has been completed to create an MR profile of BAT using T_2 weighted MRI in adults (Chen et al., 2013; Hamilton et al., 2011). It is however important to acknowledge that T_1 and T_2 weighted images of BAT are not a perfect assessment of BAT presence as there is still a high level of

cross-over between WAT and BAT, therefore biopsy may still be necessary to validate findings.

More recent methods of MRI including the use of ^{1}H chemical shift MR spectroscopy (^{1}HCSMRS) have improved our overall ability to make more accurate assessment of BAT presence within adults. ^{1}HCSMRS utilises the presence of chemical shift within a molecule that is subject to a magnetic field. Chemical shift is a measure of small precision frequency shifts as a result of the shielding/de-shielding of non-zero spin nuclei by proximal atoms (Chondronikola et al., 2018). Using this technique, it is possible to measure the unique composition of water and fat within BAT known as the fat fraction (FF). This FF can then be mapped over an image to allow visualisation of the tissue (Chondronikola et al., 2014). The use of this technique has seen wide usage in humans (Franssens et al., 2016; Hu et al., 2013; Rasmussen et al., 2013), and FF as a measure has been shown to have been reduced in BAT during stimulation, most likely as a result of increased BAT perfusion. Whilst ^{1}HCSMRS may be one of the currently most established means of BAT detection using MRI it is still important to acknowledge that FF can change over a short period and BAT and WAT can overlay making distinction between these tissues difficult even with precise assessment (Chondronikola et al., 2018).

1.10 Using infrared thermography to measure Brown Adipose Tissue Thermogenesis

1.10.1 An introduction to infrared thermography

Infrared thermography finds its origins within the military field, being developed and utilised during World War II as a means of detection. Since then however, the non-destructive, safe and generally highly portable nature of infrared thermography and infrared sensing cameras have been used as a measure of detection in a range of fields ranging from electrical and mechanical engineering to agriculture and astronomy (Rameau et al., 2013; Ring & Ammer, 2012; Thusyanthan et al., 2016; Wen et al., 2019). Thermal imaging has also seen wide usage within the field of medicine, wherein examples of its use range from detection of cancers, to sports medicine and musculoskeletal examination (Ring & Ammer, 2012).

Infrared cameras work on the principles of Planck's law (see p51). The camera itself contains a sensor which detects the radiation being emitted from the object being measured. This sensor will have been calibrated by the manufacturer against a black body allowing internal calculations to take place and the raw signal within the metadata of the image file to be stored. Using this metadata, the radiometric data can be converted into temperature data, which can in turn be converted to a colourmap image whereby each colour on the image is representative of a mapped temperature (Law, Chalmers, et al., 2018). This then is what is being displayed when thermal data is being recorded a false-colour image, an example of which can be seen in fig 2. (p58)

1.10.2 Infrared Radiation and Calculation of temperature

Infrared radiation (IR) forms part of the electromagnetic spectrum and was discovered by Sir William Herschel in 1800. Herschel demonstrated the presence of radiation on a thermometer below red light, coining the name Infra-red (Herschel, 1800). The electromagnetic spectrum also incorporates radio waves as the largest constituent, microwaves, visible light, ultraviolet light, x-rays and finally gamma rays being the smallest waves on the spectrum. IR falls just outside the visible spectrum (wave lengths between 390 and 700nm), displaying wavelengths between 700nm and 1mm (Tattersall, 2016). Importantly for the use of IR on humans it falls outside of the wavelengths considered ionising or possessing the ability to interact with atoms to cause electron loss, making it much safer than other imaging techniques such as using x-rays.

Thermal imaging utilises the relationship between temperature and long wavelength IR emitted from bodies with a temperature between -80 °C and 89 °C; a range appropriate for the majority of living beings (Tattersall, 2016). Described by Planck's law (Planck, 1959), which states that where there is a black body (or an object that absorbs and radiates heat perfectly), the radiation emitted at a given wavelength in thermal equilibrium will be completely absorbed and emit radiation dependent on its temperature. The resultant temperature of thermal equilibrium causes energy absorbed to be equal to energy released, and therefore below the equilibrium temperature a given body absorbs more energy than it emits, leading to warming and temperatures above equilibrium temperature will cause cooling (Planck, 1959). Using these principles Planck's law can then be utilised to calculate the unknown temperature of a black body in thermal equilibrium, given access to the radiometric data it produces, such as using IR sensors within the thermal imaging camera. Fig 1. (below) demonstrates this principle, whereby T represents the temperature of the black box, Bλ represents its spectral radiance (or the energy emitted at different wavelengths λ), h

is the Planck constant, kB is the Boltzmann constant and finally c is the speed of light. Whilst the black body represents a hypothetical entity, objects in reality and especially living beings will never be perfectly black, therefore the range of deviation from ideal black is measured using emissivity (ε) (Tattersall, 2016). A black body will record an emissivity of 1 whilst a perfectly reflective object will have an emissivity of 0. Objects or beings, being measured therefore will sit within this range and this will need to be considered when calculating an object's temperature using IR as a proxy for temperature and the reflected temperature will also need to be known. Human skin has a given emissivity value of 0.98 (Steketee, 1973) which remained constant for the purposes of all future experimentation.

$$B_\lambda(\lambda, T) = \frac{2hc^2}{\lambda^5} \frac{1}{e^{\frac{hc}{\lambda k_B T}} - 1}$$

Fig 1. *The above represents Planck's law (Planck, 1959) formula for calculation of temperature, represented as follows: T temperature, Bλ spectral radiance, λ wavelength, h Planck constant, kB Boltzmann constant, c speed of light.*

1.10.3 Study setup and acclimatisation

When undertaking thermography, it is important to understand the significance of controlling external variables to ensure that the data reaching the IR sensor, is representative of the body being imaged. For this reason, and as in any experimental procedure great care must be taken to ensure the study setup is appropriate for the aims of the study.

Prior to any assessment it is imperative that the camera operator is familiar with the thermal imaging camera they are using to acquire images. Most modern cameras measure with a pixel density of between 640 x 480 pixels, whilst some older models have slightly poorer

image acquisition (e.g. 320 x 240 pixels) and more expensive true high definition image capture is available for a premium. Pixel density is largely unrelated to the quality of image capture due to the IR sensor measuring radiometric data and converting this data. Sensitivity of thermal imaging cameras does tend to increase with the cost of apparatus giving them improved accuracy (with most modern camaras detecting temperature changes as low as 0.01°C), and if data analysis is being fully or semi-automated, knowing the pixel density of the camera is a key parameter for accurate analysis. Current imaging procedures are focused on taking multiple still-images over a designated time period (Law, Chalmers, et al., 2018; Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). However, owing to improvements in camera technology, it is now also possible to record longer form videos capturing images upwards of 30 frames per second allowing for a real-time assessment of temperature data. And while this method may see more use going forward the collection of large quantities of acquired data can pose further issues relating to data analysis and data storage, and not necessarily leading to more accurate or better outcomes.

1.10.4 Room Setup and camera positioning

For the purposes of experimental procedures using thermal imaging cameras, ambient temperature thermoneutrality needs to be achieved within the laboratory prior to imaging taking place. For this reason, it is important that the selected laboratory environment has appropriate temperature control. Thermoneutrality for adults in light cotton vest and shorts is approximately 22-24°C (Houdas, 1982) . It is also important to ensure that the participant is away from any direct airflow, such as that caused by air conditioning units and from other sources of infrared radiation such as lightning and heat generating objects such as radiators as these can impact the reading on the IR sensor. Owing to its portable nature, thermal

imaging has also been utilised outside of the laboratory setting to assess real world temperature changes in human subjects (M. E. Symonds, K. Henderson, et al., 2012). Even in these field scenarios it is important to ensure variables are controlled and for instances where external factors cannot be fully standardised these need to be addressed.

1.10.5 Brown adipose tissue stimulating methods and considerations

BAT is predominantly stimulated through mild cold exposure, although experimentation has been completed linking stimulation of the tissue to other means such as through pharmacological activation through the use of sympathomimetics targeting the β-3 adrenoreceptor (Cypess et al., 2015) and through dietary consumption (Yoneshiro et al., 2012). Use of IRT allows for both the assessment of acute temperature changes within the supraclavicular region, whereby users assess a change from a participant's baseline temperature upon tissue stimulation when compared to baseline. And for longer term studies in which stimulation of the tissue, the temperature change or the overall maximal temperature can be recorded across a number of visits with participants going through various interventions. Most commonly utilised, and arguably the most repeatable method for stimulating BAT for experimental purposes, is through the use of mild cold exposure. Whilst a single method has not been deemed the gold standard, cooling of an extremity such as a hand, forearm or foot with a cooling blanket or using temperature controlled water (Law, Chalmers, et al., 2018; Robinson et al., 2014; Symonds et al., 2011) away from the thermal imaging site of the supraclavicular region would be considered the most suitable as this allows for the cold stimulus to remain away from the site of measurement (reducing impact on the IR camera) and also allows for the capturing of data from a set standard point, of which the change in supraclavicular temperature can be compared. Whilst extremity cooling

is one possible method of stimulation thermal imaging technology is flexible and can be adapted to a number of settings, including use of full body cold exposure through cold room setups or full water submersion (Jang et al., 2014; Søberg et al., 2021). For all cooling methods however, it is important that the participant being imaged remains stationary throughout the image acquisition process and when any stimulus is introduced. Best practice would be to ensure the stimulus or device being used to generate a response is setup and in contact with the participant prior to starting the baseline assessment. This allows for the least movement of the participant when the stimulus is introduced. For example ensuring that the cool blanket is wrapped on the forearm of the participant and they are comfortable prior to cooling the forearm, will reduce unnecessary movement.

Another important aspect of the cooling process specifically is to ensure an effect leading to 'mild cold' exposure specifically is introduced. This will ensure sympathetic BAT innervation is taking place rather than inducing shivering or pain in participants leading to alternative thermogenic pathways being introduced. When using water as a cooling method, such as through the use of the blanket roll, water temperature should be maintained at around 15-20°C (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). This should reduce the impact or potential of non-shivering thermogenesis taking place. It is also possible to complete a standard individualised assessment for each participant, which requires the induction of shivering thermogenesis within participants and then starting assessment at a temperature which sits just above this range (van der Lans et al., 2014). Whichever method is selected it is important that the researcher is aware of the need to consistently assess participant comfort and propensity for shivering, this can be done through participant questioning and continued visual observation during imaging.

1.10.6 Participant Selection

For the purposes of thermal imaging, it is important to ensure appropriate selection of participants, especially in relation to certain non-measured variables that may impact the thermal imaging or could lead to inconsistent results. Participant screening should be considered a key factor in study setup and the use of screening questionnaires is suggested. Participant related factors which can impact thermal imaging can loosely be grouped into two categories; those which impact on the day assessment, and those which are related to the physiological or genetic aspects of the participant.

In regard to patient activities or dietary consumption which can impact the assessment on the day, it is suggested that participants refrain from the use of pharmacological agents such as beta-blockers or beta agonists (Law et al., 2014), as well as avoiding more standard dietary consumption of alcohol, smoking, caffeine, large meals or other forms of drinks which are high in sugar (Moreira et al., 2017). It is also advised that participants do not undertake any strenuous activity prior to assessment, have the chest area clean shaven (as the emissivity of hair can impact measured temperature (Fernández-Cuevas et al., 2015) and refrain from the use of cosmetics or ointments on the region being assessed as well as showering, as these can also impact the accuracy of the IR image (Moreira et al., 2017). Imaging should be held by at least 4 hours if any of these (non-pharmacological) factors are impacted.

1.10.7 Camera Setup and Factors Affecting Image Acquisition

Infrared thermography works on the premise of measuring the infrared radiation (IR) emitted by a given object, and subsequently converting this to temperature data, before recreating the temperature data as an image which can be visualised on a screen (Law, Chalmers, et al., 2018).

IRT relies on the principles of Planck's law (Tattersall, 2016), whereby the IR radiation emitted is proportional to the temperature of the object being assessed with long wavelength IR (8-15µm) emitted by objects with temperatures ranging between 80°C and 89°C. Using Planck's law the acquired radiometric data can be converted to temperature data when the emissivity; the proportion of radiation emitted by an object when compared to a purely black body, of the object is considered (Tattersall, 2016). Whilst IRT can be used to measure the human body in this way due to the range of human body temperature emitting wavelength between those discussed. It is important that suitable consideration be made when using this methodology as variables within the environment can lead to a disruption to or change of signal strength reaching the IR detector. The following should all be considered (and were considered) during all subsequent experimentation.

1.10.8 Emissivity

Emissivity is the measure of an objects ability to emit infrared radiation, which is proportional to the temperature of the object itself. Emissivity ranges from 0 to 1 where an object with 0 emissivity would be completely reflective, such as a mirrored surface and emissivity of 1 represents the so-called blackbody, an object which emits maximal radiation (Tattersall, 2016). The emissivity of human skin is approximately 0.98 and this parameter was set within the thermal imaging camera prior to the start of any experimental procedure.

1.10.9 Reflected Temperature

Reflected temperature is the measure of radiation which reaches the IR sensor which has not come from the object being measured and is therefore ambient radiation. As discussed previous, biological objects have a relatively high emissivity (0.98) and therefore background reflected radiation is generally not a factor and remains relatively low throughout experimentation, but is important to assess when carrying out procedures (Law, Chalmers, et al., 2018).

1.10.10 Relative Humidity

When completing IRT, ambient atmospheric conditions need to be considered prior to procedures. Due to the IR absorbency of water vapour, specifically in the long wavelength range. An environment with high levels of humidity can therefore lead to inaccuracies, as the object measured IR radiation can be absorbed by the water vapour prior to it reaching the sensor, therefore lowering the overall values. And subsequently, a low or alternating humidity environment, can lead to inaccuracies in reported data (Law, Chalmers, et al., 2018). For this reason it is important to measure and alleviate large fluctuations in recorded environmental humidity when undertaking IRT. For these studies, relative humidity was measured prior to procedures being undertaken, and tracked throughout the course of the experiment.

1.10.11 Object Distance

Extending the distance between the recorded subject and the IR sensor will lead to an increase in the optical path length (Law, Chalmers, et al., 2018). This increase will further increase IR recorded from the subject being absorbed by water vapour within the air. For this reason when measuring objects using IRT a shorter distance is considered best practice as

this will alleviate unnecessary absorption due to increased atmospheric humidity. For this study a distance of 2 meters was chosen as optimal and in accordance with previous studies (Law, Morris, et al., 2018). This selected distance has been shown to have low impact on overall results.

1.10.12 Atmospheric Temperature

Atmospheric temperature is a key indicator of the incident radiation that the measured object is exposed to (Law, Chalmers, et al., 2018). In this instance it is the overall amount of energy by which the subject can be exposed to and therefore absorbed and reflected to the IR sensor. It is therefore important to control atmospheric temperature and maintain an accurate and stable ambient temperature throughout the course of the experiment.

1.10.13 Physiological Factors

For the participant physiological factors, it is important that the researcher is able to gain information on age, sex, body mass, height, body mass index, ethnicity and smoking status, as all of these can impact BAT activity (Moreira et al., 2017).

For non-participant related best practice, all participants should be given standardised loose-fitting clothing such as shorts and a vest upon arrival to the imaging session. It is ideal to have the participant change into these standardised garments upon arrival and then sit within the imaging room to allow for time to acclimatise to the temperature of the study space. It is also advised that study sessions take place at the same time of day for the duration of the study as this will allow for the avoidance of participant circadian impact (Ninel Hansen et al., 2017).

1.10.14 Data Acquisition

For the purposes of data acquisition, participants should be acclimatised to the ambient

temperature of the room prior to assessment for roughly one hour or until participant temperature has plateaued (Haq et al., 2017). The experimental environment should remain at a consistent 22°C throughout the course of assessment and remain monitored by the researcher. As previously mentioned it is of high importance for the participant to remain in an upright and comfortable position throughout the whole process. This can be acknowledged by the researcher prior to undertaking any imaging through questioning. Unnecessary participant movement should be avoided at all times during acquisition as this can impact results. Participants should be sat in an upright position, with head fixed and remaining neutral and chin slightly raised to ensure visible access to the supraclavicular region (Law, Chalmers, et al., 2018). The thermal imaging camera should be setup on a tripod, facing the participant and height adjusted to ensure the image displayed on the screen encompasses the entire SCV region aiming perpendicular to the centre of the larynx of the participant throughout the recording period.

Consideration of the duration of imaging is an important component of accurate data acquisition. Whilst it is important to ensure appropriate acclimatisation of the participant to the study environment, there can often be seen a continued cooling of the SCV region when imaging starts to take place (Law, Chalmers, et al., 2018). For this reason an appropriately timed baseline temperature assessment needs to be undertaken. This should encompass at least 10 minutes of imaging of the SCV but may benefit from a longer duration if the participant is able to remain stationary for longer periods of time. An assessment should be completed during the final 5 minutes of the baseline protocol were in at least 3 measurements are taken that remain consistent. This will allow for an accurate baseline temperature of the SCV region to be obtained prior to additional stimulation occurring. When using mild cold as a stimulus, the response in the SCV region is quick to obtain, with changes

seen within the first 5 minutes of imaging post stimulation (M. E. Symonds, K. Henderson, et al., 2012) but longer periods of assessment and cooling may lead to further increase in temperature over time (Law, Chalmers, et al., 2018).

For selection of image acquisition consistency, it is important to understand the fundamentals of the camera being used. Most cameras will allow for a timelapse to be taken with images acquired over an extended period of time. Images should be collected for both baseline and stimulated time frames with an acquisition rate of no less than 4 to 10 images per minute. Modern thermal imaging cameras also offer video capture which generally works at a rate of 30 frames per second. Whilst the additional images produced can improve accuracy, the analysis of huge data sets gained from this style of image capture can pose its own issues, such as data storage and analysis leading to potential inaccuracies.

Whilst thermal data can provide a good indication of SCV region activity, it is important to also monitor other physiological outputs relating to SNS activation. These should include heart rate monitoring and blood pressure assessment. In addition and where possible it may be suitable to employ a range of sympathetic nervous system activity monitors during or between imaging such PPG stress flow analysis. And subsequent measurement of internal body temperature should be recorded consistently throughout the session.

1.10.15 Analysis of images

The analysis process relies on the conversion of the thermographic data to radiometric data which can be openly analysed. Standard thermographic image file types may vary depending on the manufacturer of the thermal imaging camera but are generally stored as Joint Photographic Experts Group (JPEG) or Tag Image File Format (TIFF) file types. For the purposes of this study and for the basis of this discussion the equipment used was

manufactured by FLIR®.

The false colour images obtained throughout the imaging process remain as a separate store of data to the radiometric data. Whilst these images can be useful for displaying large contrasts in temperature at face value, their general usage outside of this remains low. The radiometric data therefore needs to be converted to temperature data in order for the analysis process to continue. Typically, this is done using the manufacturers proprietary software such as FLIR Tools®, however these proprietary software tools tend to be limited. For this reason it is often best practice to utilise other methods for analysis, which will often require an intermediary file conversion prior to the data being available. The most common converted file formats are Comma Separated Values (.CSV), Portable Network Graphics (.PNG) or MATLAB™ file formats (Law, Chalmers, et al., 2018).

1.10.16 Region of Interest Assessment

For the purposes of BAT analysis, it is important to identify the region of interest (ROI) within the supraclavicular region. One such method involves the approximation of the contour of the shoulder between the clavicle and the sternocleidomastoid muscle, giving an indication of the lateral cervical region within which the SCV fossa sits (Law, Chalmers, et al., 2018; Robinson et al., 2014). Due to the individualised nature of this method however, manually placing markers on the anatomical landmarks during analysis would prevent the use of larger quantities of data due to the time constraints and opportunity for variability from researcher error. To make this process more suitable for larger data sets it is possible to utilise shape and region selection tools, which are commonly available within the proprietary software available with most thermal imaging cameras. Previously, studies have been conducted using rectangles, circles, triangles and other polygons to identify the ROI within the SCV (Jang et al., 2014; Law, Morris, et al., 2018; Ramage et al., 2016; Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). These methods do still however rely on the use of approximation from the part of the researcher and therefore errors in region selection can cause under or over representation of the temperature data leading to inaccuracies within data analysis. A good balance of both methods which allows for high levels of reproducibility and overall accuracy would be to employ the use of reflective markers at the appropriate anatomical locations (as described above). These markers allow the researcher to more quickly assess the data whilst aiding in reproducibility of results during analysis (figure 2 below).

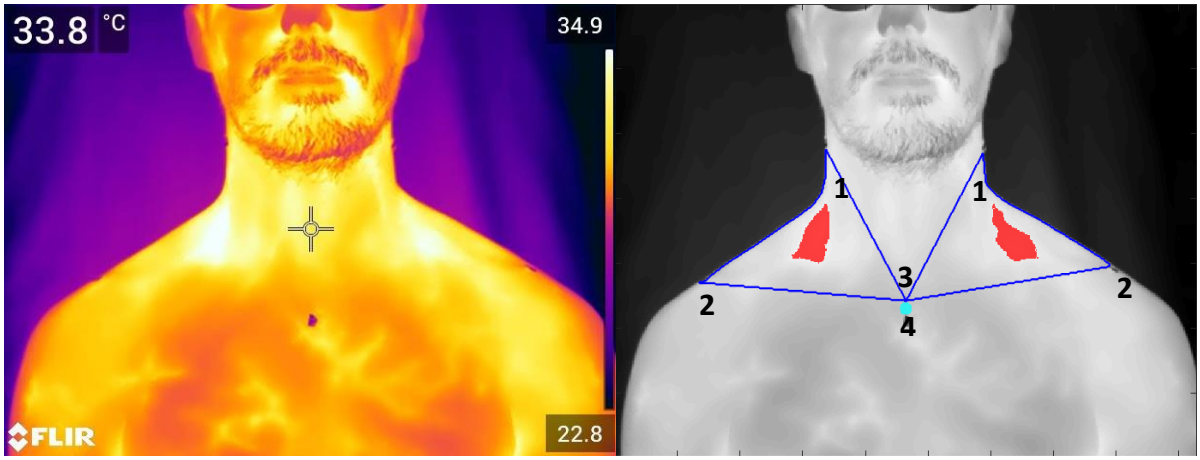


Figure 2. *Left image demonstrates the participant positioning and raw TI. Right demonstrates region of interest composed of blue lines leading to and from: 1) superolateral apices, 2) acromioclavicular apices, 3) sternal apex, 4) reference point. Red regions demonstrate the identified hotspot (highest 10%).*

Once the ROI has been established the ROI temperature can be assessed. Typically, this can be done using the manufacturer's proprietary software, which takes the temperature for the entire ROI and calculates a mean. This method however, makes the assumption that BAT contributes to the temperature across the entire region, and the hottest and coolest points within this data set are taken from individual data points from the entire region (El Hadi et al., 2016; Jang et al., 2014; Ramage et al., 2016; M. E. Symonds, K. Henderson, et al., 2012). Improving on this methodology, advances in analysis software allow for more precise targeting, which lower the reliance and potential inaccuracies as a result of researcher input. These methodology utilise automated ROI placement of the shoulder contour, with the researcher only needing to select the apices of the ROI, which can be aided with the use of reflective adhesive markers on the participant (Law, Morris, et al., 2018). Rather than relying on the average temperature across the whole ROI. Modern methods utilise and identify a BAT 'hotspot', defined within the chosen region and identified as an upper percentile of pixels within the identified region (Law, Morris, et al., 2018).

1.10.17 Error and Background Noise

In order to reduce the error term acquired when obtaining the radiometric data and aid in the reproducibility of results, it is important to consider the factors which may impact the error through background noise (Law, Chalmers, et al., 2018). Undertaking summary measures is one way of reducing error, by averaging data across thousands of pixels but may become impractical due to large quantities of data such as when using video rather than still images. When undertaking a time series method it is important to consider the error caused due to physiological changes within the participant or background noise as the imaging session progresses. Studies choosing to use only small quantities of images over shorter time periods will be the most susceptible to this kind of error and for that reason it is important to consider image acquisition timing across the whole time scale in order to gain sufficient data to reduce error. For studies involving human participants, it has been shown that skin temperature tends to stay within a smaller range (approximately 30-37°C) with little change in the mean (Law, Morris, et al., 2018).

1.10.18 Output Measures

The output measure will need to be calculated based on the ROI selected. Standard proprietary software will allow for basic output measures to be calculated based on simple shapes such as rectangles and circles and then allow users to generate minimum, maximum and mean temperature data for pixels within the selected region. For the purposes of measuring BAT activity, using these data may not be suitable as calculating the mean and using minimum and maximum temperature data can be particularly prone to under and over estimation of the impact of the BAT depot. When using the BAT 'hotspot' method using a larger ROI which doesn't assume the precise location of the BAT depot this needs to be

considered when choosing an outcome measure. Choosing the highest 10% of temperature data points from within the supraclavicular region can be used as a means to represent the BAT within the ROI (Law, Chalmers, et al., 2018). If a hotspot is selected It is important to consider that due to the non-normal distribution of the pixel data within this ROI the mean is not an appropriate representation of the thermal data. In this instance it is better to utilise the median value within the data set, which is also less prone to extremes of data error at the top and lower end of the scale (Law, Chalmers, et al., 2018). The median value of the top 10% of values within the ROI then is equivalent to the 95th percentile, a value that can be calculated easily and correlates closely to the influence of BAT within the ROI (Law, Morris, et al., 2018).

With the establishment of utilising the median 10% of the data within the selected ROI (or 95th percentile), it is important to consider the options for the output measure. Most commonly options for IRT include acquisition of the resting temperature (T_r), the temperature within the ROI upon stimulation (usually through mild cold exposure) (T_s) or the change in temperature (ΔT) e.g $T_r - T_s$. Thermal data can then be assessed using absolute values (T_{SCV}) or relative values T_{rel} e.g $T_{scv} - T_{ref}$ where T_{ref} is the thermal data acquired from a non-BAT region such as the sternum (Law et al., 2021; Robinson et al., 2016). Which allows the participant to act as their own control for the purposes of analysis which limits the impact of external factors such as skin perfusion on data acquisition.

Reference points are a useful measure when assessing BAT activity due to factors which may inhibit a radio metric response as discussed previously. Participant obesity for example has shown to inhibit T_{scv} owed in part to a potential combination of reduced BAT prevalence (Alcalá et al., 2019; Vijgen et al., 2011) and of increased subcutaneous fat within the ROI (Neves et al., 2015). ΔT then can be used to dampen the impact of these effects when

imaging those with higher BMI, and can be compared to a non-BAT reference region on the participant (T_{ref}). Alternative methods to using a selected reference region are to calculate a mean or median skin temperature (Robinson et al., 2016), again away from the BAT ROI. This can be done using skin contact probes such as thermocouples or iButtons™ which provide real-time temperature data of the specific region which they overlay and can be attached to a number of non-BAT regions as a measure of reference. This median reference data can then be compared to the median temperature data of the SCV hot-spot allowing for the acquisition of a relative temperature value (T_{rel}).

Whilst using T_{rel} (T_{SCV}-T_{Ref}) is one possible outcome measure. The use of the ΔT stimulated outcome measure can also be acquired easily wherein the stimulated temperature can be calculated following stimulation from baseline for both the SCV region and reference point (ΔT = Stimulated T – resting T) (Law, Chalmers, et al., 2018). ΔT allows for the use of the participant as their own control and using the non-BAT region reference point (through thermal data of the lower sternum or through use of skin temperature probes) allows for the standardising of results, which can then be more readily compared between participants (Law, Chalmers, et al., 2018). Finally, ΔT_{Rel} can also be utilised and has been shown to provide the closest correlation to BAT activity as measured by PET/CT (Law, Morris, et al., 2018). However, this method does assume a 'stimulated' and 'non-stimulated' state on behalf of the participant. For this reason, is it important to ensure participants have enough time within the research space to acclimatise such that a stable baseline temperature can be obtained. And even with the closest consideration there is always a possibility that a participant arrives in a maximally stimulated state and will therefore show very little change in ΔT or $\Delta T_{Rel.}$

All studies carried out using infrared thermography were completed in accordance with the methods and development undertaken by the group based in the early life research unit,

division of child health, obstetrics and gynaecology school of medicine, part of the University of Nottingham, pioneered by the work of Dr James Law et al. working in conjunction with Dr David E. Morris (Bioengineering group, faculty of engineering, University of Nottingham (Law, Chalmers, et al., 2018).

2. Hypothesis & Aims

The overall aim of this series of studies is to:

1) To Identify the effects of caffeine specifically on the SCV region when assessed using the IRT methodology.

2) Continue to offer validation and method development for the use of infrared thermography as a means to measure the impact of cold nutritional interventions on BAT.

3) To assess correlations between the human cold response, as measured using IRT and changes within the single nucleotide polymorphism TRPM8 (rs10166942 genotype) relating to the cold response and BAT.

2.1 Hypothesis

Study 1) Caffeine pilot study using a caffeinated beverage

Using a caffeinated beverage as the intervention, I hypothesise that, the supraclavicular region which overlays the most prominent brown adipose depot will respond to caffeine consumption, in a way that is detectable using the IRT method when compared to water consumption alone.

Study 2) Caffeine study using a double-blind placebo

To assess the impact of caffeine on the temperature of the supraclavicular region which overlays the most prominent BAT depot, I hypothesise that 200mg of caffeine will show a notable and detectable change in temperature within the supraclavicular region as detected using the IRT method when compared to the control intervention.

Study 3) Imaging Brown Adipose Tissue (using Magnetic Resonance and Thermal Imaging)

It is hypothesised that, if used in conjunction, forearm cooling activated brown adipose tissue thermogenesis detected using thermal imaging will co-locate with brown adipose tissue present within the supraclavicular region as demonstrated using MRI.

Study 4) Investigating how changes to single nucleotide polymorphisms can affect the cold response as measured using the IRT methodology.

I hypothesise that presence of the T allele TRPM8 rs 10166942 SNP will have a positive impact on response to mild cold exposure i.e. those with the T allele present will demonstrate a higher ΔT_{rel} when exposed to a mild cold stimulus than those with alternative genotypes

2.2 Primary and Secondary Objectives

2.2.1 Study 1) Caffeine pilot study using a caffeinated beverage

Primary Objective

The primary outcome will be the temperature change as a result of BAT thermogenesis measured using thermal imaging following the consumption of the caffeinated beverage or water.

BAT activation (the increase in temperature of the main depot of BAT in the supraclavicular region of the neck (ΔT) will be calculated by measuring the change in the temperature in the region of interest, at baseline and following consumption of caffeine or water.

Secondary objectives

Will include associated changes in physiological measurements such as heart rate and tympanic temperature, effect of influencing factors such as, anthropometric parameters, body composition and caffeine consumption.

2.2.2 Study 2) Caffeine study using a double-blind placebo method

Primary Objective

The primary objective is to study the change in heat production (ΔT) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following the consumption of caffeine compared to a placebo. Resting changes in supraclavicular BAT temperature will be measured before participants are asked to consume a caffeine supplement (200mg tablet) or placebo (200mg Lactose tablet). Measurement of supraclavicular BAT activation will be calculated from the change in the median temperature of the upper percentile of temperature points in the region of interest using previously established and published methodologies (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012).

Secondary Objectives

Secondary outcomes will include associated changes in physiological measurements such as heart rate and skin temperature, effect of influencing factors such as, anthropometric parameters, body composition, age, and caffeine consumption.

2.2.3 Study 3) Imaging Brown Adipose Tissue (using Magnetic Resonance and Thermal Imaging)

Primary Objective

To determine if regions of BAT in the neck and upper thorax can be differentiated from WAT using MRI.

Secondary Objectives

To determine if paired thermal imaging and MR imaging studies are feasible and to provide sufficient data suitable for a sample size calculation for subsequent studies.

2.2.4 Study 4) Investigating how changes to single nucleotide polymorphisms can affect the cold response as measured using the IRT methodology.

Primary objective

To use previously established data (thermal imaging) to investigate the presence of the TRPM8 SNP allele and compare this to the change in heat production (ΔTrel) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following mild cold exposure of the forearm. Resting changes in supraclavicular BAT temperature were measured before participants were subject to forearm cooling at 16°C. Measurement of supraclavicular BAT activation was calculated from the change in the median temperature of the upper percentile of temperature points in the region of interest using previously established and published methodologies (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012).

To assess correlations between the human cold response, as measured using IRT and changes within the single nucleotide polymorphism TRPM8 (rs10166942 genotype) real-time QPCR will be carried out on the genetic material from participants obtained from saliva samples.

Secondary Objectives

Secondary objectives aimed to assess the association with alterations in TRPM8 rs10166942 allele and association with secondary measures such as BMI, type to diabetes and non-alcoholic fatty liver prevalence.

Having established the link between the nervous system, the innervation of BAT, and the use of interventions to stimulate BAT this thesis aimed to gain a more comprehensive understanding of the nervous functionality of BAT and how nutritional interventions such as caffeine consumption can lead to the activation of BAT. It assessed the activation of BAT with IRT.

The analysis of caffeine induced BAT activation using IRT, allowed us to see weather nutritional supplements in the form of SNS activating compounds could elicit an acute or chronic improvement in BAT activation. Although the stimulatory effect of caffeine on metabolic rate is well established (Dulloo, 2011), most of the studies focussing on caffeine's acute thermogenic effects use relatively large doses. There is little information regarding the thermogenic effects of caffeine in amounts in general consumption (as in a cup of coffee) and the effect this has on BAT activity. This thesis aimed to measure the effect of caffeine at a dosage in common dietary consumption (89mg coffee study/200mg supplement study) and how this affects BAT activated thermogenesis, measured by IRT. It is therefore hypothesised that consumption of the

methylxanthine, caffeine, at a dosage of 200mg activates thermogenesis via stimulation of the sympathetic nervous system. It is possible that consumption of caffeine would lead to a higher level of BAT activation measured by proxy through increased temperature of the SCV region when compared to a control.

Study Protocols

Disclaimer: All work on the following projects was completed by Declan Wayne including planning, ethical approval and data acquisition. Advice on statistical analysis was given by professor Helen Budge and Professor Michael Symonds, as part of the Department of Child Health, School of Medicine, University of Nottingham.

3. Study 1: (Pilot Study) An investigation into the impact of Caffeine on BAT thermogenesis Using coffee or water as Intervention to stimulate BAT (BITSCAFF)

Abstract: In order to assess the effectiveness of coffee (caffeine) as a potential stimulator of BAT thermogenesis an initial nutritional pilot study gave participants a coffee drink or water followed by an assessment of the SCV region overlying the major BAT depot in adults using IRT. Results revealed that consumption of coffee increased the temperature within the supraclavicular region, a change not seen with the consumption of water.

3.1 Introduction

Prevalence of obesity has been linked strongly to an energy usage imbalance. Whereby there is a negative balance of caloric intake and total metabolic activity (Blüher, 2019). Current treatments are focused on the promotion of leading a 'healthy lifestyle' consisting of increasing physical activity through exercise, whilst decreasing overall caloric intake through diet, as a means to reduce the metabolic imbalance leading to a reduction in stored adipose tissue (Emily et al., 2019). These methods have so far

Prevalence of obesity has been linked strongly to an energy usage imbalance.

One alternative approach to combating obesity by increasing energy expenditure in conjunction with the previously established methods may be through the use of pharmacological interventions that promote or maintain adipose storage through increased energy expenditure through thermogenesis (Dulloo, 2011; Nedergaard & Cannon, 2010).

BAT may be one such target, as it has been shown to play an important role in energy homeostasis into adulthood (Nedergaard et al., 2007). BAT thermogenesis has been estimated to contribute significantly to overall energy usage, with an increase of BAT prevalence possibly leading to a significant increase in overall energy demand (Brooks, 2005).

BAT thermogenesis is controlled by the sympathetic nervous system through β-adrenoreceptors (Cannon & Nedergaard, 2004). Importantly for the use of pharmacological interventions, targets should aim to be BAT specific where possible to reduce the impact of side effects.

The use of caffeine is one such potential pharmacological intervention, routinely consumed and readily available, making it a desirable target. Caffeine has been linked to thermogenesis in both animal and human studies (Panchal et al., 2012; Velickovic et al., 2019). However, the mechanism of action is still unclear and human studies, especially those investigating an acute brown fat thermogenic response within the supraclavicular region are lacking. Caffeine is the worlds most populous drug and coffee is one of the most popular beverages on the planet. Due to this commonality and ease of access the consumption of caffeine found in a standard coffee drink may be a suitable initiator of BAT thermogenesis.

3.2 Primary Objective

The primary objective is to study any change in heat production (ΔT) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following the consumption of a caffeinated beverage (Nescafe 100g sachet ~89mg caffeine) or water at ambient room temperature (22°C). Resting changes in supraclavicular BAT

temperature will be measured before participants are asked to consume the caffeinated beverage (~89mg caffeine) or water. Measurement of supraclavicular BAT activation will be calculated from the change in the median temperature of the upper percentile of temperature points in the region of interest using previously established and published methodologies (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). Data from the same individuals will be compared when consuming the caffeinated beverage and water.

3.3 Study Configuration

Participants were involved in two study sessions and randomly given either:

- Caffeinated beverage using water at ambient room temperature 22°C (Nescafe 100g sachet ~89mg caffeine) or
- Water at ambient room temperature 22°C

Participants arrived at the study visit having not consumed food for 12 hours before the study session, and water, one hour before the study session. Participants were randomly assigned to study order, however blinding of the participant was not possible in this instance due to the nature of the study design being given either a coffee or water which are clearly distinguished from each other and have very clear distinguishable taste profiles.

3.4 Ethical Approval

The study was conducted in accordance with the standards of the Declaration of Helsinki following approval from the University of Nottingham Medical School Ethics Committee and informed consent was gained prior to any participant measure taking place.

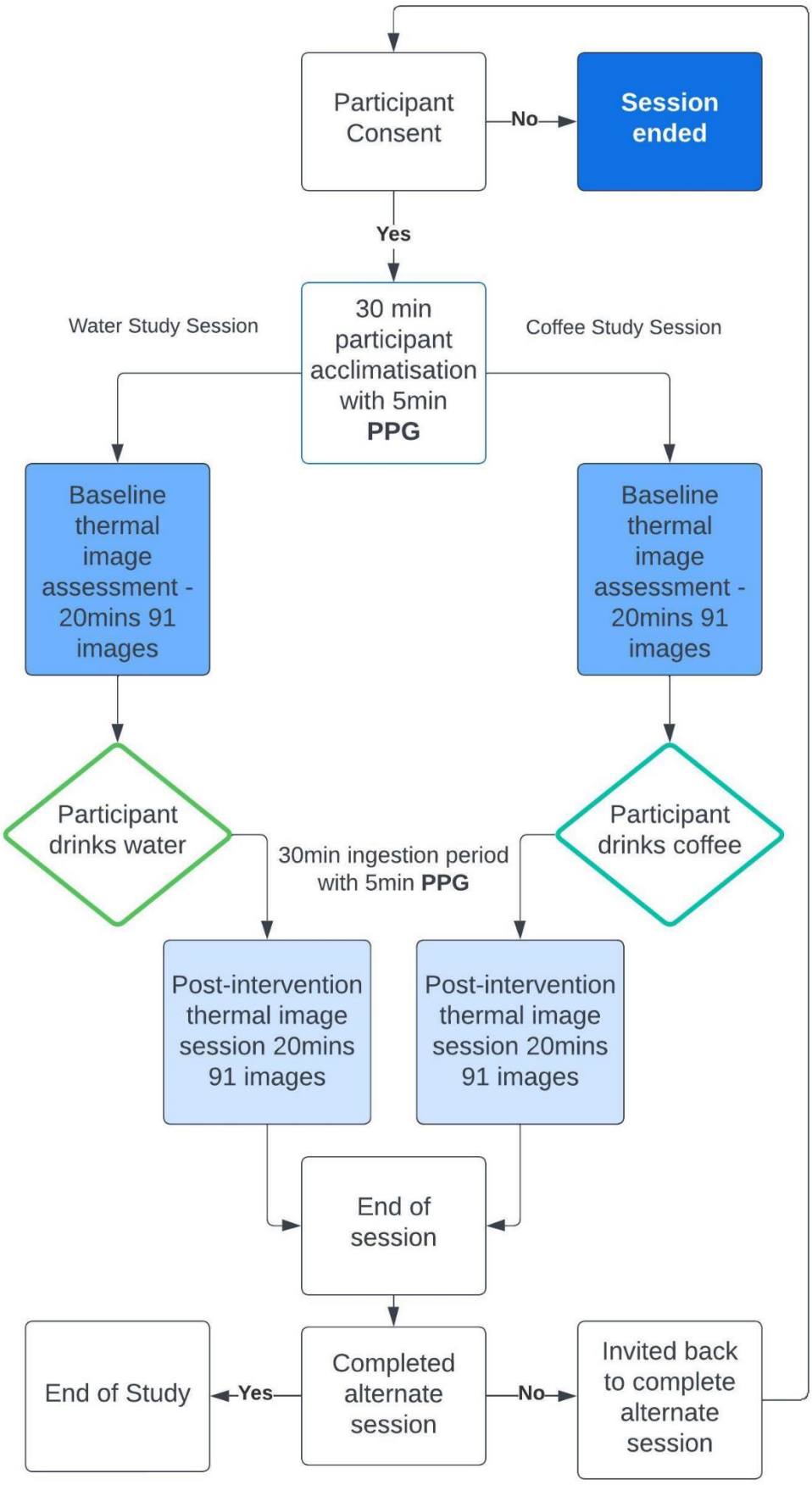
3.5 Sample Size Justification

This series of studies will allow a number of different comparisons to be made and the numbers required in each group will vary depending on the comparison being made. This is a pilot study to assess feasibility as such using previously published data In children (M. E. Symonds, K. Henderson, et al., 2012) and making use of the Power and Sample Size program (Vanderbilt Biostatistics) adults with an estimated standard deviation of 0.212, 9 participants in each of two independent groups, would give 80% power to detect the difference with a significance level of 5% for a two-tailed t-test.

3.6 Methods

This study procedure was conducted in accordance with previously completed procedures within the department of Academic Child Health (Law, Chalmers, et al., 2018) and followed IRT guidance as outlined on p56. A flow diagram detailing the final procedure, including all relevant timings can be found on p78. The following study took place after acquiring written informed consent prior to the study sessions taking place and this permission was clarified on the day of each study session.

Figure 3 *Study protocol for study 1 (Consort diagram produced in Lucidchart®)*



3.7 Room Setup and Thermal Imaging

All participants were studied at the Human Physiology Laboratory based within the Department of Academic Child Health.

The study sessions were conducted in an ambient temperature of 22°C in a thermally controlled environment this was achieved by arriving to each study session approximately 2 hours prior to each study session and setting the in-room air conditioning to this temperature. All study sessions took place in the morning starting at 10:00am, and the study took place during the months of October to February.

Thermal imaging was conducted in accordance with procedure outlined previously (p78).

3.8 Camera Setup Procedures

All imaging for this study took place using a FLIR® T540 w/24oLens.

Prior to participant arrival the camera was setup accordingly. Reflective temperature was assessed with a piece of reflective silver card with an emissivity of 0 to ensure the camera was calibrated prior to assessment taking place, room temperature and room humidity were measured consistently throughout the study using a digital thermometer and hygrometer (Thermopro TP50 Digital ThermoHygrometer Indoor room Thermometer, ThermPro®), ambient temperature and relative humidity and distance from the camera were measured and inputted into the camera prior to the participants arrival.

3.9 Anthropometric Assessment

Upon arrival to the study, for the participant's body temperature to reach a steady state in relation to the ambient room temperature, an initial acclimatisation period took place. Upon gaining further informed consent participants were initially requested to change into a loose-fitting cotton vest top and shorts which allowed for the exposure of the shoulders and neck area for imaging. Shoes were requested to be removed throughout the study. During this acclimatisation phase anthropometric assessment of each participant was carried out to measure their height using a standard stadiometer (SECA 2123 Portable Stadiometer) and weight with a set of calibrated ISO standard digital scales (Active Era® Scales) which were calibrated based on manufacturer guidelines prior to assessment, each participant was measured twice to ensure accuracy. A tympanic internal temperature assessment was taken to detect basal internal temperature prior to intervention (Braun®ThermoScan).

Basal heart rate prior to intervention of caffeine or water was then assessed using a PPG Stress Flow analysis monitor (PPG Stress Flow©: Biotekna S.r.l.®, Italy). The participant was seated with both of their forearms resting on a table. After ensuring that the sensor target area was clean and intact, sensors were placed on the right and left index finger. Participants were asked to avoid moving their fingers as this could affect the results. This process was completed over 5 minutes. After this Each participant had a further 5 minutes of sitting in order to be fully acclimatised to the room temperature before thermal imaging, completing this 20-minute acclimatisation

3.10 Participant Setup and Imaging

Several soft, self-adhesive markers were placed on the participant's skin near the area of interest (side of the neck) and on the clavicle and sternum to provide anatomical markers detectable on thermal image which facilitates subsequent analysis (See Figure 3.)

During imaging, participants were not exposed to drafts or convection currents, as this may have affected skin temperature, and participants were not placed near a fan or air conditioning outflow, near plug sockets or cables, or directly in front of reflective surfaces such as windows.

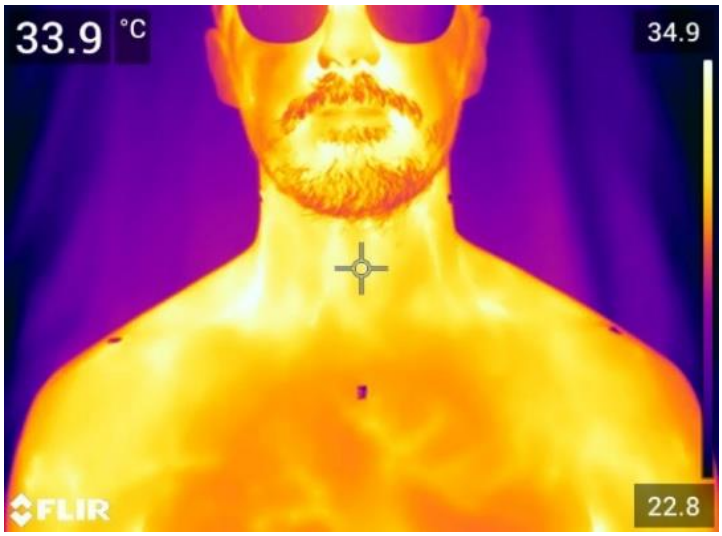


Figure 4. *Shows an example of the participant positioning and location of anatomical markers used for thermal image assessment.*

Participants were seated comfortably and approximately 1 metre horizontally from the thermal imaging camera (FLIR T540 w/24oLens), itself placed on a tripod (Jessops RR125 Hex Tripod) (approximately 1 metre vertically from the ground.

After a period of acclimatisation (20 minutes); baseline (pre-stimulation) images were

taken for 20 minutes (60 Images), prior to consumption of the randomly allocated intervention (coffee at 22°C (Nescafe 100g sachet ~89mg caffeine) or water at 22°C). The intervention beverage was temperature checked using the thermal imaging camera to ensure that it equalled ambient temperature (22°C). Following this initial imaging a 30 minute 'ingestion' period took place to allow time for the digestion of the beverage. During the final 5 minutes (25th-30th minute) of the ingestion period participants undergo a second PPG Stress Flow© analysis session for heart rate measure over 5 minutes. A second tympanic internal temperature assessment was taken to detect any change in internal temperature following intervention (Braun®ThermoScan).

Following this an additional 20 minutes of imaging, commences 30 minutes after the intervention (caffeinated beverage or water) to detect the ingested effect of the intervention on the participant. Upon completion participants were invited to return to take part in an identical study session consuming their alternative intervention.

3.11 Measurements

3.11.1 Analysis of IRT

Thermal images within this study were acquired at an interval of 1 image every 20 seconds over a period of 20 minutes, repeated for each session of imaging. They were then processed after conversion of the thermal image (TI) data to the non-proprietary portable network graphic format, marking the supraclavicular region of interest and then processing the data within those regions. The conversion of images is performed using a custom-built thermal imaging technical conversion hub. This is a Raspberry Pi-based device that takes the raw TI data and saves it in an openly accessible format from which the temperature can be calculated. Both left and right supraclavicular regions are defined by placing 4/5 markers (hence the use of sticky markers on participants during imaging) corresponding to the apices of region (see Figure 3. For details p78).

The hottest 10% of the points within the TI within each region are then identified and the medians of these points were then calculated (equivalent to the 95th percentile) (Law, Morris, et al., 2018). A reference region was also compiled from a circle 10 pixel in diameter immediately below the central apex of the region analysed, allowing for a skin temperature comparison. A moving average was then used over the time course of results reducing the impact of natural temperature variation. The primary outcome was the difference in BAT activation (TSCV and ΔTREL) following the consumption of the caffeinated coffee beverage or water. BAT activation was calculated by measuring the change in the median temperature of

the upper percentiles of temperature points in the region of interest (SCV), following stimulation by the intervention.

3.11.2 Image Analysis

Thermal images were analysed to determine the median temperature of the regions of activity, defined as the upper percentiles of temperature data in the region of interest in the neck. Visual representations of the data are displayed as coloured isotherms. The change in median temperature of the region of activity between the baseline and stimulated images was calculated (ΔTREL and TSCV).

3.11.3 Statistical Analysis

All data were analysed by Student's t test. Statistical significance was accepted at $P < 0.05$, with $*P < 0.05$; $**P < 0.01$; $*** < 0.001$. Error bars presented on graphs represent the mean ± SEM. Data were analysed using Graph Pad Prism® Software (https://www.graphpad.com).

3.12 Results

Participants included healthy volunteers (4 male; 5 female), aged 27.34 (Standard deviation (SD) 1.6 years of normal BMI (mean 23.3 (SD 2.87) kg/m2). Nine participants in each group allowed for the study to have a 95% power to detect a biologically significant mean difference of 0.2°C (Law, Morris, et al., 2018).

Table 2. *Characteristics of participants*

	Participant Group Data (Mean)	SD ±	SEM
n	9		
Age, y	27.34	1.6	0.61
Height, CM	175.2	4.37	1.46
Weight, Kg	23.67	8.23	2.74
BMI, kg/m^2	23.3	2.87	0.96

Data are presented as means SD ± and standard Error of the mean SEM

Table 3. *Further Characteristics of participants and study conditions*

	Coffee Intervention Data (Mean)	Water Intervention Data (Mean)	SD ±	*P*
Room Temperature °C	22.23	22.32	0.26	*0.3*
Room Humidity, rh%	42	42.55	7.56	0.79
Time since last meal, h	5.76	5.81	0.7	0.85
Resting HR, BPM	67.29	72.91	11.99	0.23
HR following Intervention BPM	60.36	63.78	8.75	0.3
Internal Temperature before intervetion °C	36.44	36.41	0.14	0.49
Internal Temperature After intervetion °C	36.47	36.44	0.16	0.54

Data are presented as means SD ±, P values are the result of a T-Test. HR: Heart Rate; BPM: Beats Per Minute; BMI: Body Mass Index.

Table 4. *Thermal response participant*

	Coffee	Water
TSCV Before Intervention °C	33.81 ± 0.45	34.55 ± 0.16
TSCV After Intervention °C	34.187 ±0.59***	34.55 ± 0.17
ΔTREL °C	0.4067 ± 0.28**	0.13 ± 0.12
Reference Point Temperature Before Intervention °C	31.12 ± 0.27	31.58 ± 0.14
Reference Point Temperature After Intervention °C	32.09 ± 0.31	31.67 ± 0.18

*Data are presented as means SD ±. *indicates statistical significance ***P<0.001; **P<0.01. TSCV: Temperature SCV; TREL: Relative Temperature Change SCV.*

The results indicate that the study environment and participant characteristics did not make a significant impact on the overall results of the study (Table 3 p85). Room temperature and humidity within the study environment were controlled throughout the study and showed no clear change between study sessions (p=0.3) (Table 3 p85). Participant HR (BPM) did not increase significantly between interventions and was not significantly altered as a result of the intervention or when compared across interventions. Internal temperature (tympanic) remained consistent throughout the study and was not impacted by the intervention. Participant mealtimes were consistent throughout the study and showed no significant difference between interventions (Table 3 p85).

Overall, when comparing TSCV with consumption of the caffeinated coffee beverage before and after intervention. TSCV showed a small but significant response to caffeine within this group of study participants (TSCV before intervention: 33.81 ± 0.45 °C, TSCV after intervention 34.187 ±0.59 °C, $P > 0.05$). This response was also seen when investigating ΔTREL within the same intervention (0.4067 ±0.28 °C $p < 0.001$). TSCV was not shown to be impacted with the consumption of water alone within this population group (TSCV before intervention: 34.55 ± 0.16 °C, TSCV after intervention 34.55 ±0.17 °C, $p < 0.001$). This finding was also supported by a lack of change in ΔTREL within this (water) intervention (0.13 ±0.12 °C $p > 0.05$). Associations based on anthropometric data demonstrated no significance in temperature generation within this study (in absolute TSCV or when given either intervention $p > 0.05$), there were also no significant differences or correlation between those with higher BMI and resting or stimulated HR ($p > 0.05$).

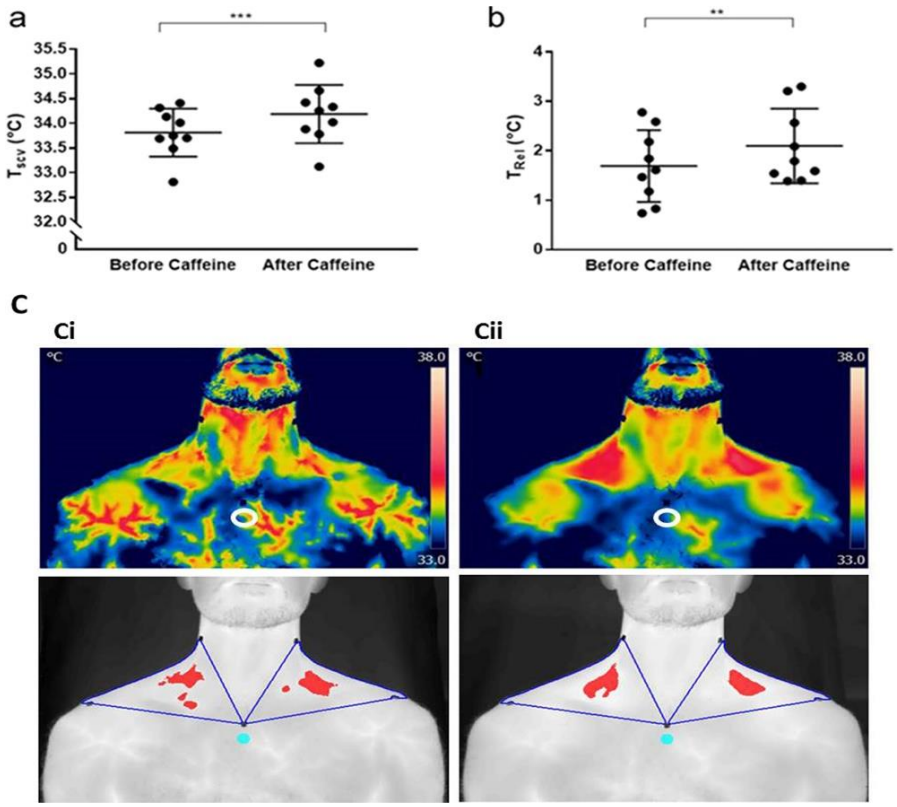


Figure 5. *Effect of drinking caffeine on heat production from brown fat in adult humans. (**a**) Caffeine resulted in a significant rise in temperature of the supraclavicular region (T_{SCV}) which co-locates with brown fat and this reflects (**b**) increase in supraclavicular temperature relative to reference body surface temperature (T_{rel}) (n=9); (**c**) Representative thermal image (i) pre and (ii) post-caffeine, as either the original FLIR image, or the transformed image high-lighting the hottest 10% of pixels. ***P<0.001; **P<0.01. White open and blue closed circles indicate the reference temperature point.*

3.13 Discussion

The primary objective of this study was to assess any change in heat production (ΔT) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following the consumption of a caffeinated beverage. Results of this investigation revealed that consumption of the caffeinated beverage (Nescafe 100g sachet ~89mg caffeine) resulted in a rise in temperature within this region, an effect that was not detected with the consumption of water alone. TSCV showed a small but significant response to caffeine within this group of

study participants (TSCV before intervention: 33.81 ± 0.45 °C, TSCV after intervention 34.187 ±0.59 °C, P > 0.05). Based on the power calculation that deemed 0.2°C the average rise on SCV temperature during stimulation, it is feasible that based on these results, that caffeine consumed as part of a standard coffee drink could increase SCV temperature overlying the main BAT depot. Importantly ΔTREL, the measure that incorporates the participant reference temperature making them a pseudo control for their own study demonstrated a statistically significant change with consumption of caffeine, a change that was not seen with the consumption of water alone.

This study showed that anthropometric assessment did not impact overall TSCV within either study. Specifically unlike previous studies which have suggested that BMI could impact SCV temperature potentially due to an increase in overlying subcutaneous WAT (Robinson et al., 2014). There was also no statistically significant indication that sex correlated with any change in SCV temperature or response to either intervention during this study. It is important to acknowledge that this was a small-scale pilot study that did not control for sex, therefore participant numbers between each sex were very small with varying anthropometric measurements and therefore it would not be suitable to make assumptions of correlation based on this.

This study has demonstrated a potential impact of caffeine on BAT activity, this data coincides with a range of publications which demonstrate a potential thermogenic effect of dietary compounds (Bracco et al., 1995; Westerterp-Plantenga, 2010; Yoshioka et al., 2003), caffeine however, has not been studied extensively in relation to BAT in adults. Currently only one such study (Yoneshiro et al., 2017) measured the impact of caffeine on BAT activity but does so in conjunction with green tea catechin. The group of Yoneshiro et al. investigated the acute and chronic thermogenic effect of a caffeine and green tea catechin containing

beverage. Catechins, also known as polyphenols are found within green tea (*Camellia sinensis*). The main catechins found within green tea are epicatechin-3-gallate (EGCG), epigallocatechin and epicatechin, whereby EGCG is the major catechin (Singh et al., 2011). Green tea catechin has been demonstrated to directly impact the sympathetic nervous system through an inhibitory effect on catechol-o-methyl-transferase (COMT) (Dulloo, 2011). COMT is responsible for the degradation of NA and EGCG has been shown to be a potent inhibitor of COMT (Chen et al., 2005). Noradrenaline plays a key role in sympathetic activation of BAT and therefore inhibition of COMT can result in an increased nervous response through prolonged NA influence.

Their study involved the use of 15 healthy male participants and utilised a combination of oxygen consumption (VO_2) and ^{18}F-FDG PET/CT to examine both the acute and chronic effect of the caffeine-catechin containing beverage. Their acute study involved participants consuming the catechin (615mg) /caffeine (79mg) containing beverage, orally and then undergoing an EE measure of VO_2 in a room at thermoneutrality over 120mins post ingestion with measurements at set intervals. The chronic study selected those who demonstrated low levels of BAT from PET/CT imaging, and they consumed the catechin/caffeine or caffeine only (control) beverage twice daily over a 5-week period. They then re-examined these participants using cold induced thermogenesis to measure VO_2 post intervention. There acute experimental results demonstrated that both the catechin/caffeine and caffeine containing beverage increased EE in those who were noted BAT positive and had negligible effect in those deemed BAT negative. They also show that the chronic study demonstrated an increase in cold induced thermogenesis with catechin/caffeine, but not caffeine alone. This demonstration of increased energy expenditure by both beverages is an interesting finding and warrants further research. Historical studies which investigated caffeine also showed the

positive thermogenic properties of caffeine in mice (Yoshioka et al., 2003) and in obese and healthy adults (Bracco et al., 1995).

The Yoneshiro et al. methodology does demonstrate the apparent deficiencies with the current assessment of BAT activity however. Firstly, PET/CT was utilised to gauge whether subjects enrolled had active BAT after cold exposure. As discussed previously this method is not a direct measure of BAT activity and thus may have misrepresented those who did and did not have active BAT. Due to the ionising nature of ^{18}F-FDG PET/CT subjects were only able to have PET/CT assessment once during the study which meant that their assessment of active BAT relied on the assumption that increased EE measured through an increase in VO_2 correlated directly to increased BAT activity. The study also utilised the oral consumption of a liquid beverage form of the catechin/caffeine combination. Whilst this may be representative of the way in which we regularly consume these substances, the act of drinking after being fasted may also contribute to thermogenesis. Water has been shown to increase thermogenesis in those who are normal weight (Bracco et al., 1995), which should be taken into consideration. They also showed a minor change in EE after caffeine consumption, but much lower than the catechin/caffeine combination. Whilst this may be the case it is possible that the amount of caffeine used within this study was not sufficient to illicit a response and therefore higher amounts of caffeine may see similar results as with a combination. These potential issues have been factored into my second project (CAFF-BAT), hence using a caffeine supplement in tablet form at 200mg rather than in beverage form. Which should negate this action.

For these reasons IRT of BAT seems to be a valid and accessible alternative to PET/CT. Whilst its use within the field is still relatively limited, one recent study (Law, Morris, et al., 2018) has

validated the technique when compared to the field leading methodology – PET/CT. The group of Law et al. utilised a combination of both IRT and PET/CT data from 8 healthy male participants who were subjected to water-jacket cooling. The results of the TI were geometrically transformed to overlay the PET/CT results, and the areas with the highest temperature and glucose uptake where compared. Their results demonstrated a close positive relationship between glucose uptake on PET/CT and the temperature change seen within the supraclavicular region upon cooling. These results offer evidence for the continued use of IRT as a valid alternative to PET/CT in the study of BAT activity.

More recently caffeine supplementation was also shown to have a stimulatory role on UCP1 in vitro (Velickovic et al., 2019). Research demonstrated that caffeine treatment of both human and murine stem-cells resulted in an increase in the overall prevalence of UCP1 and a further increase of oxygen consumption and of biomarkers that have been shown to increase metabolism (Velickovic et al., 2019); mimicking the effects of the present study in vivo. These observations comply with previous studies on mice assessing the impact of caffeine on BAT by driving the promotion of lipolysis and increasing BAT regulatory genes such as PPARϒ, PGHC-1α and PRDM16 (Yoshioka et al., 1990). These data showing then that caffeine has the potential to increase the prevalence of the major regulators of BAT adipogenesis in vitro (Kuang et al., 2008). The current study demonstrated the potential of caffeine to impact regulation of BAT and in vitro data shows further potential for caffeine's positive effect. As the current study was completed as a pilot further investigation was undertaken to further explore this acute response (see p95).

3.13.1 Study Limitations and Limitations of IRT Methodology

A range of limitations were clearly apparent throughout the study. Firstly, due to the pilot nature of the study there were no controls utilised in terms of demographic for participants accepted into the study, this was to encourage as many volunteers to take part in the trial as possible within the shortest space of time. As such participants were recruited with a wide range of age profiles and BMI, both factors which had traditionally been shown to impact SCV temperature (Robinson et al., 2014; Robinson et al., 2019) which may have impacted the final results of this study. There was a clear lack of blinding within this study which could incorporate bias into the proceedings, neither the researcher nor the participant were blinded as this would have been unfeasible due to the nature of participants consuming beverages with very distinct flavour profiles. In order to not utilise a hot beverage which could impact thermal data, the coffee was served at 22°C which was not always pleasant for participants. Consuming this beverage may have triggered a stress response which could have had an impact on BAT activity as research has discussed previously (Robinson et al., 2016). As coffee has not been investigated before It is also feasible that the consumption of the coffee may have impacted participants in a way that drinking water did not, an external factor that was not controllable during this study.

Another potential limitation of this study was the use of IRT itself as a method of detection. Whilst IRT has been validated against PET/CT (Law, Morris, et al., 2018), there is a significant lack of further research into the validation of this method specifically. Within this study IRT was also being used as a measure of BAT activity as a response to nutritional consumption which has not been validated against PET/CT. During the original validation study BAT activity

was stimulated using a very specific cooling method which involved cooling an extremity. This is an important distinction to consider, as so far there has been very little research into the reliability of using IRT as a method to detect TSCV as a result of consuming a food or drink, and in the case of this study, consumption of a drug that may impact the SNS. The validation for the IRT method was specifically targeted at forearm cooling and not nutritional interventions which may effect the validity of these types of investigations. Going forward were possible assessment using multiple forms of measurement would be more suitable, such as the joint use of IRT, MRI and PET/CT until more studies have validated the method for different experimental conditions.

It is also important to state that skin surface temperature is not a direct measure of BAT temperature. The current methodology focuses on the imaging of the supraclavicular region before and after stimulation of the tissue. This region is understandably highly vascularised and therefore it is the effect of the BAT heating the general vicinity and vasculature that is visualised through the thermal imaging camera. This means that a number of factors can affect the heat signal being received.

Adipose tissue surrounding the region is highly insulating (Law, Chalmers, et al., 2018). BAT has been shown to be affected by BMI (Robinson et al., 2014) and in this case higher BMI may mask the insulated BAT that is present giving a false negative result, which was a possible limitation of the current study. The supraclavicular fossa region assessed is highly vascularised and as such temperature changes within this region may be representative of an increased carotid blood flow rather than BAT activity, as such due to the nature of IRT detecting a heat profile there is no discernible way to distinguish the impact of this on the IR image. Some researchers have however suggested that it is possible that the evolutionary

benefit of adult BAT is to increase the temperature of blood flow through these vessels and into the brain, making these temperatures representative (M. Symonds et al., 2012).

4. Study 2: Caffeine Study Using Caffeine Supplement or Placebo – CAFF-BAT

Abstract: Following on from our study which investigated the use of coffee to stimulate BAT a more robust study was completed (prior to cancellation) In order to assess the effectiveness of caffeine as a potential stimulator of BAT. This study utilised a double-blind crossover format with participants consuming either a placebo lactose (200mg) tablet or a caffeine (200mg) tablet. Following a short ingestion period participants had their SCV BAT assessed using IRT. Results revealed that consumption of the caffeine supplement had no significant impact on the temperature of the SCV region, a finding that was also found when consuming the placebo tablet.

4.1 Introduction

There is global concern for the rapid increase in rates of obesity and overweight, especially in children and adolescents (Gepstein & Weiss, 2019). Obesity and the metabolic syndrome are the result of an over accumulation of adipose tissue, that subsequently cause a multitude of physiological and lifestyle altering comorbidities such as cardiovascular disease and an increased risk of diabetes (Huang, 2005).

Prevalence of obesity has been linked strongly to an energy usage imbalance. Whereby there is a negative balance of caloric intake and total metabolic activity (Blüher, 2019). Current treatments are focused on the promotion of leading a 'healthy lifestyle' consisting of increasing physical activity through exercise, whilst decreasing overall caloric intake through diet, as a means to reduce the metabolic imbalance leading to a reduction in stored adipose

tissue (Emily et al., 2019). It is becoming increasingly clear however that these lifestyle interventions alone are not working as intended, with research suggesting alternative interventions are required to improve long term outcomes (Greenway, 2015). For this reason, there is a requirement for the identification of alternative means of helping those who lose weight to continue to remain as such. One approach may be through the use of pharmacological interventions that promote or maintain adipose storage through increased energy expenditure through thermogenesis (Dulloo, 2011; Nedergaard & Cannon, 2010). BAT may be one such target, as it has been shown to play an important role in energy homeostasis into adulthood (Nedergaard et al., 2007). BAT thermogenesis has been estimated to contribute significantly to overall energy usage, with an increase of BAT prevalence possibly leading to a significant increase in overall energy demand (Brooks, 2005) making it a promising target for pharmacological intervention.

BAT thermogenesis is controlled by the sympathetic nervous system through β-adrenoreceptors (Cannon & Nedergaard, 2004). The neural pathway which regulates BAT in humans is not well established, however it has been studied extensively in rodent models (Morrison & Nakamura, 2019). Importantly for the use of pharmacological interventions, targets should aim to be BAT specific where possible to reduce the impact of side effects. Rodent models have demonstrated that there is some overlap between the neural pathways controlling both BAT and the cardiovascular system (Morrison & Nakamura, 2019). However, the cardiovascular system appears to be controlled mostly through a separate mechanism to that which regulates thermoregulation (Rathner et al., 2008). Sympathetic BAT regulation has been shown to be regulated through premotor neurons in the rostral raphe pallidus neurons (Rathner et al., 2001), an alternate area from those which control the baroreflex response, which are regulated in the rostral ventrolateral medulla (Morrison, 1999). It is this distinction

that could lead to progress within this area of research as nuclei specificity will allow for the most appropriate pharmacological target. However, there is still a lack of evidence describing the complete mechanism of BAT activation through specific nuclei and especially in humans. The use of caffeine is one such potential pharmacological intervention, routinely consumed and readily available, making it a desirable target. Caffeine has been linked to thermogenesis in both animal and human studies (Panchal et al., 2012; Velickovic et al., 2019). However, the mechanism of action is still unclear and human studies, especially those investigating an acute brown fat thermogenic response within the supraclavicular region are lacking.

The caffeine supplement or placebo study (CAFF-BAT) was designed based on the results obtained from the 'caffeinated beverage' pilot study. Whilst the pilot protocol was successful, a few minor changes have been made for the caffeine supplement study, to ensure the research was as robust as possible and comfortable for participants. All changes have been detailed below and the final study protocol can be seen in Figure 6 (p109).

4.2 Primary Objective

The primary objective for this study is to study the effect of the consumption of caffeine to demonstrate a potential thermogenic response (ΔT, TSCV, ΔTREL) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following the consumption of caffeine compared to a placebo in adults.

4.3 Secondary Objectives

The secondary outcomes are:

To assess the potential impact of arthrometric data on the SCV BAT activation using thermal imaging.

To assess the value of the Biotekna® PPG Stress Flow system as a measure of nervous

system activity

4.4 Sample size and justification CAFF-BAT

Using data from our previous studies, (Robinson et al., 2014); and the recently completed
EVOO-BAT study H10112015SoM CHOG PhD) which demonstrated a mean SCV temperature difference of 0.1^{o}C and a standard deviation of 0.15^{o}C, we will aim to recruit 25 participants to give 80% power to detect a difference with a significance level of 5%, allowing 25% drop out between the two sessions and completing the study once 20 participants have completed both study sessions.

4.5 COVID-19 Amendments

Due to the progress of the COVID-19 pandemic and the in-person nature of this experimental procedure, the n number and goal for the power calculation was not reached for this study. The study was terminated pre-emptively and in accordance with University of Nottingham COVID-19 procedures.

4.6 Participant Selection

15 male participants took part in the study. The study criteria chose to assess only males, due to the scope of the project and previous research demonstrating the potential difference in BAT response between males and females (Law et al., 2014; Robinson et al., 2019). Due to the small scope of this study ethical advice was given that in order to incorporate females into this study, study sessions would require a female present at all times. Due to the small scope and nature of this project this was not feasible, however should be considered in all future work. All participants were studied at the Human Physiology Laboratory based within

the Department of Academic Child Health. The study was approved by the Nottingham University Faculty of Medicine and Health Sciences Research Ethics Committee (39-1807) and performed in accordance with the declaration of Helsinki. Written informed consent was provided prior to participation from all participants.

4.7 Eligibility criteria for CAFF-BAT Study

Inclusion criteria

Participants will be eligible for inclusion if they have all the following characteristics:

- Body Mass Index (BMI) between 18.5-24.9 kg/m^2
- Healthy adults (male)
- Aged between 18 and 30 years old

Exclusion criteria

Participants will not be eligible for inclusion if they have any of the following:

- Are allergic or intolerant to caffeine or lactose.
- Have a diagnosis of hypothyroidism, hyperthyroidism or diabetes, or are taking betablocker oral medication as these conditions may affect BAT activation.
- Have a pacemaker or epilepsy as these individuals are unable to have their body composition measured through electrodes.
- Body Mass Index (BMI) over 24.9 kg/m^2

4.8 Study Configuration

4.8.1 Study Design

The study utilised a crossover design. Participants were involved in two study sessions and during these sessions were randomly given either:

- Caffeine supplement tablet form (Bulk Powders PureSeries 200mg) or
- Lactose placebo tablet during their study visit

Each participant was randomly assigned to a study order and the researcher and participants were blinded to which the intervention at each study visit (Figure 6 p9107).

Participants were asked to arrive to the study visit having not consumed food for 12 hours before the study session, and water one hour before the study session.

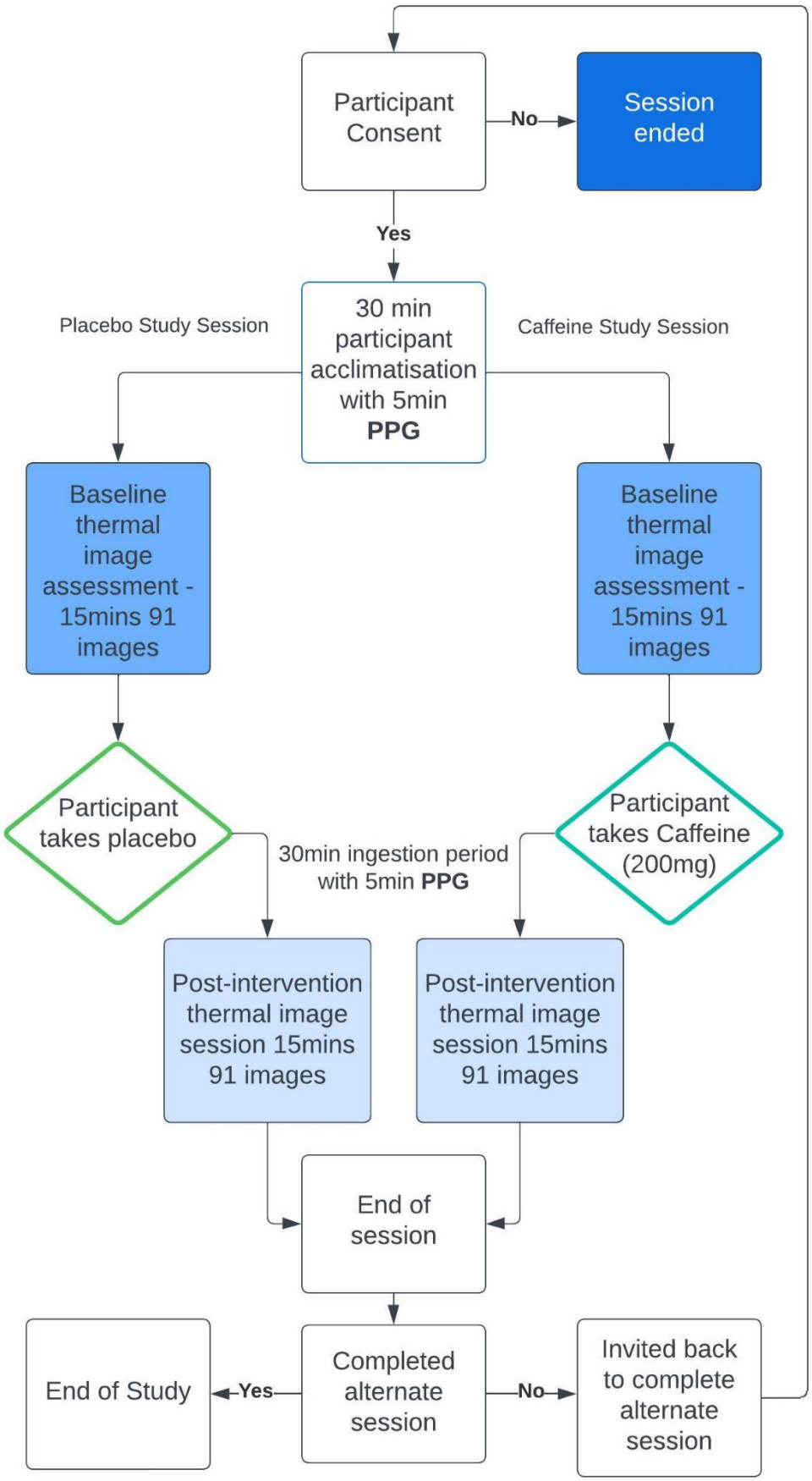
4.8.2 Blinding procedure further Information

This was a double-blind study as such the researcher and participants were not informed which tablet they had been given during each visit. Both tablets were placed in envelopes labelled A and B by a third party who wrote down the combination for each participant. A random number (letter) generator was then used to determine the order in which they were given each envelope during the two study sessions. The researcher was not aware of the order of interventions until the study was concluded.

4.9 Methods

This study procedure replicated the study procedure for the BITSCAFF study (p84). Any changes to this procedure will be noted and discussed below. Some changes were made to the procedure to improve participant comfort and acquire additional anthropometric data and have been detailed within this section. A flow diagram detailing the final procedure, including all relevant timings can be found on p102. All of the following took place after acquiring written informed consent prior to the study sessions taking place and this permission was clarified on the day of each study session.

Figure 6 *Study 2 protocol Design – (Consort diagram produced in Lucidchart®)*



4.9.1 Room Setup and Thermal Imaging

All participants were studied at the Human Physiology Laboratory based within the Department of Academic Child Health.

All study sessions took place in the morning starting at 10:00am until Approximately 12:00pm, and the study took place during the months of February to November.

4.9.2 Study Questionnaire

The initial requested action for each participant on the study day visit is the completion of a questionnaire (Appendix 1).

As the activation of brown fat varies according to ethnicity (Bakker et al., 2014), participants were asked to self-report their 'ethnicity' during their first visit using a standardised ethnicity classification. The standard classification used was that utilised by the UK Home Office in collecting census data from the UK domiciled population. Participants were be asked to choose which, from a list of standard ethnicities, they feel best describes their own ethnicity. Ethnicity data for the participants can be seen in Table 4.

As brown fat activation varies according to age (M. E. Symonds, K. Henderson, et al., 2012), participants were asked to self-report their age range during their first visit. Participants were also asked to express their day-to-day caffeine consumption per day, this was a general assumption on their behalf and based on how many caffeinated products they believed they consumed each day on a standard regular basis. This was to gage how consistently each participant consumed caffeine from any

source. This was a selected measure as the impact of caffeine has been shown to decrease with continued usage (Goldstein et al., 2010).

4.9.3 Measurements

The protocol for measurement collection for this study followed those seen in the 'caffeinated beverage' pilot study with the exception that participants undertook a full PPG assessment during the acclimatisation stage by use of the PPG Stress Flow monitor (PPG Stress Flow©: Biotekna S.r.I.®, Italy) (detailed below and see Figure 6 p95).

As participants adjusted to the study room environment (acclimatisation period), basic anthropometric measurements of height and weight were made using a standard stadiometer (SECA 2123 Portable Stadiometer) and weight with a set of calibrated ISO standard digital scales (Active Era® Scales) and internal in ear temperature was measured using a digital in-ear thermometer (Braun®ThermoScan, Braun®).

To gauge the impact of the intervention on the nervous system functionality, heart rate variability and overall activity of the autonomic nervous system was evaluated using an optoelectrical plethysmograph. A plethysmograph (PPG) (PPG Stress Flow©: Biotekna S.r.I.®, Italy) was used to measure changes of the volume within an organ, in this case the heart. From this blood flow (F) the volume change (dV) was calculated over time (dt), F= dV/dt (Julio et al., 1997) by the device resulting in numerical data that can be interpreted to show nervous system activity as a percentage. Reflection from the finger was used for the PPG, by having each participant place a pulse oximeter on their left and right forefinger. The device then measured the change in

the volume by using a light-emitting diode (LED) which illuminates the skin with light and then measures the amount of the light transmitted and/or reflected (Julio et al., 1997). During this study we used 'sympathetic activation %' a Biotekna® measure of sympathetic activity which records a % activity for sympathetic (SNS) and parasympathetic (PNS) activity during assessment (See appendix 2. for example of Biotekna® analysis), as a proxy for the sympathetic response during this test. The technique has been validated and is already used on many sites of the body of healthy or intensive care patients to find thrombosis, airway resistance and to monitor anaesthesia, and mental stress (Julio et al., 1997). This assessment was completed during the initial climatization phase over the course of five minutes. The PPG stress flow device also measured heart rate (BPM) during this time.

In total the participant was acclimatised within the research space for at least 20mins prior to any thermal imaging taking place.

With participant permission, thermally reflective skin markers were stuck to the apices of the region of interest (See Fig 2. P58 for details). These markers are used to aid in the ROI selection tool during image analysis. Reflective markers are clearly visible on the thermal images which allows for a consistent and reproducible way to target the correct surface anatomy for each participant.

4.9.4 Thermal Imaging Session 1 – Baseline

Prior to assessment reflective temperature, room temperature, humidity and distance from the camera were measured and camera settings adjusted as necessary. The room was maintained at a consistent 22°C throughout with the temperature monitored through the course of the experimental procedure.

Once the participant was sat comfortably and given time to acclimatise to the ambient temperature (for approximately 30mins) the first baseline thermal imaging session began. This session took place over the course of 15mins rather than the previously established 20mins. The reduction in imaging time was decided as a result of participants finding it difficult to sit comfortably for multiple 20-minute imaging sessions, this resulted in discomfort for the participants and may have lead to increased movement during the later stages of imaging. Participants were seated approximately 1 metre horizontally from the thermal imaging camera (FLIR T540 w/24°Lens), itself placed on a tripod (Jessops RR125 Hex Tripod) approximately 1 metre vertically from the ground. baseline (pre-stimulation) images are taken for 15 minutes (91 images).

4.9.5 Ingestion Period

Once baseline assessment had been completed participants were then requested to ingest their randomly assigned intervention; either 200mg caffeine supplement or lactose placebo in tablet form. At this stage the participant remained seated for 30 minutes to allow time for the intervention to take effect in accordance with published work detailing the speed at which caffeine is metabolised (Heckman et al., 2010). During the final 5 minutes of the ingestion period (from 25min onwards) the participant undergoes further PPG assessment for 5 minutes, which concludes the ingestion period.

4.9.6 Thermal Imaging Session 2 – Measuring Stimulation

Upon completion of the ingestion phase, a second session of thermal imaging took place. This was performed in accordance with the method described in Thermal Imaging Session 1. Participants are imaged for a further 15mins, acquiring a total of 91 images during this phase. The completion of this phase is the end of session 1 for the

participants.

4.9.7 End of Session and Recall

This concludes the session. As part of the cross-over design participants were then requested to return to complete the same session again having been given their 2nd randomly allocated intervention. It is important to note that both the researcher and the participant where blinded throughout this process. For more details on the session progression please see figure 6 (p109).

4.9.8 Image Analysis

Thermal images within this study were acquired at an interval of 1 image every 10 seconds over a period of 15 minutes. They were then processed after conversion of the thermal image (TI) data to the non-proproprietary portable network graphic format, marking the supraclavicular region of interest and then processing the data within those regions. Both left and right supraclavicular regions are defined by placing 4/5 markers (hence the use of sticky markers on participants during imaging).

The hottest 10% of the points within the TI within each region was then identified and the medians of these points were calculated (equivalent to the 95th percentile) (Law, Morris, et al., 2018). A reference region was also compiled from a circle 10 pixel in diameter immediately below the central apex of the region analysed, allowing for a skin temperature comparison. A moving average (period 3) was then used over the time course of results reducing the impact of natural temperature variation. The primary outcome was the difference in SCV temperature following the consumption of caffeine or placebo. BAT activation was calculated by measuring the change in the median temperature of the upper percentiles of temperature points in

the region of interest, the supraclavicular region, following stimulation by the intervention. Data from both sessions was then compared to detect a change due to the intervention. During the procedure resting TSCV was defined as the mean TSCV taken from the final minute of the baseline imaging session. The primary outcome was to measure the relative change (Trel) (using the sternal reference point) for both intervention by deducting the baseline temperature from the mean TSCV post intervention. The group has previously demonstrated that the relative supraclavicular temperature is closely associated with BAT activity as measured by PET/CT (Law, Morris, et al., 2018).

Multiple secondary analyses were undertaken to establish links between anthropometric data and those data gained by PPG in relation to thermal data.

4.9.9 Statistical Analysis

All data were analysed by Student's t test. For data that was not normally distributed the non-parametric Mann Whitney test was used to assess the statistical significance between both groups. Pearson correlation coefficients were calculated for associations between variables. Statistical significance was accepted at $P < 0.05$, with *$P < 0.05$; **$P < 0.01$; *** < 0.001. Error bars presented on graphs represent the mean ± SEM. Data were analysed using Graph Pad Prism Software (https://www.graphpad.com). A Medical Statistician from the University of Nottingham's School of Medicine's Statistical Resource 'Clinic' advised on the preparation of the data and the data analysis before the commencement of data recording.

4.10 Results

Results obtained from this study were incomplete due to the COVID-19 pandemic and will be reported as complete, although the n number to power the study was not reached due to University of Nottingham COVID-19 policy on in person research.

4.10.1 Study Environment Data

Data analysis revealed room conditions (room humidity %, average temperature °C) within the study environment remained consistent throughout the study period (Table 5 below.).

	Caffiene Intervention Data (Mean)	Lactose Placebo Data (Mean)	SD ±	*P*
Room Temperature °C	22.27	22.38	0.27	*0.21*
Room Humidity, rh%	39.77	41.22	9.58	0.66

Table 5. Study 2 environment data

4.10.2 Demographic and Anthropometric Data

Upon cessation of the project participants included a total of 15 male healthy volunteers aged 24.8 (SD) 3.6 years with a mean BMI of 22.38 (SD 2.42) kg/m2). Questionnaire data revealed a total demographic breakdown incorporating stated ethnicity; 1 Indian, 2 Chinese, 8 White British and 4 Other white background. Mealtimes were recorded prior to the study taking place and showed no significant change between repeated study sessions. Caffeine consumption data revealed that on average participants were consuming 1.13 (SD ± 1.2) caffeine containing products per day (for example a standard cup of tea of coffee). Internal temperature (Tympanic) was also unaffected by the intervention ($p > 0.05$ See tables p111 for further details.

Stated Ethnicity	Total Participants
Indian	1
Chinese	2
White British	8
Other White Background	4

Table 6. Ethnicity data for study 2

	Participant Group Data (Mean)	SD ±	SEM
n	9		
Age, y	27.34	1.6	0.61
Height, CM	175.2	4.37	1.46
Weight, Kg	23.67	8.23	2.74
BMI, kg/m^2	23.3	2.87	0.96

Table 7. anthropometric data for study 2 SD (Standard deviation) SEM (Standard error of mean)

	Caffeine Intervention Data (Mean)	Lactose Placebo Data (Mean)	SD ±	*P*
Time since last meal, h	10.5	10.12	1.98	0.57
Resting HR, BPM	68.9	70.46	7.92	0.46
HR following Intervention BPM	65.46	65.62	4.96	0.9
Internal Temperature before intervetion °C	36.3	36.17	0.33	0.17
Internal Temperature After intervetion °C	36.1	36.09	0.51	0.92

Table 8. Further participant data – SD (Standard deviation)

4.10.3 Assessment of Thermal Images

Results from the assessment of thermal images showed that the 200mg caffeine intervention had no statistically significant effect of the temperature within SCV region which co-locates with BAT during this study (TSCV for caffeine intervention baseline: 34.43 ± 0.22°C, TSCV after caffeine intervention 34.51 ± 0.25°C P = 0.24). This effect was also not present within the lactose placebo (200mg) sample (TSCV for lactose intervention baseline: 34.45 ± 0.43°C, TSCV after lactose intervention 34.48 ± 0.57°C P = 0.68) as expected.

On continued analysis, Trel was compared between interventions and revealed no significant change within SCV temperature (Trel caffeine: 2.05 ± 0.325°C, Trel Placebo 1.86 ± 0.175°C P = 0.077). Average temperature change was higher within the caffeine intervention when compared to placebo but did not reach statistical significance.

The thermal data for ΔTrel (which incorporates the reference temperature) was also compared between both interventions. ΔTrel following intervention showed no significant change in SCV temperature (ΔTrel caffeine: 0.24°C, ΔTrel placebo 0.072°C p = 0.29) however overall, average temperature change was higher within the caffeine group but again did not reach statistical significance.

4.10.4 Further Assessment of Thermal Images – Caffeine responders and non-responders

Following standard assessment of thermal images, further investigation took place investigating whether it was possible to detect a 'response' to caffeine when compared to the placebo trial in each participant. Data values were taken from post intervention Trel for both interventions and a relative change was found by deducting the placebo data from the

caffeine data for each individual participant. A ‘responder’ gave a positive response to this calculation i.e., positive result over 0.1 °C vs a negative result. Within the 15 participant population 9 showed a response to caffeine (responder average Trel 0.35 ± 0.10 °C) whereas 6 were considered non-responders (average Trel -0.14 ± 0.071 °C).

4.10.5 Biotekna® PPG sympathetic activity assessment

Biotekna PPG system was used to measure HR (BPM) and sympathetic activity during both interventions. Participant HR (BPM) did not increase significantly during either intervention or when compared between interventions ($p > 0.05$) (see table 7 p111). An actual SNS response was measured prior to and after consumption of caffeine (average SNS activity before consumption of caffeine: 58.57 ± 8.85%, average SNS activity after consumption 57.02 ± 6.95%) a change that was not statistically significant ($p = 0.58$). A relative assessment of change in SNS activity was also measured before and after consumption of the placebo or caffeine intervention and compared between both interventions. Average relative SNS activity was significantly changed according to this metric (average relative SNS placebo: -14.6 ± 5.92, average relative SNS activity caffeine -1.54 ± 5.21 $p = 0.0007$) with caffeine showing a higher relative change than the consumption of the placebo.

4.10.6 Assessment of anthropometric data and thermal images

Associations based on anthropometric data demonstrated no significance in SCV temperature within this study (as a measure of absolute TSCV or when given either intervention $p > 0.05$), there were also no significant differences or correlation between BMI, height, resting or stimulated HR when compared to TSCV or TREL ($p > 0.05$). Consumption of caffeine based on questionnaire was also compared to TSCV and Trel and in all occasions showed no significant correlation ($p = >0.05$)

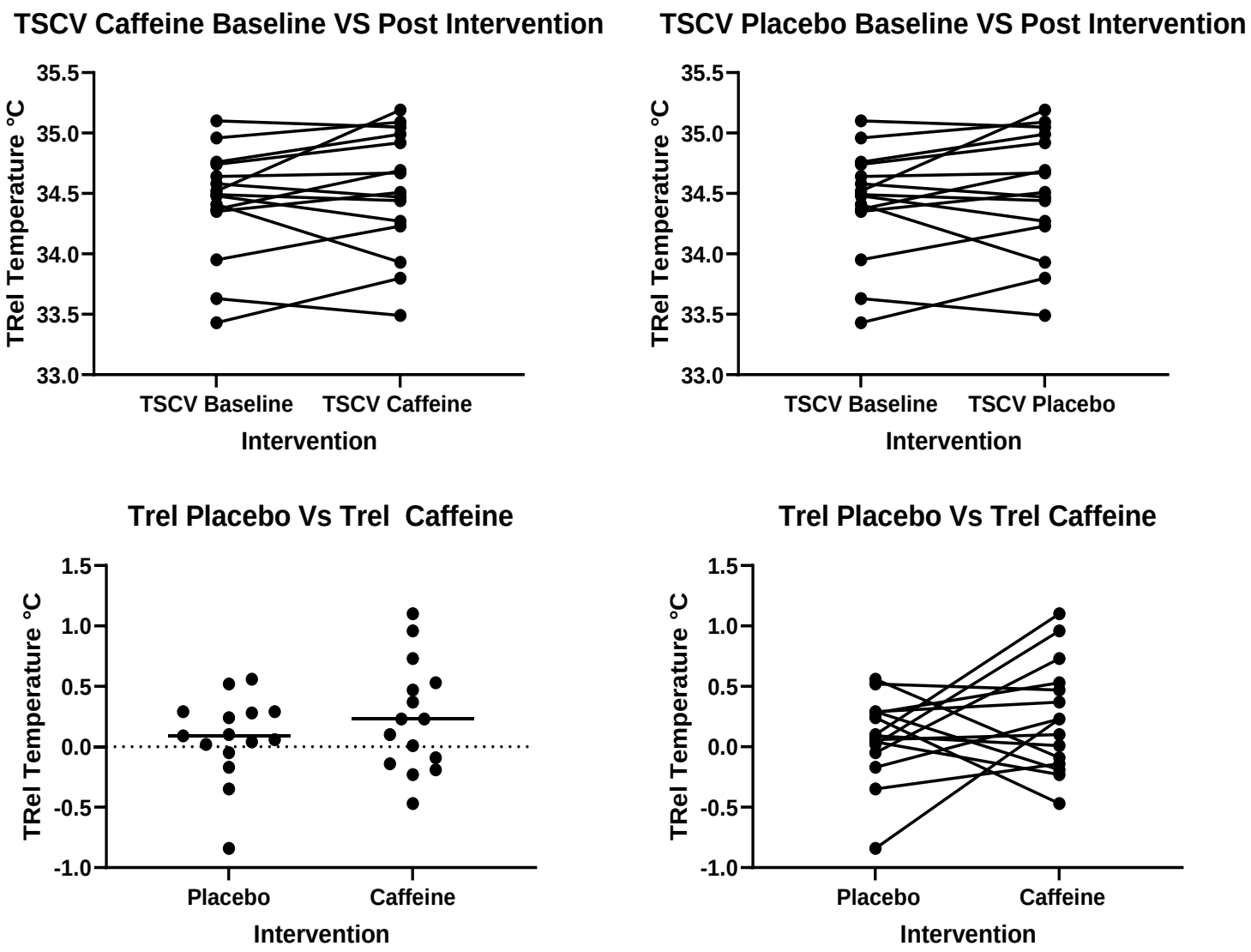


Figure 7. (Left to right) Shows data from thermal imaging study; 1. (left top row) Shows TSCV post intervention data caffeine 2. (right sight top row) Shows TSCV data Placebo. 3. (left bottom row) 3. Shows TRel with consumption of placebo vs caffeine 4. (right bottom row) Shows TRel data for placebo vs caffeine data - lines to represent matched pairs.

4.11 Discussion

The primary objective of this study was to assess whether caffeine consumption could have an effect on the temperature of the SCV region overlying the BAT depot within this area of adults. Results of this investigation revealed that the 200mg caffeine supplement had no statistically significant impact on the temperature of the SCV region following intervention with caffeine as measured using IRT. TSCV region data were analysed and compared within each group (comparing baseline with intervention data) and showed a general minor increase in average TSCV overall but did not reach statistical significance. This result was also apparent within the placebo group but to a lower degree. Based on our power calculation and from previous studies (Robinson et al., 2014)EVOO-BAT study H10112015SoM CHOG PhD) which demonstrated a mean SCV temperature difference of 0.1^{o}C and a standard deviation of 0.15^{o}C upon stimulation, these results demonstrated no significant change within the SCV temperature as a result of the intervention. A comparison of ΔTrel; which utilises the sternal reference temperature, also revealed no statistically significant evidence to reveal that caffeine significantly effected SCV temperatures. A comparison of the caffeine intervention and placebo data did demonstrate a higher increase in overall temperature within the caffeine group when compared to the placebo group (ΔTrel caffeine: 0.24°C, ΔTrel placebo 0.072°C). The increase in the caffeine group in accordance with previous work (Law et al., 2021; Robinson et al., 2014) would demonstrate a potential thermogenic response, however since power was not met for this study this data cannot be verified in this way.

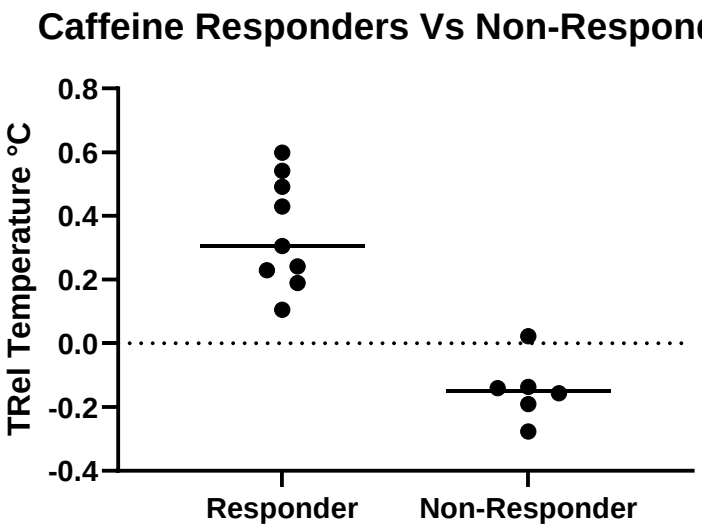
Within this study age and anthropometrics were generally controlled as to attempt to improve validity based on this specific demographic. For this reason it was therefore not surprising that BMI demonstrated no significant impact on our overall results. BMI has been

shown to impact TSCV BAT response inversely in previous studies (Law et al., 2021) however, due to the recruitment process for this study all participants fell within the 'healthy range'. Along these terms, age also demonstrated no significant impact on overall TSCV or Trel within this population, a measure which has also previously been suggested to impact BAT activity, possibly linked to an age related loss of brown adipocyte regenerative capacity over time (Graja & Schulz, 2015).

On further investigation, thermal data was compared between participants and a trend towards so called 'responders' and 'non-responders' may have been present within this study. Within this sample set 9 out of the 15 participants demonstrated a thermogenic response to caffeine (+0.1°C or more Trel), that was either not apparent with or greater than that seen with consumption of the placebo. Our previous investigation did reveal a general trend for caffeine having a potential thermogenic impact on BAT activity within the SCV a finding that was supported by in vitro studies (Velickovic et al., 2019). Caffeine is also widely consumed within the field of sports as a supplement to improve productivity and sports science research (Southward et al., 2018) has illuded to the fact that caffeine's ergogenic effect may vary depending on the individual themselves and could have a genetic component, something that would not have been detected within this trial. Caffeine response is also dependent on adrenoreceptor and phosphodiesterase inhibition. It is well established that continuous caffeine consumption can lead to a dulled catecholamine response and reduced lipolysis over time (Dekker et al., 2007), which may have impacted the results. Within this study however, analysis of participant survey responses when asked to describe their average daily caffeinated beverage consumption showed that 'responders' demonstrated an overall higher average consumption of caffeine per day according to survey responses (responder average 1.88 ±1.03 p/day non-responder 0.75 ± 0.5 p/day) and those

with the highest overall response were also those who notably consumed the most caffeinated products each day according to their own reported data. This finding does contradict the idea of a reduced response to caffeine with continuous consumption, however based on the age range of this group, this dulling response may not have been present at this stage and a range of other factors unexplored may have come into play here. It is also important to note that neither of these results showed statistical significance when assessed for correlation and are therefore inconclusive.

Figure 8. Graph showing a comparison of caffeine responders and non-responders



The Biotekna PPG data demonstrated no effect or change in HR (BPM) over the course of this investigation. Whilst it is well documented that caffeine can lead to an increase in heart rate and blood pressure (Turnbull et al., 2017), PPG assessment was carried out prior to and 25 minutes after the intervention had been consumed. Due to this it is highly likely that the HR detected post intervention was not sustained over an average for this time period and due to the nature of the assessment participants were requested to move as little as possible, which would also prevent a noticeable elevation in HR. When investigating the SNS response based on the BioTekna® system parameters, actual SNS activity did not change on either

intervention day to meet statistical significance, however comparing the relative SNS activity between the studies the caffeine study showed a slightly higher overall average SNS activity. Use of the Biotekna® stress flow device was completed in conjunction with Biotekna® Italy, as a potential product that could be utilised alongside TI in order to create a more complete assessment of SNS activity during investigations. An increase in relative response on the system does follow expectations based on the impact of caffeine on nervous system activity, however as a metric it may not be useful as responses tended to be varied and when comparing results during sessions, when SNS activity should have been impacted showed no change making use of this device inconclusive and require further trials.

One limitation of this study was documented from the outset of this section, unfortunately due to the cancellation of projects as a result of the COVID19 pandemic, the study was not sufficiently powered to accurately represent findings among similar individuals within the overall population and therefore impacted overall validity. Whilst data analysis was completed on the data available and statistical outcomes have been represented, without meeting power for this investigation, overall outcomes must be considered inconclusive. The results however could be built on for future studies.

Participant selection for this study was much more strict than previous studies, with an emphasis on reducing variation in age, anthropometric factors (BMI) and sex, all of which have been shown to impact TSCV when using IRT as a measure of BAT thermogenesis (Law, Morris, et al., 2018; Law et al., 2021; Robinson et al., 2014). This decision was made in order to improve reliability and investigate a specific response as a proof of concept that could be assessed further in future studies. This decision however does also limit the usability of data as it is especially targeted and only demonstrates results within a specific demographic. It is especially important to incorporate both sexes within human trials in order to create a

representative sample of the general population. It is for this reason that all future work should aim to allow for the assessment of females, and procedures could be put in place in order to make this possible. The questionnaire utilised for this study aimed to get a better understanding of participants general caffeine consumptions habits, this was far too general overall and only gave a very surface level idea of caffeine consumption habits. Considering the potential impact of diet and of caffeine consumption, future studies should aim to incorporate a more robust system of dietary tracking for participants, such as a food diary. This would allow improved robustness when investigating the impact of caffeine consumption on TSCV.

Whilst the experiment did not show conclusive evidence or demonstrate a long-term impact of caffeine consumption. Results, especially those in 'caffeine responders' showed a potential acute impact of caffeine on the SCV temperature co-locating with BAT thermogenesis that warrants further investigation.

4.11.1 Study Limitations

In terms of methodology, aside from the general limitations of using IRT to assess BAT already discussed (See p99 limitations of IRT) it is important to evaluate the effectiveness of the timings chosen and decisions made in study order for this project. It is possible that future sessions may wish to begin imaging immediately after consumption of the caffeine or placebo intervention rather than following the ingestion period. Research has demonstrated that caffeine can have a total half-life of around 5 hours and a blood caffeine peak anywhere between 15-45 minutes following consumption (Heckman et al., 2010). It is possible that some of our participants may have deviated from this generalised metabolic time frame and therefore any response to caffeine could have been missed. In order to prevent this a

consistent phase of imaging may have been a solution starting immediately after consumption. However, this brings up further issues as based on prior assessment (see study 1 p74) it was noted that participants found sitting for extended periods in a still position became uncomfortable, and therefore extending this period over 50mins could bring up ethical concerns in terms of participant welfare. In future it may be appropriate to measure this immediate effect using alternative measures such as using digital temperature data loggers within the SCV region such as ibuttons® (Thermochron®,AUS). Use of data loggers would need to be able to measure very small changes in temperature and were not available at the time of the study. This study took place over a number of months which incorporated multiple seasons and therefore weather and temperature profiles. It is highly likely that BAT not only has diurnal properties but also due to the nature of mild cold stimulating the tissue itself, there may be a seasonal change in BAT activity or prevalence. Whilst recruitment and imaging was not feasibly completed for this study within a single time of year it may have impacted the results overall and due to logistical reasons was not able to considered.

5. Study 3: Imaging Brown Adipose Tissue (using Magnetic Resonance and Thermal Imaging) – BAT-Thermal-MRI Study

Abstract: Adipose tissue is a complex paracrine and endocrine organ distinguished by two varying subtypes, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT contains unilocular lipid and a displaced nucleus, whilst BAT contains multiple lipid droplets and a high level of vascularisation. This distinction makes BAT a potential target of assessment using MRI. This project aimed to assess thermal images in conjunction with MRI in order to determine if regions of BAT in the neck and upper thorax can be differentiated from WAT using MRI by collecting both thermal and MR images. This was a feasibility study to assess correlation between TI data and MRI data, it was also intended to provide data to enable power calculations to be made for future projects. Results: This project was cancelled due to University of Nottingham COVID policy, as such no data was obtained, however further research could be conducted in this area using this protocol as a basis for future work.

5.1 Introduction

Adipose tissue is a complex paracrine and endocrine organ distinguished by two varying subtypes, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT contains unilocular lipid and a displaced nucleus, whilst BAT contains multiple lipid droplets and a high level of vascularisation.

5.1.1 Brown Adipose Tissue

Brown adipose tissue (BAT) is a highly metabolically active tissue and an essential component of mammalian survival following birth into the cold extremes of the extra-uterine environment. BAT can generate heat at a rate not seen in any other fat tissue and utilises a unique protein, uncoupling protein 1 (UCP1), to do so (Cannon & Nedergaard, 2004). UCP1 uncouples adenosine triphosphate (ATP) production at the mitochondrial membrane from the oxidation of glucose with the energy being dissipated as heat (Saito et al., 2009).

The existence of BAT in rodents and newborn humans is been well documented (Cannon & Nedergaard, 2004). However, studies have now demonstrated the existence of metabolically active BAT in healthy adult humans (Nedergaard et al., 2007). BAT is most abundant in those who are younger and not obese and has a positive impact in avoiding the development Type 2 diabetes (Saito et al., 2009). Although brown adipose tissue (BAT) persists beyond the neonatal period into adulthood, it's functions outside the newborn period are unknown. A large and growing body of evidence has confirmed the presence of supraclavicular BAT in adult humans (Zingaretti et al., 2009).

For this reason, BAT may be a suitable target in preventing obesity and associated diseases, as only 50g of active BAT has been estimated to burn approximately 20% of the basal caloric needs (Lefeuvre et al., 1987). BAT thermogenesis is activated by acute and chronic cold stimulation (M. E. Symonds, K. Henderson, et al., 2012; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) via the release of norepinephrine by the sympathetic nervous system (SNS). The SNS is the main inducer of BAT thermogenesis via β-3 receptors (Cannon & Nedergaard, 2004).

5.1.2 Imaging BAT

The morphological characteristics of WAT and BAT tissues allow for signal contrast making them an appealing target for identification using magnetic resonance imaging (MRI) and magnetic spectroscopy (MS) (Deng et al., 2018). We are currently lacking appropriate methodologies for the detection of BAT in humans. Specifically, standard methods available to assess BAT activity of positron emission tomography combined with F-18 fluorodeoxyglucose (FDG-PET-CT) (Nedergaard et al., 2007), single photon emission tomography (SPECT) scanning with radiotracers and/or tissue biopsy (Cypess et al., 2013) are unsuitable for use in children or for repeated use in adults. MRI may offer a valid alternative for studying BAT in both adults and children.

Members of the Division of Child Health, Obstetrics and Gynaecology (University of Nottingham) have developed an imaging technique which uses non-invasive infrared thermography (thermal imaging) to measure the temperature change in the specific area of the neck overlying BAT (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). This has recently been shown to give comparable results to PET-CT (a field gold standard method of assessment) (Law, Morris, et al., 2018). Infrared thermography overcomes the problems of studying BAT in healthy volunteers as it is completely non-invasive, acceptable to participants, cost effective, simple and repeated measurements are also acceptable (Jang et al., 2014). , in conjunction with thermal imaging which has demonstrated increased supraclavicular temperature upon cooling (Law, Morris, et al., 2018)

This technique of infrared thermography has been used in recent studies (M. E. Symonds, K. Henderson, et al., 2012),(Robinson et al., 2014),(Law, Morris, et al., 2018) and ongoing studies at the University of Nottingham [e.g. Medical School Ethics Committee Reference

(Ref. D39-1807 SoM Child Health). These studies have shown that this methodology is feasible, acceptable to volunteers and highly reproducible.

Current MRI methodologies for the detection of BAT involve measuring blood perfusion, water diffusion, temperature, substrate metabolism and fat content (Karampinos D.C., 2018). The morphological distinction between BAT and WAT, including the levels of intra and extra cellular water present within each cell allow for targeting of these tissues using methodology which include proton magnetic resonance spectroscopy (^{1}H-MRS) (Koskensalo et al., 2017), chemical-shift encoded water-fat, and chemical-shift techniques. These techniques distinguish the tissue sub types by revealing distinctions between the water and fat ratio within the cell (known as the Larmor resonance frequency), which differ between BAT and WAT (Karampinos D.C., 2018).

BAT has a lower fat signal fraction than WAT due to its smaller lipid content which is believed to also be an indicator of its current level of metabolic activity due to its use of triglyceride to activate thermogenesis (Karampinos D.C., 2018). The widespread availability of these techniques have led to a range of research groups demonstrating feasibility of utilising the fat-fraction as a means of BAT detection within the supraclavicular region of adults (Deng et al., 2018). This pilot study will focus on the fat fraction MR imaging method, with other non-invasive MR methods if appropriate, to distinguish BAT from WAT and will determine if regions of BAT co-locate with areas of functional heat production visualised using thermal imaging.

5.2 Objectives

Primary Objective

- To determine if regions of BAT in the neck and upper thorax can be differentiated from WAT using MRI by collecting both thermal and MR images.

Secondary Objectives

- To determine if paired thermal imaging and MR imaging studies are feasible
- To provide sufficient data suitable for a sample size calculation for subsequent studies utilising these techniques.

5.3 Study Configuration

The study will comprise one study session. Participants will undergo supraclavicular thermal imaging at baseline and after forearm cooling. Followed by an MRI scan of the same region.

5.4 Ethical Approval

This study gained full approval from the University of Nottingham Medical School Ethics Committee and informed consent would be gained prior to any participant measurement taking place.

5.5 Sample Size Justification and Statistical Outcomes

This study has not been powered to detect differences in BAT volume or supraclavicular heat production. It is a pilot study designed to develop a novel method of detection of BAT on MRI in human adult volunteers in whom heat production from the neck can be demonstrated on

thermal imaging. This study will, therefore, provide data to inform sample size calculations for subsequent adequately powered future studies using these techniques.

For thermal imaging, the primary outcome will be the difference in brown adipose tissue activation (ΔT) following forearm cooling. BAT activation is calculated by measuring the change in the median temperature of the upper percentiles of temperature points in the region of interest, the supraclavicular region, following stimulation (e.g. (Law, Morris, et al., 2018).

If brown adipose tissue is detected on the MRI image, it's location will be mapped to the area of heat production on the thermal image to see if the region of the median upper percentile temperature detected through thermal imaging co-locates the region of brown adipose tissue on the MRI scan. This methodology has previously been used to show that the area of heat production on thermal imaging maps to the region identified on radiolabelled studies using PET-CT (11).

A Medical Statistician from the University of Nottingham's School of Medicine's Statistical Resource 'Clinic' will advise on the preparation of the data and the data analysis before the commencement of data recording.

5.6 Inclusion Criteria

- Adults (age ≥ 18 years)
- Healthy
- Normal BMI (18.5-25 kg/m^2)
- Able to full comprehend the informed consent procedure

5.7 Exclusion Criteria

- Inability to complete MRI safety questionnaire and / or informed consent process
- known contraindication to MRI scanning (for example pacemaker/implanted defibrillator, Intracranial vascular clip, implanted programmable device, intra-ocular metallic fragment etc.)
- Known claustrophobia
- Pregnant
- Cranioplasty, craniofacial reconstruction, fixed dental brace or other craniofacial metalwork that is likely to cause significant image degradation or artefact

5.8 Methods

This study procedure will be conducted in accordance with previously completed procedures within the department of Academic Child Health (Law, Chalmers, et al., 2018) and previously completed studies (See Study 1 and Study 2) and will follow IRT guidance as outlined on p157. A flow diagram detailing the final procedure, including all relevant timings can be found on p138.

5.8.1 Questionnaire

After informed written consent has been received, the initial action requested of each participant on the study day visit will be the completion of a questionnaire (appendix 1). As the activation of brown fat varies according to ethnicity (Bakker, 2014), we will ask participants to self-report their 'ethnicity' during their first visit using a standardised ethnicity classification. The standard classification used will be that utilised by the UK

Home Office in collecting census data from the UK domiciled population. Participants will be asked to choose which, from a list of standard ethnicities, they feel best describes their own ethnicity (appendix 1).

5.8.2 Anthropometric measurements and thermal imaging

As the activation of brown fat varies according to age (M. E. Symonds, K. Henderson, et al., 2012) we will ask participants to self-report their age range during their first visit in the age bands. Participants will be asked to choose into which age band, from a list of age ranges, their age fits.

An initial anthropometric assessment of each participant will be carried out to measure participant height using a standard stadiometer and weight using a set of calibrated ISO standard digital scales. Participants will be requested to change into a loose-fitting vest top which will allow for the exposure of the shoulders and neck area as this is the area that we wish to image and needs to be clear of any clothing. Participants will be given appropriate privacy to change.

Upon completion of this step, participants will be requested to sit comfortably for 20 minutes to acclimatise to the room temperature. Thereafter, (around 20 minutes into the study visit) participants will have their resting BAT temperature measured by thermal imaging for 15 minutes. Participants will then have their forearm cooled in a cooling sleeve (Cincinnati Sub-Zero Products, Cincinnati, OH) at a 'mild cold' temperature of 16 °C, to initiate a potential brown fat thermogenic response, whilst imaging takes place for a further 15 minutes. This session should take approximately 50-60 minutes.

This concludes the thermal imaging session and participants will be taken to the MRI suite for imaging.

5.8.3 MRI Imaging

Participants will be requested to remove all metal items and be provided with a locker before entering the MR imaging suite. All scanning sequences used will fall within the National Radiological Protection Board guidelines for MRI examination. The participant will have the MRI scan in a 1.5Tesla, 3Tesla or 0.5T open clinical scanner in the University of Nottingham Medical School MRI facility. The scan will be performed by a fully qualified research radiographer or a researcher trained to operate the scanner according to local protocols. The MRI scan will not last more than 1-hour and no intravenous contrast or other agent will be administered.

A detailed breakdown of imaging procedures can be found on (P138 figure 9 and p139 figure 10).

Figure 9. *Shows the order of proceedings for the thermal imaging component of this study*

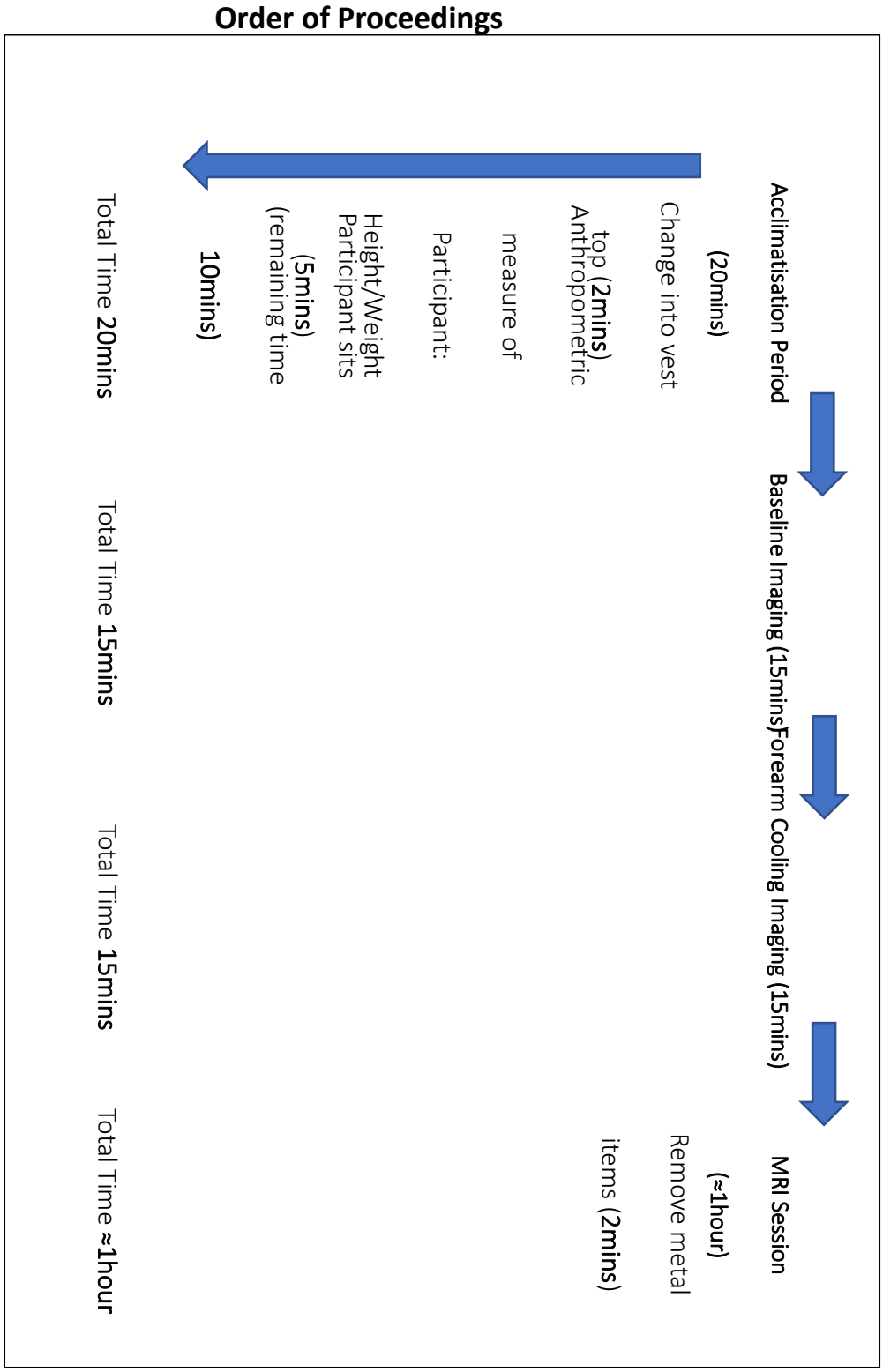
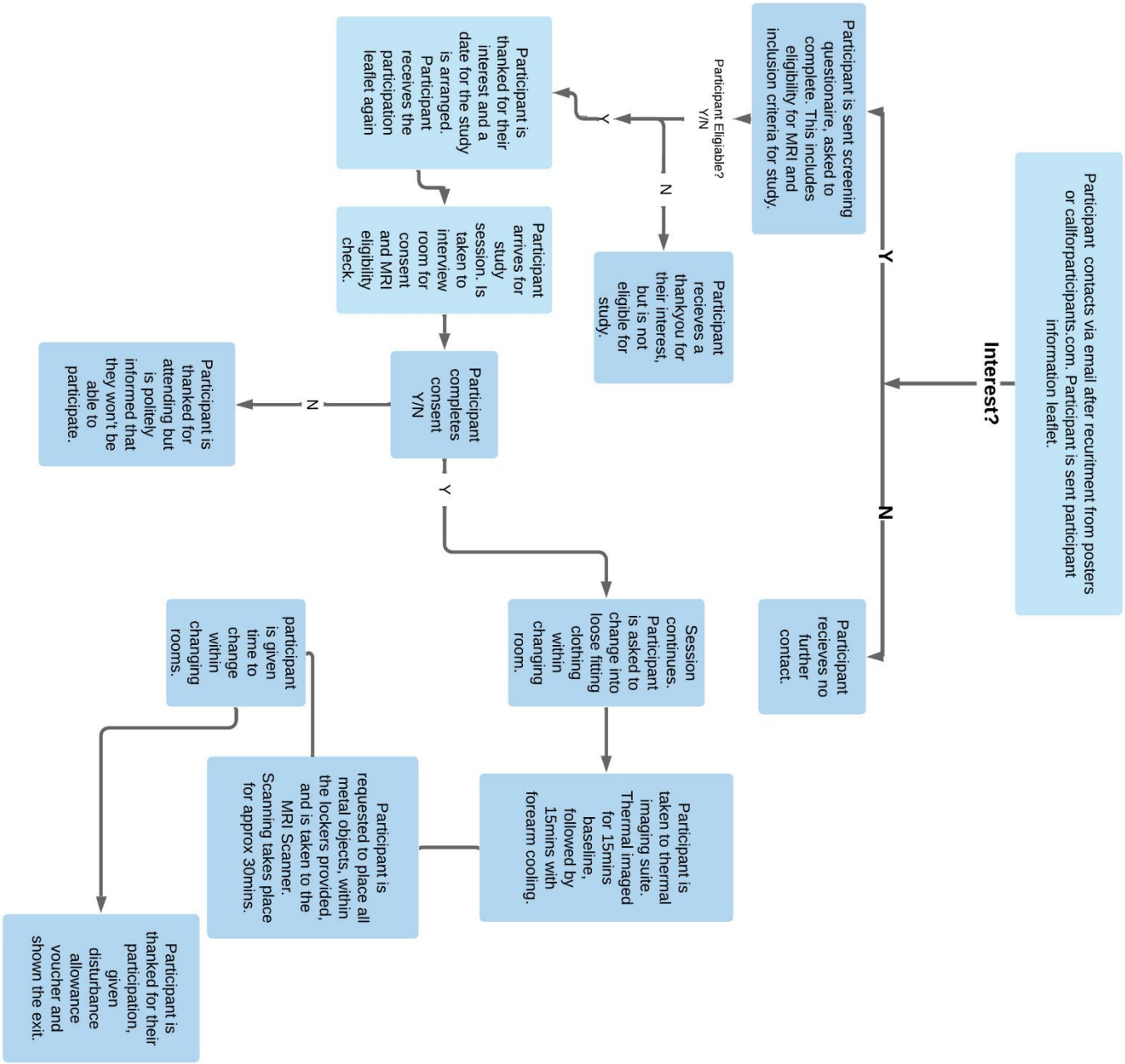


Figure 10. *Shows the order of proceedings for the full study including a breakdown of participant recruitment and MRI*



5.9 Results

As a result of the COVID-19 outbreak, results were not collected for this project, in accordance with University of Nottingham policy all studies involving human participants were cancelled. The study still represents a large amount of work and I would highly recommend that the department continue to work in this area as the combination of MRI and thermal imaging is a very important step in validating thermal imaging as a suitable method of measuring BAT activity in adults and children.

6. Collaborative and small-scale proof of concept Studies

Disclaimer: The study detailed within this section were completed as a result of collaboration. The thermal images, anthropometric data and saliva (DNA) samples utilised in this project were collected by Dr Jane Chalmers part of the Nottingham University Hospitals NHS Trust, NIHR Nottingham Digestive Diseases Biomedical Research Unit (NDDBRU) and the NIHR Biomedical Research Centre, School of Medicine, Nottingham as part of a separate research project. My own contribution to this work included the investigation into genetic traits associated with ethnicity and TRPM8 specifically, my goal was to assess BAT thermogenesis related SNPs using the data collected by Dr Jane Chalmers and her team. Unfortunately further genotyping and investigation for this project was not possible due to the COVID19 pandemic.

6. Study 4: The Assessment of Brown Adipose Tissue Thermogenesis Using Thermal Imaging and Associated Single Nucleotide Polymorphisms

Abstract: Our ability to detect ambient environmental temperature is a key component in the body's ability to maintain a stable internal environment through shivering and non-shivering thermogenesis. Temperature sensation is caused by the activation of transient receptor potential (TRP) ion channels and specifically TRP subfamily M member 8 (TRPM8) has been shown to play a central role in cold sensation and thermoregulation. Studies in small mammals have shown that cold adaptations and phenotypic changes to the thermoreceptor may impact the thermogenic response. The TRPM8 SNP rs10166942 encodes a cold receptor that is activated upon mild cold exposure and serves a potential role in physiological thermoregulation and adaptation to cold environments. BAT is reliant on thermoreceptors, specifically those responsible for mild cold detection to function. This study aimed to investigate the impact of this genetic trait through genotyping in relation to mild cold exposure by investigating the presence of the allele frequency of this SNP in relation to the BAT response to cold as measured by TI. The presence of the T Allele was strongly associated with increased BAT activity (ΔT_{rel}) in our sample set.

All studies carried out using infrared thermography were completed in accordance with the methods and development undertaken by the group based in the early life research unit, division of child health, obstetrics and gynaecology school of medicine, part of the University of Nottingham, pioneered by the work of Dr James Law et al. working in conjunction with Dr

David E. Morris (Bioengineering group, faculty of engineering, University of Nottingham (Law, Chalmers, et al., 2018). This project was performed in conjunction with the sample acquisition, participant recruitment and thermal imaging work completed by Dr Jane Chalmers (a separate project) part of the Nottingham University Hospitals NHS Trust, NIHR Nottingham Digestive Diseases Biomedical Research Unit (NDDBRU) and the NIHR Biomedical Research Centre, School of Medicine, Nottingham.

6.1 Introduction

6.1.1 Genetic Origins of Cold Adaptation

Whilst human origins stem from Africa over millions of years, their adaptation and successful colonisation of colder environments outside of this continent are much more recent; approximately 50,000 years (Key et al., 2018). Harsher climates, especially those which force populations to live in extreme cold conditions, have led to a number of genetic adaptations, including alleles in genes relating to thermogenesis and fatty acid metabolism (Cardona et al., 2014; Fumagalli et al., 2015).

Our ability to detect ambient environmental temperature (thermosensation) is a key component in the body's ability to maintain a stable internal environment through shivering and non-shivering thermogenesis and therefore warm and cold receptors that innervate and detect changes within the environment are a core component of thermoregulation (Key et al., 2018).

6.1.2 Cold Temperature Thermoreceptors

Temperature sensation is caused by the activation of transient receptor potential (TRP) ion channels (Basbaum et al., 2007) and specifically TRP subfamily M member 8 (TRPM8) has been shown to play a central role in cold sensation and thermoregulation (Basbaum et al.,

2007; Peier et al., 2002). Expressed in pain and temperature sensitive neurons of the dorsal root ganglia (Peier et al., 2002), as well as in the prostate and liver (at much lower levels) (Wang & Siemens, 2015).

TRPM8 is sensitive specifically to 'mild cold' temperatures ranging from 15°C-30°C (Julius et al., 2002), subsequently, temperatures which also impact non-shivering BAT thermogenesis (Contreras et al., 2017). Within this temperature range the channel passes an inward cationic current, based on the severity of the ambient temperature (Basbaum et al., 2007).

TRPM8 has been closely linked to the cold response and subsequently thermoregulation through multiple studies which have demonstrated that deletions within the gene result in diminished responsiveness to cold challenges (Dhaka et al., 2007; Knowlton et al., 2011) and in rodents substitutions within the TRPM8 transmembrane domain have been shown to reduce cold sensitivity (Matos-Cruz et al., 2017).

TRPM8 is located on the short arm of chromosome 2 and demonstrates genetic diversity with potential polymorphisms demonstrating phenotypic alterations (Henström et al., 2017). The single nucleotide polymorphism (SNP rs10166942, C/T chr2:234825093 in hg 19) upstream of the gene has been shown to potentially lead to alterations in transcription factor binding and variation within phenotype (Henström et al., 2017). Currently, the strongest association for rs10166942 is for migraine, specifically those with the C allele has been shown to be protective (Anttila et al., 2013). The role of TRMP8 in thermoregulation and cold perception then are well established, and studies in small mammals have shown that cold adaptations and phenotypic changes to the thermoreceptor may impact the thermogenic response.

6.1.3 Genetic Links to Thermogenesis and temperature regulation in TRPM8

In humans these links are not well established, however, climate and global latitude have been shown to impact the TRPM8 allele (Key et al., 2018). Through genome wide analysis (Key et al., 2018) rs10166942 has shown extreme population differentiation. Specifically, the T allele has been shown to vary in frequency dependent on geographic location (% population); 0.05 in Africa, 0.36 East Asia, 0.48 South Asia, 0.83 Europe (Key et al., 2018). This distribution of the T allele is closely correlated to latitude and subsequently temperature at a rate that goes above and beyond what would be explained by traditional means such as shared ancestry.

There is increasing evidence that the South Asian population may be increasingly at risk of obesity, and obesity related co-morbidities, specifically type-II diabetes than the Caucasian population (Shah & Kanaya, 2014). The South Asian population have been shown to have higher levels of insulin resistance, impaired β-cell function and an overall genetic predisposition to diabetes when compared to Caucasians at lower BMI (Narayan & Kanaya, 2020). Risk factors for these conditions are currently being increased throughout Asia due to globalisation, with less physically demanding jobs and an increase in consumption of foods rich in fats and high in sugar (Narayan & Kanaya, 2020), a trend that is continuing to take place upon migration to more globally developed countries (Ramachandran et al., 2010). BAT activity has been linked to whole body energy expenditure, fat storage and obesity related metabolic conditions (Dulloo, 2011). The TRPM8 SNP rs10166942 encodes a cold receptor that is activated upon mild cold exposure and serves a potential role in physiological

thermoregulation and adaptation to cold environments. BAT is reliant on thermoreceptors, specifically those responsible for mild cold detection to function. This study aimed to investigate the impact of this genetic trait in relation to mild cold exposure in both South Asians and Caucasians using the thermal imaging method, as a potential means of influence on BAT function which may increase susceptibility to obesity related comorbidities in this population.

6.2 Hypothesis & Aims

I hypothesise that the presence of the TRPM8 variant can influence BAT activity, and alteration in the allele may specifically influence BAT activity in the South Asian Sample set when compared to the Caucasian sample set.

6.3 Objectives

Primary Objective

The primary objective was to study the change in heat production (ΔTrel) from the skin overlying the main depot of BAT in the supraclavicular region of the neck following mild cold exposure of the forearm. Resting changes in supraclavicular BAT temperature were measured before participants were subject to forearm cooling at 15°C. Measurement of supraclavicular BAT activation was calculated from the change in the median temperature of the upper percentile of temperature points in the region of interest using previously established and published methodologies (Robinson et al., 2014; M. E. Symonds, K. Henderson, et al., 2012). To assess correlations between the human cold response, as measured using IRT and changes within the single nucleotide polymorphism TRPM8 (rs10166942 genotype) real-time QPCR was carried out on the genetic material from participants obtained from saliva samples.

Secondary Objectives

Secondary objectives aimed to assess the association with alterations in rs10166942 alleles and association with secondary measures such as BMI.

Disclaimer: The following method is a representation of the work completed by Dr Chalmers. I had no input on this stage of the project. All participant recruitment, thermal imaging and DNA samples were acquired by Dr Chalmers. This project was completed havening met ethical approval for collection of data in both the UK and in India, under the strictest ethical guidance and followed throughout. Statistical analysis decisions, such as the power size calculation for the IRT study was completed by Dr Chalmers with guidance form an experienced medical statistics expert from the University of Nottingham and based on the results of previous thermal imaging work (Law, Morris, et al., 2018; Robinson et al., 2019).

6.4 Ethical Approval

The study was approved by the Nottingham University Faculty of Medicine and Health Sciences Research Ethics Committee (IRAS Project ID 224087) and performed in accordance with the declaration of Helsinki. Written informed consent was provided prior to participation from all participants. (Please note Indian cohorts were recruited as part of a separate study "Dietary, lifestyle, genetic factors, dyslipidaemia and insulin resistance associated with NAFLD and its severity in a population based cohort in South India"
Ethical approval granted 17/01/13 from Sree Gokulam Medical College and Research Foundation Instutional Ethics Committee (REC ID:04/36/01/2013
Ethical approval for the Indian cohort for this study (SA-NAFLD) granted 19/05/2017) from Sree Gokulam Medical College and Research Foundation Institutional Ethics Committee (REC ID:26/299/05/2017)

6.5 Sample Size

Sample size calculations were undertaken to ensure ability to identify differences for multiple variables between groups. For the assessment of BAT activity, using an estimated standard

deviation of 0.368, 20 participants per group will be required to give an 80% power to detect an expected biologically significant change of 0.3°C with a significance level of 5% (one sided independent t-test) a sample size of 20 participants per group was selected. *This power calculation was completed by Dr Chalmers prior to completion of the genetic investigation project and was based on previous work from the group investigating thermal imaging as a measure of BAT activity.*

6.6 Participant Selection

The total number of participants recruited for the study was 120. These participants where split into two groups dependent on the country in which they reside. The cohorts where then split further into groups dependent on race and presence of NAFLD.

The groups were as follows:

UK Cohort:

20 UK South Asian NAFLD

20 UK Caucasian NAFLD

20 UK South Asian Controls

20 UK Caucasian Controls

South Indian Cohort:

20 India NAFLD

20 India Controls

6.7 Inclusion and Exclusion Criteria

6.7.1 Inclusion Criteria

. Adult participants aged 18 years or over

. Male

. Able to give informed consent

UK cohorts: Self-identified as ether “White” or “South Asian” during previous study (REC:13/EM/0123)

. For NAFLD cohort: Fatty/echo-bright liver on ultrasound

6.7.2 Exclusion Criteria

. Females

. Known or suspected cirrhosis on clinical/histological/radiological grounds

. Current or recent history of significant alcohol consumption (>14 units per week)

. Other documented causes of chronic liver disease including:

- Hepatitis B or C infection

- Drug-induced liver disease

- Alcohol-related liver disease

- Autoimmune hepatitis

- Wilson’s disease, Haemachromatosis

- Primary biliary cirrhosis, Primary Sclerosing Cholangitis

. Currently taking medication that can induce steosis (corticosteroids, Amiodarane, Tamoxifen, Methotrexate)

. Evidence of any other unstable or untreated clinically significant immunological, endocrine, haematological, gastrointestinal, neurological, neoplastic or psychiatric disease

. Presence of hair within the region of interest (neck) that cannot be removed prior to study day.

6.8 Materials & Methods

6.8.1 Measurements

Participants who chose to take part in the study were asked to attend having fasted from midnight (clear fluids only) and having abstained from alcohol, caffeine and strenuous exercise in the preceding 24 hours. Upon arrival participants were checked against the inclusion/exclusion criteria to ensure their participation was adequate to meet the goals of the project. After informed consent was taken, participants had a brief medical history check (focusing on metabolic syndrome related issues). Participants then underwent abdominal ultrasound of the liver to ensure they were entered into the relevant cohort for the study. Anthropometric measures of height, weight, waist and hip circumference were taken using a standard stadiometer (Marsden, Rotheram, UK) and weight with a set of calibrated ISO standard digital scales (Seca, Hamburg, Germany) and internal in ear temperature was measured using a digital in-ear thermometer (Braun®ThermoScan pro 600, Braun, Germany). Blood samples were acquired for the purposes of investigating biomarkers of BAT and insulin resistance (not applicable to this current study) and saliva samples were collected to measure cortisol levels and for genetic analysis (SpeciMAX saliva collection kit, Thermofischer®, Germany).

6.8.2 Thermal Imaging Methodology

Imaging took place within the Human Physiology Laboratory in the Queen's Medical Centre Campus (for those who were part of the UK cohort) part of the Division of Academic Child Health or within the Sree Gokulam Medical College, Trivandrum (Trivanathapuram,India). The Human Physiology Laboratory is temperature controlled and ambient temperature remained at 22°C throughout. The room was allowed to reach ambient temperature prior to any imaging sessions taking place and temperature was monitored throughout proceedings. Participants were requested to change into standardised light fitting cotton clothing prior to thermal imaging. With participant permission, thermally reflective skin markers were stuck to the apices of the region of interest. These markers are used to aid in the ROI selection tool during image analysis. Reflective markers are clearly visible on the thermal images which allows for a consistent and reproducible way to target the correct surface anatomy for each participant. They were allowed time to acclimatise to the room temperature prior to imaging. Participants were seated upright approximately 1 meter away from the thermal imaging camera (FLIR B425, FLIR Systems,AB, DAnderyd Sweden). Infrared thermal images of the supraclavicular region were then recorded to acquire a baseline temperature of the supraclavicular region which overlays the BAT region, at a rate of 4 per minute for 20 minutes. A cold stimulus was then introduced to the participants right forearm via a cooling blanket set to 14°C (Cincinnati Sub-Zero Products, Cincinnati, OH). This cooling continued for a further 20 minutes. Importantly shivering was assessed by the researcher and the participants as this would constitute shivering thermogenesis and impact the results. Participants were also asked to report any pain sensation to the researcher during the

cooling phase. This concludes the thermal imaging phase of the protocol. Cohorts from the India study underwent the same study procedure.

6.8.3 Genotyping Study Procedure

Single nucleotide polymorphism genotyping was conducted in accordance with the methods outlined by applied biosystems® (Life technologies corporation, Carlsbad, CA) using TaqMan® probes and NanoDrop® technology.

6.8.4 SNP Probe and Plate Setup

Genetic data was acquired through saliva samples taken from participants during study sessions and stored by the NDDBRU. Saliva samples were processed and stored in short-term aliquots at -80 degrees centigrade prior to analysis.

Once thawed Primary DNA samples were assessed for purity and suitability and extracted using NanoDrop® spectrophotometry.

The PCR reaction mix was then prepared following guidance from the manufacturer (applied biosystems®) methodology.

Prior to making the master solution, all reagents were lightly vortexed.

For the 96 well plate 5.0 µL of 2xTaqMan® (Life technologies corporation, Carlsbad, CA) Master Mix was added to 0.5 µL assay working stock (SNP; rs10166942 Life technologies corporation, Carsbad, CA) and 4.5 µL Nuclease-free water per well. For each run a stock solution was prepared and appropriate sample added to each well. Once added to a single tube the solution was vortexed again to ensure all reagents were suitably mixed. The SNP stock solution was then added to the 96 well plates and 4.5 µL of each gDNA sample was delivered to each well 2 wells of no template controls were added to the 96 well plate for

comparison. The plates were then covered with MicroAmp® (Life technologies®) Optical Adhesive Film. The plates were then briefly spun in a centrifuge to ensure the solution reached the base of each well and to eliminate any air bubbles caused by pipetting.

6.8.5 Real Time PCR Setup

Real-Time PCR was completed on the plates using the 'standard' thermal cycling mode of the StepOne® Real-Time PCR software system (ThermoFisher Scientific, Waltham, MA) which employed the following thermal cycling conditions; Enzyme activation occurred at 95°C for 10 minutes, denaturation at 95°C for 15 seconds and Annealing/Extension at 60°C for 1 minute, with 40 cycles total as suggested by the manufacturer of the SNP probe solution (Life technologies®). 5% of samples were randomly selected and re-ran for accuracy (in a separate plate). Samples that did not give readings were eliminated from analysis.

6.8.6 DNA Analysis

Upon PCR amplification proceedings a post-PCR plate read was completed using the StepOne® Software package. This software plots R_n values based on fluorescence signals from each well, which determines which alleles is present within each sample. In all cases control wells showed no results. The software plots the results of the allelic discrimination data as a scatter plot of Allele 1 (VIC® dye) and Allele 2 (FAM™ dye) with heterozygous and homozygous Alleles being visible for each data point. Each well is represented as a singular plot point and allele data can be extracted from the plot as a .txt or .xml file that can easily be read in Microsoft Excel. As the wells were labelled with each sample prior to running the analysis the data was easily readable giving the allele data for TRPM8 SNP rs10166942 for each participant.

6.8.7 Image Analysis

Image analysis was completed in accordance with the method described on p90. The change in mean temperature of the region of activity between the baseline and 5 minute and 1-minute measurements was calculated as the final measure of BAT activity (ΔT).

6.8.8 Statistical Analysis

Data was pooled from the UK and India cohorts. BAT activity and genetic data was assessed in 24 (native south Asians 10 NAFLD, 14 control) 32 UK based South Asian (17 NAFLD, 15 control) and 51 UK Caucasian (21 NAFLD, 30 control) male participants. All statistical estimates are supported with 95% confidence intervals. The level of statistical significance will be set at $P<0.05$ (two-tailed). A Statistician from the University of Nottingham's School of Medicine's Statistical Resource 'Clinic' advised on the preparation of the data and the data analysis before the commencement of data recording.

6.9 Results

Analysis of the genetic data demonstrated that the T Allele frequency among Caucasians and South Asians was 0.8 and 0.48 respectively and sits in accordance with expected values for the allele frequency within these demographic populations. The presence of the T Allele was strongly associated with increased BAT activity (ΔT_{rel}) in our sample set (0.49 +/- 0.22 vs 0.33 +/-0.21 **p** = 0.006). On univariate analysis of the data, the T allele was shown to be associated with higher BMI (27.2 vs 24.7 **p** = 0.017), prevalence of diabetes (41.6% vs 16.7% **p** = 0.046) but lower likelihood of NAFLD (39.3% vs 72.2% **p** = 0.01). Through multiple logistic regression modelling, adjusting for BMI, BAT activity, T allele was negatively associated (odds ratio 0.06 (0.008, 0.43; **p** = 0.005).

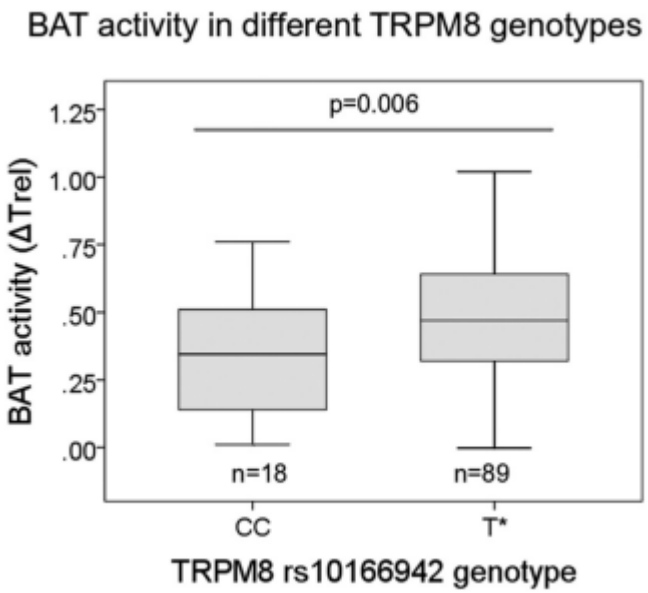


Figure 11. *shows the effect of T allele frequency among total assessed population in relation to BAT activity (ΔT_{rel}) as measured using thermal imaging*

6.10 Discussion

Data shows that possession of the TRPM8 dominant T Allele variant may be associated with increased BAT activity in this cohort study. This data and others (Key et al., 2018) have demonstrated a racial alteration in the presence of the T allele specifically. The T allele shows much higher prevalence within regions which are further north in latitude which coincides with decreasing temperatures, and was shown within the demographic data of this study (0.8 Caucasian vs 0.48 South Asian). For this reason, it is possible that the T allele for TRPM8 gave an evolutionary advantage to those in colder climates. Whilst, this is no longer a factor for survival, TRPM8 and the efficiency of cold sensitivity in those carrying the T allele may result in an increase in BAT activity, which in turn could lead to an increased basal energy demand, aiding in a healthier energy balance in this population and lower propensity to metabolic disease risk. It is established that a range of endo and exogenous ligands are responsible for BAT activation (Bargut et al., 2016). The cold receptor TRPM8 responds to mild cold temperatures and pharmacological agents that mimic the cold sensation such as menthol (Basbaum et al., 2007). Cold stimuli have been shown to enhance brown fat activity through the activation of β-adrenergic signalling, a process mediated by UCP1 (Cannon & Nedergaard, 2004). It has been shown however that mice lacking β-receptors have unchanged BAT function with lower levels of UCP1 (Lehr et al., 2006), demonstrating that other mechanisms of action could be involved with activation of thermogenesis. TRPM8 has been shown to be expressed in BAT and can be activated by mimicking the mild cold effect through pharmacological stimulation. An effect that has also been shown to protect against high fat diet induced obesity in mice, a finding similar to those found when using adrenergic stimulants to induce BAT stimulation in similar rodent models (Xiao et al., 2015).

These assessments have not been made in humans however, and evidence relating thermoregulation and racial factors are lacking. Whilst it is highly unlikely that any single nucleotide polymorphism will impact phenotype dramatically, epidemiological data has demonstrated the increase in obesogenic related ailments within the South Asian population specifically at a lower BMI than in the Caucasian population (Shah & Kanaya, 2014).

Other factors which predispose South Asians to obesogenic related co-morbidities, which link closely to BAT thermogenesis include increased insulin resistance and increased total body adiposity, both factors that have shown to be improved with adulthood active BAT (Saito et al., 2009).

Increased insulin resistance within the South Asian population has been linked to adaptations during fetal development (Hu, 2011). The so called “thrifty genotype” hypothesis suggests that individuals that are able to store and more efficiently utilise consumed energy, will have an increased chance of survival during times of famine (Reddon et al., 2018). With the changing economic situations in the South Asian population, their ability to store excess energy may now be detrimental and could predispose insulin resistance in south Asian teens and adults (Ramachandran et al., 2007). Specifically, when compared to their Caucasian counter parts South Asians, have demonstrated significantly higher insulin levels and NAFLD (Petersen et al., 2006).

Increased total body fat is another area in which the South Asian population have demonstrated higher rates when compared to Caucasians when compared specifically to a lower overall BMI; a trend seen across multiple Asian subcontinents (Deurenberg-Yap et al., 2000). South Asians have a higher total body fat mass at lower BMI, for both visceral and subcutaneous fat (Lear et al., 2007). Adipose tissue plays an important endocrine role in the regulation of IR and obesity related co-morbidities (Lear et al., 2007), therefore increased

adiposity could explain this increase in metabolism related ailments across this population specifically. For the purposes of thermal imaging increased adiposity overlaying the ROI may have impacted the overall detectable rate of BAT activation or temperature increase within the SCV region. Previous data has shown the importance of subcutaneous adipose tissue within the area and how increased adiposity can lead to a dampened radiometric data reading (Law, Chalmers, et al., 2018).

Ultimately, a host of factors will play a role in this predisposition, however alterations in the genetic makeup of ones ability to regulate cold and in brown fat activity may be one factor which shifts the balance towards obesity related co-morbidities within this population.

7. Summary of Projects, Further Discussion of Study Limitations and Implications for future research

7.1 Study 1) Using Thermal Imaging to Assess the Effect of Caffeine on Brown Adipose Tissue Thermogenesis – Pilot Study

In this study the consumption of the caffeinated beverage significantly increased the temperature of the supraclavicular region co-locating with BAT. This effect was seen as an increase of TSCV and Trel which was not seen with the consumption of water alone (Caffeine TSCV before intervention: 33.81 ± 0.45 °C, TSCV after intervention 34.187 ±0.59 °C, $P > 0.05$ TREL 0.4067 ±0.28 °C $p < 0.001$)

The study was set up as a pilot and which limited the validity of the study. Primarily the sample size and study demographic were not controlled. Whilst the n number was reached the study utilised both male and female participants. BAT has been shown to be affected by sex (Law et al., 2014) and there have been numerous studies demonstrating the impact of sex on regulation of body weight (Shi & Clegg, 2009) and as such should have been controlled. During this pilot investigation sex was not controlled as a means of giving the study the best opportunity to recruit participants. BMI and age were also not controlled upon participant application for reasons discussed previously, both factors which are known to impact BAT activity (Vijgen et al., 2011). Increased subcutaneous adipose tissue within the supraclavicular region has been shown to impact the radiometric data observed through the thermal imaging camera due to a dampening process (Law, Chalmers, et al., 2018) and as such may have impacted the results. Age is also a major factor in the maintenance of active SCV BAT and should have been controlled or a larger sample size may have been beneficial as a means of assessing how age impacted the thermal data from the coffee drinking pilot study. As the study group was so small making any conclusions of the impact of any of the

anthropometric data would be difficult, in future more participants should be recruited to fully investigate the results shown in this study.

The research process also did not have any blinding due to the nature of investigating a commonly consumed beverage and as such participants and the researcher where aware of the intervention taking place. Whilst the researcher did complete all data analysis in accordance with protocol, unblinding can introduce bias to the research and should always be avoided where possible. Further investigation into this study could aim to measure the effect of coffee by utilising a decaffeinated alternative as this would have been much closer in taste to the caffeinated version and therefore could have enabled blinding.

Overall, the results obtained were interesting and in conjunction with in vivo data (Velickovic et al., 2019) showed a promising relationship between BAT and caffeine that had not been tested previously. This study formed the basis for the double-blinded caffeine placebo study.

7.2 Study 2) Using Thermal Imaging to Assess the Effect of Caffeine on Brown Adipose Tissue Thermogenesis

Results from the study demonstrated that the 200mg caffeine intervention showed no significant change of the supraclavicular region temperature (TSCV $p = 0.24$) which co-locates with BAT. On continued analysis, the thermal data for Trel post intervention was compared between both groups (placebo vs caffeine). Trel post intervention showed no significant change in SCV temperature ($p = 0.077$). Data from the study showed no overall statistically significant increase in temperature within the supraclavicular ROI upon consumption of caffeine, however the average temperature across both Trel and TSCV was increased with the consumption of caffeine when compared to the placebo. Discussed previously, it was also possible that there was a clear response to caffeine in some participants when compared to

placebo. This is an interesting finding and would warrant further investigation. Studies have previously discussed the concept of BAT positivity and BAT negativity (Maliszewska et al., 2022), which in short implies that it is feasible that due to the variance in human physiology, and through aspects discussed within this thesis (such as ethnicity) it is possible that some participants within this study did not possess active BAT or that the BAT they did possess was not sufficient to elicit a thermogenic response during this study. In order to investigate this further it would be worth utilising a mixed method approach (as suggested in study 3) which would utilise thermal imaging and MRI in conjunction to validate the presence of BAT within the SCV region. This could then be compared to thermal data giving us a better indication as to whether those giving a positive temperature reading do possess sufficient BAT within the region to reliably stimulate that response or if other factors (such as those discussed p92) become a factor.

7.3 Study 4) The Assessment of Brown Adipose Tissue Thermogenesis Using Thermal Imaging and Associated Single Nucleotide Polymorphisms

For this study analysis of the genetic data demonstrated that the T Allele frequency among Caucasians and South Asians was 0.8 and 0.48 respectively and sits in accordance with expected values for the allele frequency within these demographic populations. The presence of the T Allele was strongly associated with increased BAT activity (ΔT_{rel}) in our sample set (0.49 +/- 0.22 vs 0.33 +/-0.21 $\mathbf{p}$ = 0.006). On univariate analysis of the data, the T allele was shown to be associated with higher BMI (27.2 vs 24.7 $\mathbf{p}$ = 0.017).

Whilst the data for this study was interesting there were a noticeable number of limitations and issues with the study design that would need to be addressed to validate any subsequent data.

A major component of the study was to assess thermal data taken from two continents (UK, Nottingham and Sree Gokulam Medical College, Trivandrum, India). This may have been an issue as thermal imaging is an extremely sensitive process and whilst it is clear that all measures were put in place to ensure thermal imaging protocol between regions was maintained, including ensuring the study environment temperature and RH was maintained in both locations, in reality it would not have been possible to keep all aspects the same as the imaging suite being used would have been different. The study was also originally designed (due to the goal of the original study by Dr Chalmers) aimed to assess those with higher BMI. The healthy participant cohort however was relatively open and age difference between groups could be fairly large due to the nature of these co-morbidities generally occurring at higher age ranges.

As discussed previously, BMI and age can both impact the thermal data received (Law, Chalmers, et al., 2018) which may have impacted the overall results, especially when comparing thermal to genetic data. The homozygous non-dominant allele was also not present in a large frequency. Although data for the frequency of the occurrence of the alleles was in accordance with global genotype data and fit with the research the study was based on (Key et al., 2018) it left only 18 participants within the non T allele group (CC) vs 89 who had a T allele for TRPM8 present (89). This meant that when comparing thermal data the groups where largely imbalanced. For future research, expanding the assessment of thermal and genetic data across a wider cohort such that the thermal imaging groups are equal may give a more accurate representation of the impact of the T allele on thermal data. The choice of cooling at the stimulant may also be improved, specifically with the use of a menthol pharmacological substance, as this would ensure specific targeting of the TRPM8 receptor. Overall, these projects have further demonstrated the use case for assessing brown fat

activity using thermal imaging. The combination of studies demonstrates the value and potential for a wide array of research to be undertaken using infrared thermography as a proxy measure for BAT activity and the future potential for caffeine to be investigated as a means of stimulating SCV BAT.

7.4 Implications and Future Research

Further work was set to be carried out on this project which would have further validated the methodology, using MRI in conjunction with thermal imaging was planned to be completed and was unable to be carried out which may have validated previous work, as well as demonstrating a novel mixed methods approach to the assessment of SCV BAT activity and presence. The work that has been completed has continued to provide evidence for the continued use of thermal imaging as a methodology, which is currently lacking in the field. Future research should continue to utilise IRT to investigate nutritional supplementation as a means of activating SCV BAT. In order to validate this further, more work needs to be done to validate the presence of SCV BAT within participants who show a demonstratable response to mild cooling stimuli or nutritional interventions. MRI would be a good way to achieve this, and used in conjunction could make for a genuine non-invasive alternative to the use of PET/CT in future research.

8. Appendix

Appendix 1 CAFFBAT Study Protocol and Questionnaire

Appendix 1

Study Questionnaire; Participant demographics. Study No:_____

We are asking for the following information because the activity of brown fat can be different in people of different ethnicity and of different ages. This information will be held securely, and you will not be identified from it.

1. Which of the following do you feel best describes your ethnicity:

Indian	
Pakistani	
Bangladesh	
Any other Asian ethnic	

Please tick

Asian or Asian British

Black or Black British

Caribbean	
African	
Any other Black ethnic background	

Mixed

White and Black	
White and Black	
White and	
Any other Mixed ethnic	

Chinese or other ethnic group
Chinese
Any other ethnic group

White	
British	
Irish	
Any other White ethnic	

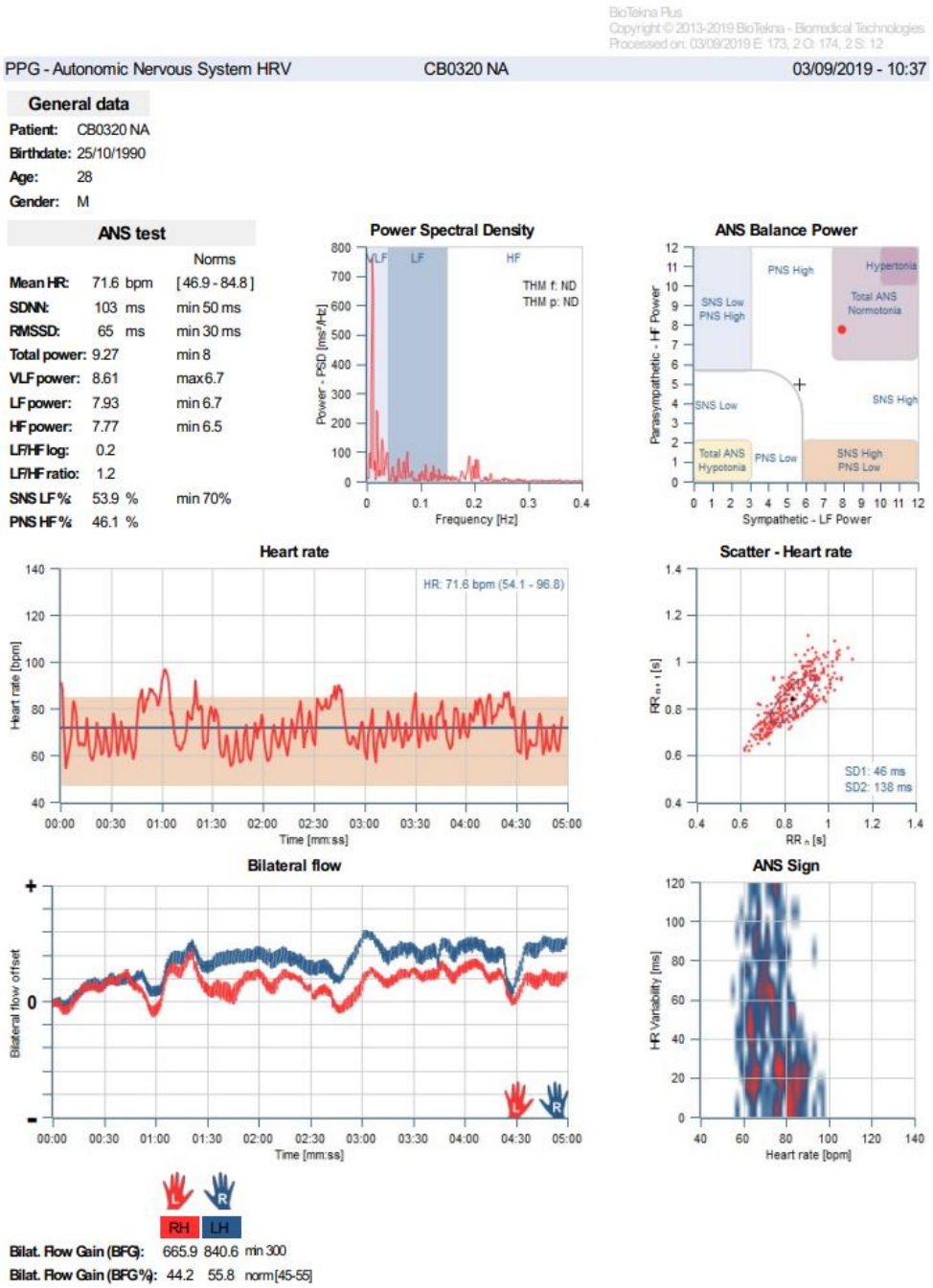
2. Which of the following age ranges does your current age fit?

	Please tick
Aged 18-21	
Aged 22-25	
Aged 26-30	

3. How often would you say you consume a caffeinated beverage (tea, coffee, energy drink etc.)

	Please tick
1 or <1 Per	
2 to 3 times per	
Over 3 times per	

Appendix 2 Example of Biotekna® PPG Stress Flow Analysis



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