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The Thyroid and Glycaemic Endocrine Influences on Brown Adipose Tissue and its Measurement Using Infrared Thermography

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Other authors' declaration

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The thyroid & glycaemic endocrine influences on BAT and its measurement using IRT

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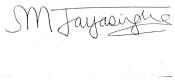
Astle V 	30/04/2020	**Jayasinghe M** 	04/05/2020
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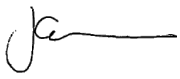
Chalmers J 	30/04/2020	**Rabiner E** 	01/05/2020
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Dhillo WS 	30/04/2020	**Salem V** 	30/04/2020
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Izzi-Engbeaya C 	01/05/2020	**Tan T** 	30/04/2020

Table of Contents

Abstract

Brown, and brown-like, adipose tissue provides a potential target for treatments to increase energy expenditure and the consumption of lipids and glucose. In humans, active depots have been shown to persist into adulthood where, previously, they were thought to atrophy in childhood. Due to the presence of uncoupling protein-1 (UCP1) within the inner mitochondrial membrane, brown adipocytes possess the capability to prevent the energy released during cellular respiration from being stored as adenosine triphosphate, instead allowing the energy to be released as heat. Since heat energy is, eventually, dissipated from the body, the result is energy expenditure. Why the body possesses such a tissue specifically designed to waste energy is explained by the benefit conferred by the ability to produce heat on demand, in response to cold challenge. The major alternative heat generating method, shivering, can respond instantly but produces vastly less heat for the same effort. Beyond the acute shivering response, which cannot be maintained for prolonged periods, brown adipose tissue is an essential contributor to thermal homeostasis.

Brown adipose tissue is innervated by the sympathetic nervous system (SNS), acting on B3-adrenoreceptors to stimulate intracellular lipolysis, increased free fatty acids (as well as glucose uptake), upregulation of UCP1 and, hence, heat production. This process is modulated both at the level of the hypothalamic SNS activity and at the intracellular level by thyroid hormones. Thyroid hormones act on the hypothalamus to increase brown adipose tissue stimulation and the biologically active thyroid hormone, triiodothyronine, concentrations are increased within the BAT cell by conversion from the inactive form, thyroxine, by 5’deiodinase-2.

To examine the efficacy of potential stimulators, an effective way of measuring brown adipose tissue activity is required. Positron emission tomography-computed tomography

(PET-CT) measures glucose uptake and therefore, under cold stimulation, has allowed brown adipose tissue activity to be quantified using the surrogate measure of glucose uptake and for brown adipose tissue volumes to be calculated. However, PET-CT is unable to measure brown adipose tissue in the fed state due to glucose uptake of muscles, and the radiation exposure means it is not suitable for use in children, large studies or repeat measurements. It is of particular interest to understand the attributes of brown adipose tissue in children since this is the period of maximal brown adipose tissue activity and the time in which physiological parameters are established with lifelong effects.

Since the major output of BAT is heat, attempts have been made to measure the heat that is transferred from the BAT depot to the skin either by use of thermocouples, such as iButtons™, or infrared thermography. These methods aim to address some of the limitations of PET-CT as they are non-invasive, readily available and do not expose the participant to ionising radiation. Furthermore, they directly measure the major output of BAT activity, i.e. heat, rather than measuring an input, e.g. glucose uptake, which may not always reflect the activity of the tissue.

The thesis presented here aimed to validate IRT against PET-CT for the measurement of BAT activity and to improve the efficiency of IRT image analysis by automation before using IRT to demonstrate the effects of a) glucose dysregulation and b) thyroid hormone dysfunction on human BAT *in vivo*.

While infrared thermography was clearly able to demonstrate a supraclavicular hotspot, it was questioned whether this truly reflected BAT and whether changes in the thermal signature were indicative of BAT activation as many other factors influence skin temperature. In addition, image analysis was limited by the time-intensive process of manual image analysis. I, therefore, designed a semi-automated method of image analysis,

coded using MATLAB™ by a colleague in the University's Faculty of Engineering. This method was 86% faster than the previous method, without any increase in variation on repeated analysis.

I then undertook an analysis of thermal images and PET-CT scans from the same individuals and compared them. Following image alignment, the "hotspot" of glucose uptake on PET-CT overlaps with the thermal "hotspot" from infrared thermography and glucose uptake was correlated well with changes in the temperature of the skin overlying the BAT depot.

Having established and validated the image analysis method, a series of cold-water swimmers were imaged before and after their event. Exposure to this mixed maximal stimulation showed dramatic preservation of the BAT hotspot compared to the sternal reference region (3.5±1.6°C higher post-swimming compared to baseline), although further work is needed to identify the relative effects of the different stimulation components.

I have then shown that girls with autoimmune hypothyroidism have reduced increase in supraclavicular temperature relative to a sternal reference point compared to healthy controls (hypothyroid: 0.1±0.1°C; control: 0.2±0.2°C; $p = 0.04$) yet, within those participants with hypothyroidism, TSH concentration was associated with increased relative supraclavicular temperatures ($r = 0.7$, $p = 0.01$). In children with diabetes compared to healthy controls stimulated relative supraclavicular temperatures were lower (diabetes: 35.0±0.6°C; control: 35.4±0.5°C; $p = 0.04$) and a smaller change in relative supraclavicular temperature following stimulation, after adjusting for BMI (diabetes: 0.1±0.1°C; control: 0.2±0.2°C; $p = 0.03$). In these studies, data was not available on thyroid function test results or glycaemic status for healthy volunteers which future work should look to address in addition to measuring and controlling for pubertal status.

In conclusion, this thesis demonstrates that infrared thermography is a valid and reproducible way to measure BAT activity in the non-invasive and affordable way. The increase in image analysis rate provides the opportunity for future work to look at larger groups, longer imaging periods or increased image frequency even to the level of thermal videos and, thereby, reveal finer detail about the mechanisms and potential stimulators. Further advances in image analysis techniques will aim to fully automate the process.

In children with diabetes, there is an indication of a similar reduction in BAT activity, potentially due to the differences between exogenous and endogenous insulin, such as endogenous insulin not being suppressed by sympathetic nervous system activity. Further work is also required to better understand the complex interaction between BAT and the thyroid-axis with many questions remaining. In girls with autoimmune thyroid disease, BAT activity appears to be reduced but TSH appears to stimulate BAT activity suggesting that the disease process itself may have a negative effect on BAT response but this may be mitigated by high TSH levels.

Abbreviations

^{18}F-FDG	^{18}F-fluorodeoxyglucose
ADP	Adenosine diphosphate
AR	Adrenoreceptor
AT	Adipose tissue
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BeAT	Beige adipose tissue
BMI	Body mass index
BRAIDT	Brown Adipose Tissue Activation In Children with Diabetes or Thyroid Dysfunction
cAMP	Cyclic adenosine monophosphate
CT	Computed tomography
DIO2	Type II iodothyronine deiodinase
DIT	Diet-induced thermogenesis
FFA	Free fatty acids
HPA	Hypothalamic-pituitary-adrenal
HPT	Hypothalamic-pituitary-thyroid
IRT	Infrared thermography
MRI	Magnetic resonance imaging
Myf	Myogenic factor
NA	Noradrenaline
NEAT	Non-exercise activity thermogenesis
NS	Not significant
OSA	Obstructive sleep apnoea

PET-CT	Positron emission tomography-computed tomography
RMR	Resting metabolic rate
ROI	Region of interest
SCV	Supraclavicular region
SNS	Sympathetic nervous system
SUFE	Slipped upper femoral epiphysis
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
T3	Triiodothyronine
T4	Thyroxine
TEA	Thermic Effect of Activity
TEE	Thermic Effect of Eating
T_{Ref}	Median temperature in the reference region
T_{Rel}	Relative temperature ($T_{SCV} - T_{Ref}$)
T_{SCV}	95th percentile of temperature in supraclavicular region of interest
TSH	Thyroid-stimulating hormone
UCP	Uncoupling protein
UK	United Kingdom
WAT	White adipose tissue
WHO	World Health Organization
ΔT	Change in temperature

List of Figures

Other figures are listed within the respective articles.

List of Tables

Tables are referenced within the respective articles.

Section 1 Introduction

Obesity remains a major challenge to international public health in both developed and developing countries (Collaboration 2016; Ng et al. 2016); increasing obesity is a leading factor in the prediction that the current generation of children will be the first in recent times to have a shorter life expectancy than their parents (Olshansky et al. 2005). Yet the mechanisms underpinning an individual's resistance or susceptibility to body weight gain remain poorly understood, and effective treatments elusive.

Brown adipose tissue (BAT) has been the recent focus of intense research as a possible novel mechanism to increase energy expenditure and improve metabolic profiles, following its rediscovery in adult humans in 2009 (Sacks 2009; Celi 2009; Virtanen et al. 2009; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009). BAT is able to uncouple the oxidation of glucose and fatty acids from the production of adenosine triphosphate (ATP), liberating the chemical energy stored in substrate molecules as heat (Cannon and Nedergaard 2004). Since heat is ultimately lost to the environment, increased BAT activation can result in increased energy expenditure and favourable weight control, at least in rodents.

Increased BAT activity in response to a high-fat diet buffers the expected gain in weight (Rothwell and Stock 1979) and stimulation can lead to weight loss (Ghorbani, Claus, and Himms-Hagen 1997), at least in rodents, although the situation in humans is less clear (Yoneshiro et al. 2013). Increased BAT activity can improve glycaemic control and plasma lipid levels, improving metabolic health (Stanford et al. 2013; Liu et al. 2013). Brown adipocytes (BAs) express insulin receptors (IRs) (Tanti et al. 1986) and glucose-transporters (such as GLUT-4) (Omatsu-Kanbe, Zarnowski, and Cushman 1996) on their surface, and insulin-independent uptake allows BAT to remove glucose from the

circulation even in the presence of reduced plasma insulin concentrations (T1DM) and increased insulin resistance (T2DM) (Section 1.2.6).

BAT is responsive to the endocrine system, especially thyroid hormones. Thyroxine (T4) is a key modulator for BAT activity, and BAs contain high levels of Type II iodothyronine deiodinase (DIO2). DIO2 converts triiodothyronine (T3) to T4, leading to a locally hyperthyroid environment necessary for optimal BAT function (Carvalho et al. 1991). Hypothyroid animals become rapidly hypothermic, primarily from an inability to initiate BAT adaptive thermogenesis (Bianco and Silva 1987) and thyroid status has a profound effect on BAT activation in humans (Skarulis et al. 2010).

Studies of BAT in humans are difficult and restricted. Much of our knowledge, therefore, depends on extrapolation from animal data, in particular from rodent studies. This requires a fallible assumption of similar underlying cellular physiology; moreover, there are significant differences in thermogenic requirements. The anatomical distribution of BAT depots makes them difficult to sample directly in humans, and there is, therefore, a need for reliable validated *in-vivo* imaging techniques. The current gold standard is ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography/computed tomography (PET-CT), which measures the uptake of radiolabelled glucose. However, this is invasive, and the high radiation dose makes it inappropriate for large studies, repeated imaging or imaging in children. Consequently, studies tend to be on small numbers of healthy volunteers or rely on imaging performed for clinical reasons, typically as part of oncological investigations. These are not optimised for BAT identification and are unlikely to be representative of the non-morbid population. Specifically, studies on children are scarce and information on the behaviour and changes in BAT during this crucial developmental period is lacking.

This current work aims to establish reliable methodologies for the study of human BAT, both *in-vivo* and *in-vitro*, and to utilise these to explore the effect of the glycaemic and thyroid axes on BAT activity in humans.

1.1 Obesity

1.1.1 Definition

Obesity in adults is typically defined as a body mass index (BMI) of greater than or equal to 30kg/m^2, with an overweight individual having a BMI of 25kg/m^2 or more (Global Reference List of 100 Core Health Indicators 2015). BMI as a parameter to define obesity has the appeal of being easy to measure, being reproducible and using readily available equipment. BMI does not measure fat-mass directly, but it correlates well with direct measures (Gallagher et al. 2000; Loprinzi et al. 2015). However, two individuals with the same BMI do not necessarily have the same body fat percentage or the same risk of obesity-associated morbidity and mortality (Gallagher et al. 1996; Kannel et al. 2002; Deurenberg, Yap, and van Staveren 1998; The Global 2016). Other measures of obesity such as waist circumference, skinfold thickness or bioelectrical impedance are available and imaging modalities, such as computed tomography (CT) or magnetic resonance imaging (MRI), may be able to give a better prediction of the risk of obesity on health by measuring the distribution of AT including its proximity to vital organs (Burkhauser and Cawley 2008; Fox et al. 2007).

In children, BMI naturally varies with age and so fixed thresholds are not useful. Instead, BMI charts are available which compare a child's BMI to the normal distribution for children of the same age and gender. Clinically, a child above the 98th centile for BMI is considered obese and one between the 91st centile and 98th centile, overweight; epidemiological studies typically use cut-offs of 95th and 85th centiles respectively (Scientific Advisory Committee on Nutrition (SACN) and the Royal College

of Paediatrics and Child Health (RCPCH) Joint Statement on Defining Child Underweight, Overweight and Obesity in the UK 2012). These definitions are not universal (Cole et al. 2000; de Onis et al. 2007).

1.1.2 Epidemiology

Obesity is fundamentally a result of modern industrialised lifestyle with the ready availability of cheap energy-dense food and increasingly sedentary behaviour (French, and, and Jeffery 2001; Caballero 2007). The second half of the twentieth century saw a rapid rise in rates of obesity, with a skew of the normal distribution curve to the left (Finucane et al. 2011). Overall, the worldwide prevalence of obesity is currently estimated to be 12% in adults and 5% in children (Collaborators et al. 2017). In the United Kingdom, it is estimated to be 28% and 15%, respectively (Conolly and Craig 2019). The highest rates are typically seen in developed countries, but rapidly changing diets and internationalisation is causing the fastest rates of rise in some of the world's poorest countries (Collaboration 2017, 2016; Finucane et al. 2011). Although it is seen across the spectrum, obesity is more prevalent in lower socio-economic groups (Collaborators et al. 2017; Conolly and Craig 2019; Molarius et al. 2000).

There is much debate about why data from the last few years suggests that this rapid rise is slowing or has halted (Health Survey for England, 2018: Overweight and obesity in adults and children data tables 2018), at least in developed and richer countries (Collaboration 2017). It may be that the increased awareness of the risk of obesity has halted the rise with a combination of government intervention and individual response (Lissner et al. 2010; Schmidt Morgen et al. 2013). Alternatively, the presence of a spectrum of susceptibility may mean that there is a saturation of susceptible individuals, thus an increasingly obesogenic environment may have relatively little effect on the overall prevalence of obesity (Schmidt Morgen et al. 2013).

1.1.3 Causes

At the most basic level, obesity is the result of a chronic net positive energy balance, when energy intake (from diet) exceeds energy expenditure. Total energy expenditure comprises (Poehlman 1989):

- Resting metabolic rate (RMR), from cellular function at rest, including smooth muscle activity, circulatory requirements and respiratory function.
- Thermic effect of activity (TEA) which results in additional requirements above the RMR, from voluntary activity causing increased skeletal muscle energy expenditure.
- Facultative (or adaptive) thermogenesis, of which BAT is a major component.
- Thermic effect of eating (TEE; also known as diet-induced thermogenesis, DIT), required to digest and metabolise food.

However, the simplicity of the equation *Energy Balance = Energy In – Energy Out* belies the complexity of energy balance. Some individuals appear to be able to eat more freely without appearing to expend more energy, and yet be resistant to weight gain; the reasons for this are multifactorial and complex and not yet fully appreciated (Blundell et al. 2005). A substantial part may be recording bias: study participants routinely under-record intake (Black et al. 1993). The proportion of sedentary time may be more important than exercise (Owen et al. 2010) as non-exercise activity thermogenesis (NEAT) (i.e. not from the resting metabolic rate, eating or sports-like exercise) (Levine 2002) may have more effect on daily energy expenditure than relatively short bursts of more intense physical activity. Increased energy expenditure from enhanced BAT mass and activity is also associated with lower BMI (van Marken Lichtenbelt et al. 2009; Saito et al. 2009; Cypess et al. 2009) and the factors affecting BAT activity are explored further below.

Finally, at the population level, genome-wide studies have demonstrated gene combinations that are associated with an increased susceptibility for weight gain (Thorleifsson et al. 2009; Rankinen et al. 2006).

The many components of obesity and the cumulative effect of small, repeated actions (whether snacking or fidgeting) make this a complex issue.

1.1.4 Effects

Obesity is associated with a range of adverse health conditions and poor outcomes (Kitahara et al. 2014), affecting every system in the body. This includes the metabolic syndrome (a combination of T2DM, high blood pressure and obesity) which is associated with a high risk of cardiovascular disease (Manson et al. 1990; Lakka et al. 2002). Although some of the effects of obesity, such as arthritis, are a direct result of increased weight, many are due to the maldistribution of AT and ectopic deposits.

AT evolved to store surplus energy in time of plenty, against scarcity and famine. In health, AT is an essential component of the endocrine system, responding to major hormone axes including the hypothalamic-pituitary-thyroid (HPT) axis and hypothalamic-pituitary-adrenal (HPA) axis, and secreting signalling molecules, termed adipokines, such as leptin and adiponectin (Galic, Oakhill, and Steinberg 2010; Kershaw and Flier 2004; Halaas et al. 1995).

AT is normally distributed in the subcutaneous depot. When this becomes saturated, ectopic deposits begin to develop, most notably around visceral organs and the cardiovascular system (Stefan et al. 2008). Excess visceral AT is a major risk factor for cardiovascular and metabolic sequelae (Taksali et al. 2008; Björntorp 1991) and longitudinal increases in this depot worsen the risk of developing metabolic syndrome (Tu, Humphries, and Lear 2017). However, there is also some evidence that subcutaneous AT

stored in the abdominal and truncal distribution results in significantly more insulin resistance than gluteal and femoral depots (Patel and Abate 2013) although this remains debated (Wong, Janssen, and Ross 2003).

The multi-organ systemic effects of obesity are reflected in the wide variety of health problems experienced by obese individuals. When depots are saturated, AT enters a pro-inflammatory state (Bawadi et al. 2016), releasing cytokines and attracting macrophages and other components of the immune system, further inflaming the tissue (Chawla, Nguyen, and Goh 2011; Jin Young et al. 2014; Xu et al. 2003; Boutens and Stienstra 2016). Lipid enters the circulatory system, leading to plaques and eventually both arterial and venous cardiovascular disease. The systemic sub-acute inflammatory state, along with changes in oestrogen and other hormone levels increases the risk of certain cancers (such as uterus, gallbladder, kidney, thyroid, and cervix) (Bhaskaran et al. 2014). Within the endocrine system, obesity is associated with end-organ insulin-resistance (Xu et al. 2003); initially, the pancreas increases insulin secretion to compensate but eventually, insulin resistance exceeds its capacity to respond and T2DM results.

In childhood, the effects of obesity are two-fold. Firstly, obese children are at significantly increased risk of becoming obese adults (Nader et al. 2006; Freedman et al. 2001). Childhood is a period of development and metabolic programming (Sebert et al. 2011; Hales and Barker 2001; Parsons, Power, and Manor 2001); early experiences and learning shape later behaviours, making good nutritional behaviour essential for later health. In addition, having an obese parent doubles the risk of a young child becoming an obese adult (Whitaker et al. 1997), demonstrating the importance of breaking this inter-generational cycle.

Secondly, obese children are at increased risk of childhood morbidity and mortality. There has been an increase in T2DM in children (Haines et al. 2007; Dabelea et al. 2014) and obese children are also at increased risk of conditions such as obstructive sleep apnoea (OSA) (Alonso-Alvarez et al. 2014; Alonso-Alvarez et al. 2015; Sulit et al. 2005) and asthma (Figueroa-Muñoz, Chinn, and Rona 2001; Gilliland et al. 2003; Sulit et al. 2005) (with an associated mortality risk), mental health disorders (Schwimmer, Burwinkle, and Varni 2003; Halfon, Larson, and Slusser 2013) and musculoskeletal problems, including Blount's Disease and slipped upper femoral epiphysis (SUFE) (Murray and Wilson 2007; Halfon, Larson, and Slusser 2013). Effectively combatting childhood obesity has a tangible benefit to the child and a multiplying benefit both to the individual and to society for future health improvement and economic savings.

1.1.5 Treatments

Treatments for obesity aim either to reduce energy intake or to increase energy expenditure. The simplest options are reduced energy intake by dietary modifications, and regular physical activity to increase energy expenditure. In the United Kingdom, only one medication, orlistat, is currently approved for the treatment of obesity (Treatment summary: Obesity 2019). Orlistat reduces intake by decreasing intestinal absorption of dietary lipid. Surgical interventions typically aim to reduce intake by reducing the capacity of the stomach, thereby causing earlier satiety.

Non-surgical interventions typically have poor long-term outcomes. Initial weight loss is often achieved, but there is a tendency to revert to established behaviours and weight maintenance is far more difficult to achieve (Lemmens et al. 2008; Godino et al. 2016). Multi-disciplinary approaches which are consistent with individuals' psychosocial image and societal norms may have more success (Hunt et al. 2014), but these programmes require careful tailoring to individual groups. Surgical interventions are more effective, but

they are costly and relatively risky (Lemmens et al. 2008). Within the United Kingdom, surgery is reserved for individuals with severe obesity and a co-morbid condition, who have failed non-surgical weight loss measures and who are fit for surgery and is only available as part of an intensive weight management strategy.

Weight management strategies in children are broadly similar but with some fundamental differences. Firstly, most effective interventions address the child as part of the family unit (Oude Luttikhuis et al. 2009). Since childhood is a period of growth, the aim is usually for weight maintenance by dietary improvements and increasing physical activity, rather than for weight loss; as the child's height increases, there is a fall in their BMI without the risks of affecting growth and development associated with restrictive diets. Although medication (in children over 12 years old) and surgery may be considered, this is only done in exceptional circumstances, with surgery limited to those who have, at least nearly, achieved physical maturity, as evidence of benefit and risks are limited (McDuffie, Calis, Booth, et al. 2002; McDuffie et al. 2004; McDuffie, Calis, Uwaifo, et al. 2002).

Critically, the currently available weight management strategies are of limited long-term effectiveness for children or adults. In the context of ongoing dietary and activity management, BAT stimulation, whether environmental or pharmacological, could increase daily energy balance from a small net positive amount (with resultant weight gain) to a small net negative amount, helping to achieve and maintain weight loss, increasing the effectiveness of the currently available treatments.

1.1.6 Metabolic Health

As noted above, much of the morbidity associated with obesity is metabolic in origin (Section 1.1.4). Improvements to the metabolic profile can result in substantial health

improvements, seen in the wide-spread use of statins to lower LDL cholesterol which reduces coronary heart disease even without weight loss (Baigent et al. 2005).

Although obesity is a significant risk factor for poor health, a group of individuals, who seemed resistant to the usual metabolic sequelae (Primeau et al. 2011; Karelis 2008), were identified and termed “metabolically healthy obese” (Blüher 2010), representing up to 20% of obese individuals (Ferrannini et al. 1997). Adipose tissue (AT) in this group had a different anatomical distribution to other obese individuals (Bi et al. 2015; Patel and Abate 2013) and their metabolic profile (e.g. insulin resistance, glycaemic control and plasma lipid levels) was comparable to normal-weight individuals (Karelis et al. 2005; Karelis 2008). They remained insulin sensitive (Ferrannini et al. 1997), without hypertension, and with favourable lipid and inflammatory profiles (Karelis et al. 2005; Karelis and Rabasa-Lhoret 2008). However, these individuals show atherosclerotic changes compared to healthy lean controls (Oflaz et al. 2003) and longer-term follow-up beyond ten years has suggested that this is a transient phenotype (Kramer, Zinman, and Retnakaran 2013; Goossens 2017). Instead, it is more realistic to consider individuals to have weight threshold, beyond which they will increasingly develop ectopic fat deposition in the liver, muscles and pancreas which will lead to the development of type 2 diabetes and the metabolic syndrome, demonstrated by the reversal of type 2 diabetes in the DIRECT study by loss of a fixed 10-15 kg, independent of the starting weight (Lean et al. 2018; Lean et al. 2019).

1.2 Brown Adipose Tissue

1.2.1 Introduction

AT can be broadly divided into two types. White adipose tissue (WAT) acts essentially as a store of chemical energy in the form of lipid, although it is now also recognised as a major endocrine organ (Galic, Oakhill, and Steinberg 2010; Kershaw and Flier 2004). In

contrast, BAT is a highly metabolically active tissue and a key component of facultative non-shivering thermogenesis. As well as "classical" discrete depots, brown-like adipocytes are seen within WAT, described as beige adipose tissue (BeAT, Section 1.2.2) (Nedergaard and Cannon 2014).

The ability of BAT to produce significant quantities of heat, despite its small volume, is conferred by the presence of uncoupling protein (UCP)-1 (Meyer et al. 2010), a protein unique to BAT (Nedergaard et al. 2001). UCP-1 spans the inner mitochondrial membrane and acts as part of the mitochondrial respiratory chain (Klingenberg and Huang 1999). In the absence of UCP-1, the chemical energy from substrates such as lipids and sugars is converted from ATP from ADP via oxidative phosphorylation (Bengtsson, Cannon, and Nedergaard 2000). UCP-1 interrupts the formation of ATP and the resulting energy is released as heat (Klingenberg and Huang 1999; Cannon and Nedergaard 2004; Nedergaard et al. 2001).

BAT, known to be present throughout life in small mammals with large surface area to volume ratios (Adamsons, Blumberg, and Joelsson 1969; Hardman and Hull 1972), was previously believed to involute in humans during early childhood, once its role in preventing hypothermia in the transition of the new-born from the warm intrauterine to the cool extrauterine environment had been fulfilled (Jung et al. 1988; Cunningham et al. 1985). However, in 2009, several studies demonstrated the presence of BAT in human adults (Sacks 2009; Celi 2009; Virtanen et al. 2009; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009), renewing interest in it and raising the possibility of its role in obesity prevention. Recently, some authors have questioned whether, in isolation, BAT stimulation could produce significant weight loss (Chechi, Nedergaard, and Richard 2014), but its potential role as a combined therapy and in improving metabolic health remains widely discussed.

1.2.2 Beige adipose tissue

BeAT describes brown-like adipocytes within WAT. Their presence appears to depend on exposure to a suitable stimulus (e.g. a cold environment). Classical BAT and BeAT have been shown to have different cellular signatures and cell lineages (Petrovic et al. 2010).

In humans, the situation is less well defined (Jespersen et al. 2013) with the molecular signature of cells from discrete BAT depots resembling BeAT (Wu et al. 2012; Sharp et al. 2012) or BAT (Cypess et al. 2013), dependent on age and location. It is likely that, in the human new-born, which requires protection from hypothermia, the major BAT depots are analogous to classical BAT in rodents (Lidell et al. 2013). In the modern world, humans are largely protected from significant cold exposure and, with a small surface area to volume ratio reducing relative heat loss, the requirement for adaptive thermogenesis is much diminished. This may result in atrophy of classical BAT with BeAT induced as required, consistent with the seasonal variation in the prevalence of BAT (Au-Yong et al. 2009; Astle et al. 2014).

It also remains to be established whether BeAT is due to transdifferentiation of mature white adipocytes (Barbatelli et al. 2010), diverted differentiation of common WAT/BAT precursors (Lee et al. 2012), or a dormant population of BAT-specific precursors, responsive to thermal challenge (Wang et al. 2013; Bartelt et al. 2011) (Figure 1).

1.2.3 Embryology

In humans, AT, including BAT, first appears in the second trimester of gestation (weeks 14 to 24) (Poissonnet, Burdi, and Garn 1984; Merklin 1974), with proliferation in preparation for birth, followed by a postnatal decline, consistent with other large mammal models (Symonds, Pope, and Budge 2015; Symonds 2013; Lean et al. 1986). In contrast, WAT is largely a postnatal development in rodents.

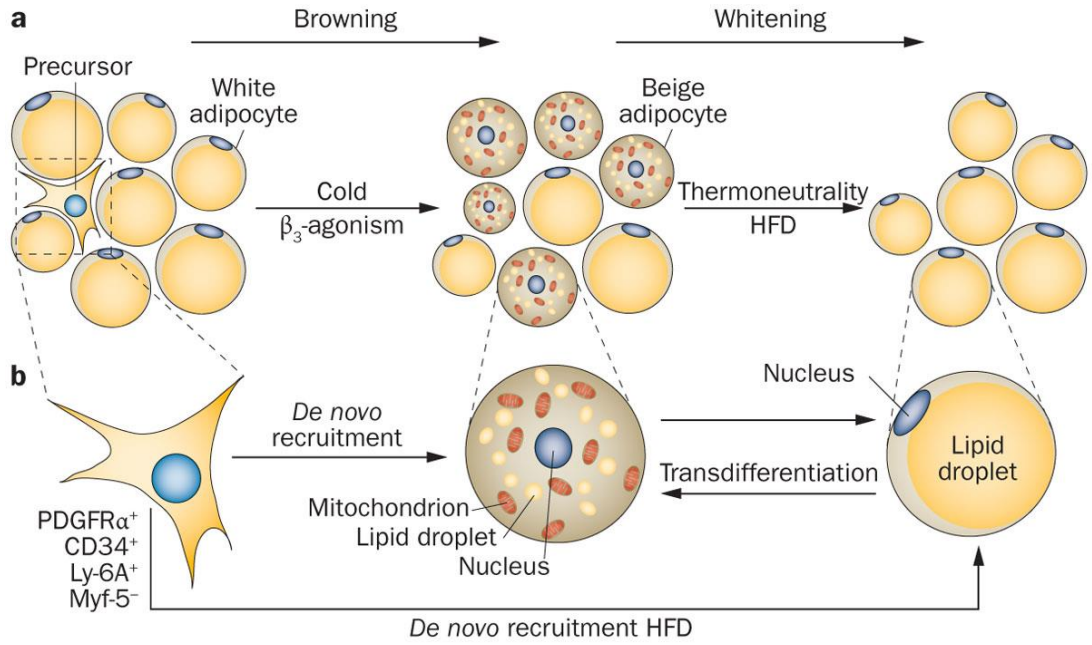

Figure 1 Derivation of Beige adipose tissue
(from Bartelt and Heeren (2014), reproduced with permission of Springer Nature © 2013) Browning within white adipose tissue is a reversible process, dependent on the environmental conditions of the cells. The appearance of brown-like cells may be from de novo recruitment of precursors or transdifferentiation of mature white adipocytes.

The precise embryological origins of BAT in humans remain debated. Both WAT and BAT derive from mesenchymal stem cells (MSCs) which themselves derive from the mesoderm (Billon, Monteiro, and Dani 2008). MSCs are able to differentiate into osteocytes, chondrocytes, adipocytes and myocytes (Billon, Monteiro, and Dani 2008). Lineage studies in rodents show that classical brown adipocyte precursors are myogenic factor (Myf)-5 positive, in common with myocytes, whereas inducible brown adipocytes were Myf-5 negative, in common with WAT (Seale et al. 2008). However, as discussed above (Section 1.2.2), human studies have shown a more mixed picture.

1.2.4 Anatomy

The locations of BAT depots reflect the physiological function of thermogenesis. In rodents and new-born infants, the major depot is the interscapular one (Heaton 1972; Sacks and Symonds 2013; Cypess et al. 2009). Significant depots are also found around the major vessels and organs where they are able to transfer heat rapidly and efficiently around the

body (Cypess et al. 2013). This includes the suprascapular depot, in close proximity to the vessels supplying the head and brain, and epicardial AT (Sacks et al. 2013). The major human BAT depots are shown in Figure 2.

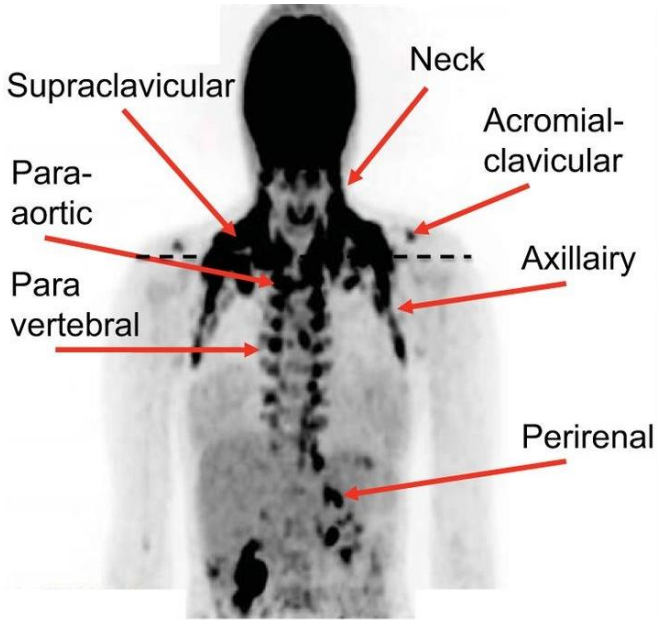

Figure 2 Uptake of ^{18}F-FDG in BAT on PET image, demonstrating significant BAT depots.
Significant BAT depots (labelled) are demonstrated by symmetrical uptake of ^{18}F-FDG, in the cervical, supraclavicular/acromioclavicular, axillary, paraaortic, paravertebral and perirenal regions (from van der Lans et al. (2014), reproduced with permission of The American Physiological Society © 2014).

1.2.5 Histophysiology

In contrast to white adipocytes, which are monolocular with relatively few mitochondria and a peripheral nucleus, BAs are rich in mitochondria, (which gives them their characteristic colour under the microscope), with multiple smaller lipid droplets and a central nucleus.

BAT is innervated by the sympathetic nervous system (SNS) (Thureson-Klein, Lagercrantz, and Barnard 1976; Alexander and Stevens 1980). SNS stimulation leads to noradrenaline (NA) release which acts on β3-adrenoreceptors (ARs). The resulting increased intracellular levels of cyclic adenosine monophosphate (cAMP) causes lipolysis of lipids stored within the intracellular lipid vacuoles and an increase in free fatty acids

(FFA), which promote the expression and activation of UCP-1 (Klingenberg and Huang 1999). UCP-1 is situated on the inner membrane of the mitochondria (Klingenberg and Huang 1999) where it facilities the dissipation of the proton gradient usually found across the mitochondrial membrane. This increase oxidation of substrates, and consequently heat production.

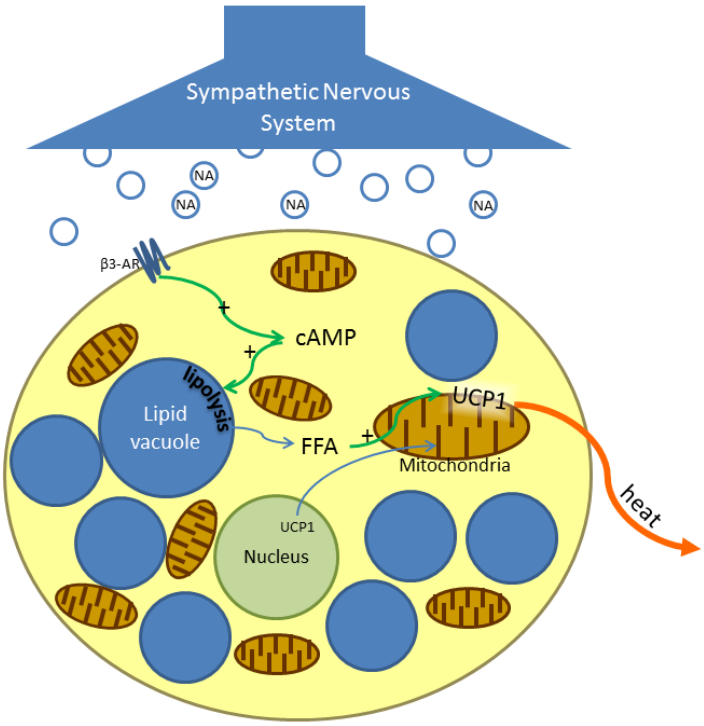

Figure 3 Representation of a brown adipocyte.
Noradrenaline (NA) release from sympathetic nervous system synapses acts on β3-adrenoreceptors (β3-AR) to cause a rise in intracellular cyclic adenosine monophosphate (cAMP) levels. This results in the breakdown of triglycerides stored within the lipid vacuoles to free fatty acids (FFA) and, in turn, increased uncoupling protein (UCP)-1 expression. Increased FFA concentrations also activate UCP-1 within the inner membrane of the mitochondria which uncouples the mitochondrial respiratory chain from the production of adenosine triphosphate (ATP), releasing the energy stored in substrates as heat (adapted from Law et al. (2014)).

1.2.6 Effect of glucose & insulin on BAT

BAT can utilise significant quantities of glucose relative to its weight, contributing to the overall clearance of glucose from the circulation, and is, therefore, an important component of glucose homeostasis. In a resting state, BAT glucose uptake is mainly

mediated via insulin but, when stimulated, uptake is mediated via NA (Orava et al. 2011; Marette and Bukowiecki 1989).

BAs express IRs on their cell surface (Tanti et al. 1986). When insulin levels are high (e.g. following feeding), insulin binds to its receptors, causing GLUT-4 proteins, a key glucose transporter stored intracellularly, to be relocated to the cell membrane, and increasing GLUT-4 expression (Omatsu-Kanbe, Zarnowski, and Cushman 1996). This significantly increases glucose uptake into the cell. In this anabolic state, glucose is not required for thermogenesis and, instead, is either stored directly as glycogen or metabolised for fatty acid or triglyceride synthesis (McCormack 1982). Without IRs on BAs, BAT atrophies but pancreatic beta-cell mass and insulin secretion are also reduced (Guerra et al. 2001), suggesting a bidirectional synergy between BAT and glycaemia which has further been shown to have a circadian rhythm (Lee et al. 2016).

In contrast to the fed state, both physiological hypoinsulinaemia during fasting and the relative insulin-deficiency of obesity are associated with reduced surface expression of GLUT-4 and reduced glucose uptake of the tissue (Smith et al. 1992). Patients with T1DM tend to maintain slightly higher blood sugar levels than healthy controls in order to avoid the risks of hypoglycaemia but are therefore also relatively hypoinsulinaemic. Reduced GLUT-4 abundance due to hyperglycaemia at the presentation of T1DM (Yki-Järvinen and Koivisto 1986) or later (Yki-Järvinen, Helve, and Koivisto 1987) causes peripheral insulin resistance, which normalises (at least initially) with effective insulin treatment, but it is not clear what the long-term effect on BAT of T1DM is.

In contrast, glucose uptake during adaptive thermogenesis is largely insulin-independent (Shibata and Nagasaka 1984; Shibata et al. 1989). The exact mechanism is not clear, but NA leads to increased FFA levels and UCP-1 activation (Section 1.2.5). In UCP-

1 knockout mice, NA-mediated (but not insulin-mediated) glucose uptake was lost (Inokuma et al. 2005), and inhibition of fatty acid oxidation also reduces glucose uptake (Marette and Bukowiecki 1991). Whether NA induces translocation of GLUT receptors and/or increases their functional activity remains to be identified, yet this insulin-independent mechanism is of interest due to the insulin resistance of obesity. The potential of insulin-independent pathways can be seen by the ability of a BAT transplant to restore glycaemic control without the need for exogenous insulin in a mouse model of T1DM (Gunawardana and Piston 2012).

1.2.7 Effect of thyroid hormones on BAT

BAT is primarily activated by the SNS and thyroid hormones act as a key modulator of this activation. T4 is the major circulating thyroid hormone and can be converted locally by outer-ring deiodination to the active form, T3, by DIO2 or by inner-ring deiodination to the inactive form, reverse-T3, by DIO3.

Circulating T3 acts on the hypothalamus to increase SNS stimulation (Lopez et al. 2010; Reiter et al. 1990). Within the brown adipocyte, SNS stimulation causes an increase in DIO2 activity with resulting high local concentrations of T3 to produce a localised hyperthyroid environment (Bianco and Silva 1987; Carvalho et al. 1991). These high levels saturate the cell's thyroid hormone receptors which, in turn, causes an increase in UCP-1 expression (de Jesus et al. 2001), as well as inducing β-oxidation and mitophagy (Yau et al. 2019).

Animal models of hypothyroidism show hypothyroidism prevents BAT activity (Weiner et al. 2016) and leads to a marked reduction in the ability to respond to cold stress by increasing non-shivering thermogenesis and removal of the ability of the cell to convert T4 to T3 results in a marked reduction in BAT function (de Jesus et al. 2001; Bianco and Silva

1987; Carvalho et al. 1991). Conversely, hyperthyroidism exaggerates BAT responses (Weiner et al. 2016).

Studies in humans are limited and conflicting. There is no association between serum thyroid hormone concentrations and BAT activation (Orava et al. 2011; Ouellet et al. 2012) in healthy volunteers, which is not surprising as intracellular levels of T3 are unlikely to be affected. There may be an association in particular groups of patients, such as those with anorexia nervosa (Bredella et al. 2012), but the effects of anorexia on the hypothalamus mean it is not possible to extrapolate to the general population. Retrospective analysis of PET-CT scans undertaken for clinical reasons provide large datasets not otherwise available (Zhang et al. 2014) but are likely to significantly under-represent BAT activity and prevalence, as discussed below (Section 1.3.1). The lack of biochemical thyroid function data, and reliance on thyroid glucose uptake as a surrogate marker for the former, prevents firm conclusions being drawn.

Functional BAT is present in patients with hypothyroidism (Broeders et al. 2016; Kim et al. 2014; Gavrila et al. 2017; Broeders et al. 2018) but whether treatment with thyroxine increases (Broeders et al. 2016) or decreases (Kim et al. 2014) BAT activity is unclear. Even iatrogenic thyrotoxicosis (during treatment for thyroid cancer) appears to have only a modest (Broeders et al. 2016, 2018), if any (Gavrila et al. 2017), effect with the differences perhaps due to the markedly different biochemical thyroid function profiles of the participants between studies.

1.3 Imaging of Brown Adipose Tissue

1.3.1 Positron Emission Tomography-Computed Tomography

The rediscovery in 2009 that BAT is present in adult humans followed the observation of symmetrical uptake on ^{18}F-FDG PET-CT scans (Hany et al. 2002) that would resolve if the

participant was kept warm (Christensen, Clark, and Morton 2006; Nedergaard, Bengtsson, and Cannon 2007). ^{18}F-FDG PET-CT remains the gold standard for imaging of BAT but has several drawbacks. Firstly, exposure to ionising radiation means it is not suitable for repeated imaging, for large studies of healthy volunteers, or for children. Secondly, and more fundamentally, the use of radio-labelled glucose means that only BAT that is actively taking up glucose is demonstrated. Although glucose is an important BAT substrate, free-fatty acids are the major source of chemical energy, and BAs contain multiple lipid vacuoles able to supply FFA. Therefore, for BAT to be demonstrated on an ^{18}F-FDG PET-CT, BAT must be active and actively taking up glucose and, even then, the precise relationship between ^{18}F-FDG uptake and thermogenesis has not been fully established. Indeed, under adrenergic stimulation, ^{18}F-FDG uptake is maintained in a UPC1 KO murine model despite diminished thermogenesis (Hankir et al. 2017). Conversely, in the post-prandial state, high insulin levels result in the rapid uptake of ^{18}F-FDG by muscles, impairing the ability to detect BAT uptake on PET-CT (Vosselman et al. 2013).

Other radio-labelled molecules, such as ^{11}C-acetate and ^{18}F-fluoro-thiaheptadecanoic acid (a fatty acid tracer), have been used to try and address this issue (Ouellet et al. 2012), so more suitable options may become available. However, the factors determining FFA uptake in BAT have not yet been consistently elicited to enable their wider use (U Din et al. 2017; Blondin et al. 2017; Blondin et al. 2015; Ouellet et al. 2012) and further work is needed to determine the optimal kinetic model (Richard et al. 2019).

1.3.2 Magnetic Resonance Imaging

MRI as a technique for BAT analysis in rodents was reported in the early 1990s (Osculati et al. 1989; Sbarbati et al. 1991; Osculati et al. 1991) and was first published in humans in 2012 by Chen, Cypess et al. (Chen et al. 2012). The technique is based on the ability to

distinguish BAT from WAT due to the higher water-to-fat ratio of BAT and its greater density of mitochondria and blood vessels.

MRI has some advantages over PET-CT: it avoids the use of ionising radiation, and functional MRI can estimate BAT metabolic activity. In humans, MRI is better suited to targeted imaging of a specific anatomical region (such as the neck) since whole-body scans are time-intense and, hence, expensive. Imaging of obese patients requires special wide-bore scanners and is sometimes not possible. The techniques and protocols for MRI of BAT are still being refined (Abreu-Vieira et al. 2019; Deng et al. 2018) but, as protocols develop and equipment improves, its use is likely to increase.

1.3.3 Infrared Thermography

Law J, Chalmers J, Morris DE, Robinson LJ, Budge H, Symonds ME (2018). The use of infrared thermography in the measurement and characterization of brown adipose tissue activation. *Temperature* 5(2), 147-61

The first part of the following article was primarily written by Jane Chalmers. The rest, from "Background of IRT" onwards, was primarily written by me.

The use of infrared thermography in the measurement and characterization of brown adipose tissue activation

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URL: http://www.nottingham.ac.uk/research/groups/earlyliferesearch

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Abstract

Interest in brown adipose tissue has increased in recent years as a potential target for novel obesity, diabetes and metabolic disease treatments. One of the significant limitations to rapid progress has been the difficulty in measuring brown adipose tissue activity, especially in humans. Infrared thermography (IRT) is being increasingly recognized as a valid and complementary method to standard imaging modalities, such as positron emission tomography-computed tomography (PET/CT). In contrast to PET/CT, it is non-invasive, cheap and quick, allowing, for the first time, the possibility of large studies of brown adipose tissue (BAT) on healthy populations and children. Variations in study protocols and analysis methods currently limit direct comparison between studies but IRT following appropriate BAT stimulation consistently shows a change in supraclavicular skin temperature and a close association with results from BAT measurements from other methods.

Key terms: Brown adipose tissue, thermal imaging, infrared thermography, human, rodent, PET-CT

Abbreviations:

BAT	Brown Adipse Tissue
BMI	Body mass index
IRT	Infrared thermography
JPEG	Joint photographic experts group
PET/CT	Positron emission tomography-computed tomography
ROI	Region of interest
SCV	Supraclavicular
T	Temperature
T_r	Resting temperature
T_{ref}	Reference temperature
T_{rel}	Relative temperature
T_s	Stimulated temperature
T_{SCV}	Supraclavicular temperature
UCP1	Uncoupling protein 1
WAT	White adipose tissue
ΔT	Change from resting temperature

Brown adipose tissue

BAT was first documented in 1551 by the naturalist Conrad Gessner, who described tissue found in the interscapular region of the marmot as "neither fat nor flesh – but something in between".[1] Having initially been identified solely by its color (due to its granular, mitochondria-rich cytoplasm), BAT was only conclusively demonstrated as being a separate pathological entity to white adipose tissue (WAT) in the early 2000's – where it was shown that bone morphogenic proteins were involved in the differentiation of adipocytes into white or brown adipose tissue.[2] BAT is unique in that it contains uncoupling protein 1 (UCP1) on the inner surface of its mitochondrial membrane which, when activated, allows rapid dissipation of energy by the free flow of electrons, producing heat[3] and playing a pivotal role in thermoregulation, particularly in infancy. There are also discrete regions of UCP1 containing cells within WAT, that have been defined as beige adipocytes[4-7] although these may have the capacity to utilize other modes of uncoupling.[8]

Historically, BAT was thought to disappear with age, however, the persistence into adulthood of BAT in small depots, at sites similar to those found in infants, was noted by the pathologist Heaton in the 1970s.[9] It is likely that further insights were apparent at this time but were largely neglected.[10]

The largest BAT depots are found surrounding the vasculature of the neck,[11] thought to play a role in thermoregulation of the blood to and from the brain.[12] It is the activity of these depots, noticed on PET/CT imaging of adults,[13,14] which promoted the resurgence of interest into BAT activity early in the millennium. The estimated volume of BAT varies depending on the conditions and quantification methods,[11] which are not standardized.[15]

BAT activity is controlled by the action of norepinephrine (from the sympathetic nervous system) on β_3-adrenoreceptors[16] and is inhibited by vagal nerve stimulation[17] (from the parasympathetic nervous system). Norepinephrine has a number of actions on BAT to increase its activity; promoting proliferation of preadipocytes, differentiation of mature adipocytes,[18] directly upregulating the expression of genes coding for UCP1,[19] increasing mitochondrial mass[20] and preventing apoptosis.[21] The activation of BAT through this pathway can be acute (i.e. in response to abrupt cold exposure or a meal[22]) or can be a result of enhanced BAT recruitment, increasing thermogenic capacity over a period of time.

As implied above, there are many stimuli to BAT activation (whether acute or chronic). The most documented is the activation of BAT in response to cold, which probably underpins the evolutionary survival of mammals in the

neonatal period. Extreme cold exposure results in the production of heat primarily through shivering, and although BAT is also activated, it contributes to overall energy expenditure far less.[23] At lesser degrees of cooling, BAT is the main source of heat production. Over a period of time, following repeated cold exposure, or prolonged acclimation to a low temperature (over weeks), the recruitment of BAT can result in non-shivering thermogenesis to maintain a stable body temperature (adaptive thermogenesis).[24,25]

BAT is also activated through diet. As early as the nineteenth century it was noted that when humans over-ate, weight gain did not mirror the additional calories ingested,[26] suggesting an increase of energy expenditure in response to food.[27] The hypothesis that this was due to BAT activation was supported when studies on UCP1 knock-out mice showed an increase weight gain compared to wild-type mice housed at the same temperature.[28]

In addition to environmental factors, the activity of BAT is also affected by different drugs, mainly those that impact the sympathetic system, such as cocaine and amphetamines[29] as well as serotonergic drugs.[30] Other drugs have been studied to assess their ability to increase BAT activity through other means. For example, thiazolidinediones have been shown to induce "browning" of WAT in mouse models,[31] whilst raised triiodothyronine has been shown to be associated with higher activity of BAT, both in cell cultures (through increased upregulation of UCP-1 expression and mitochondrial biogenesis[32]) and in clinical practice (as demonstrated by an increased basal metabolic rate and non-shivering thermogenesis in response to thyroxine treatment post thyroidectomy[33]).
Although a relatively wide range of different factors have been found to impact on the activity of BAT, the best way to measure that activity in a research environment is contentious. The pros and cons of different modalities (including thermal imaging) in humans are discussed below.

Assessing BAT activity

There are many methods of assessing the activity of BAT that have been utilized experimentally. Broadly speaking they can be divided into direct and indirect measures of activity.

Direct examination of BAT requires biopsy samples (usually taken from the supraclavicular (SCV) depot under CT or PET/CT guidance following activation through cooling). There are a number of different direct methods of assessing activity including the measurements of gene expression markers (UCP1 mRNA,[34] lipoprotein lipase mRNA[35]), gene protein products (UCP1 or BAT lipid content), or measures of mitochondrial respiration[36,37]. The requirement of biopsy samples for all these methods limits the use of direct

measures of BAT activity to animal models or small human studies.

Indirect measures of BAT activity are more widely used in clinical research, although which method is optimal remains a matter of debate. The principal function of BAT is to produce heat, and as such, calorimetry may be considered the most appropriate method of measuring the heat production from BAT. This is usually performed through indirect calorimetry that measures gas exchange, i.e. oxygen consumption and carbon dioxide production, calculating energy expenditure using the Weir equation ($EE(J) = 15.818VO_2(l/min) + 5.176VCO_2(l/min)$). Indirect calorimetry has therefore been utilized as a method of measuring an increase in cold-induced energy expenditure in numerous human studies.[38,39] However, this rise in metabolic rate in response to cold does not necessarily indicate an increase in BAT activity. A number of studies have shown that UCP1 knock-out mice maintain the ability to increase their metabolic rate in response to cold – indicating a lack of specificity to BAT.[40,41] The use of indirect calorimetry alone as a measure of BAT activity may not be sufficient.

PET/CT

The revival in interest in BAT physiology over the last 10 years followed the demonstration that the symmetrical uptake seen on PET/CT[13] would resolve if the participant was kept warm[14,42] and the proof that this was due to BAT present in adult humans.[43-47] PET/CT, specifically ^{18}F-FDG PET/CT, has since been considered the gold standard for imaging and measuring BAT in vivo in humans. ^{18}F-FDG PET/CT relies on the high glucose uptake rate of activated brown adipocytes. PET detects the radioactivity of the decaying ^{18}F-FDG in cells, whilst CT measures the density of tissue (distinguishing BAT from WAT).

The utility of PET/CT is limited most significantly by the necessary exposure to relatively high levels of ionizing radiation (~8mSv). Studies of healthy individuals are therefore ethically limited to small numbers and, although some studies have employed repeated PET/CT BAT measurements on the same individuals, these studies are particularly limited to very small numbers at most,[48,49] reducing confidence in the results. In addition, the amount of BAT identified depends on external factors such as PET resolution, the region of interest (ROI) chosen and the PET threshold criteria and may have been being significantly underestimated.[11] Larger studies can be undertaken by looking at images taken for clinical indications but conditions and protocols are optimized to minimize BAT identification so these studies find much smaller prevalence of BAT[46,50] than those that use cooling protocols,[45,47] and cannot necessarily be extrapolated to healthy

populations. Other practical limitations of PET/CT include the availability and cost of equipment and scanning time. A PET/CT scanner costs upwards of $100,000; a single research scan for BAT quantification typically costs around $450 and takes 1.5 hours from administration of ^{18}F-FDG to completion.

In addition to the practical concerns, there are also methodological questions. The most common tracer used in PET/CT imaging of BAT is ^{18}F-FDG. Whether glucose uptake is a good surrogate measure of BAT activity remains to be answered. Although BAT[51] and beige cells[52] do utilize circulating glucose for oxidation, ^{18}F-FDG uptake is unaffected even in UCP1 KO mice where BAT thermogenesis is diminished.[53] Further to the concerns around substrate uptake being equated with cellular activity, PET/CT is also not suitable for studying the effect of a meal. Following a meal, insulin release causes glucose uptake by muscle reducing the contrast with BAT uptake, making interpretation difficult[54] and risking under-estimation of BAT.[55] Since the field of BAT research is largely ultimately looking towards novel obesity and diabetes interventions, the inability to study the effects of meals is a significant limitation that should not be overlooked.

The non-destructive, non-invasive properties of infrared (IR) and, being part of the electromagnetic spectrum, its ability to travel in a vacuum, have meant it has found a use in a wide range of disciplines including conservation and heritage,[56] astronomy,[57] mineralogy[58] and engineering.[59,60] IRT was first used for medical purposes in the 1950s for the identification of breast malignancy.[61] While mammography and ultrasound overtook IRT in this particular application, it has since then gone on to be used for a wide range of medical uses[62] including sports medicine,[63] arthritis,[64,65] and a renewed interest in malignancy.[66,67]

Background of IRT

IRT is the process of constructing an image of an object's temperature by first measuring the IR radiation being emitted, then converting that radiometric data to a temperature and, finally, displaying the temperature data as an image. The IR radiation emitted is related to the temperature of the body with long-wavelength IR (8-15μm) emitted by objects whose temperature is between -80°C and 89°C, including the human body.[68] Planck's Law can be used to convert radiometric data to temperature data when accounting for emissivity of the body being measured.[68,69] In addition, adjustment must be made for any attenuation of the signal prior to it reaching the detector, for instance, due to water vapor in the atmosphere (a function of humidity and distance).[68] The variables used in the conversion are listed in Table 1 along with an outline of their effect.

Table 1 Variables which influence conversion of radiometric to thermal data

Variable	Effect
Relative humidity	Water vapor is the main atmospheric absorber of IR in the LWIR range. High humidity, therefore, results in less IR reaching the sensor.
Object Distance	Increasing the distance between the object and the camera increases the optical path length and hence the attenuation due to any water vapor present. For the distances typically used in measuring BAT (<2m) the effect will be minimal.
Emissivity (ε)	Emissivity is the proportion of radiation emitted by an object compared to an ideal black body. Emissivity of human skin is 0.98.
Atmospheric temperature	Atmospheric temperature is required to calculate the incident radiation that the object is exposed to (that is, the amount of energy available to be absorbed by the body).
Reflected temperature	Reflected temperature is used to estimate the proportion of the radiation arriving at the sensor that is from background radiation, i.e. not emitted from the object. For imaging biological objects, emissivity is high (0.95-0.98) and so background reflected radiation is relatively low.
External IR window compensation	This is not applicable unless the camera is imaging an object from which it is segregated. This may be desirable in, for instance, the imaging of newborn infants in an incubator.

BAT: brown adipose tissue; IR; infrared; LW: long-wavelength

IRT of BAT

IRT offers a complementary detection method for BAT activity and can overcome some of the drawbacks of PET/CT. The non-invasive nature of thermal imaging and its avoidance of ionizing radiation make it highly suitable for BAT measurements in healthy cohort or children and young people. Children as young as three years old can participate in short imaging protocols.[70,71] The flexible and non-harmful nature of IRT make serial measurements of large numbers of individuals feasible and ethical, allowing greater power through paired, rather than independent, analysis.

IRT requires very little equipment and good quality measurements can be made if sufficient care is taken in the setup. Changes in SCV temperature (T_{SCV}) can be measured by five minutes of stimulation,[70] although further changes are seen with prolonged cooling.[72] Environmental temperature will affect results[70] but this need not prevent studies being conducted outside the laboratory environment and they have successfully been undertaken in people's homes[70] and at locations within schools.[71]

In contrast to PET/CT, IRT measures heat directly which is the key outcome of thermogenesis and has been used successfully to study meal effects.[73-75] The practical and ethical freedoms of IRT compared to PET/CT thereby confer the ability to create large datasets which will provide increased confidence in results as well as allow others to check that results are reproducible.

IRT was used to study BAT as early as the late 1970s to show a thermogenic response to ephedrine in an individual[27] although this was subsequently found to only measure changes in blood flow. As techniques developed, BAT hotspots were consistently located, especially in rodents, and some quantification was attempted but was not compared to other measures[76] and was limited by the low spatial resolution of the cameras available.[77] Following the confirmation in 2009 of BAT in human adults,[44-47] IRT was re-examined as a potential technique to measure BAT activation.[70,74,78] Rodent studies demonstrate consistently strong associations between BAT activity measured using thermal imaging and other methods such as 18F-FDG uptake[79] or metabolic demand,[80,81] whether using paired analysis[79,80] or comparing between groups.[81] However, it remains to be shown whether these results would translate to humans who have a much lower body surface area:volume ratio, tend to live at thermoneutrality[82] and have significantly different metabolic demands.[83,84]

As the largest and most active BAT depot in humans,[11,85] the SCV region has been the area most often studied using IRT. It occupies a superficial location below the subcutaneous adipose tissue in the SCV fossa within the lateral cervical region of the neck making it highly suitable for this modality.[85,86] In early life, there is an active interscapular depot[9,87] which was felt to represent the closest human depot to classical BAT as found in rodents[85,88] and is similarly amenable to thermal imaging[76] but which rapidly declines in prevalence with age.[9] However, mice also possess a classical BAT depot in the SCV region with a gene profile similar to that of human SCV BAT.[89]

Lee et al. showed that IRT had promise as a method for measuring BAT activity in humans, demonstrating that cold stimulation resulted in increased relative sparing of the SCV temperature, at least in some individuals,[74] and it was clear that the SCV "hotspot" was projected in the anatomical area close to BAT on PET/CT. Early studies showed a specific and reproducible warming of the SCV region following cool stimulus, consistent with BAT activation[70,71] but did not directly compare IRT with PET/CT in the same individual.[70,74] Paired studies failed to show a correlation between SCV temperature and BAT volume[49] or positive BAT status on PET/CT[50] but only

analyzed single images at one[50] or three time-points.[49] The use of single images does not capture the dynamic nature of the changes seen on IRT[70,72] and is vulnerable to bias from external and environmental factors.[90] Moreover, the use of clinically indicated PET/CT scans, which typically use protocols to reduce BAT "artefact" (e.g. by keeping the room warm) resulted in less than 10% of participants being defined as BAT positive.[50] In contrast, protocols designed specifically to measure BAT activity demonstrate positive scans in the majority of patients.[49] Furthermore, studies analyzed thermal images either using triangles to approximate the SCV region[50] or using circles to define the SCV hotspot, which requires a subjective decision about the precise placement.[49] It has now been confirmed that BAT on PET/CT and the hotspot on IRT co-localize closely and that IRT measurements are strongly correlated with measurements on BAT activity on PET/CT.[72]

Despite these promising findings, caution must be exercised so as not to underestimate the complexity of the relationship between skin surface temperature and BAT heat output, reflected in recent efforts to standardize the collection and analysis of data.[90] The key function of BAT is efficient heat production to enable thermoregulation.[3] Consistent with this, BAT depots are richly vascularized and are generally located near to major vasculature.[86,91] The location of the major BAT depot within the SCV fossa is presumably to contribute to cerebral blood temperature,[15] consistent with its proximity to carotid arteries. Heat is transported from the activated tissue around the body and specifically caudally, but sufficient heat is transmitted to the surface of the skin to be detected.

Heat that is radiated to the external environment must first be conducted through the subcutaneous adipose and the dermal tissues. Many factors, other than BAT activity, may, therefore, alter the heat signal measured using IRT. Adipose tissue is highly insulating[92] and increased adiposity reduces skin temperature.[93] Body mass index (BMI) is inversely associated with SCV temperature in children[71] and to what extent this an effect of attenuation of the heat signal or reduced BAT activity is yet to be fully elicited. The percentage of body fat in any given anatomical area is inversely correlated with skin temperature in that area and subscapular skinfold thickness specifically is negatively associated with lower local skin temperature.[94] The insulative and dissipating effect of adiposity can be corrected for either by calculating general indices of body composition, such as BMI,[50] or by specifically measuring skinfold thickness[94] close to the ROI, for instance subscapular. At the population level, skinfold thickness and BMI are strongly

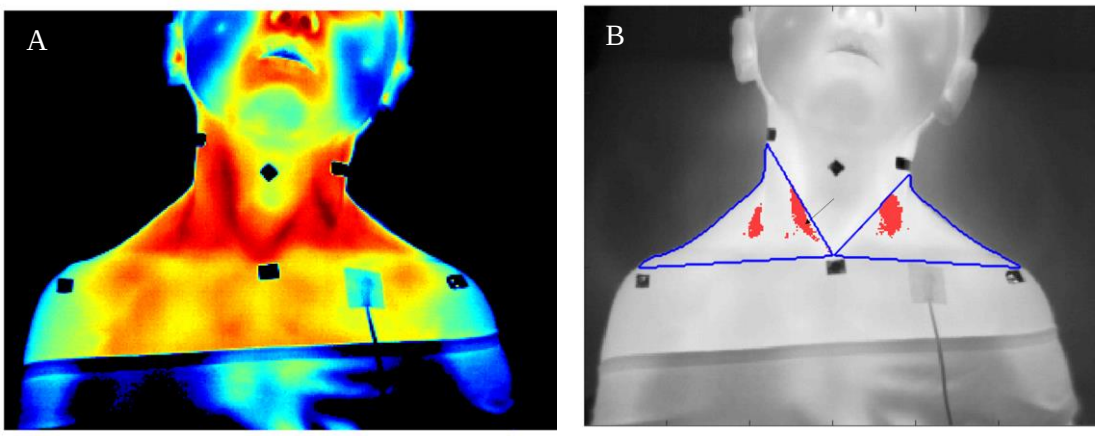

Figure 1 (A) Original thermal image (B) Thermal image after processing
Thermal image of a 7-year-old boy. (A) false color image and (B) grey-scale image with the SCV ROIs outlined in blue and "hotspot" (upper decile of values) in red. The participant's right ROI contains two distinct red areas of which the medial one (arrow) close to the projection of the carotid artery. ROI: region of interest; SCV: supraclavicular.

correlated[95-98] but the variation between individuals[96-98] may mean a direct measure is necessary.

On the other hand, BAT is not the only local source of heat in the neck and, notably, increased blood flow in the carotid arteries may be expected to confound IRT measurements. Relative SCV temperature increases even in the absence of cardiovascular changes (Law et al., unpublished) but images from some individuals show "hot spots" which appear to follow the path of the carotid vessels (Figure 1). Further work is needed to establish even more rigorous methodologies to isolate thermal changes related to BAT activity, but there is no evidence currently that this is a significant issue at a practical level.

IRT is also limited to measuring SCV and interscapular BAT depots due to their convenient anatomical location. While the interscapular depot in the newborn is classical BAT, it is argued that the molecular signature of the supraclavicular depot in adults is more similar to beige depots in rodents.[5,85,88] Whether inducible beige depots, located within WAT, possess a greater or lesser combined potential to improve metabolic health than classical BAT depots remains unanswered.[6,99] Their diffuse nature, however, prevents them from being measured using thermal imaging or PET/CT currently.[100,101] If the supraclavicular depot is truly beige, changes in its activation may mirror browning in other potential beige locations, allowing measurements of its activity to be used as a surrogate for whole body changes.

Study protocols using IRT

Standardization of IRT protocols remains poor and is a significant barrier to comparing results. Prior to commencing imaging measurements, it is essential that the participant is fully rested and acclimatized. Activity can affect skin perfusion.[102,103] Acclimatization is usually reported (Table 2 in supplementary data) but duration varies from 5 minutes[70] to 2 hours.[104] Changes in environmental temperature can likewise affect recordings[70] and room temperatures are consistently reported. Except for when participant cooling is being achieved by reduced air temperature, they are usually within the thermoneutral zone (approximately 22-24°C, in the absence of wind or airflow, for adult participants, in light cotton shorts and t-shirt[105] but affected by individual factors such as adiposity, age and possibly gender;[82,105,106] the effect of variation in one of more of these factors limits direct comparison between studies).

To maximize the IR radiation measured by the thermal camera, the camera should be perpendicular to the ROI and the ROI should be as large as possible within the field of view. The externally rotated shallow angle of the SCV fossa from the coronal plane means that the optimal camera position would be rotated down and inwards. This would mean only one side could be measured by a single camera and the position would be difficult to reproducible reliably. An acceptable compromise of positioning the camera level with the participant at the level of the larynx is adopted in most studies[49,50,70-74,104,107-110] but it should be realized that this is at the expense of reduction in the IR radiation being captured by the camera and, therefore, the calculated temperature will be below the true temperature (Figure 2). The camera should be moved along an imaginary line perpendicular to the coronal plane and passing through the larynx to ensure that the deltoid muscles are close to the border of the field of view but not extending beyond it so that the ROI occupies a maximal area while maintaining the necessary anatomical landmarks. In addition, the participant should maintain a standard body position with the head in a neutral position and the shoulders abducted and this should be consistent between participants.

Acute stimulation can be achieved in several ways including cooling,[49,70,71,74,75,104,109-111] pharmacological agents,[27] diet[73] or mental stress.[107] Conversely, relaxation can inactivate BAT.[112] Due to its core function of thermogenesis, the most commonly used methods of stimulation involve cooling. This may be achieved by either decreasing the air temperature,[49,74,104] using water either in direct contact with an extremity,[70,71,75,104,110,111] or using a water-cooled blanket or vest.[109] Water temperature from 5°C[110,111] to 15-20°C

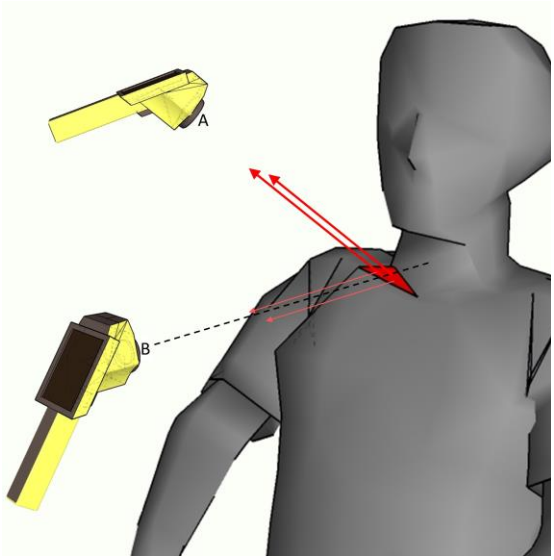

Figure 2 Representation of infrared radiation emitted by the supraclavicular region
Radiation from the supraclavicular region (red triangle) is emitted maximally perpendicular to the region in the direction of a camera at position A (dark red arrows), which is elevated and rotated relative to the camera at position B. Position B is on a reproducible imaginary line that passes through the larynx and is perpendicular to the coronal plane (black dotted line). A camera at position B will underestimate the true skin temperature of the supraclavicular region due to reduced infrared radiation being measured (light red arrows).

has been used.[70,71,74,75,104] Ice-water (4-5°C) is significantly below the temperature at which shivering thermogenesis is activated[24] and exposure will lead to maximal SNS activation due to pain.[113] Even when shivering is not observed,[110] its absence should be confirmed by both participant report and electromyography measurements as well.[15,38,114] Shorter protocols may not allow significant time to measure activation and potentially fail to find a change in activation where one exists.[111] More acceptable cooling protocols detect changes within five minutes.[70,71,75] Several studies measuring BAT activity with PET/CT have utilized individualized protocols to maximize cold stimulation[24,55,115-117] and attempt to correct for factors such as obesity. To date, individualized protocols have not been used in IRT studies. While PET/CT measures activity at a given time point (usually relative to a non-BAT tissue such as muscle), much of the information from IRT is in the change over time (i.e. from resting, in response to stimulation). Individualized protocols are usually determined on the day of imaging, as they may vary from day to day, but cooling beyond the onset of shivering on the same day would risk affecting resting IRT measurements. Nevertheless, particularly with the interest in measurements on obese participants, measures to correct for adiposity will be increasingly important and require further consideration.

Analysis of thermal images

Like the variation seen in imaging protocols, analysis of the resulting images is highly heterogeneous (Table 2 in supplementary data). This is exacerbated by the fact that thermal imaging cameras will usually save radiometric images in a proprietary file format that encourages the use of the manufacturer's own software. Apart from reducing the ease of collaborations, this also risks data in images taken using obsolete formats rapidly becoming inaccessible. For example, FLIR, a major thermal imaging supplier, currently stores its raw thermal

photographic experts group (JPEG) file of a false color image that is representative of the thermal data contained within (Figure 3A). While the pseudocolored image is readily accessible in image processing software, the raw radiometric data cannot easily be obtained without the use of FLIR™ proprietary software. The radiometric data stored in the file, along with other stored variables (Table 1) allow the image to be viewed as temperature information when accessed using the proprietary software (Figure 3B). In this environment, basic analysis and color maps and altering the value of recorded variables. Depending on the software being used, images may also be saved in an alternative, more accessible format such as comma-separated values (.csv) or MATLAB™ (.mat) files, where the pixel data is now stored as temperature values for further analysis.

An alternative, if using FLIR™ cameras, is to utilize their software development kit that contains a compiled binary code file (a MATLAB™ .mex file) that allows radiometric data to be read directly into MATLAB™ without prior conversion to another format. This enables many still images and video formats to be read, including historic ones. Finally, images can be stored in a futureproof, openly accessible format such as a 16-bit portable network graphics format.[72] Conversion of large numbers of images can be undertaken automatically and rapidly and the resulting images can be easily imported into any programming environment and converted to temperature data with the required flexibility to modify recorded variables.[72]

Once the image has been opened within the desired environment the ROI needs to be identified and analyzed. Original

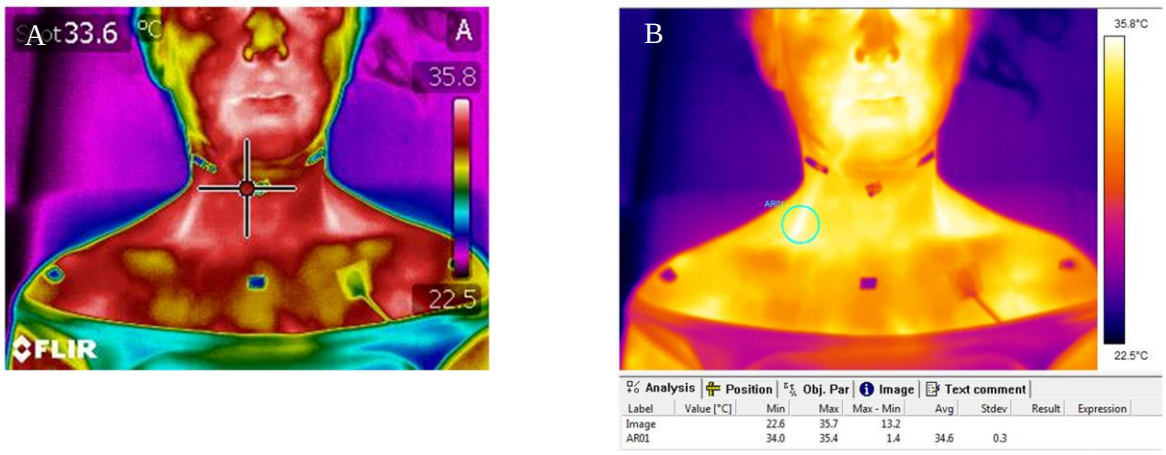

Figure 3 Thermal image saved by a FLIR™ camera (A) viewed with standard image-viewing software and (B) opened in FLIR™ ResearcherIR™

(A) The JPEG false-color image is static and overlaid with crosshairs, FLIR™ logo and any other information on the screen of the camera when captured. Thermal values can be estimated by reference to the colourmap, but cannot be precisely calculated. (B) The same image with FLIR™ ResearcherIR™ is interactive and ROIs can be defined (e.g. cyan circle) from which basic data such as minimum, maximum and average temperature can be derived (white box at bottom). JPEG: joint photographic experts group; ROI: region of interest.

methods using large rectangular ROIs[70] were superseded by careful manual approximation of the contour of the shoulder between the clavicle and sternocleidomastoid muscle[71] (Figure 4A), approximating the lateral cervical region within which the SCV fossa is situated using anatomical landmarks identifiable on the thermal image, and which are therefore reproducible. Data was exported from proprietary software as CSV files to calculate the average value of the BAT "hotspot" within this larger area.[70,71] These methods, however, preclude analysis of large numbers of images which is desirable for many studies. To simplify the identification of the ROI, studies have used circles[49,74,104,108] (Figure 4B), triangles[50,110] (Figure 4C) or even squares[111] (Figure 4D) to define the SCV area. Unfortunately, this relies on a variable degree of subjective placement and/or poor approximation to the contour of the neck,[110] and may result in apparent inclusion of the background region.[111] To simplify the calculation of the metric, basic measures of a whole ROI can be made using the manufacturers' software (Figure 4B). However, the average[50,104,110,111] necessarily assumes BAT contributes to the whole ROI and the maximum[49,50,104] relies on the temperature at a single point.

Improved computational methodologies have started to try and address these shortcomings. The ROI can now be calculated automatically with the user identifying just the apices,[72] reducing the subjective placement of the ROI outline and allowing close approximation to the shoulder contour (Figure 4E). This method is currently used to calculate the median value of the upper decile of points within the ROI (equivalent to the 95th percentile). While a range of values has been used,[70,71,73,107,109] measurements made activity measured by PET/CT.[72] An alternative option using a seeding approach has been suggested[75] which has the benefit of identifying a discrete single hotspot but requires a subjective decision about a key parameter (T_t). This is currently limited by the computational power required and the increased using 10% correlate well with BAT complexity does not appear to offer a significant advantage over a percentile approach in practice at the moment.

Options for IRT outcome measures include resting temperature (T_r), stimulated temperature (T_s), change in temperature ($\Delta T = T_r - T_s$), with each measure able to be calculated as an absolute value (i.e. T_{SCV}) or relative to a reference point ($T_{rel} = T_{SCV} - T_{ref}$). For the purposes of this review, ΔT refers to the change from resting, but it is used by some to mean the difference between the SCV and reference,[49] here called T_{rel}.

Reduced T_{SCV} in cohorts with increasing BMI[93] is due to a combination of reduced

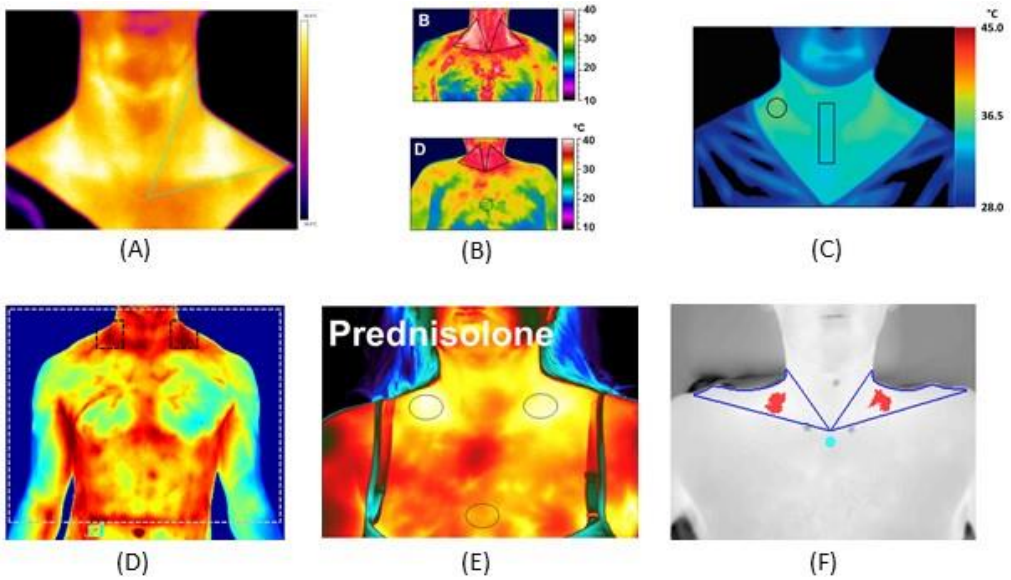

Figure 4 Examples of different methods used to analyze thermal images
(A) Identification of SCV ROI (cyan line) using FLIR™ ThermaCAM™ Researcher Professional polygon tool (reproduced using the methodology from ref. 62). The process shown here for the left side would be repeated for the right. The ROI is then exported as a CSV file for further analysis. (B) Two thermal images using triangles to approximate the ROI. Subjective placement can result in a variable ROI being selected and reduced reproducibility. Unusually the hotspot in the upper image appears to have a maximal temperature of approximately 40°C. © Institute of Physics and Engineering in Medicine. Reproduced by permission of IOP Publishing. All rights reserved.[110] (C) Thermal image from a child with a diagnosis of hypothyroidism. A wide temperature range on the color-scale reduces contrast in the SCV ROI impeding interpretation of image. Reference area (black rectangle) taken across jugular and sternal region. Reproduced by permission of Oxford University Press.[108] (D) Square SCV and reference ROI which include background of image. © 2017 The Obesity Society. Reproduced by permission of John Wiley and Sons.[111] (E) Circular ROI used for SCV and reference ROI. Reproduced under CC-BY license.[104] (F) Thermal image analyzed in MATLAB™ with using semi-automated process to define SCV ROI, SCV hotspot and reference ROI. Reproduced under CC-BY license.[72] CSV: comma-separated values; ROI: region of interest; SCV: supraclavicular.

BAT[115,118] and increased insulation.[93,94] This compound effect of obesity can be moderated by using ΔT, rather than T_r or T_s, and by comparing the SCV ROI to a non-BAT area on the same individual (i.e. T_{rel}). This approach effectively uses the participant as their own control and may explain why the best correlation with PET/CT is ΔT_{rel} ($r^2 = 0.583$; $p = 0.027$),[72] rather than T_r or T_s, and why T_{rel} offers a better prediction of BAT status on PET/CT than T_{SCV}.[49] Given the significantly different components of BAT function being measured by these two methods (glucose uptake versus thermogenic capacity), better agreement is unlikely.

Different approaches to identifying a reference value have been attempted, but these can broadly be grouped into two general approaches: either a non-BAT region on the thermal image is used,[49,50,73,74,104,108-111] or a measure of skin temperature is made by direct contact methods (thermocouples or iButtons™ [71,107]). Using a non-BAT region on the

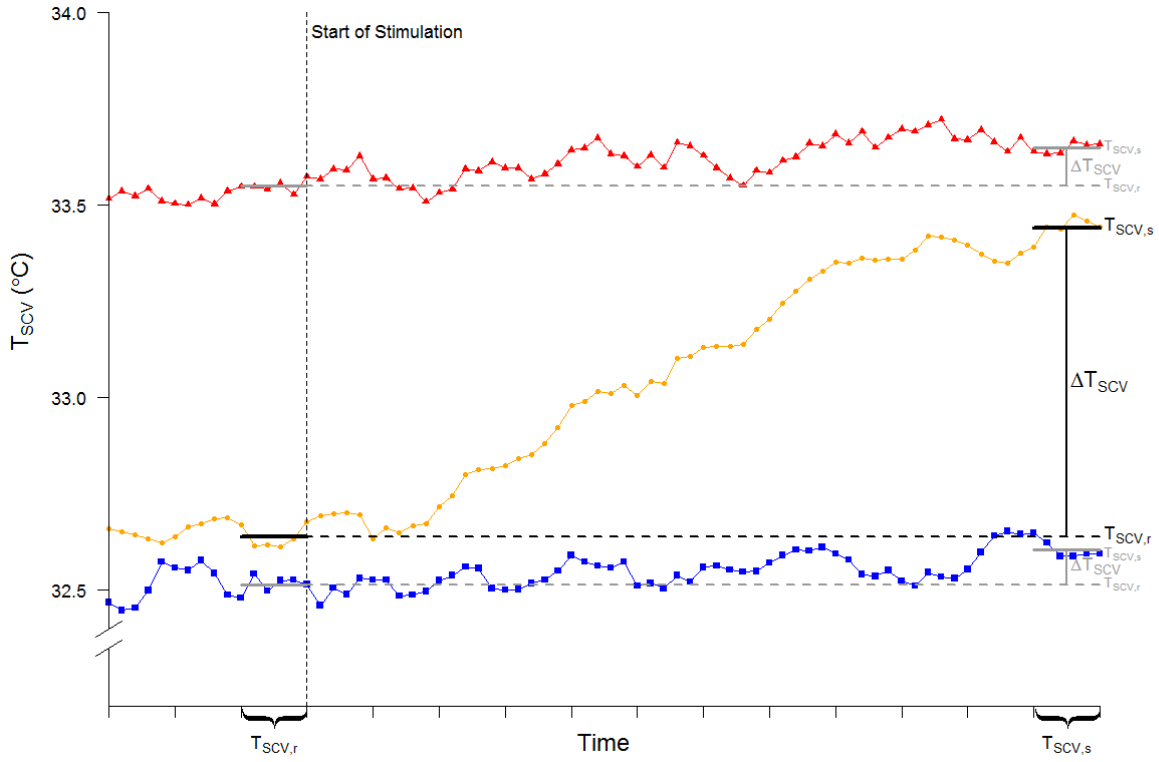

Figure 5 Simulated data from a mock IRT study.
Participant A (blue) has little active BAT – they, therefore, have a low resting SCV temperature($T_{SCV,r}$) and little rise (ΔT_{SCV}). Participant B (yellow) has activatable BAT which shows reduced activity at rest (low $T_{SCV,r}$) but a large response to the stimulation and therefore a large ΔT_{SCV}. Finally, participant C (red) has highly activated BAT; they show a small ΔT because their BAT is already maximally activated. Since resting temperature is affected by factors other than BAT activity, IRT may not be able to distinguish maximally activated BAT from inactivated BAT. BAT: brown adipose tissue; SCV: supraclavicular; T_r: resting temperature; ΔT: change in temperature from resting.

thermal image has the advantage that it can be added or altered retrospectively if the relevant area was imaged originally. However, it is worth noting the relative scarcity of female participants (Table 2 in supplementary data) and that the frequent use of a reference point including the chest area is less likely to be acceptable for females. In addition, use of contact measurement allows mean skin temperature to be calculated over a larger anatomical distribution.[107,119]

While this approach reduces the effect of differences in obesity (as well as gender, age and other variables), T_{SCV}, either at rest or stimulated, may be an important measure of effective BAT activity in itself. Theoretically, a participant who has very active BAT, even at rest, may not increase their T_{SCV} by much in response to stimulation as there is a maximal threshold temperature.[73] Consequently, it may be difficult to distinguish this from someone with inactive unresponsive BAT (Figure 5). Where the intention is to take serial measurements to look at changes in BAT over time in the same individual, whether in longitudinal or interventional studies, comparison of the change in T_r or T_s from baseline would avoid the

complexity in interpreting ΔT and, without the ionizing radiation and cost limitations of PET/CT, such study designs are now feasible.

Summary

Developments in IRT over the last six years have established it as a significant and validated tool for the measurement of BAT activation in humans as well as other animals. The ready availability of affordable equipment and lack of ionizing radiation make possible for the first-time large studies on cohorts not previously accessible and lead to novel insights intoBAT function and physiology. However, significant challenges remain to ensure reproducibility and consensus statements on imaging acquisition and analysis are urgently required to allow results to be compared.

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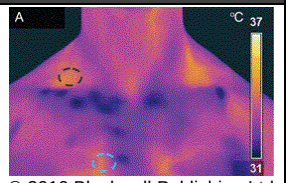
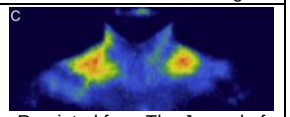
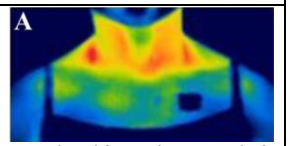
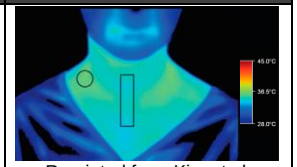
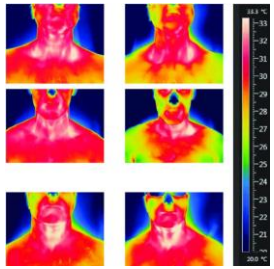
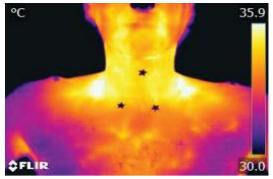
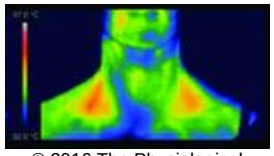
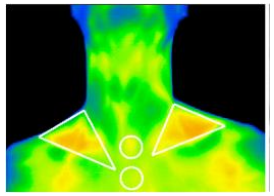
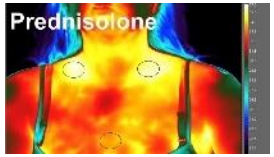
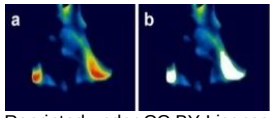
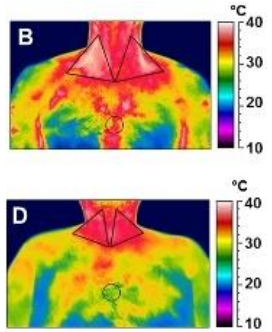
Supplementary Data

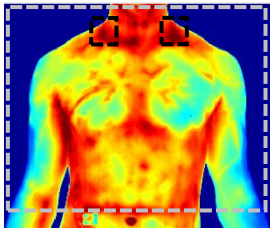
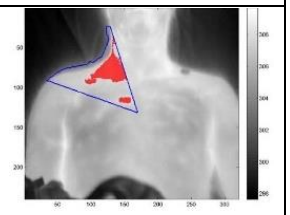
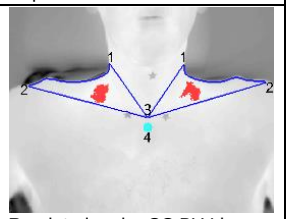
Table 2 Summary of major studies using IRT to study BAT in humans

Stimulation	Cooling	Room Temperature (°C)	Subjects	Analysis	Accl.	Results	$T_{SCV,r}$ (°C)	$T_{rel,r}$ (°C)	$T_{SCV,s}$ (°C)	$T_{rel,s}$ (°C)	ΔT_{SCV} (°C)	ΔT_{rel} (°C)	Paper	Example images
Air cooling; meal	17°C room for 30min	17	1 healthy M; age 32y	Number of images not stated. Images taken at 3 timepoints. Circles over SCV and mediastinum. Measure not stated.	NK	T_{SCV} reduces less than T_{ref} with cold; T_{SCV} rose to T_r with meal (T_{ref} no change)	[34.1]	0.7	cold/meal [33.2/34.3]	1.8/[1.7]	-0.9/[0.2]	1.1/[1.0]	Lee et al. (2011)	 © 2010 Blackwell Publishing Ltd
Water cooling	Both feet or single hand in 19-20°C water for 5min	19-21	3-8y (n=7), BMI 16.9 ± 0.95; 13-18y (n=12), BMI 22.6 ± 0.76; 35-58y (n=7) BMI 25.2 ± 1.45.	1 image/min for 10 min. Square SCV ROI from nipple to mandible with 10% hotspot. Ref temp from iButtons™. FLIR™ QuickReport™ to define ROI. Exported for calculation of mean of hotspot using MS Excel™ macro.	1 hr	T_{SCV} increases with cool challenge, ΔT decreases with age	[34.1]	UC	[34.5]	UC	0.62 (3-8y) 0.25 (13-18y) 0.2 (35-58y)	UC	Symonds et al. (2012)	 Reprinted from The Journal of Pediatrics, 161/5, Symonds et al., Thermal Imaging to Assess Age-Related Changes of Skin Temperature within the Supraclavicular Region Co-Locating with Brown Adipose Tissue in Healthy Children, 892-98. © 2012 with permission from Elsevier
Water cooling	Single hand in 20°C water for 5min	21.8	55 healthy 25M, 30F; BMI% 58.8±2; age 6-11.2y	1 image/min for 12 min. Polygon SCV ROI with 25% hotspot. Ref temp from iButton™ on chest. FLIR ThermaCAM™ Researcher Pro to define ROI and exported as CSV for calculation of median of hotspot.	5 min	T_r showed significant negative correlation with BMI; ΔT showed significant negative correlation with BMI (R only)	L/R 35.4/35.4	UC	UC	UC	0.1/ 0.1	UC	Robinson et al. (2014)	 Reprinted from The Journal of Pediatrics, 164/2, Robinson et al., Body Mass Index as a Determinant of Brown Adipose Tissue Function in Healthy Children, 318-22, © 2014 with permission from Elsevier

Stimulation	Cooling	Room Temperature (°C)	Subjects	Analysis	Accl.	Results	$T_{SCV,r}$ (°C)	$T_{rel,r}$ (°C)	$T_{SCV,s}$ (°C)	$T_{rel,s}$ (°C)	ΔT_{SCV} (°C)	ΔT_{rel} (°C)	Paper	Example images
Hypothyroid	NA	22	1F; severe hypothyroid; BMI 18.6; age 11.5y	1 image pre-and and 1 post-MRI (~30 min apart). Circular SCV ROI. Rectangular suprasternal ref ROI. Measure not stated. Analysis tool not stated. Emissivity set at 0.95.	NK	As shown no right. "Resting" is defined here as treated and "Stimulated" as when hypothyroid.	37.1	ref 36.4 *rel 0.7*	36	ref 34.3 *rel 1.7*	*1.1*	*1*	Kim et al. (2014)	 Reprinted from Kim et al. Presence of Brown Adipose Tissue in an Adolescent with Severe Primary Hypothyroidism, 2014, 99/9, E1686-E1690 by permission of Oxford University Press
Air cooling	2 hours in 19°C room	19	17 healthy 12M, 5F; BMI 25.4±5.9; age 36±8y	No. of images not stated. Images taken at 0, 60, 120 min. Circular SCV ROI. Circular ref ROI on chest. ROIs defined using FLIR Researcher IR and calculate max temp within ROI.	NK	T_{SCV} trend to higher in PET+ at baseline and 2 hr. ΔT not sig changed. Left higher than right in PET+ and -, but volume not different. T_{SCV} not correlated with SCV BAT vol.	PET+/- L 32.8/31.5 R 32.4/31.4	L 1.2/0.6 R1.0/0.5	L 32.3/31.3 R 31.6/31	L 2.1/0.7 R 1.4/0.4	*L 0.5/0.2 R 0.8/0.4*	*L 0.9/0.1 R 0.4/-0.1*	Jang et al. (2014)	 Reprinted under CC BY License
Water cooling; glucagon	Cooling vest at 8°C for 100min	22-25	11 healthy M only; BMI 20.5-25.2; age 20.8-39.8y	Images every 30 secs for 10 min at 3 timepoints. Triangular SCV ROI with 10% hotspot. Triangular ref ROI over deltoid. ROIs defined using Research IR to define ROI and exported as CSV for calculation of mean of hotspot.	30 min	Change in T_{SCV} to cold in BAT+ not BAT- group. No change to glucagon/vehicle. Fall in deltoid to all stimulation (greatest with cold).	PET+/- [35.0/34.7]	UC	[35.3/34.8]	UC	0.4/"no change"	ΔT_{ref}: Cold -1.9, Ctrl -1.1	Salem et al. (2016)	 Reprinted under CC BY License

Stimulation	Cooling	Room Temperature (°C)	Subjects	Analysis	Accl.	Results	$T_{SCV,r}$ (°C)	$T_{rel,r}$ (°C)	$T_{SCV,s}$ (°C)	$T_{rel,s}$ (°C)	ΔT_{SCV} (°C)	ΔT_{rel} (°C)	Paper	Example images
Mental arithmetic	NA	22.8±0.2	5 lean, F only; BMI 19.8-24.6; age 21.7-29.2y	3 images every 10 min over 50 min. Polygon SCV ROI with 5% hotspot. Ref defined as mean skin temperature (7 sites using iButtons™). FLIR™ ThermaCAM™ Researcher Pro to define ROI and exported as CSV for calculation of median of hotspot.	30 min	Anticipation of stress raised T_{rel}	MA/Rest: 34.9/34.6	[3.1/2.8]	[35.0/34.9]	[3.6/3.6]	[0.1/0.3]	[0.5/0.8]	Robinson et al. (2016)	 © 2016 The Physiological Society
None	NA	21	102 patients, 43% F; BMI 26±5; age 58±17y	Single image. Triangular SCV ROI. Circular ref regions in jugular and sternal area. Mean and max of whole ROI using varioSCAN software.	15 min	Positive BAT on PET group had higher T_{SCV}; lost significance when normalised for BMI. Significant negative correlation with BMI/SCAT and T_{SCV}	PET+/- 35.0/34.6	No difference if presternal or jugular ROI with PET+/-	ND	ND	ND	ND	Gatidis et al. (2016)	 Reprinted under CC BY License
Water and air cooling; pred	Hands in 15°C water for 10min then moved from 24°C to 16°C room	24 then 16	5M/4F; BMI 21.8 ± 0.6; age 22.7±1.3y	No. of images per timepoint not stated. Taken every 10 or 20 min. Circular ROI on SCV and sternum (ref). Mean and max of whole ROIs using FLIR ResearcherIR™.	2hr	No change in T_{SCV} with cold water, but T_{SCV} increased with cold room (but not T_{ref})	pred/ctrl [36.2/36.2]	[1.3/1.3]	[35.8/35.5]	[2.2/2.0]	Water: 0.0/0.1 [Total: -0.4/-0.7]	[0.8/0.6]	Ramage et al. (2016)	 Reprinted under CC BY License

Stimulation	Cooling	Room Temperature (°C)	Subjects	Analysis	Accl.	Results	$T_{SCV,r}$ (°C)	$T_{rel,r}$ (°C)	$T_{SCV,s}$ (°C)	$T_{rel,s}$ (°C)	ΔT_{SCV} (°C)	ΔT_{rel} (°C)	Paper	Example images
Water cooling; capsinoid	Both hands & feet in 18°C water for 5min	24	24 healthy lean M only; BMI 20±0.3; age 23±0.4y	Single 5min video for cold and video every 30 min for 2.5 hours for capsinoid. Averaged across 150 frames (5secs). Seeded region growing technique used to calculate size of region and temperature used to calculate heat energy output relative to baseline. No control region used.	45 min	High-BAT group (on energy output) had smaller ΔT but larger area. ΔT_{SCV} +0.28 to +0.43oC	UC	ND	UC	ND	L/R High BAT group: 0.41/0.43; Low BAT group: 0.28/0.34	ND	Ang et al. (2017)	 Reprinted under CC BY License
Water cooling	Both hands in 5°C water for 20min	24	30 M only; 14 lean (BMI <22.9±1.8; age 24±2.2y); 16 obese (BMI 33.3±1.7; age 29±6y)	Number of images not stated. Triangular SCV region. Circular ref region on sternum. Mean of whole ROI calculated using FLIR R&D software.	1 hr	T_{SCV} increase in lean, but not obese; no change in T_{rel}.	Lean/obese 34.3/33.6	ref 33.1/32.1, *rel 1.2/1.5*	35.2/33.6	ref 33.3/32.0, *rel 1.9/1.6*	*0.9/0.0*	*0.7/0.1*	El Hadi et al. (2016)	 © Institute of Physics and Engineering in Medicine. Reproduced by permission of IOP Publishing. All rights reserved

Stimulation	Cooling	Room Temperature (°C)	Subjects	Analysis	Accl.	Results	$T_{SCV,r}$ (°C)	$T_{rel,r}$ (°C)	$T_{SCV,s}$ (°C)	$T_{rel,s}$ (°C)	ΔT_{SCV} (°C)	ΔT_{rel} (°C)	Paper	Example images
Water cooling	Both feet in ice water for 2min	22-23	14 healthy M only; BMI 22-32; age 2-40y	2 pre- and 2 post-stimulation images analysed in MATLAB™. Square SCV ROI possibly including background, not centred on hotspot. Large square ref ROI of whole torso, possibly including background. MATLAB™ to calculate mean of whole ROI.	30 min	No change in T_{rel}.	UC	UC	UC	UC	UC	Pre-/ Post-feed -0.05/-0.04	Peterson et al. (2017)	 © 2017 The Obesity Society
Mixed meal; HC+ISO	NA	23-26	8 healthy M only; BMI 20.6–24.2; age 18-34y	4 images/min* for 2.25 hrs. SCV ROI down to nipple with 25% hotspot. Circular ref region on chest. Median of ROI (ref) and hotspot (SCV) calculated using UoN MATLAB™ algorithm.	30 min	Meal increased T_{SCV}/T_{rel}. HC also increased. ISO increased but max threshold reached.	Saline/HC [35.5/35.9]	[1.3/1.0]	[36.0/36.5]	UC	[0.5/0.6]	UC	Scotney et al. (2017)	 Reprinted under CC BY License
Water cooling	Cooling vest at 8°C for 100min	22-25	8 healthy M only; BMI 19.3-23.0; age 18-35y (subset of Salem et al. 2017)	Images every 5 secs for 10 min at 3 timepoints. SCV ROI to sternal notch with 10% hotspot. Circular ref ROI upper sternum. Median of ROI (ref) and hotspot (SCV) calculated using UoN MATLAB™ algorithm.	30 min	Glucose uptake on PET/CT positively correlated with ΔT_{SCV}	Cold/Ctrl 35.1/35.7	1.9/1.3	35.3/35.7	2.6/1.4	*0.2/0.0*	*0.7/0.1*	Law et al. (2017)	 Reprinted under CC BY License

Numbers in square brackets are measured from graphs presented in the paper. Numbers in italics are calculated from other numbers given in the paper. * Personal communication (not published). Accl.: Acclimatisation; BMI: body mass index; CSV: comma-separated values; F: female; HC: Hydrocortisone; ISO: isoprenaline; L: left; M: Male; NA: not applicable; ND: not done; NK: not known; Pred: prednisolone; R: right; ref: reference; rel: relative; ROI: region of interest; SCV: supraclavicular; Tr: resting temperature; Ts: stimulated temperature; UC: unable to calculate; UoN: University of Nottingham; y: years old; ΔT: change in temperature (Ts-Tr).

1.4 Aims of the project

The overall aim of this thesis is to:

1) Validate the method of infrared thermography for the measurement of brown adipose tissue against the current gold standard, PET-CT, and to optimise the current methods of image analysis.
2) Identify the effects of glucose dysregulation and thyroid hormone dysfunction on human BAT *in vivo*.

I hypothesise that:

- Measurements of BAT from IRT will correlate closely with measurements from PET-CT.
- Automation of image analysis will allow more efficient analysis and reduce variability between researchers.
- High glucose concentrations will result in increased BAT activation.
- The ability of BAT to respond to stimulation will be reduced by sub-physiological thyroid hormone levels.

In order to test these hypotheses, specifically, I will:

- Refine and develop current IRT techniques to assess BAT activation.
- Compare measurements of BAT activity from IRT to measurements from PET-CT.
- Review IRT results in a group of healthy volunteers following an intense cold-exercise challenge.
- Use IRT to compare BAT activation in children with T1DM or with hypothyroidism to healthy control participants.

Section 2 Methods and materials

2.1 Development of infrared thermography methodology

Assessment of current and novel IRT techniques was undertaken to ensure the quality, efficiency and validity of the techniques employed in the case-control studies.

In conjunction with Dr David E. Morris (Bioengineering Research Group, Faculty of Engineering, University of Nottingham), improvements to the imaging analysis method were developed, with the initial code written by Dr Morris and developed by Dr Morris and me.

Our novel method allowed images to be analysed in a custom-built app within the MATLAB programming environment and the simple and rapid identification of five apices of the regions of interest (Section 3.1). Departmental researchers undertook training in both the previous and novel image analysis methods allowing a comparison between the two methods to be undertaken (Section 3.1).

The novel method was then used to re-analyse images from a previously published study (Salem et al. 2016) to compare the outputs from IRT and PET-CT, including detailed mapping techniques to examine the anatomical overlap of the “hotspots” (Section 3.2).

Finally, a real-world study of the effect of an extreme stimulation (cold-water swimming) was undertaken to demonstrate supraclavicular temperature changes in a potentially maximal setting, prior to undertaking more moderate stimulation in patient groups.

2.2 Summary of infrared thermography methodologies

Details of the specific study protocol, method of image acquisition and image analysis method for each study are included in the respective papers (Section 3) and the appendices for imaging of healthy volunteers (Appendices, section 5.1.2) and patients (Appendices, section 5.2.2). Study imaging sessions consisted of three distinct phases 1) setup and acclimatisation; 2) image acquisition and stimulation and 3) image analysis. The background to these methods and the reasons for the choices made are discussed in detail in Section 2.3 and summarised in brief below.

2.2.1 Setup and acclimatisation

Prior to the arrival of the participant, the thermal imaging laboratory – or study location – was prepared. This included ensuring the room was at a steady thermoneutral temperature (22-24°C) and that imaging equipment and assessment was away from both sources of infrared radiation (heat) and airflow.

When the participant arrived, they were asked to change into the appropriate study clothing to initiate acclimatisation. They were encouraged to be relaxed and to remain as still as possible. Once any pre-imaging measurements (e.g. height and weight) and data (e.g. medical, dietary or physical activity history) had been obtained, participants were asked to sit upright in a comfortable and sustainable position opposite the thermal imaging camera. At this point, they were asked to be entirely still to encourage full acclimatisation. Additional monitoring (e.g. pulse oximetry or thermocouples) and/or thermal makers (to allow identification of surface anatomy on thermal images) were attached before imaging.

2.2.2 Image acquisition and stimulation

Thermal images were acquired at a set rate, limited by the capability of the thermal imaging camera to 4 frames per minute. Images were acquired during baseline (pre-

stimulation) and then during stimulation (cooling blanket [Sections 3.2 and 3.5]) or an arm in cold water [Section 3.4]), or after stimulation (cold-water swimming, Section 3.3). Where cooling stimulation was applied to a limb, this continued for 5-10 minutes.

2.2.3 Image analysis

Images were analysed to export thermal data from within the supraclavicular region of interest bounded inferiorly by the clavicle, medially by the sternocleidomastoid muscle and, laterally, by the contour of the neck.

The primary output from each image was the temperature of the median value of the top decile of pixels within the region of interest, equivalent to the 95th percentile. This was compared to the median temperature of the reference region on the sternum to calculate a "relative temperature". Time series were plotted and changes from baseline to stimulated temperature calculated for each session.

2.3 Infrared thermography of brown adipose tissue

Law J, Morris DE, Budge H, Symonds ME (2018). Infrared Thermography. In: Brown Adipose Tissue. Handbook of Experimental Pharmacology. 251: 259-82

Infrared Thermography

James Law, David E. Morris, Helen Budge, and Michael E. Symonds

Contents

Abstract

Historically, brown adipose tissue has been elusive and not easy to detect, hence its relative obscurity in human physiology until its rediscovery in 2009. At that point, it was proven that the symmetrical artefacts frequently detected on positron

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Handbook of Experimental Pharmacology 251, https://doi.org/10.1007/164_2018_137

emission tomography-computed tomography (PET-CT), which resolved if the environment was kept warm, were brown adipose tissue deposits. PET-CT has remained the stalwart of human brown adipose tissue research and is still considered the gold standard. However, PET-CT exposes the participant to ionising radiation, limiting studies to large, but retrospective, review of clinical imaging or a small-scale, but prospective, design. Within this context, alternative imaging modalities have been sought. Due to the heat-generating properties of brown adipose tissue, infrared thermography is a natural candidate for measuring its activity and the supraclavicular depot is relatively superficial, allowing detection of the heat signature. Infrared thermography is a non-invasive, non-contact technique for measuring temperature remotely. Recent developments in image analysis techniques have facilitated the use of infrared thermography to study brown adipose tissue activation in populations, and in ways, not previously feasible.

Keywords
Brown adipose tissue · Brown fat · Image analysis · Infrared radiation · Infrared thermography · Region of interest identification · Thermal imaging

1 Introduction

Historically, brown adipose tissue (BAT) has been elusive and not easy to detect, hence its relative obscurity in human physiology until its rediscovery in 2009 (Celi 2009; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). At that point, it was proven that the symmetrical artefacts frequently detected on 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography-computed tomography (PET-CT), which resolved if the environment was kept warm, were BAT deposits. PET-CT has remained the stalwart of human BAT research and is still considered the gold standard. However, PET-CT exposes the participant to a high dose of ionising radiation (~8 mSv). Such studies either rely on retrospective review of clinical imaging (Cypess et al. 2009) or are small-scale prospective studies (van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). Retrospective studies are able to review results from large numbers of patients, but clinical imaging adopts methodologies to minimise BAT detection, resulting in an underestimate of prevalence and activity, and is largely performed on non-healthy populations. Prospective research studies, while able to maximise BAT detection and be undertaken on healthy volunteers, are ethically limited to small numbers of volunteers with no, or limited, repeat imaging and exclude potentially vulnerable groups, such as children. In addition, 18F-FDG PET-CT is not suitable for postprandial imaging as the signal uptake into muscle precludes detection of BAT (Vosselman et al. 2013; Vrieze et al. 2012). Direct measurement of BAT, by biopsy, is similarly limited to small-scale studies due the proximity of BAT to important anatomical structures such as major vessels.

Within this context, alternative imaging modalities have been sought. Due to the heat-generating properties of brown adipose tissue (Cannon and Nedergaard 2004), infrared thermography (IRT) is a natural candidate for measuring its activity.

2 Infrared Radiation and Temperature

Infrared (IR) radiation is part of the electromagnetic spectrum – a continuum of wavelengths (Fig. 1) – and includes radio waves (which have wavelengths of as much as a few hundred metres) and gamma rays (which have wavelengths of less than 0.01 nm). Wavelengths of less than 10 nm are able to ionise atoms with which they interact (i.e. cause the loss of an electron) and are termed ionising radiation. The visible spectrum is in between these extremes, with wavelengths of between 390 and 700 nm (Tattersall 2016), adjacent to the IR region. IR radiation was discovered by Sir William Herschel in 1800 who showed an effect on a thermometer of radiation below red light (Herschel 1800), hence 'infrared' (although this term was not used until later). All objects with a temperature greater than absolute zero (−273.15°C) emit IR radiation dependent on their temperature (Planck 1914). The IR spectrum is subdivided for practical purposes and there are a few different classification systems in use. Thermal imaging utilises the portion commonly referred to as long-wavelength IR which is emitted by objects whose temperature is between −80 and 89°C, a range relevant to humans and other living creatures.

The relationship between IR radiation and temperature is defined by Planck's law (Eq. 1). Planck's law describes the radiation emitted at a given wavelength by a black body in thermal equilibrium, where a black body is an object that absorbs and radiates heat perfectly and does not possess any reflective property. Such a body will, therefore, absorb all radiant energy that reaches it and emit radiation depending on its temperature. The equilibrium temperature is the point at which the energy being absorbed is equal to the energy being emitted: below this temperature, the body will absorb more energy than it emits and will, therefore, warm; above this temperature the body will emit more energy than it absorbs and will, therefore, cool (Fig. 2).

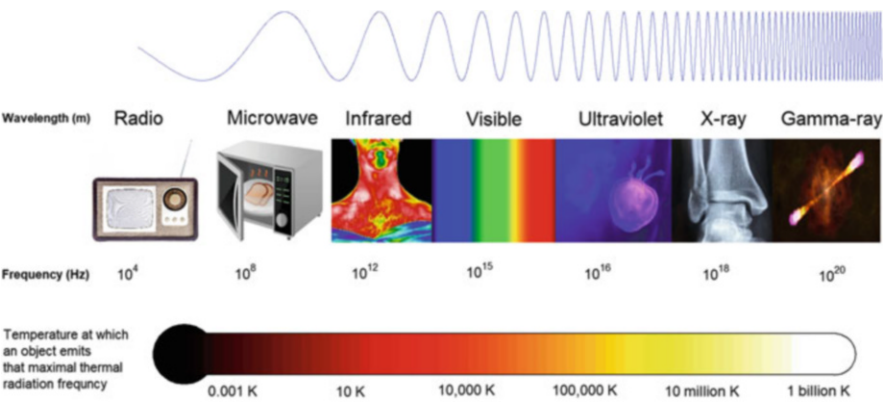

Fig. 1 Electromagnetic spectrum from radio waves to gamma rays. K: Kelvin. Images of radio, microwave, ultraviolet and X-ray examples reproduced under CC0 licences. Image of gamma ray example credited to NASA

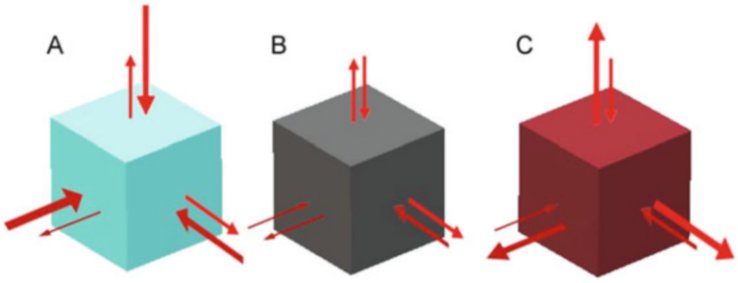

Fig. 2 (**a**) Black body object below thermoneutral point where radiation absorbed (arrows in) is greater than radiation emitted (arrows out) resulting in temperature increasing towards thermoneutral point. (**b**) At thermoneutral point, radiation absorbed is equal to radiation emitted and temperature equilibrium is maintained. (**c**) If the black body object is above the thermal equilibrium temperature, more radiation is emitted than absorbed, and the temperature falls towards the thermoneutral point

Planck's law can be used to solve for an unknown temperature of a black body in thermal equilibrium from the radiometric data measured by IR sensors. A black body at temperature, T, will have a spectral radiance, B_λ, which describes the energy emitted at different wavelengths, λ.

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

where h is the Planck constant, k_B is the Boltzmann constant, and c is the speed of light.

A black body is a hypothetical entity. Real objects deviate from being ideal black bodies by a greater or lesser amount and the degree of deviation is denoted by the emissivity (ε). Black bodies have an emissivity of 1 and a perfect reflector would have an emissivity of 0. Real-world objects lie somewhere between these two extremes. To calculate an object's temperature, the conversion equation must allow for its emissivity. For example, given an object with $\varepsilon = 0.7$, 70% of the radiation measured would be radiated from the object and 30% would be reflected from the environment. Therefore, to calculate the unknown temperature of an object, the object's emissivity and the apparent reflected temperature must be known. The apparent reflected temperature is measured using a reflector.

The emissivity of human skin is usually taken to be 0.98 and is constant across the long-wavelength IR range (Steketee 1973). As only 2% of the measured radiation from human skin is reflected, errors in measured environmental and reflected temperature will have a relatively small effect on the calculated skin temperature.

Some radiation emitted by the object will be lost between the object and the receiver. Most notably, water absorbs IR radiation (Roberts et al. 1976) and so the amount of water vapour between the object and the receiver affects measurements. The effect can be mitigated by having knowledge of the object-receiver distance, the relative atmospheric humidity and the atmospheric temperature. Similarly, if the

object and receiver are separated by an external 'window' (i.e. an object that is at least partially transparent to IR radiation), the properties of the window must also be known and allowed for. For measurement of brown adipose tissue (BAT), the IR camera is typically less than 1 m from the participant being imaged and the participant and camera are not separated by an IR window. Over such short distances, the absorption of IR radiation by water vapour is minimal.

Finally, maximal radiation is emitted perpendicular to the surface of the object. The plane of the receiver should ideally be placed parallel to the plane of the object along an imaginary line perpendicular from the object with the plane of the detector. Any deviation from this optimal position will reduce the signal being detected and will result in an underestimate of temperature.

Key Points

- Knowledge of emissivity and reflected temperature are necessary to calculate the unknown temperature of an object of interest.
- The emissivity of human skin is 0.98.
- Knowledge of atmospheric temperature and relative humidity are necessary to calculate the loss of signal between the object and sensor.

3 Infrared Thermography

Work during the Second World War accelerated the development of IRT making it a practical reality. It has many beneficial properties that have allowed it to find uses in a wide range of disciplines. IR radiation can be measured remotely and without destruction of the object being studied. This makes its measurement a useful tool both in conservation and heritage work (Candoré et al. 2012) and in structural or electronic engineering (Clark et al. 2003; King et al. 2000); being part of the electromagnetic spectrum, it can travel in a vacuum, allowing the measurement of celestial objects in astronomy (Baldwin et al. 1973). Within medicine, it was used for the identification of breast malignancy in the 1950s. For this particular purpose, alternative modalities of ultrasound and mammography became dominant, but IRT continues to be used in many health-related areas (Ring and Ammer 2012) such as sports medicine (Hildebrandt et al. 2012) and arthritis (Collins and Cosh 1970; Ring and Collins 1970), and its use in malignancy continues to be of research interest (Arora et al. 2008; Kontos et al. 2011).

A modern IR camera measures the radiation arriving at a sensor array that has been calibrated by the manufacturer against a black body and stores the raw signal within the metadata of the image file (see Sect. 6.1). Using the principles outlined above and the calibration constants determined by the manufacturer, the radiometric measurements are converted to temperature values in the preferred units (degrees Celsius, degrees Fahrenheit or Kelvin). Finally, a colourmap can be defined where

A

20476	20525	20576	20626	20676	20727
20178	20227	20277	20326	20376	20426
19885	19933	19982	20031	20080	20129
19595	19643	19691	19740	19788	19836
19310	19358	19405	19452	19500	19548

B

35.79	36.03	36.28	36.52	36.76	37
34.34	34.59	34.83	35.07	35.31	35.55
32.9	33.14	33.38	33.62	33.86	34.1
31.45	31.69	31.93	32.17	32.41	32.66
30	30.24	30.48	30.72	30.97	31.21

C

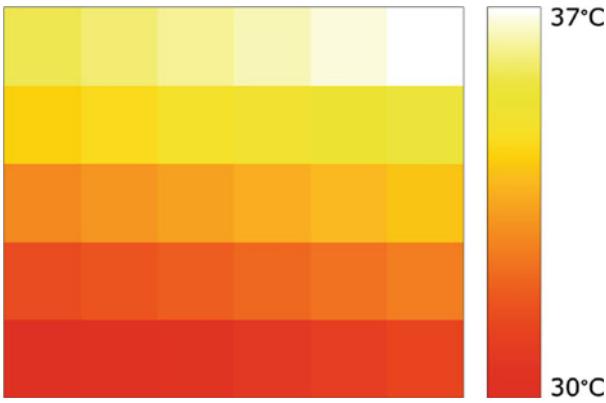

Fig. 3 Conversion of (**a**) example radiometric data to (**b**) temperature data (degrees Celsius) and displayed as (**c**) a thermal image with associated colour bar and scale alongside

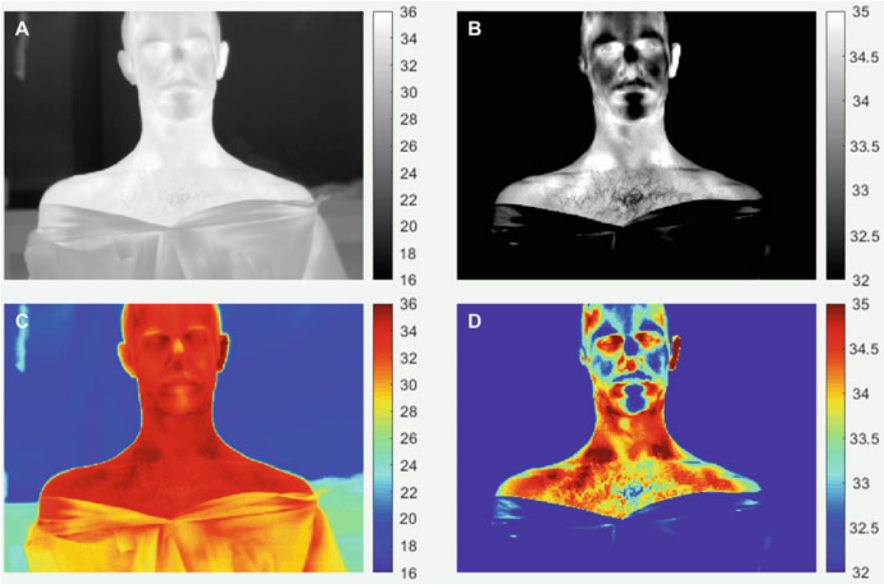

Fig. 4 False-colour images of the same thermal image (**a**, **b**) in greyscale and (**c**, **d**) using the 'jet' palette (MATLAB™) with (**a**, **c**) a wide temperature range and (**b**, **d**) a narrow temperature range. Comparison of **a** and **b** demonstrates the importance of choosing an appropriate temperature range to ascertain detail within the region of interest. The 'jet' colourmap does not have uniform luminance and can cause the false appearance of thermal contrast (e.g. the bottom section of background appears to contrast with the participant's clothing more in **c** than is evident in **a**)

each temperature value is assigned a colour value, allowing the data to be graphically displayed as a false-colour thermal image (Fig. 3).

The colourmap and range can be adapted as required (Fig. 4). The default setting in image viewing software will be to scale the colourmap over the full range of temperature in the image. The background of the image is likely to be cooler than the participant and of little interest. Setting a narrow temperature range is more appropriate for BAT analysis as the dynamic range of the area of interest is limited and because a wide temperature range will reduce the visual thermal resolution of the image.

The choice of colourmap can markedly affect the appearance of the final image and can create the appearance of demarcated regions and features where they do not exist. This can be the case with commonly used colourmaps such as 'jet' (MATLAB™, MathWorks, Natick, MA, USA) and 'rainbow' (FLIR Systems, Wilsonville, OR, USA) and is due to peaks in the luminance of the colourmap. The best colourmaps are sequential ones with a uniform luminance gradient – and the simplest of these is greyscale.

Summary of Stages of Infrared Thermography

1. The radiometric signal arriving at a sensor array is measured and the value stored.
2. The raw signal is converted to temperature values.
3. Temperature data is displayed as a false-colour image.

4 Use of IRT in Studies of BAT

IRT was first used to investigate BAT in the 1970s to demonstrate a thermogenic response to ephedrine (Rothwell and Stock 1979). The non-invasive, non-contact nature of IRT makes it suitable for use in large-scale studies, for repeat imaging in longitudinal and intervention studies, and it has been used for imaging of children as young as 3 years old (Symonds et al. 2012). It can be used to measure the real-time effect of acute interventions including meals or dietary components (Ang et al. 2017; Lee et al. 2011; Scotney et al. 2017), as well as the effect of chronic interventions.

BAT depots in humans are located in multiple anatomical locations (Heaton 1972; Leitner et al. 2017). Of these, the supraclavicular (SCV) BAT depot is most amenable to imaging with IRT due to its relatively superficial location, underneath the subcutaneous adipose tissue in the SCV fossa. IRT measures *surface* temperature and so, for the heat signature of the BAT depot to be detected, at least a proportion of the heat must be transmitted through the tissues to the surface. The more insulating tissue present (for instance, increased subcutaneous adipose tissue), the less heat that will be transmitted to the surface. This can pose a challenge if trying to compare results between individuals directly or if intra-participant changes are sought in a longitudinal study where adiposity has varied through the study duration. Methods to measure local subcutaneous adipose thickness and directly adjust IRT measurements are not yet established, but validated indirect adjustments are discussed below (see Sect. 6.4).

Since the evolutionary purpose of BAT is adaptive thermogenesis (Cannon and Nedergaard 2004), the location of the SCV BAT depot close to the major vessels of the neck suggests a role in maintaining cranial temperature (Smith and Horwitz 1969). Therefore, at least some of the heat must be transmitted into the body and not available to be measured with IRT. In addition, the skin surface temperature changes are affected by other homeostatic mechanisms, most notably blood flow (Braverman 2000), controlled by arteriovenous anastomoses under sympathetic nervous system (SNS) and hormonal regulation (Ootsuka and Tanaka 2015) in response to temperature changes, hypovolaemia and arousal. It is important, therefore, that these factors are controlled when interpreting skin temperature in measurements of BAT activity.

Despite these potential limitations, IRT is reproducible (Haq et al. 2017) and has been shown repeatedly to be able to detect heat changes following cold stimulation (Law et al. 2017a), typically exposure of a limb to cold water or a cooling blanket

(see Sect. 5.4). In addition, IRT has been shown to correlate well with PET-CT (Law et al. 2017b). 18F-FDG PET-CT measures glucose uptake, whereas IRT measures heat production, which are not equivalent, and so perfect correlation would not be expected. Optimal methods for detecting BAT are still being refined and, at the moment, comparison between studies can be difficult due to variations in methodologies and the details reported (Law et al. 2017a). Within the general field of physiological measurements using IRT, sports and exercise medicine have led the way in developing consensus opinion (Moreira et al. 2017) and many of the recommendations (Table 1) are equally valid when using IRT for BAT measurements.

5 Acquiring IR Thermographs

In addition to the methodological advantages outlined above, IRT has the benefit of requiring minimal, relatively inexpensive equipment, compared to PET-CT and MRI. However, the same rigour and care needs to be observed when collecting data using IRT as with other imaging techniques. The rapid expansion of the use of IRT, largely driven by industrial uses, has brought down the price and increased the specification of equipment, with a resultant increase in image quality and definition (Fig. 5).

- Modern cameras typically measure between 320 × 240 and 640 × 480 pixels, although true high-definition options are available.
- The option for radiometric video is standard on many cameras allowing 30 frames per second, or greater, to be captured, compared with still images of four to six per minute previously.
- Increased sensitivity means that changes of as little as 0.01°C can be measured.

The camera settings must be checked and adjusted for each session as necessary, including distance from the SCV region to the camera, emissivity (0.98 for human skin) and environmental temperature and humidity (measured using a thermohygrometer).

5.1 Environment

Laboratory-based studies should be undertaken in a temperature-controlled environment at thermoneutrality [approximately 22–24°C for an adult in light cotton shorts and vest, away from airflow (Houdas and Ring 1982)]. The assessment should be undertaken away from both sources of infrared radiation (including lighting and heating) and airflow (such as air-conditioning) (Moreira et al. 2017). The nature of IRT means that, where desired, the equipment is highly portable, allowing real-world studies to be undertaken in the field (Robinson et al. 2014; Symonds et al. 2012), but even here it is important to control as many variables as possible and acknowledge where it has not been possible to do so.

Table 1 Recommendations from a Delphi consensus statement on the measurement of human skin temperature with thermographic imaging in sports and exercise medicine

1) The relevant individual data of the participants must be provided.
Note: These could include, but are not limited to, age, sex, body mass, height, body mass index, ethnicity and whether they are smokers or not. An indication of physical activity profile (e.g. frequency, duration, intensity and activity description) should be reported.
❑ Yes ❑ No ❑ Unclear
2) Participants should be instructed to avoid alcohol beverages, smoking, caffeine, large meals, ointments, cosmetics and showering for 4 h before the assessment. Also, sunbathing (e.g. UV sessions or direct sun without protection) should be avoided before the assessment.
Note: This should be confirmed verbally before the assessment. The use of any medicinal treatments or drugs should be recorded. Any condition that could not be avoided should be reported.
❑ Yes ❑ No ❑ Unclear
3) Extrinsic factors affecting skin temperature (e.g. physical activity prior to the assessment, massage, electrotherapy, ultrasound, heat or cold exposure, cryotherapy) should be clearly described.
❑ Yes ❑ No ❑ Unclear
4) Ambient temperature and relative humidity of the location where the assessment took place must be recorded and reported as mean ± standard deviation.
❑ Yes ❑ No ❑ Unclear
5) The assessment should be completed away from any source of infrared radiation (e.g. electronic devices, lightning) or airflow (e.g. under an air conditioning unit).
Note: Any condition that could not be controlled should be reported.
❑ Yes ❑ No ❑ Unclear
6) The manufacturer, model and accuracy of the camera used should be provided.
Note: When available it is recommended to provide the maintenance information of the equipment (e.g. when and where it completed the last calibration).
❑ Yes ❑ No ❑ Unclear
7) An acclimation period in the examination room should be completed.
Note: This item is only applicable for initial baseline measurements or basal analysis.
❑ Yes ❑ No ❑ Unclear
8) If necessary the camera should be turned on for some time prior to the test to allow sensor stabilisation following the manufacturer's guidelines.
❑ Yes ❑ No ❑ Unclear
9) Conditions of image recording such as mean distance between object and camera and percentage of the region of interest within the image should be detailed.
❑ Yes ❑ No ❑ Unclear
10) The camera should be positioned perpendicular to the region of interest.
❑ Yes ❑ No ❑ Unclear
11) Emissivity settings of the camera must be reported.
Note: 0.98 of emissivity is suggested for a dry clean skin surface.
❑ Yes ❑ No ❑ Unclear
12) The time of day at which the images were taken should be reported.
❑ Yes ❑ No ❑ Unclear

(continued)

Table 1 (continued)

13) The standard body position of the subject and the regions of interest must be well described and appropriately selected. A visual example (with temperature scale presented and scale of colours properly configured) is recommended.
❑ Yes ❑ No ❑ Unclear
14) If the skin is dried (e.g. to remove surface water), the drying method should be clearly described.
❑ Yes ❑ No ❑ Unclear
15) The evaluation of thermograms and collection of temperature from the software should be clearly described.
❑ Yes ❑ No ❑ Unclear

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5.2 Participants

Participants should be screened for factors likely to affect BAT activity or skin temperature and excluded unless explicitly required for the study. These should include smoking (Grassi et al. 1992) and medications such as beta-blockers or beta-agonists (Alexander and Stevens 1980; Law et al. 2014; Thureson-Klein et al. 1976). The region to be imaged should be hair-free, as the emissivity of hair results in a lower measured temperature (Fernández-Cuevas et al. 2015). In addition, they should be asked to refrain from showers; from consuming alcohol, caffeine-containing drinks or large meals; and from strenuous activity for at least 4 h prior to imaging (Moreira et al. 2017) and to avoid use of cosmetics and ointments (Engel 1984; Fernández-Cuevas et al. 2015; Steketee 1976). Where feasible, assessment should be undertaken at a consistent time of day to avoid potential circadian variations in BAT activity (Redlin et al. 1992). A detailed account of the factors influencing skin temperature is provided by Fernández-Cuevas et al. (2015).

Participants should be asked to don standardised study clothing, usually consisting of light cotton shorts and vest, as soon after arrival as practical. This allows the participant to begin to acclimatise to the room and, therefore, they should remain in the study room for the duration of this period. The vest should be of a style and fit to allow complete exposure of the neck and shoulders above the line of the clavicle. Basic anthropometric and characteristics must be measured and recorded including height, weight, age, gender, ethnicity, smoking status and medications.

5.3 Positioning

Following initial acclimation, the participant should be invited to sit in an upright position. The head should be in a near-neutral position, slightly extended if necessary to expose the SCV region. The participant should be positioned in such a way to avoid movement during the imaging period. Firstly, this should be such that they can be expected to remain comfortable for the duration of the imaging period; and,

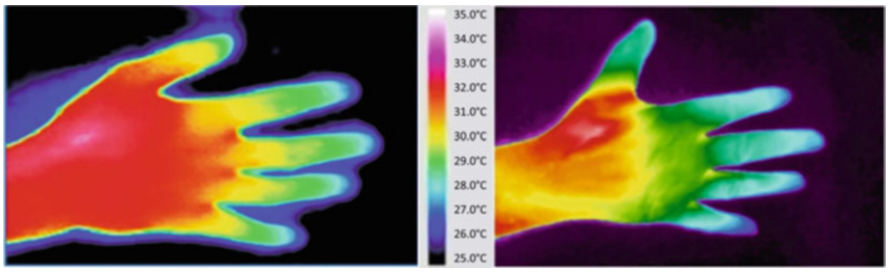

Fig. 5 Infrared thermograph of the dorsum of a hand taken in 1990 (320 × 240 pixels) and at in 2011 (640 × 480 pixels). From Ring and Ammer (2012) © Institute of Physics and Engineering in Medicine. Reproduced by permission of IOP Publishing. All rights reserved

secondly, they should be able to avoid needing to move when the stimulus is introduced (see Sect. 5.4).

They should be directly facing the thermal imaging camera, which should be secured on a tripod able to allow adjustment in height and rotation in all axes. Prior to commencement of imaging, the camera should be positioned such that the receiver is perpendicular to an imaginary line itself perpendicular to the centre of the larynx (Fig. 6). To optimally measure the radiation from the SCV fossa, the camera would ideally be placed along a line perpendicular to that plane, which is externally and cranially rotated relative to the coronal plane. However, the resultant variable and subjective positioning of the camera would reduce reproducibility and would only allow unilateral imaging. The recommended position is a compromise between consistency and maximising detection of radiation. It should, therefore, be recognised that results will underestimate the true SCV temperature.

5.4 Stimulation

BAT stimulation can be altered in several different ways, such as by diet (Kim et al.2015), pharmacological agents (Scotney et al. 2017) or temperature. Stimulation may occur acutely (over minutes) or chronically (over days and weeks). IRT study design can be adapted to measure both acute changes (e.g. increase from a resting baseline) and chronic changes (e.g. measuring change in resting or change in stimulated temperatures at serial study sessions).

Perhaps the most frequently used stimulus is acute cold. This is in the form of either a cold room (Jang et al. 2014; Lee et al. 2011; Ramage et al. 2016) or a cooling stimulus [for instance, cold water (Ang et al. 2017; El Hadi et al. 2016; Peterson et al. 2017; Ramage et al. 2016; Robinson et al. 2014; Symonds et al. 2012) or a cooling blanket (Law et al. 2017b; Salem et al. 2016)] applied directly to the skin but away from the SCV region. An extremity, such as a foot (Ang et al. 2017; Peterson et al. 2017; Symonds et al. 2012; Virtanen et al. 2009) or hand (Ang et al. 2017; El Hadi et al. 2016; Ramage et al. 2016; Robinson et al. 2014; Symonds et al. 2012), is often

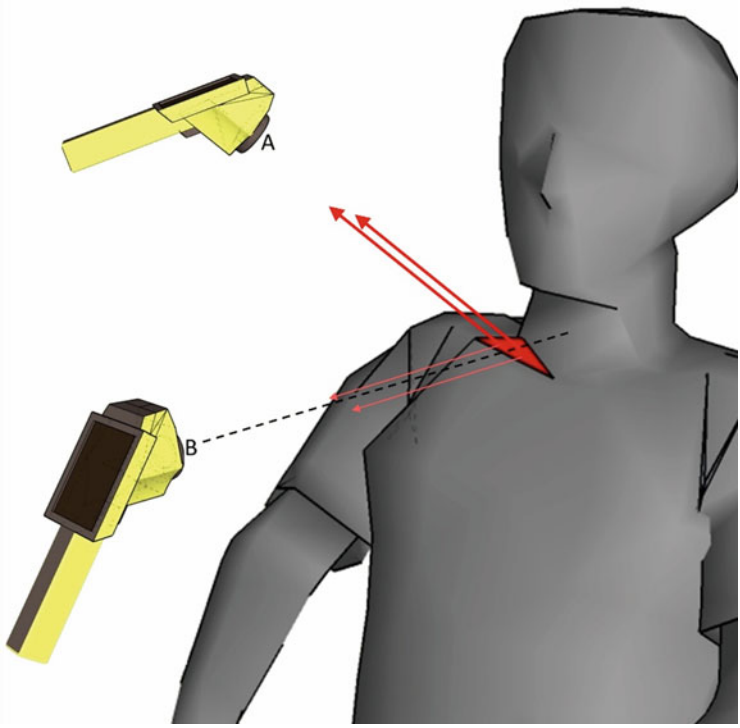

Fig. 6 Representation of infrared radiation emitted by the supraclavicular region (red triangle) which is maximally detected by the camera at position A. While the camera at position B will detect less radiation and therefore underestimate the true skin temperature in the region of interest, the position is reproducible and therefore recommended. From Law et al. (2017a) reproduced by permission of Taylor & Francis Ltd., http://www.tandfonline.com

used as the site of cooling. If a cooling stimulus is to be used, care should be taken to avoid change of body position when the stimulus is introduced. In the case of cold water, the hand (or foot) should therefore be positioned in an empty receptacle prior to image acquisition period. For the same reason, if a cooling blanket is to be used, this should be put in place at the start of imaging, without flow and at a neutral temperature initially.

Cooling should be sufficient to achieve SNS BAT activation, but not so extreme as to induce shivering, an alternative adaptive thermogenesis mechanism, or pain, an alternative SNS activation pathway. Where a standardised temperature is used, the water is typically 15–20°C (Ang et al. 2017; Ramage et al. 2016; Robinson et al. 2014; Symonds et al. 2012).

The alternative is to use an individualised protocol. In this case, the participant is cooled to the point of shivering, then the stimulus temperature is gradually increased until shivering ceases (van der Lans et al. 2013; Vosselman et al. 2012). Shivering should be defined as any one of three measures: participant report, researcher

observation and electromyogram (van der Lans et al. 2014). The temperature at which shivering ceases is then used as the participant's individualised stimulus temperature. Although there is much that is appealing about this approach, and it is recommended in PET-CT studies of BAT (van der Lans et al. 2014), caution must be exercised when applying it to IRT studies. If the set point is established on the day, there may be an insufficient washout period and the earlier cooling period may affect the later study session. PET-CT measures substrate uptake at a given point and so is less affected by the effect of earlier cooling than IRT which compares change from baseline (see Sect. 6.4). Shivering points for individuals vary over time, so a set point established prior to the study day may not be valid at a later date (Davis 1961).

5.5 Imaging Duration

There is considerable variation in the length of imaging periods utilised. Acclimatisation to the study room temperature is achieved after an hour (Haq et al. 2017). The change in SCV temperature during acclimatisation is likely to vary depending on the environment that the participant was most recently in. Despite the initial acclimation period, there is frequently a cooling of the skin temperature upon commencement of imaging in our experience, thought to be due to the cessation of voluntary muscle activity in the sedentary state in which imaging is undertaken. For this reason, a prolonged baseline period of imaging is recommended. A baseline period of at least 10 min is necessary to achieve a steady SCV temperature prior to introduction of an acute stimulus. To demonstrate achievement of a steady state, at least three measurements in the last 5 min of the baseline period are required.

The initial response to cold stimulus is rapid. Five minutes of stimulation is sufficient to detect a change from baseline following the introduction of a direct contact cold stimulus (Symonds et al. 2012), but longer periods of up to an hour may show a further increase (Law et al. 2017b). Detailed longitudinal information about the response to a cold environment challenge is not available, but studies have reported results between 30 min (Lee et al. 2011) and 2 h (Jang et al. 2014; Ramage et al. 2016).

Images should be collected at regular intervals throughout the baseline and stimulation periods. Still images should be collected at a rate of at least four to six images per minute, as the camera will allow. Less frequent capture is likely to permit significant movement between images which both reduces image consistency and increases thermogenesis from voluntary muscle movement. Video is captured at typically up to 30 frames per second and may allow more detailed changes to be seen, but the increased data set created may add complexity to the data analysis phase (see Sect. 6).

5.6 Additional Measurements

To interpret fully the data from the infrared thermographs, additional physiological measurements are necessary. These should include measures of SNS activation including heart rate and blood pressure. In addition, core temperature should be measured at the start and end of the session as a minimum, and at regular intervals of longer imaging sessions.

Additional skin temperature measurements are also valuable to use to compare to the changes in BAT temperature (discussed further in Sect. 6.4).

6 Image Analysis

6.1 Conversion

The details of the file structure of thermographs will vary depending on the manufacturer and equipment used, and the researcher will need to be familiar with the particular system in use. The information discussed here is based on experience with FLIR® equipment but will be applicable to many others.

Thermographs are stored in a variety of file formats including, but not limited to, JPEG and TIFF. The radiometric data is stored distinctly from the displayed false-colour image and may be obfuscated to a greater or lesser extent to encourage use of proprietary software for image analysis. The displayed false-colour image cannot be analysed in any meaningful way- and temperature data can only be approximately estimated by comparison to a colour bar. The radiometric data within the file must be accessed and converted to temperature data before progressing. This may be done by utilising proprietary software. Free versions will typically have limited functionality especially of the type required for analysis of BAT. Commercially available versions have a wider range of tools, and it is important to ensure that those required are included.

Alternatively, data can be accessed outside of manufacturers' proprietary software, and this usually requires a conversion step first. Options for conversion include:

- Opening images within proprietary software that allows saving in a variety of formats, such as comma-separated values files (CSV) or MATLAB™ data files (MAT).
- Conversion to an openly accessible format such as portable network graphics (PNG) (Law et al. 2017a, b) using a Python-based script, or equivalent, where the raw radiometric data is stored as the image rather than in the metadata (see Sect. 3).
- FLIR®, for instance, provides a MATLAB™ executable file that allows images taken on FLIR®-manufactured devices, including videos, to be imported into the MATLAB™ programming environment directly, following which data can be analysed in a flexible way.

As well as allowing more flexible analysis, conversion also protects against data loss from proprietary file formats becoming obsolete. This is a significant risk as manufacturers continue to develop new cameras with new file formats and may stop supporting the current ones.

6.2 Region of Interest Identification

Once the image has been opened within a suitable environment, it must be analysed to identify the region of interest (ROI). The shape and border used to define the ROI varies significantly between publications. Early studies used circles or simple polygons to identify the region of interest (Jang et al. 2014; Kim et al. 2014; Lee et al. 2011; Symonds et al. 2012). Where small ROIs over the SCV fossa have been used, the output is usually a summary measure of the whole ROI (e.g. mean or maximum temperature) (Gatidis et al. 2016; Jang et al. 2014). This approach has the problem of requiring subjective placement.

Alternatively, a larger ROI can be identified and a BAT "hotspot" is defined within it: a summary measure of the hotspot being calculated as the output measure (Robinson et al. 2014; Symonds et al. 2012). The latter approach has progressed from simple ROIs to more refined options. Current techniques include the automatic identification of the shoulder contour (Fig. 7) to allow rapid and accurate analysis of large numbers of images (Law et al. 2017b; Scotney et al. 2017). The hotspot is then defined either as an upper percentile of pixels within the ROI (Symonds et al. 2012) or using a watershed method, with a summary measure calculated. Alternatively, a seeded-region growing algorithm approach can be used to define the area of BAT activity, which avoids imposing a fixed size or sharp cut-off (Ang et al. 2017). However, this limits the number of images analysed due to the computational time required and has not yet demonstrated a definite advantage over simpler methods.

6.3 Averaging

As with any other imaging modality, or measurement, each radiometric datum is subject to an error term. While summary measures will reduce the error by averaging across thousands of pixels, time series demonstrate variation around the underlying trend due to physiological rhythms, inherent noise in the measurement or a combination of the two (Fig. 8). Studies that use only single or isolated images (Gatidis et al. 2016; Kim et al. 2014; Peterson et al. 2017), or only acquire one image per minute (Robinson et al. 2014; Symonds et al. 2012), are, therefore, exposed to significantly reduced reproducibility. In contrast, radiometric video, while increasing the noise in each individual frame, allows frames to be flattened in the z-axis (Fig. 9), increasing accuracy. In general, averaging across n frames will reduce the noise (here defined as standard deviation) by $\sqrt{n}$. When considering the noise in data, it is usually useful to compare the noise to the mean as noise is frequently expected to increase proportionately to the mean. This can either be undertaken by calculating a

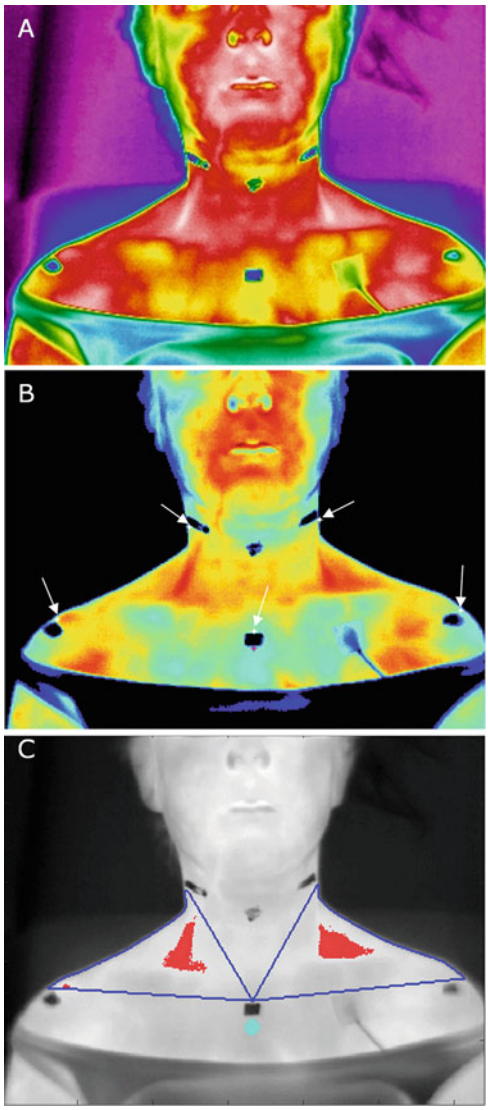

Fig. 7 Contour of region of interest, including border of the shoulder, automatically identified following manual identification of apices. (**a**) Original thermal image (**b**) apices identified (white arrows) (**c**) output image highlighting ROI contour (blue), BAT 'hotspot' (red) and sternal reference region (cyan)

signal-to-noise ratio (where SNR is the mean divided by standard deviation) or a coefficient of variation (CV% is the standard deviation divided by mean, multiplied by 100). As an example, the same absolute accuracy would not be expected from a heavy goods vehicle weighing bridge as would be expected from a set of laboratory scales, but the ratio between the signal (weight) and noise may be similar.

Comparison of this form is less straightforward when dealing with temperature because it is not a linear proportionate scale, i.e. a temperature of 15°C is not half of a

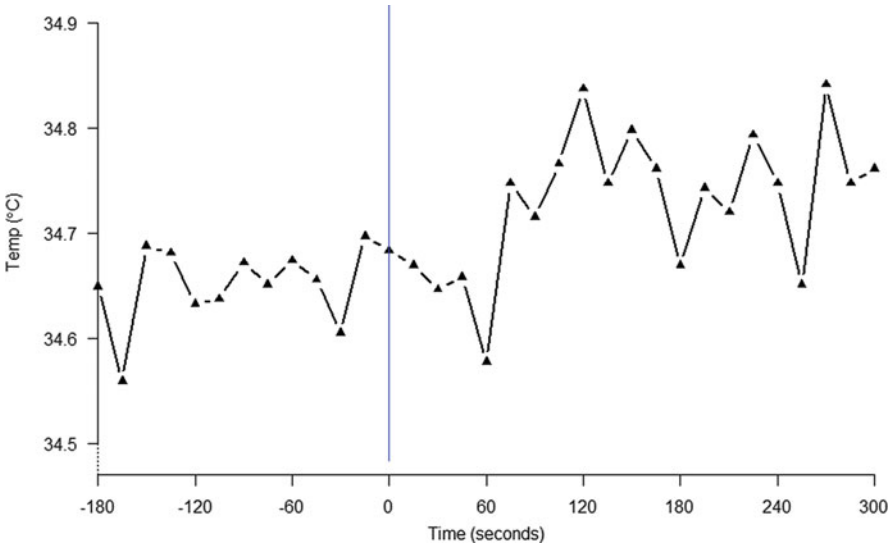

Fig. 8 Time series of infrared thermography measurements from a single participant, with four images per minute over 5 min of stimulation showing a rising trend following introduction of a cold stimulus (blue line) but with significant noise

temperature of 30°C. It follows then that the error in a measurement with a mean temperature of 15°C would not be expected to be half of the error in a measurement with a mean temperature of 30°C. Secondly, human skin temperature is generally in a small dynamic range of ~30–37°C (Law et al. 2017a). There is little change in the mean, and so changes in the SNR or CV are primarily determined by changes in the noise rather than the signal. In theory, a signal could be calculated from the raw radiometric data or from absolute temperature (Kelvin), but this would further emphasise the small range of temperature measurements.

6.4 Measures

Whether or not attempts are made to reduce noise by averaging frames, once the region of interest has been identified, an output measure will need to be calculated. The output measure selected will depend to an extent on the ROI chosen and the software being used. Most proprietary software will allow at least rectangular and elliptical ROIs to be drawn and, from these, provide a minimum, maximum and, possibly, mean temperature. If the ROI has been chosen to represent a small area over a potential BAT deposit, these may not be unreasonable options but are all susceptible to influence from extreme values, especially minimums and maximums. In addition, each image will need to be analysed by hand and the output recorded

manually, risking transcribing errors and setting a practical limit on the number of images analysed.

If a larger ROI has been selected, from which the BAT 'hotspot' forms a smaller part, this should be reflected in the outcome measure chosen. The top 10% of temperature pixels within a SCV ROI as previously described (see Sect. 6.2) can be considered to represent the BAT hotspot. Even if the temperature of pixels in the ROI is normally distributed, the temperature of pixels in the hotspot would not be, and so the mean is no longer an appropriate point measure. Instead, the median value is chosen, which has the additional advantage of being less susceptible to influence from extreme values. The median value of the top 10% of values is equivalent to the 95th percentile of the original ROI, which can be easily calculated by any suitable software such as Excel, R (both by exporting the CSV files of the ROI) or MATLAB™.

As discussed above (see Sect. 4), the temperature of the SCV BAT outcome measure (T_{SCV}) can be influenced by factors other than BAT activity, such as obesity (increased insulation) and environmental temperature (skin perfusion). Perfusion can be accounted for by comparing T_{SCV} to a reference skin temperature (T_{Ref}). Most easily, this is undertaken by selecting a reference region within the thermal image over a non-BAT area such as the sternal (Law et al. 2017b) or deltoid regions (Fig. 7), but these are limited to the frame of the image. An alternative is to calculate

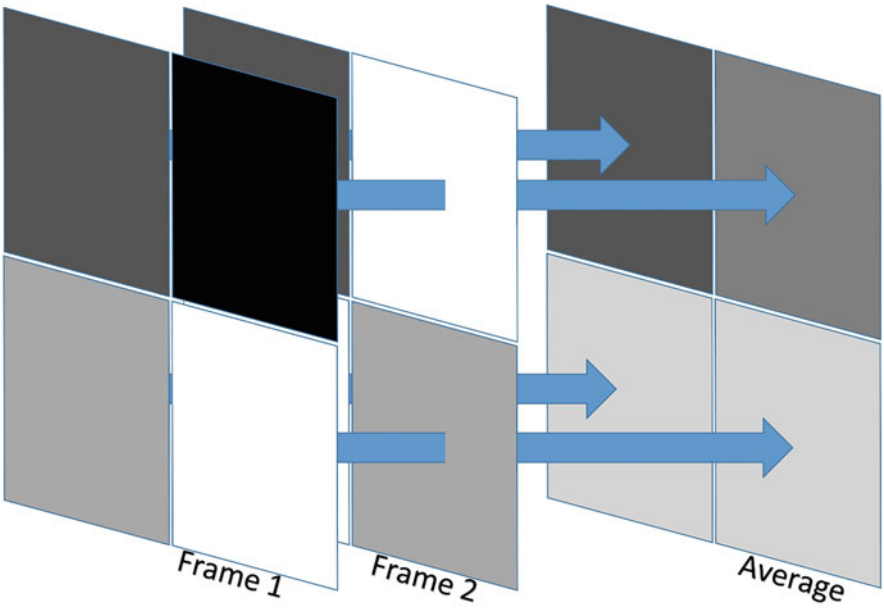

Fig. 9 Simplified example of effect of frame averaging. Frame 1 and Frame 2 are images of a homogenous radiation source and each consist of a 2 × 2 array of 2-bit (0–3) encoded pixels represented using a greyscale. Each pixel in the averaged frame is the mean of the corresponding pixels in Frames 1 and 2, reducing the variation in measurements

a mean skin temperature (Robinson et al. 2016) using contact skin temperature probes (such as iButtons™ or thermocouples attached to real-time display) to measure multiple points simultaneously and continuously. The median temperature of the non-BAT area or the mean skin temperature (T_{Ref}) can then be compared to T_{SCV} to calculate a relative temperature (T_{Rel}) (Eq. 2).

$$T_{Rel} = T_{SCV} - T_{Ref} \tag{2}$$

Relative supraclavicular temperature (T_{Rel}) calculated as the difference between absolute supraclavicular temperature (T_{SCV}) and a reference temperature (T_{Ref}).

Finally, change following stimulation (ΔT) can be calculated from baseline resting temperature (Eq. 3). Note that in some publications, ΔT is defined as the difference between BAT and non-BAT skin temperature, here called T_{Rel}.

$$\Delta T = \text{stimulated } T - \text{resting } T \tag{3}$$

Change in temperature (ΔT) from resting following stimulation.

ΔT further uses each participant as their own control, standardising results and better allowing direct comparison between individuals. ΔT_{Rel} has been shown to correlate closely with BAT activity measured using PET-CT (Law et al. 2017b). ΔT_{Rel} relies on being able to measure both a rested ('off') state and a stimulated ('on') state. A participant who has maximally activated BAT at baseline may show no further increase with stimulation and, therefore, provide indistinguishable data from the participant who has minimal active BAT in both resting and stimulated states (Law et al. 2017a). This is particularly pertinent where individualised cooling protocols (see Sect. 5.4) are being determined on the day of stimulation, potentially resulting in a failure to fully induce a resting state in BAT activity at baseline. For studies of chronic BAT activation, comparison of changes in stimulated temperature over the duration of the study would mitigate these potential pitfalls. Acute studies are able to produce reliable results where the above issues are carefully considered in the design.

7 Summary

Infrared thermography utilises the intrinsic properties of infrared radiation to infer temperature and reconstruct the heat properties of a scene. The supraclavicular BAT depot is relatively superficial and the heat-generating property of this major BAT depot can be detected directly using inexpensive and readily available equipment. Cooling produces a reproducible increase in the temperature of the skin overlying the supraclavicular BAT depot which can be determined by careful identification and analysis of the region of interest. By comparing the results to a non-BAT reference region and calculating change from resting temperature, results from IRT show strong correlation with PET-CT. In this way, the non-invasive, non-contact methods

of IRT open the possibility of studying populations and interventions not previously possible.

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Section 3 Results

3.1 Semi-automated analysis of images

Law JM, Morris DE, Robinson LJ, Symonds ME, Budge H (2020)

Semi-Automated Analysis of Thermal Images Increases Speed of Analysis without Increasing Variation in Results. ***Submitted to Current Research In Physiology***

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Semi-automated analysis of supraclavicular thermal images increases speed of brown adipose tissue analysis without increasing variation in results

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Abstract

Interest in brown adipose tissue remains high a decade after it was determined to be present outside of the neonatal period. In vivo imaging, however, has remained a challenge due to the lack of a imaging modality suitable for large healthy-volunteer studies, post-prandial investigations and vulnerable groups, such as children. Infra-red thermography is increasingly accepted as valid, non-invasive and flexible alternative but there is a wide approach to analysis between different groups. Defining the region of interest with anatomical borders rather than using a simple polygon may have advantages in terms of consistency but makes image analysis slower, limiting some applications. Our novel semi-automated method, using a custom-built graphical user interface, allows an 86% improvement in speed of image analysis (54.9 (38.3-71.4) seconds/image) without increases in variation between analysers or with repeated analysis. The improved efficiency demonstrated makes feasible larger studies, longer imaging periods or increased image acquisition frequency, providing an opportunity to study novel features of brown adipose tissue function.

Keywords: Brown adipose tissue; brown fat; infrared thermography; thermal imaging; automation; method comparison; Matlab; human.

Introduction

Brown adipose tissue (BAT) is a heat-generating tissue which oxidises glucose and fatty acids through mitochondrial respiration without the usual resultant production of adenosine triphosphate. This is due to the presence of a unique protein, uncoupling protein (UCP)-1, in the mitochondrial membrane (Cannon and Nedergaard, 2004). The last decade has seen a renewed interest in BAT following the demonstration of its presence outside of the neonatal period, hinting at a potentially novel mechanism to aid weight management by increasing calorie expenditure, beyond the usual increase in voluntary physical activity (Celi, 2009, Virtanen et al., 2009, Cypess et al., 2009, van Marken Lichtenbelt et al., 2009).

Measuring BAT activity remains challenging with the gold standard method of PET-CT, using either glucose (18F-fluorodeoxyglucose) or fatty acid (e.g. 18F-fluro-6-thiaheptadecanoic acid or 11C-acetate) isotopes (Labbé et al., 2016, Ouellet et al., 2012). PET-CT provides a static measurement of what is a dynamic tissue and, also, cannot be used to look at the effects of meals due to the rapid uptake of the isotope into muscle in the postprandial period (Hankir et al., 2017). In addition, the high dose of ionising radiation makes PET-CT unsuitable for use in large studies of healthy volunteers and in groups such as children.

Due to the heat-generating property of BAT, infrared thermography is a natural alternative to PET-CT for measuring BAT activity, especially given the relatively superficial location of the supraclavicular depot which is the largest BAT depot in humans (Leitner et al., 2017). Infrared thermography measures infrared radiation emitted by an object and converts infrared data to temperature which is then presented as a false-colour image using a user-defined colour-map. Images can then be analysed the determine temperature properties of all, or part of, the image. By measuring the heat signature of the supraclavicular region before, and during, the introduction of a suitable stimulus, such as cold, the response of the supraclavicular BAT to the stimulus can be ascertained (Law et al., 2019, Ang et al., 2017, Lee et al., 2011, Haq et al., 2017). Infrared thermography has been validated as an alternative to, and shows a strong correlation with, PET-CT (Law et al., 2018b, Lee et al., 2016, Boon et al., 2014, Jang et al., 2014, Haq et al., 2017).

A variety of image analysis methods have been used by groups with some opting to define the contour of the region of interest (ROI) using simple regular shapes and others utilising anatomical borders (Law et al., 2018a). The latter has advantages in terms of reproducibility and accuracy but was time-consuming and, therefore, exerted a practical limit on the number of images that could be analysed.

We, therefore, custom-built an image analysis application with the intention of streamlining the analysis process while continuing to use an ROI defined by anatomical borders and

without an increase in variation between repeat-analysis.

Methods

A pragmatic retrospective evaluation was undertaken. All thermal images were acquired on a FLIR B425 camera (FLIR Systems) using standard settings (Emissivity 0.98) as previously described (Law et al., 2019, Law et al., 2018b, Robinson et al., 2014, Robinson et al., 2016). Members of the research group analysing thermal images were trained in two methods of image analysis, a manual method using FLIR's proprietary software, ResearchIR version 4 (FLIR Systems AB, Danderyd, Sweden) (Robinson et al., 2014, Robinson et al., 2016), and a semi-automated method using a custom-designed graphical user interface (GUI) within the MATLAB (MathWorks, Natick, MA, USA) programming environment (Law et al., 2019, Law et al., 2018b). Training on both methods was delivered consistently by a single individual to all researchers. Analysers practised analysing training images until they were confident in both techniques and then analysed further sets of images to demonstrate competence and consistency. Each analyser analysed two sets of 31 images using both methods and then reanalysed a subset of eight images from each set a further two times.

Images

Each analyser was asked to analyse two sets of images (Set A and Set B) on three occasions (Visit 1, Visit 2 and Visit 3). On the first visit, an extended set of 31 images were analysed in each set (62 images in total). On the subsequent two visits, a subset of eight images was analysed from each set (16 images in total). Set A comprised a set of images where the participant had moved a moderate amount between frames; set B comprised a set of images where the subject had moved very little between frames. On the first visit, analysers were randomly assigned to analyse images using the manual (M) or the semi-automated (SA) method first and then alternated the methods used first at the second and third visit.

Manual (M) Method

Thermal images of the supraclavicular region were acquired and saved in the proprietary FLIR JPEG format, as previously described (Robinson et al., 2014). This format saves the infrared data in the file metadata along with the values of variables required to calculate the temperature of the object in the image (Tattersall, 2015). The image was opened in ResearchIR which displayed the temperature data in an alterable and processable format.

The inbuilt polygon tool was used to define a contour around a region of interest (ROI). Two ROIs were drawn: one around the left supraclavicular region and one around the right (Figure 1A). Once the ROIs had been defined, the temperature data of the pixels within each was exported as a comma-separated value (.csv) file which was given a file name indicating the image is related to and whether it was the left or right side. Finally, a FLIR session file (.irs) was saved. The session

file saved the workspace, including the polygon (and any other measurement tools), and a link to the image being viewed to allow the analysis to be reproduced later. If errors were later found to have been made during the ROI-saving process, the session file allowed the workspace to be reopened and the ROI saved using the analyser's original polygon.

The process was then repeated for the next image. The polygon defining the ROI was maintained by the software when the next image was selected. The analyser chose to adapt the previous polygon (by translation along the x- and y-axes and by the movement of points) to fit the ROIs on the new image or delete it and define a new one. Where the image was similar, small changes to the original polygon were quicker and acceptable but tended to become increasingly less appropriate with each subsequent image and worked poorly if there was more movement between images.

Once all the images in the set had been analysed, the analyser opened each comma-separated value file in Microsoft Excel. Each cell of the spreadsheet corresponded to a pixel in the original image. The analyser was then required to process the data using a short succession of keystrokes to run a custom-built macro. The macro ranked the cells from warmest to coolest and then identified the output required, namely 95th percentile (Law et al., 2018b). The analysers than saved the file output as a new file.

An automated checking process was developed (using R) to confirm that all the expected filenames were present and that the file labelled as containing the data from the left ROI was different to the one labelled as containing data from the right ROI (i.e. that the same ROI had not been exported twice). Other errors, such as cross-labelling images would not have been detected and cannot be excluded.

Semi-automated (SA) Method

As previously published (Law et al., 2018b), thermal images, saved in FLIR's proprietary JPEG format, were converted into an open, portable network graphic (.png) format with the raw radiometric data stored as a greyscale image and the variables required for conversion to thermal data stored in the metadata.

Using the MATLAB GUI (Figure 1B), the analyser identified the five apices of the region of interest (i.e. the sternal notch, the acromioclavicular processes, and the intersections of the sternocleidomastoid muscles with the contour of the neck) on each image. The "threshold" temperature is set to a default of 30°C to distinguish the person in the image (temperature above the threshold) from the background (temperature below the threshold) and can be adjusted by the researcher if required.

The points from the previous image remained in position when the next image was displayed and could be adjusted by either dragging or clicking with the mouse.

Once apices had been defined for all images in the set, the ROI was automatically defined and

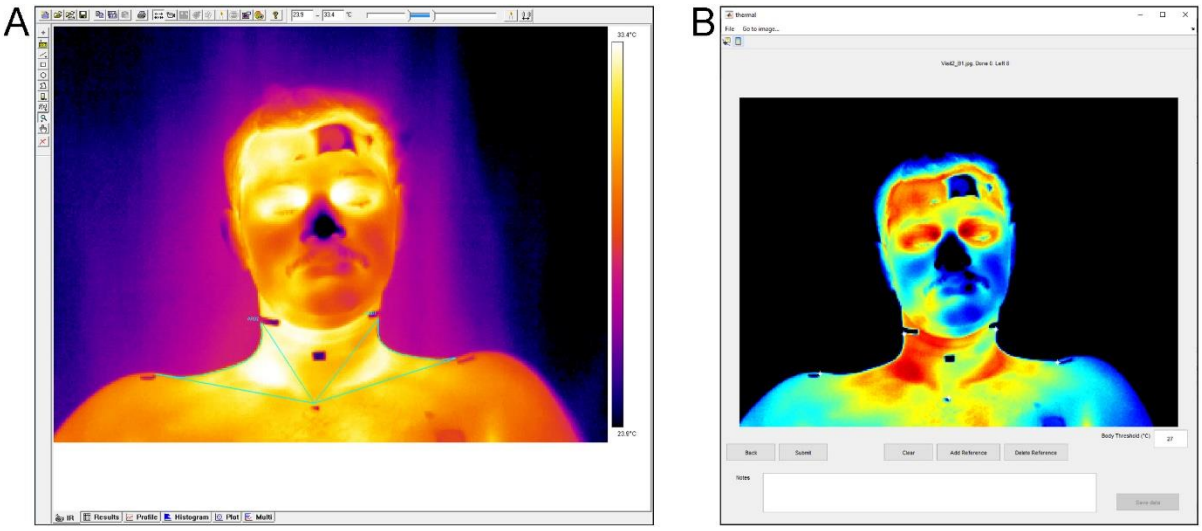

Figure 1 (A) Example of analysis using FLIR ResearcherIR software. The borders of the contour were a straight line approximating the sternocleidomastoid muscle, from the point where it intersects the contour of the neck to the suprasternal notch; a straight line approximating the clavicle, from the suprasternal notch to the acromioclavicular process; and the contour of the neck between the preceding two lines, manually approximated by the research by a series of short straight lines. (B) Example of analysis from MATLAB app showing apices (white stars) identified by analyser.

processed, and the output saved as a single comma-separated file with each row corresponding to an image and outputs in columns. In our case, an output of the 95th percentile of the ROI was used (Law et al., 2018b) but the code can be easily adapted to produce any output(s) required.

Statistics

As above, the BAT "hotspot" was defined as the pixels with temperature values in the upper decile within each ROI and the output value (TSCV) was the median value of the hotspot, equivalent to the 95th percentile of the region (Law et al., 2018b).

On the first visit, analysers noted how long it had taken them to analyse each complete set of 31 images for each method to determine the mean length of time to analyse each image using the different methods and whether movement of the imaged participant affected the analysis rate. To determine the effect of Method (M or SA) and Set (A: moderate movement or B: little movement) on analysis speed (seconds per image), a 2-way repeated-measures ANOVA was undertaken.

The intra-analyser variability for each researcher and the inter-analyser variability for each image was determined for both methods. Use of the co-efficient of variation was not appropriate as temperature (unless measured in Kelvin) is not measured using a ratio scale. Since, in this context, the mean temperatures are very similar, the population standard deviation, $\sigma = \sqrt{\frac{\sum(X-\mu)^2}{n}}$, of the repeated measures was used as the measure of consistency.

The intra-analyser variability was calculated as the standard deviation of TSCV for the three repeated measures of the eight images in Set A and the eight images in Set B. Two 3-way repeated-measures ANOVAs were undertaken, one for the left ROI and one for the right, to

determine the effect of Method, Set and Analyser. To check the robustness of the results, 2-way repeated measures ANOVA were then undertaken for each combination of Set A and Set B and of the left ROI and right ROI to review the effect of Method and Analyser.

Similarly, the inter-analyser variability, defined as the population standard deviation of TSCV from the third visit of the seven researchers, was calculated for each method for the sixteen images (8 in Set A and 8 in Set B). Two 3-way repeated-measures ANOVAs were undertaken, one for each ROI, to determine the effect of Method, Set and Image. The robustness of the results was checked via the same four 2-way repeated measure ANOVAs as above, to review the effect of Method and Image.

For each analysis, a full factorial model was initially fitted with the equivalent main effect model used subsequently if the interaction term(s) was/were not statistically significant ($p>0.05$). Tukey's post-hoc comparison test was used to determine which levels were statistically significantly different and the direction of the difference.

Statistics were performed using R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

Results

Seven analysers – all post-graduate research students affiliated with the Division of Child Health, Obstetrics and Gynaecology of the University of Nottingham Medical School – completed all three visits.

Speed and errors

The interaction between Method and Set in the 2-way repeated measures ANOVA full factorial model was not significant ($p=0.28$). The subsequent main effects model showed Method was highly significant ($p<0.0001$) and Set tended towards significance ($p=0.08$) (Figure 2). Post-hoc analysis showed SA was significantly faster than M (SA: 8.6 sec/image; M: 63.5 sec/image; difference 54.9 (38.3 to 71.4) sec/image; $p<0.0001$) and that the set of images with movement (Set B) trended towards being significantly faster than the set with more movement between images (Set A: 45.5 sec/image; Set B: 30.8 sec/image; difference 15 sec/image (-2 to 32); $p=0.08$).

In addition, errors were commonly made by analysers when using M. Errors included missing files (in 4 sets), the same region of interest saved as both the left and right file (in 8 sets), and files being duplicated (in 2 sets). In total, 12 out of 42 (29%) sets of images analysed using M had errors identified. One error was identified for the sets analysed using SA, where the threshold temperature had not been set correctly.

Intra-analyser variation

The initial 3-way repeated measures ANOVA full factorial models showed significant interactions between all combinations of Method, Analyser and Set ($p<0.0001$) for both the left and right ROIs. Method ($p<0.0001$),

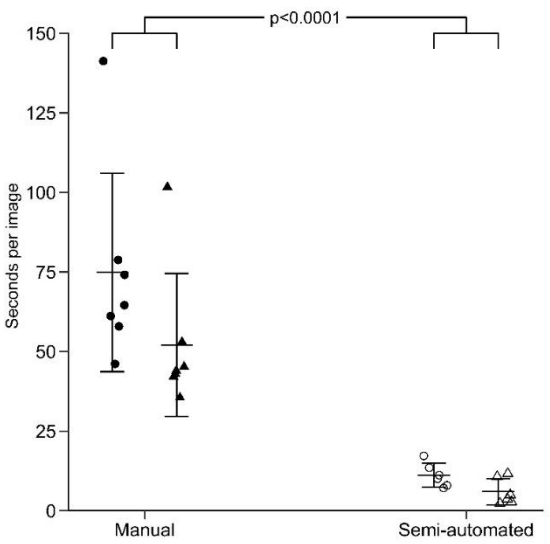

Figure 2 Mean time taken per image for each researcher to analyse 31 images using the manual (filled) and semi-automated (open) methods for two sets of images. Set A (circles) had a moderate amount of movement of the participant between images; Set B (triangles) had very little movement. Lines indicate mean±SD.

Set (p=0.007) and Analyser (p<0.0001) were all independently highly significant for the left ROI and Method (p<0.0001) and Analyser (p<0.0001), but not Set (p=0.733), were highly significant for the right ROI. All four 2-way repeated measures ANOVA full factorial models (Set A: left ROI and right ROI; Set B: left ROI and right ROI) confirmed a significant interaction between Method and Analyser (p<0.0001), i.e. that the effect of Method was different for different analysers, and that Method and Analyser were independently highly significant for both sets and both ROIs (Set A: left ROI p=0.040, right ROI p=0.0001; Set B: left & right ROI p<0.0001).

Post-hoc analysis, as well as simple observation (Figure 3A & C), demonstrated one analyser was a significant outlier and had performed poorly with M (interactions between the outlier and other analysers: p<0.0001; all other interactions: p>0.1). To test the robustness of the results, the analysis was rerun with that data excluded (Figure 3B). A further two analysers had high variability in one and two images using M and a third analyser had high variability in one image using SA (Table 1) which were not excluded.

Repeat analysis of the 3-way full factorial model, with the outlier excluded, showed no significant effect of Method for either ROI (right: p=0.377; left: p=0.596). Set was significant for the right (Set A: σ=0.019; Set B: σ=0.007; difference: 0.012 (0.007-0.017); p<0.0001), but not left (p=0.125), ROI. Analyser trended towards significance on the right ROI (p=0.055) and was significant on the left (p=0.021). Only Set & Analyser showed a significant interaction on the right (p=0.021) but all interactions were significant for the left except Method & Set (Supplementary Material). Two-way ANOVAs confirmed that the interaction between Method & Analyser was not significant for the right ROI (Set A: p=0.779; Set B: p=0.939) but was on the left for Set A (p=0.005) and trended toward significance for Set B (p=0.088). Two-way ANOVAs were, therefore, run both with and

Table 1 Images where intra-analyser variation (population standard deviation) was greater than 0.1.

Analyser	**1**	**1**	**2**	**5**
Image	4	7	7	5
Method	M	M	M	SA
Set	A	A	A	A
Variation (right ROI)	0.141	0.043	<0.001	0.073
Variation (left ROI)	0.189	0.141	0.141	0.108

M: manual method; SA: semi-automated method; ROI: region of interest.

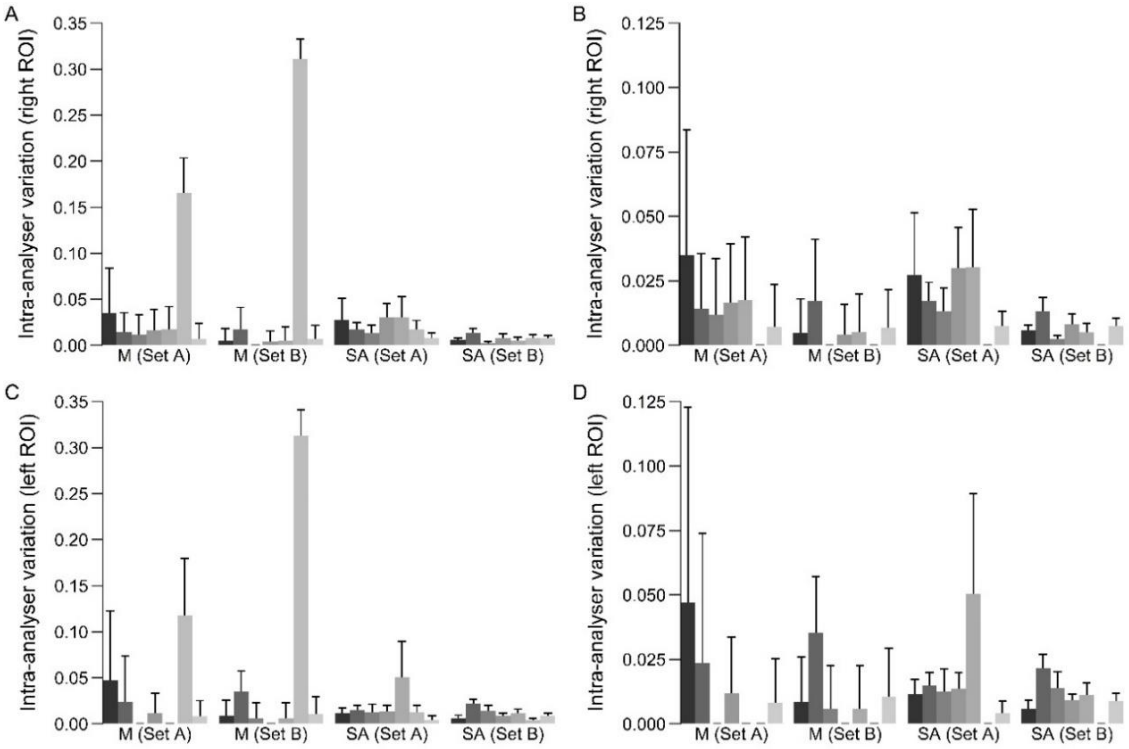

Figure 3 Variation (population standard deviation) in repeated measures of the same image by the each researcher on three occasions (intra-analyser variation) comparing the manual (M) and semi-automated (SA) methods for two sets of images (Set A: minimal movement between images; Set B: moderate movement between images). (A) right region of interest from all analysers and (B) excluding outlier; (C) left region of interest from all analysers and (D) excluding outlier. Column shading represents individual researchers. Data presented as mean+SD of variation.

without the interaction term and the results consistently showed there was no effect of Method on intra-analyser variation but there was an effect of Analyser, except on the left for Set A (Supplementary Material).

Inter-analyser variation

The initial 3-way repeated measures ANOVA full factorial models for both the left and right ROIs showed a significant effect of Method ($p<0.0001$) and Set ($p<0.0001$) (Figure 4A & C) but not Image ($p>0.2$) and a significant interaction only between Method & Set ($p<0.0001$). After removal of the outlier, as above, there remained a significant effect of Set on both the right (set A: $\sigma=0.035$; set B: $\sigma=0.012$; difference 0.023 (0.009-0.037); $p=0.003$) and left (set A: $\sigma=0.048$; set B: $\sigma=0.016$; difference 0.032 (0.012-0.051); $p=0.003$) (Figure 4 B & D) but not of Method ($p>0.4$) and there were no significant interactions (Supplementary Material). Therefore, the main effects model was calculated with consistent results. Similarly, no effect of Method, Image or the interaction term was seen when 2-way full factorial models were calculated for the left and right ROIs for each set separately nor for Method or Image in the 2-way main effects model.After removal of the outlier analyser, one image had high variability (Table 2).

Table 2: Images where inter-analyser variation (population std dev) was greater than 0.1

Image	**7**
Method	M
Set	A
Variation (right ROI)	0.037
Variation (left ROI)	0.141

M: manual method; SA: semi-automated method; ROI: region of interest.

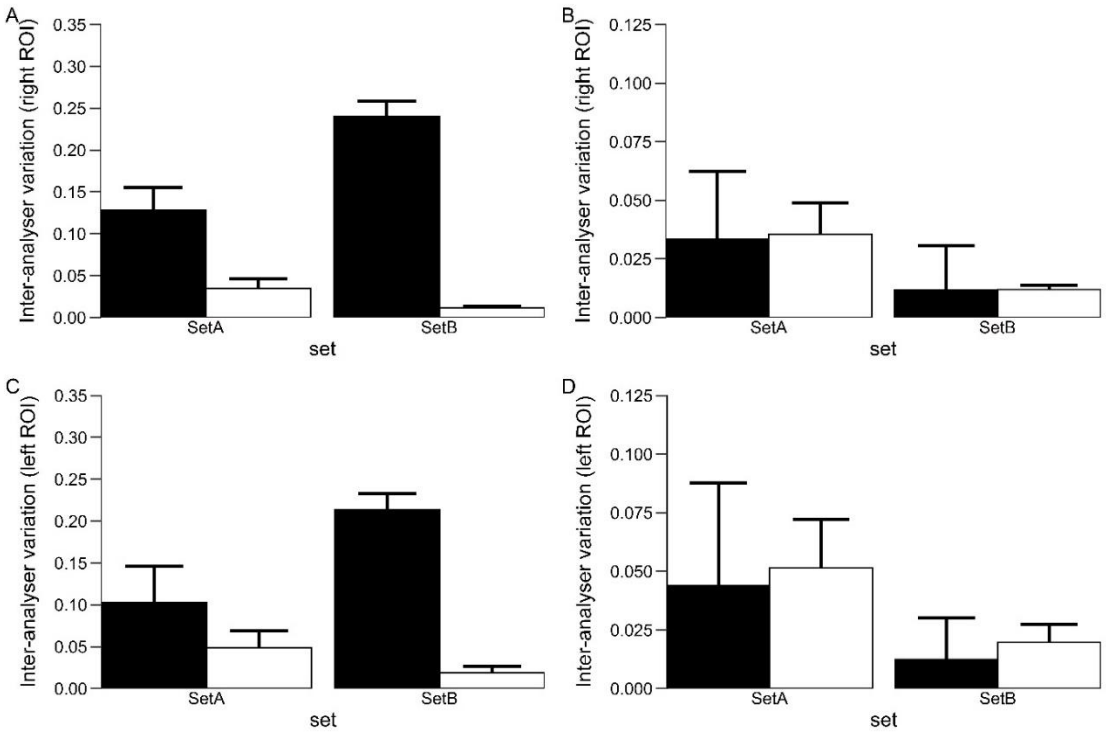

Figure 4 Variation (population standard deviation) in measures of the same image by multiple analysers (inter-analyser variation) comparing the manual method (filled bars) and semi-automated method (open bars) for two sets of images (Set A: minimal movement between images; Set B: moderate movement between images). (A) right region of interest including all analysers and (B) exlcuding outlier; (C) left region of interest from all analysers and (D) excluding outlier. Data presented as mean+SD of variation.

Discussion

SA showed an 86% increase in speed compared to M with no loss of consistency either between analysers or on repeated analysis by the same researcher. Images with more movement between them increased inter and intra-analyser variability and trended towards slower analysis. The relevance of the increase in efficiency is not limited to making it easier for analysers, but rather makes practical the analysis of larger sets of images. This makes longer imaging sessions, studies with more participants and, perhaps most excitingly, studies with increased image acquisition frequency a realistic prospect. The latter has the potential to include analysis of thermal videos. By averaging the raw pixel data of rapidly repeated images, the noise can be reduced enhancing the detection of subtler signals, like the methods used for MRI and CT image acquisition. Smaller temperature changes and cyclical patterns within the overall trend may then be able to be detected, presenting opportunities to detect hitherto undetectable responses. Further work, not presented here, has taken the automated analysis one-step further to create a fully automated system, capable of analysing hundreds of frames per minute. As well as the efficiency savings, the fully automated system will precisely track the anatomical position of the apices image-by-image, where the human user must decide whether the point has moved from the previous image and whether an adjustment is required.

Despite training prior to analysing these sets of images, one of the seven analysers had a distinctively higher variability when using M

and a further two analysers had higher variability ($\sigma>0.1$) in one or more images. In contrast, only one analyser had a high variability for a single image analysed using SA. Even after removal of the outlier analyser, one image analysed with M had a high inter-analyser variation, but none analysed using SA did. There is, therefore, an indication of fewer outliers being generated when using SA. Although the difference in favour of SA was lost when the outlying analyser was excluded, there was no evidence of any increase in either in inter or intra-analyser variability, accepting that the possibility of a false-negative conclusion must be considered due to the pragmatic nature of the analysis.

There were some significant differences between the findings for the left and right ROI, particularly in the interaction terms. Due to the small sample size, there should be caution determining that an interaction is not significant due to a non-significant p-value, hence both full and main effects models were compared for robustness. However, the intra-analyser interaction between Set & Analyser was significant for both sides, suggesting how well each analyser performed was variable depending on whether they were analysing images where the participant moved a little or a moderate amount. Indeed, set was frequently found to be significant with both more inter and intra-analyser variability and slower analysis when the participant was moving between images. This emphasises the importance of optimising image quality and consistency during the acquisition process as results can be degraded in an unpredictable manner and may be one of the factors differentiating groups who are able to consistently utilise thermal imaging successfully. Furthermore, Analyser was significant in all intra-analyser 2-way ANOVAs, except the left ROI for set A, indicating that Analysers tended to be consistent in the amount of variability between repeated analyses. Further work could examine whether analysers with higher variability could improve their results with further training but, again, this highlights a further source of variability which may affect results.

Further work will consider the options to reduce variability further. On some images, body habitus can make it difficult to identify the anatomical landmarks retrospectively. We have, therefore, developed thermally opaque skin markers which can be placed on the participant prior to the start of image acquisition. We expect this to increase the accuracy and consistency of the choice of apical placement by analysers at a cost of slowing down analysis if analysers make multiple small adjustments to markers because all little movements between images are more evident. We have, therefore, developed a fully automated method capable of detecting marker placement.

In conclusion, our novel semi-automated method drastically improves the speed of image analysis and reduces the risk of errors without increasing variability and should, therefore, be used in preference to manually defined complex polygons. Use of this method

allows larger studies, longer imaging periods and increased image acquisition frequency which will allow novel study designs to further understand BAT function.

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Data availability: The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request. Original images are not available to preserve the confidentiality of participants.

Resource availability: The software used for infrared thermographic analysis in the current study is available from the corresponding author upon reasonable request

3.2 Validation of Infrared Thermography for Measurement of Brown Adipose Tissue Activity in Humans

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Thermal Imaging Is a Noninvasive Alternative to PET/CT for Measurement of Brown Adipose Tissue Activity in Humans

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Obesity and its metabolic consequences are a major cause of morbidity and mortality. Brown adipose tissue (BAT) utilizes glucose and free fatty acids to produce heat, thereby increasing energy expenditure. Effective evaluation of human BAT stimulators is constrained by the current standard method of assessing BAT—PET/CT—as it requires exposure to high doses of ionizing radiation. Infrared thermography (IRT) is a potential noninvasive, safe alternative, although direct corroboration with PET/CT has not been established. **Methods:** IRT and ^{18}F-FDG PET/CT data from 8 healthy men subjected to water-jacket cooling were directly compared. Thermal images were geometrically transformed to overlay PET/CT-derived maximum intensity projection (MIP) images from each subject, and the areas with the most intense temperature and glucose uptake within the supraclavicular regions were compared. Relationships between supraclavicular temperatures (T_{SCR}) from IRT and the metabolic rate of glucose uptake (MR(gluc)) from PET/CT were determined. **Results:** Glucose uptake on $MR(gluc)_{MIP}$ was found to correlate positively with a change in T_{SCR} relative to a reference region ($r^2 = 0.721$; $P = 0.008$). Spatial overlap between areas of maximal $MR(gluc)_{MIP}$ and maximal T_{SCR} was 29.5% ± 5.1%. Prolonged cooling, for 60 min, was associated with a further T_{SCR} rise, compared with cooling for 10 min. **Conclusion:** The supraclavicular hotspot identified on IRT closely corresponded to the area of maximal uptake on PET/CT-derived $MR(gluc)_{MIP}$ images. Greater increases in relative T_{SCR} were associated with raised glucose uptake. IRT should now be considered a suitable method for measuring BAT activation, especially in populations for whom PET/CT is not feasible, practical, or repeatable.

Key Words: brown adipose tissue; thermal imaging; infrared thermography; PET/CT

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Obesity is a leading global health concern, with no effective drug treatments currently available. An adverse metabolic profile, including insulin resistance and raised blood lipids, is a common pathway to many of the consequences of obesity. Brown adipose tissue (BAT) is a major effector of adaptive thermogenesis and an attractive antiobesity drug target (*1*). It is a highly metabolically active organ that utilizes glucose and free fatty acids and is able to release chemical energy efficiently as heat by uncoupling mitochondrial respiration from adenosine triphosphate production (*2*). Increased BAT activity results in raised energy expenditure, improved glycemic control, and an improved blood lipid profile (*3*).

In recent years, after the rediscovery of BAT in adult humans (*4–8*), it has been the focus of intense research. However, replication of the promising results from animal studies has been slow (*9*). This is due, in part, to difficulties in measuring BAT activity directly in humans. Because of the variable anatomic position of BAT, close to major vessels, safe and routine biopsy is difficult, causing imaging to become the preferred method of BAT quantification. The standard method of BAT imaging remains PET/CT, which exposes participants to a significant radiation dose and is therefore not suitable for studies with repeated measures, large numbers of healthy volunteers, or children. Measurements using PET/CT are also limited to fasting subjects (*10*).

An alternative imaging technique, infrared thermography (IRT), makes use of the heat-emitting properties of BAT and the relatively superficial position of the supraclavicular depot, one of the largest BAT depots in adults (*7*). Using IRT, several research groups have shown a specific rise in supraclavicular temperatures (T_{SCR}) after introduction of a cool stimulus (*11–14*). IRT has the advantage of being able to measure real-time activation and can be used to gather repeated measures in large numbers of healthy subjects irrespective of age and nutritional status (*11*).

To date, IRT has not been validated against the current gold standard, ^{18}F-FDG PET/CT. Although the results from multiple previous IRT studies are consistent with the measurement of BAT, the lack of data directly comparing results between PET/CT and IRT in the same subjects has remained a limitation of the technique. Using novel IRT analysis methods, as well as PET/CT analysis techniques additional to those used by Salem et al. (*13*), we explored the anatomic and functional relationship between alternative measures of BAT in individual participants. For the first time, to our

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knowledge, we show here that the area identified as overlying BAT using IRT closely corresponds to the area of maximal glucose uptake on PET/CT and that the higher the glucose uptake on PET/CT the better the agreement.

MATERIALS AND METHODS

Subjects

To determine the correlation between BAT activity measured by PET/CT and IRT, 8 healthy men known to be BAT-positive on PET/CT (*13*) (mean age, 23.5 y; range, 18–35 y; mean body mass index, 22.0 kg/m^2; range 19.3–23.0 kg/m^2) underwent PET/CT and IRT sessions as part of a study approved by the London Central Ethics and Research Committee (13/LO/0925), registered with ClinicalTrials.gov (NCT01935791), and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from subjects before enrollment in the study. Participants who were BAT-negative on the initial PET/CT scan (*13*) were not included in the analysis because of the small size of the group ($n = 3$).

Study Visits

As previously described (*13*), participants attended an initial IRT session and an initial PET/CT session. For both visits, the volunteers wore a pair of light cotton trousers with a cooling vest surrounding the torso, away from the supraclavicular region. After acclimatization, cold water at 8°C (*13*) was pumped through the cooling vest to stimulate BAT.

In summary, during the thermal imaging session, images were captured during acclimatization; over the first 10 min of cooling (initial period), which represents the period of maximal BAT activation from the resting state (*12*); and after prolonged stimulation (final period) (Fig. 1). Images were acquired using a FLIR T440bx infrared camera (FLIR Systems). During the PET/CT visit, 180 MBq of ^{18}F-FDG were injected after water-vest cooling, and a 60-min dynamic emission scan was obtained with an axial field of view from mandible to mid thorax (*13*).

All volunteers attended a second thermal imaging session without cold stimulation (IRT vehicle session), and 4 volunteers who were BAT-positive on the first PET/CT scan underwent a further PET/CT imaging session, without cold stimulation (PET/CT vehicle session). Thermal and PET/CT images were acquired as before.

Analysis of IRT

Thermal images (TIs) acquired by IRT at 5-s intervals were analyzed from each period of imaging for both IRT sessions. We developed a semiautomated method for analysis of TIs to allow the efficient analysis of large numbers of images in a systematic and reproducible manner by first converting the TIs to the nonproprietary Portable Network Graphic format, then identifying the supraclavicular regions of interest (ROIs), and finally processing the data within the ROIs.

The TIs were converted using our custom-built Thermal-Imaging Technical Conversion Hub, a Raspberry Pi–based device that extracts the raw data from the TI and saves it in an openly accessible format from which temperature data can be calculated (*15,16*). Our method for converting the raw radiometric data to temperature data produces results identical to those obtained by FLIR's proprietary software (data not shown).

Left and right supraclavicular ROIs were defined as previously described (*12*). In contrast to previous methods (*12*), this simply required each image (Fig. 2A) to be labeled with 5 key points, corresponding to the apices of the ROIs (Fig. 2B). The medial and inferior borders of the ROI were defined as straight lines between the appropriate apices. The contour of the neck was defined programmatically by identifying the temperature gradient between the volunteer and the background. This approach represents a significant improvement on previously published methods, in which the lateral border of the ROI is approximated by a simple straight line (*17*) or is painstakingly identified between manually plotted points (*12*).

The hottest 10% of points within each ROI were identified, and the medians of these points were calculated (equivalent to the 95th percentile) (*11,12,18*). Corresponding graphical output allowed clear visualization of the identified hotspot (Fig. 2B). A reference region consisting of a circle 10 pixels in diameter immediately below the central apex of the ROIs was analyzed for comparison. A moving average (period 5) was applied to the resulting time series to reduce the effect of natural variation in measurements. The main outcome measures for IRT were base T_{SCR} (mean of the first minute of stimulation), peak T_{SCR} (maximal T_{SCR} within a given period), and ΔT_{SCR} (peak T_{SCR} − base T_{SCR}), all calculated for the right ROI (*11*) over the first 10 min of cooling relative to the reference region (*11,18*). Secondary outcomes included analysis of absolute T_{SCR} values as well as comparison of left and right ROIs.

Videos of change in skin temperature over time, relative to the baseline image, were compiled. Pixels were averaged over 3 sequential images, and sequential frames were registered to the baseline image by applying an automatically calculated geometric transformation consisting of translation, rotation, and scale. Unchanged pixels were displayed as white, colder pixels as progressively blue and then black, and warmer pixels as progressively red and then yellow (Supplemental Video 1; supplemental materials are available at http://jnm.snmjournals.org).

Analysis of PET/CT

Regional estimates of the metabolic rate of glucose [$MR(gluc)_{BAT}$] were calculated as previously described (*13*). In addition, 2 coronal maximum-intensity-projection (MIP) images for each volunteer were calculated from the PET/CT data for each visit, one for the CT image (CT_{MIP}) and one for the MR(gluc) values ($MR(gluc)_{MIP}$) (*19*). MIP images are 2-dimensional representations of 3-dimensional data, with the value of each point determined by the maximum intensity of the data along a point-line perpendicular to the projected plane, that is:

$$CT_{MIP}(x, y) = \max_z(CT(x, y, z)), \text{and}$$

$$MR(gluc)_{MIP}(x, y) = \max_z(MR(gluc)(x, y, z)).$$

A composite of the 2 MIP images was used in the graphical output (Fig. 2). The region of the greatest 10% of $MR(gluc)_{MIP}$ was identified, and the median of these values was calculated for comparison with the results of IRT.

PET/CT/IRT Comparison

To identify similarities and differences between the data acquired from PET/CT and the data acquired from IRT, the TI was warped onto the $MR(gluc)_{MIP}$ image from the same participant (Fig. 2). The comparative TI was a mean composite of the last 3 images

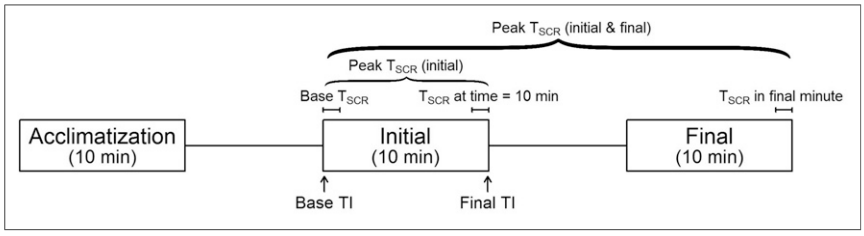

FIGURE 1. IRT imaging protocol annotated with IRT outcomes. Acclimatization = imaging period during acclimatization; initial = imaging period during first 10 min of stimulation; final = imaging period after stimulation for ~1 h.

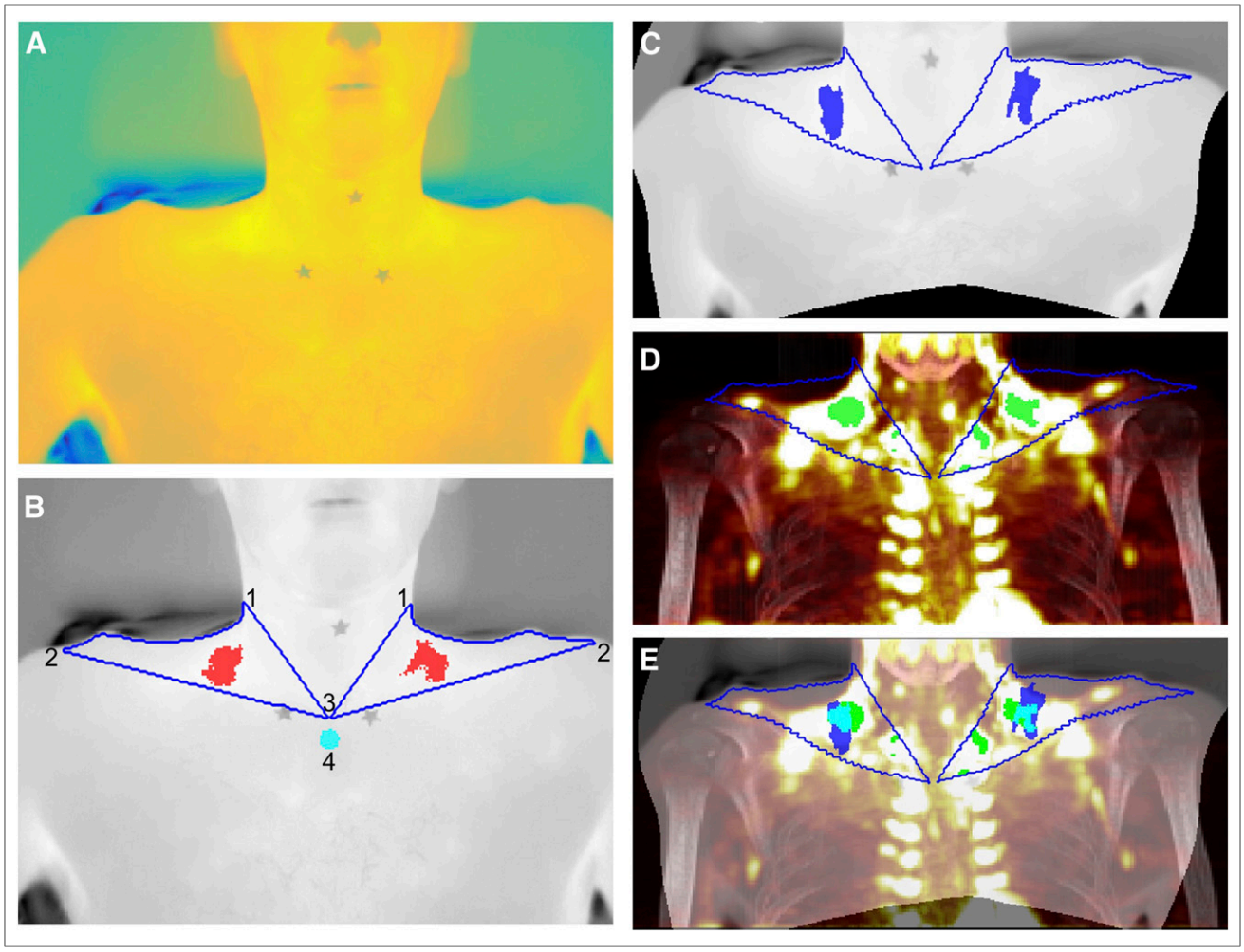

FIGURE 2. Mapping of TI to PET/CT. (A) Apices of ROIs identified on original TI. (B) Contour of ROI (blue) and hottest 10% of pixels (red) identified, with contour of neck precisely defined using automated process: left and right superolateral apices (1), acromioclavicular apices (2), sternal apex (3), and reference area (4). (C) TI after warping by lwm transformation calculated after identification of control-point pairs. (D) Warped contour superimposed on MIP with most intense 10% of pixels highlighted (green). (E) Final composite image with warped TI superimposed on MIP image and hottest pixels from TI (blue), most intense pixels from MIP (green), and overlap (cyan) demonstrating close anatomic proximity.

from the initial imaging session (i.e., after 10 min of stimulation) (Figs. 2A and 2B). The TI and $MR(gluc)_{MIP}$ images were displayed beside each other, and control-point pairs were defined, identifying corresponding anatomic points. Seventy control-point pairs were identified in the supraclavicular region of each pair of images, with additional nonsupraclavicular points depending on the framing of the images. A locally weighted mean transformation (*20*) mapping was created by inferring a second-degree polynomial at each control-point based on the 16 closest points and using a locally weighted average of these polynomials. This mapping was applied to the TI (Fig. 2C).

An ROI was identified on the $MR(gluc)_{MIP}$ defined by applying the locally weighted mean transformation to the contour of the ROI of the TI and superimposing this on $MR(gluc)_{MIP}$. The greatest 10% of values within the ROI were identified for the $MR(gluc)_{MIP}$, and the median of these points was calculated in a similar manner to that described for the TI (Fig. 2D).

The percentage spatial overlap between TI and $MR(gluc)_{MIP}$ hotspots was calculated and displayed as an image (Fig. 2E). Since the TI and $MR(gluc)_{MIP}$ hotspots are, by definition, the same size, the overlap is the number of pixels that the hotspots have in common divided by the total number of pixels of either hotspot).

Statistical Analysis

After conversion of the TI to the Portable Network Graphic format, further graphical analysis was performed using MATLAB, version 2016a (The Mathworks Inc.). The apices were identified using a custom-built graphic-user interface within MATLAB, and a second script was written to undertake the analysis. The inbuilt MATLAB function *imregister* was used to register sequential images to compile the videos, *cpselect* was used to identify control-point pairs, and *fitgeotrans* was used to fit an lwm transformation object to the control-point pairs. Trends in T_{SCR} over time and correlations between variables were analyzed using R: A Language and Environment for Statistical Computing, version 3.2.3 (R Core Team).

RESULTS

TI Analyses

Analyses of TIs from the initial imaging period of the cooling session showed that participants had a base T_{SCR} of 35.1°C ± 0.3°C at the start of stimulation, with a peak temperature of 35.2°C ± 0.3°C during the first 10 min of stimulation (change during first 10 min of stimulation [$\Delta_{10}T_{SCR}$], 0.1°C ± 0.03°C) (Table 1). Base T_{SCR} was

TABLE 1
IRT Analysis: Absolute T_{SCR} and T_{SCR} Relative to Sternal Reference Region

T_{SCR} (°C)	Vehicle session	Cold session	*P*
Absolute			
Base	35.7 ± 0.07	35.1 ± 0.29	0.09
Peak (initial)	35.8 ± 0.06	35.2 ± 0.29	0.09
Change over first 10 min of cooling	0.1 ± 0.03	0.1 ± 0.03	0.64
Peak (initial and final)	35.9 ± 0.08	35.4 ± 0.25	0.06
At 10 min of cooling	35.6 ± 0.07	35.1 ± 0.30	0.11
In final minute of cooling	35.7 ± 0.08	35.3 ± 0.25	0.09
Relative			
Base	1.3 ± 0.13	1.9 ± 0.24	0.01
Peak (initial)	1.4 ± 0.13	2.2 ± 0.27	0.01
Change over first 10 min of cooling	0.2 ± 0.03	0.3 ± 0.05	0.04
Peak (initial and final)	1.5 ± 0.14	2.7 ± 0.29	0.001
At 10 min of cooling	1.4 ± 0.14	2.2 ± 0.26	0.004
In final minute of cooling	1.4 ± 0.13	2.6 ± 0.27	0.0004

Data are mean ± SEM from 8 men.

1.9°C ± 0.2°C above the sternal reference region, with a peak difference of 2.2°C ± 0.3°C during the first 10 min of stimulation ($\Delta_{10}T_{SCR}$, 0.3°C ± 0.05°C). The peak T_{SCR} of the initial imaging period alone was significantly lower than the peak T_{SCR} during the initial and final imaging periods combined (initial only, 35.2°C ± 0.3°C; both, 35.4°C ± 0.2°C; $P = 0.03$). Similarly, the mean T_{SCR} over the last minute of the initial period of stimulation was significantly lower than the mean T_{SCR} over the last minute of the final imaging session (35.1 ± 0.3°C vs. 35.3 ± 0.2°C, respectively; $P = 0.03$).

There were trends toward a decreased base T_{SCR} from the vehicle compared with the cooling session (vehicle, 35.7°C ± 0.07°C; cooling, 35.1°C ± 0.3°C; $P = 0.09$) and lower peak T_{SCR} (vehicle, 35.8°C ± 0.06°C; cooling, 35.2°C ± 0.3°C; $P = 0.09$) but no difference in $\Delta_{10}T_{SCR}$ (vehicle, 0.1°C ± 0.03°C; cooling, 0.1°C ± 0.03°C; $P = 0.64$). When compared with the reference region, however, base T_{SCR} (vehicle, 1.4°C ± 0.15°C; cooling, 2.1°C ± 0.23°C; $P = 0.008$), peak T_{SCR} (vehicle, 1.5°C ± 0.15°C; cooling, 2.4°C ± 0.26°C; $P = 0.006$), and $\Delta_{10}T_{SCR}$ (vehicle, 0.1°C ± 0.03°C; cooling, 0.3°C ± 0.04°C; $P = 0.007$) were all significantly increased by cooling.

Overlap

Representative images of the process are shown in Figure 2. TI hotspots and $MR(gluc)_{MIP}$ hotspots were in the same anatomic area of the ROIs (Fig. 2E). The overlap between the area of maximal glucose uptake on $MR(gluc)_{MIP}$ and the warmest pixels on TI was 29.5%, respectively (range, 11.6%–55.5%; Table 2). Images from participants who underwent a PET/CT vehicle session demonstrated that, in the absence of cooling activation of BAT, the overlap between the hotspots was significantly less (cooling, 27.9% ± 6.2% overlap; vehicle, 6.3% ± 3.1% overlap; $P = 0.009$) (Table 2).

Relationship Between Hotspots on PET/CT and IRT

The linearity of the relationship between the IRT and PET/CT outcomes from cooling sessions is given in Table 3. There were strong positive correlations between relative $\Delta_{10}T_{SCR}$ and both $MR(gluc)_{MIP}$ ($r^2 = 0.721$; $P = 0.008$) and $MR(gluc)_{BAT}$ ($r^2 = 0.583$; $P = 0.027$). Absolute T_{SCR} measurements and relative base and peak T_{SCR} did not significantly correlate with $MR(gluc)_{MIP}$ or $MR(gluc)_{BAT}$.

All 4 participants who had both cold-activated and vehicle PET/CT scans had an increase in both relative $\Delta_{10}T_{SCR}$ and $MR(gluc)_{MIP}$ during cooling, compared with the control session (Fig. 3).

TABLE 2
Percentage Spatial Overlap Between MIP and TI

Participant	Overlap (%)	
	Cold	Vehicle
A	31.6	
B*	11.6	0.1
C*	36.6	8.4
D	55.5	
E	14.3	
F*	38.5	14.1
G*	24.9	2.6
H	22.9	
Mean ± SEM	29.5 ± 5.1	
*Mean ± SEM	27.9 ± 6.2	6.3 ± 3.1

Individual values from 8 men showing percentage spatial overlap between area of maximum supraclavicular MR(gluc) on $MR(gluc)_{MIP}$ image and area of maximum T_{SCR} on TI. Overlap for participants who underwent both cold and vehicle PET/CT scans (*) was significantly reduced in vehicle sessions (cold, 35.4% ± 5.1%; vehicle, 4.7% ± 2.8%, $P < 0.001$).

TABLE 3
Correlation Between IRT Outcome Variables and PET/CT Measures

Parameter	IRT outcome (right ROI)	MR(gluc)$_{MIP}$	MR(gluc)$_{BAT}$
Relative	Base T_{SCR}	0.280 (NS)	0.003 (NS)
	Peak T_{SCR} (initial)	0.386 (NS)	0.032 (NS)
	$\Delta_{10}T_{SCR}$	0.721*	0.583†
Absolute	Base T_{SCR}	0.118 (NS)	0.443‡
	Peak T_{SCR} (initial)	0.087 (NS)	0.406‡
	$\Delta_{10}T_{SCR}$	0.214 (NS)	0.056 (NS)

*$P < 0.01$.
†$P < 0.05$.
‡$P < 0.1$.
NS = not statistically significant.
Values are r^2, square of Pearson coefficient for 8 male volunteers.

Stronger correlations with PET/CT outcomes were seen for the right ROI than the left (Table 4). The left ROI demonstrated a strong positive correlation between MR(gluc)$_{MIP}$ and relative $\Delta_{10}T_{SCR}$ ($r^2 = 0.698$; $P = 0.010$) but not with other relative IRT outcomes or with MR(gluc)$_{BAT}$.

DISCUSSION

IRT is increasingly being used to assess BAT activation and has found an important role in the imaging of healthy volunteers and children, in repeated imaging, and in studies not conducted on fasting subjects (*10*)—situations in which exposure to ionizing radiation must be minimized. Despite this increasing use, definitive evidence that IRT can measure BAT activity has been lacking. Even studies that have utilized both techniques have done so in a way that does not allow direct comparison (*21*). We demonstrate here that there is anatomic overlap of the area of maximum temperature measured using IRT and the projected area of maximum glucose uptake on PET. IRT correlates strongly with MR(gluc) from PET/CT. Indeed, since ^{18}F-FDG PET/CT measures glucose uptake and not thermogenic capacity per se (*22*), greater correlations would not reasonably be expected.

The measure of BAT function obtained by PET/CT is therefore different from that obtained by IRT: glucose uptake as opposed to heat production. The question of which measure is the optimum index of BAT function remains to be fully answered. We observed that subjects who were BAT-positive on PET/CT exhibited a similar magnitude of BAT function on IRT. Furthermore, PET/CT is usually conducted on fasting subjects, because after the subject eats, uptake in BAT is largely masked by the much greater uptake in skeletal muscle (*10*). It has been suggested that eating, per se, stimulates BAT function (*23*), and to date, we have been able to detect a positive IRT result in all subjects measured ($n > 200$). Taken together, these findings indirectly support those from repeated PET/CT, in which most subjects appear to be BAT-positive on at least one scan (*24*). In addition, as with the reduced BAT activity in obese adults as measured with PET/CT (*25*) we have previously observed a reduction in BAT activity with increasing BMI percentile in children (*11*). Future work comparing differences between IRT and PET/CT measures may indeed offer novel insights into different components of BAT activation.

Previous papers have often used absolute T_{SCR} as the primary outcome. However, many factors may affect T_{SCR} and even cause it to fall (*18*). In many cases, the magnitude of the effect on T_{SCR} is sufficient to overcome any counteracting factors, but it is clear that true, relative warming is best demonstrated by comparing the T_{SCR} with a reference region. We have used a region on the sternum, but alternatives, such as mean skin temperature (*18*), may allow more subtle effects to be revealed. However, even with the addition of a simple reference region, the true relationship between the two

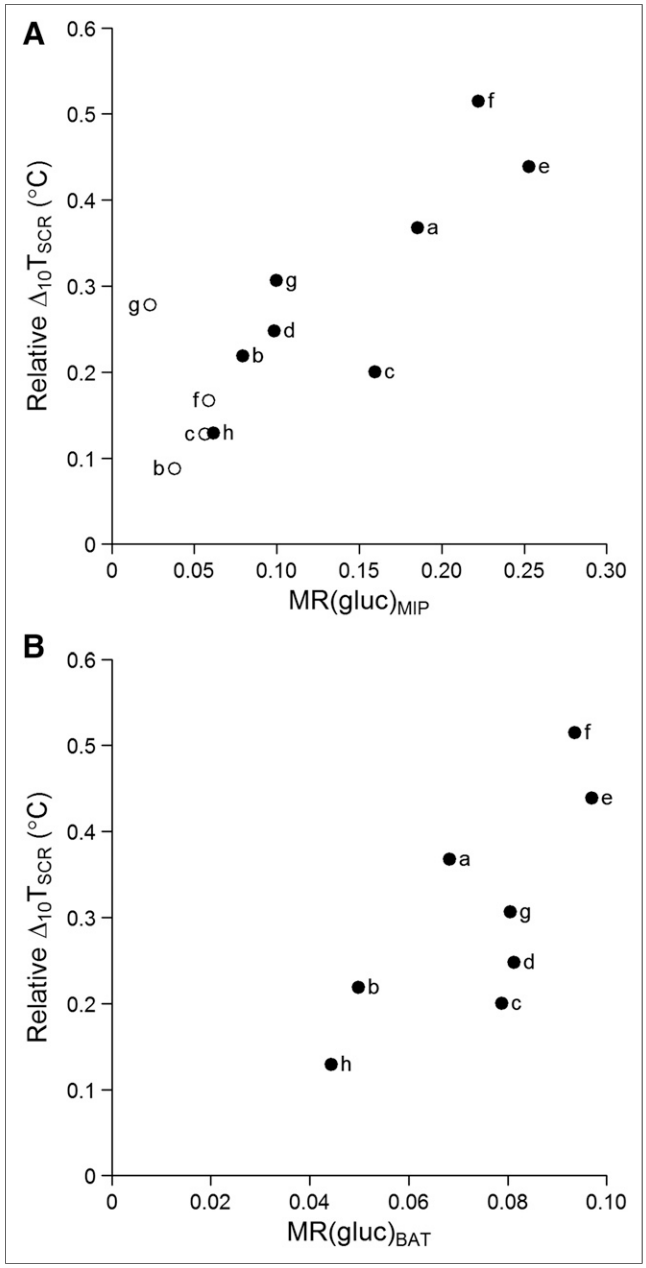

FIGURE 3. $\Delta_{10}T_{SCR}$ (relative to sternal reference) against glucose uptake on PET/CT. Correlation is shown between BAT activity measured with IRT, median MR(gluc)$_{MIP}$ hotspot value ($r^2 = 0.721$; $P = 0.008$) (A), and MR(gluc)$_{BAT}$ ($r^2 = 0.583$; $P = 0.027$) (B). ○ = control; ● = cooling.

TABLE 4
Correlation Between IRT Outcome Measures for Left and Right ROIs and PET/CT Measures

PET/CT outcome	IRT outcome (relative to reference region)	Left ROI	Right ROI
$MR(gluc)_{MIP}$	Base T_{SCR}	0.172 (NS)	0.280 (NS)
	Peak T_{SCR} (initial)	0.239 (NS)	0.386*
	$\Delta_{10}T_{SCR}$	0.698†	0.721†
$MR(gluc)_{BAT}$	Base T_{SCR}	0.001 (NS)	0.003 (NS)
	Peak T_{SCR} (initial)	0.002 (NS)	0.032 (NS)
	$\Delta_{10}T_{SCR}$	0.337 (NS)	0.583*

*$P < 0.05$.
†$P < 0.01$.
NS = not statistically significant.
Values are r^2, square of Pearson coefficient for 8 male volunteers.

measures of BAT activity becomes evident. This is perhaps not surprising when videos of change in skin temperature relative to baseline are viewed (Supplemental Video 1). These show the specific warming of the supraclavicular region relative to surrounding skin temperatures and, therefore, the importance of framing the participant to include the superior portion of the sternum as a reference.

We have previously seen a greater rise in the T_{SCR} of the right ROI (*11*). In addition, we show here that the outcome measures using the right ROI correlate better with BAT glucose uptake on PET/CT than do the outcome measures using the left ROI or a combined measure. The reasons merit further investigation and could be due to either anatomic or functional differences. There is no evidence of a difference in the volume of BAT between the left and right sides on PET/CT (*26*), but functional symmetry has not been investigated in non-IRT modalities, which could reveal whether asymmetry represents a true difference in activity or, for instance, an artifact or a slight difference in the anatomic position of BAT between the left and right sides.

Base T_{SCR} was measured as an average of the first minute of stimulation. That T_{SCR} is already higher in participants during the cold session demonstrates the rapidity of the BAT response to a cold stimulus. We show here that the response seen within the first 10 min is sufficient to ascertain an individual's BAT activity, correlating well with glucose uptake on PET/CT; however, contrary to previous reports (*12*), prolonged stimulation is associated with a further rise in T_{SCR}. In addition, a proper acclimatization period is essential for the accurate measurement of BAT activity using IRT and our data (not published) show that this period should be 20 min, during which time the participant should be in the same environment and clothing as for the study and remain as still as during imaging.

The area of maximal glucose uptake on a coronal projection MIP image from ^{18}F-FDG PET/CT closely corresponds to the warmest area within the supraclavicular ROI measured using IRT. In the 8 participants known to be BAT-positive on PET/CT, a high degree of overlap was demonstrated between the most intense glucose uptake on PET/CT and the warmest area on IRT.

The main limitation of these analyses is the small number of participants available, reducing the power to detect correlations between IRT and PET/CT outcomes. Larger studies are not ethically feasible, especially in healthy volunteers, because of the risks of exposure to the ionizing radiation associated with PET/CT. We recommend that studies using IRT to measure BAT activity should report the right-sided ROI relative to a reference region, and authors should include a statement about the acclimatization period that was used.

Advances in IRT are allowing for more detailed assessments of changes in superficial temperature associated with changes in the underlying BAT. Radiometric sensors are becoming increasingly accurate, with some sensors now able to distinguish a change of only 0.01°C, and the resolution of images is improving, with cameras able to acquire truly high-definition images. These improvements will allow the subtler changes to be better defined, especially when combined with more sophisticated calculations of reference values for mean skin temperature. To take full advantage of these improvements, together with improvements in image acquisition rates, a fully automated analysis method should be considered. In addition, an automated standardized analysis method would reduce variability between groups. The establishment of IRT as a valid method of measuring supraclavicular BAT activity in humans will make studies feasible that until now have not been possible.

CONCLUSION

IRT provides a safe, credible, and quantifiable alternative to PET/CT that can now be used in a wide range of population groups for the measurement of BAT activity.

DISCLOSURE

No potential conflict of interest relevant to this article was reported.

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3.3 Cold-Water Swimming

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Research Article

Cold-Water Swimming Activates Brown Adipose Tissue in Humans as Detected by Infrared Thermography

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Abstract

Objective: Brown adipose tissue is a thermogenic organ with an important life-long role in temperature and metabolic regulation. Enhanced brown adipose tissue activity is a possible treatment modality for obesity and diabetes. We report a unique "real-life" field study of enhanced activity of brown adipose tissue in response to the acute cold challenge from open-water swimming.

Methods: Participants were healthy, non-smokers recruited from those attending a Chill-Swim® 250m open-water (temperature 7˚C) swimming event on Lake Windermere, UK on 11thDecember 2016. Resting brown adipose tissue activity was assessed using infrared thermography centered on the supraclavicular region, together with baseline heart rate, blood pressure and tympanic membrane temperature. Repeat observations and thermal imaging were undertaken immediately after completion of the swim.The study group consisted of two men and two women with a body mass index of 25.8±2.0 kg/m^2.

Results: The decline in temperature of the brown adipose tissue region was substantially less that of other areas following the cold-water swim. This resulted in significantly higher supraclavicular temperature relative to a sternal reference region (3.5±1.6˚C; p=0.02), indicating activation of supraclavicular brown adipose tissue.

Conclusion: Regular exercise in cold water could be a strategy to increase brown adipose tissue activity and thus confer improved metabolic health on participants. It would thus be a further life-style change that could aid the prevention of obesity.

Keywords: Brown Adipose Tissue; Thermal Imaging; Thermogenesis; Water

Introduction

Brown adipose tissue (BAT) is a thermogenic organ with an important role in temperature regulation and glucose homeostasis [1]. It is maximally functionalat birth and its activity gradually decreases with age [2]. The primary BAT depot in adult humans is the supraclavicular one [3] where changes in skin surface temperature can be used to assess functional responses [4, 5]. Raised temperature of these supraclavicular depots reflects an increase in the activity of uncoupling protein (UCP)1 [6], located on the inner membranes of mitochondria within brown adipocytes [7]. When activated, UCP1 uncouples adenosine triphosphate production from mito-

chondrial respiration[7], thereby releasing physiologically significant amounts of energy as heat [8].

Reduced BAT is associated with obesity [9]. Recently, a temporal association between rising global environmental temperatures and increased prevalence of diabetes, irrespective of obesity, has been suggested [10]. Such a relationship could be mediated by reduced BAT activity. Positron emission tomography-computed tomography (PET-CT) studies have shown that BAT glucose uptake (considered a measure of BAT activity) can be stimulated by acute cold exposure [11-13], although this response may be confined to individuals who are more responsiveto cold and, thus, defined as 'BAT positive'. Cold-water swimming, of sufficient intensity and duration, leads to lower blood glucose and insulin concentrations [14] and an early increase in oxygen uptake [15]. Rodent studies support an increase in BAT activity following cold-water swimming [16].

The assessment of assess BAT activity PET-CT imaging techniques is constrained by the need for participants to be exposed to appreciable amounts of radiation, to be fasted and to remain within the medical facility in which the detector is located. These constraints are all overcome by the use of thermal imaging [15] which has now been validated in comparison with PET-CT with which it correlates well [17-19]. We, therefore, conducted a proof-of-concept "real-life" study of healthy participants undertaking swimming in cold water on the same winter's day in the United Kingdom and demonstratedthe feasibility of measuring BAT activity outside of the laboratory setting. It was hypothesized that there would be an acute increase in BAT activity after the cold-water swim, providing an index of the maximal thermogenic capacity of BAT following an acute physiological challenge.

Methods

Ethical Approval

The study was approved by the University of Nottingham, School of Medicine Medical Ethics Committee and undertaken in accordance to the revised Declaration of Helsinki, except for registration in a database. All volunteers provided written, informed consent prior to participation.

Study Design and Participants

Participants were recruited from those attending a Chill-Swim® event, involving a 250m open water swim in Lake Windermere, UK. Any participant who was medically fit was eligible for the study. The study was advertised to those attending the event and study information was provided to those who expressed an interest. All participants underwent a brief assessment by a registered medical physician including a targeted medical history and baseline observations. Due to the nature of the event, participants followed their own pre-swim routine and were not required to fast or to avoid any specific food or drinks. The average temperature of the water on the day of the study was 7°C and participants were immersed in the water for 4.8±1.0 minutes for the 250m swim. We needed to study 3 subjects,to give a power 0.8 witha significance level of 0.05to detecta difference of at least 3.5±1.4°C in the change in relative supraclavicular temperature (T_{SCV}) from before to after cold-water swimming.

Procedures

Participants were assessed in the hour preceding the start time of their swim. Following a period of acclimatizationin a room of constant temperature (20°C), baseline heart rate (Radical-7®, Massimo Corporation, Irvine, CA, USA), blood pressure (Microlife BP 3AC1-1, Taipei, Taiwan) and tympanic temperature (ThermoScan® 5, Braun GmbH, Kronberg, Germany) were recorded. Participants wore standard tracksuit bottoms covering their swimming kit; any straps of swimming costumes participants were placed below the shoulder to ensure the upper sternum and ROI were uncovered. Resting BAT activity was assessed as supraclavicular temperature (T_{SCV}) and the temperature of a reference point at the top of the sternum (T_{Ref}) using infrared thermography (IRT) (FLIR T440bx infrared camera, FLIR Systems, West Malling, UK). Measurements were taken at 15 second intervals for 3 minutes and a mean value over the 3-minute period calculated. Thermal imaging data was analyzed as previously described [17, 20, 21]with T_{SCV} representing the median of the hottest 10% pixels within bilateral regions of interest and T_{Ref}, the median temperature of the sternal reference point. Peripheral and central skin temperature over the dorsum of the hand and subclavicular region, respectively, were measured each minute using wired skin probes (M1024254 skin temperature probe, GE Healthcare). Immediately following the swim, participants were re-assessed using the same protocol.

Statistics

Two-tailed paired t-tests were used to compare physiological measurements and BAT parameters pre- and post-cold-water swim. The significance level was set at $p<0.05$. Values are reported as mean ± SD unless otherwise stated. All statistical analyses were performed with R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

Results

Five participants were recruited and underwent baseline imaging. One participant did not complete the swim so was excluded from the analysis. Therefore, two women and two men completed the study. Meanage and BMI were 44.6 (SD 3) years and 25.8 (SD 2.0) kg/m^2, respectively. No participant had fasted. Three of the four participants had consumed caffeine within the preceding 4 hours.

There was a pronounced change of skin temperature in all

subjects after the cold-water swim and thisresulted in a higher temperature of the supraclavicular region relative to other areas of the body (Figure 1). This reflected a much greater reduction in temperature at the sternal reference point compared with the supraclavicular region. This was accompanied by a decline in tympanic membrane (pre: 36.3±0.5˚C; post: 34.2±0.1˚C; p=0.002), central skin (pre: 32.5±0.6˚C; post: 28.1±1.9˚C; p=0.01) and peripheral skin temperatures (pre: 28.0±2.1˚C; post: 21.6±0.4˚C; p=0.01) following cold-water swimming. Consequently, the temperature overlyingthe region in which BAT is located was 3.5±1.6°C higher, relative to the sternal reference point, after cold-water swimming (Figure 2A). In contrast, there was no evidence of a change in the mean difference between central and peripheral skin temperatures (Figure 2B; pre: 4.5±2.6˚C; post: 6.5±2.1˚C; p=0.22). There was no evidence of any significant changes in blood pressure or heart rate (Table 1) althoughthe study was not powered to identify changes in cardiovascular measures.

Participant	Systolic BP (mmHg)		Diastolic BP (mmHg)		Pulse (bpm)	
	Pre	Post	Pre	Post	Pre	Post
A	134	139	78	103	72.1	67.4
B	164	149	99	110	97.9	97.3
C	178	178	95	93	87.5	69.8
D	129	144	70	98	59.4	70.7
Mean ± SD	151±24	152±18	85±14	101±7	79±17	76±14

Table 1. Cardiovascular parameters of four participants pre- and post- cold water swim.
BP: blood pressure; SD: standard deviation; bpm: beats per minute; mmHg: millimeters of mercury. No significant difference was demonstrated between pre and post cold-water swimming measurements.

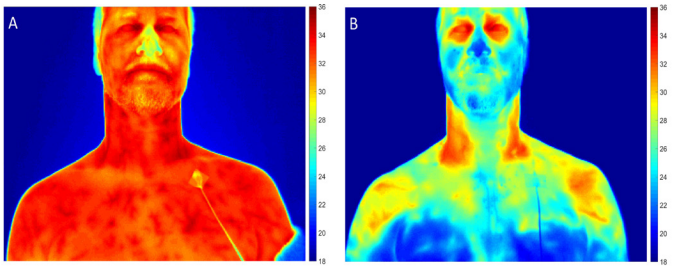

Figure 1. Representative example of a thermal image of the same participant (a) prior to cold water swim and (b) after cold water swim.

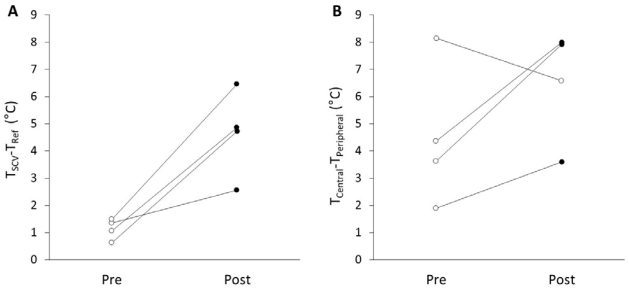

Figure 2. Difference between A) supraclavicular temperature (T_{SCV}) and sternal reference temperature (T_{Ref}) of individual participants before (empty circles; 1.1°C) and after (filled circles; 4.6°C) cold-water swimming (difference 3.5 (1.0-6.0)°C) and B) central (sub-clavicular) and peripheral skin temperatures, before (empty circles; 4.5°C) and after (filled circles; 6.5°C) cold-water swimming (difference 2.0 (-2.2-6.2)°C).

Discussion

We demonstrate in a "real-life" study that the decline in skin temperature following a 250m swim in cold water is significantlyless near supraclavicular BAT than at other sites, indicative of enhanced BAT activity. Consequently, the change in temperature within the supraclavicular region following exposure to cold water was 3.5˚C less than the temperature fall in an adjacent reference point,where relative change in T_{SCV} is correlated with BAT activity measured with PET-CT [17]. The magnitude of the change was sufficient to demonstrate a statistically significant change despite relatively small numbers of participants. The sternal reference point used for comparison is the central anatomical position used by doctors to assess central perfusion. Changes due to cold-induced variation in perfusion between central and peripheral skin regions will,therefore, have minimal effects on T_{SCV}-T_{Ref}. In contrast, a greater difference would be expected between thecentral sub-clavicular skin temperature and the peripheral dorsum of the hand skin temperature but, if anything, there was less difference found. Whilst the lack of a significant change in the central-peripheral skin temperature gap (Figure 2B) is, in part, due to one participant having a higher temperature gap before, and a smaller gap after, the cold water swim, we found no evidence that the change in relative BAT $T_{SCV}$is a result of skin perfusion changes. The latter would be expected to result in the most extreme skin temperature changes at peripheral sites. Interestingly, the participant with the narrowing of the central-peripheral temperature gap had the greatest rise in relative temperature change over BAT.

The small numbers in the study limit the inferences that can be made about cardiovascular measurements but mean systolic blood pressure and pulse were similar before, and after, the intervention. Diastolic blood pressure,representing increased peripheral resistance, was higher, although the difference was not statistically significant as this study was not powered for this secondary outcome. While blood pressure

was high in two participants (Table 1), this is not entirely unexpectedgiven the likely high activity of the sympathetic-adrenal nervous system with "race nerves"[22]. This may have led to increased BAT activity,as seen in pheochromocytoma patients [20] but, importantly,the similar heart rate and systolic blood pressure before, and after, the interventionimplies this is unlikely to have had a significant effect on BAT activation.

Previous studies in a thermoneutral environment have demonstrated around a 1.5˚C change in BAT temperature after either eating a standard meal, following hydrocortisone or isoprenaline [23]. Given the magnitude of cold exposure and decline in core body temperature despite appreciable amounts of shivering observed in participants, it is likely that we recorded the maximal rise in BAT temperature. We have demonstrated the clear effect of anintensemixed stimulation of cold and exercise, but the individual contribution of each component, and whether they interact in an additive or synergistic way, remains to be determined. While the mechanism of BAT activation from cooling is well established in both rodents and humans [24], the mechanism of exercise-induced BAT thermogenesis has been the subject of several recent reviews but remains the subject of debate, at least in humans [25-27]. Rodent studies have provided potential pathways [28-30] and there is much synergy in humans where this has been established [28, 30], providing a strong indication that results from rodent studies may translate, at least in part, to human physiology. Chronic exercise increases beige and brown depots [31], increases UCP1 [29] and improves glucose homeostasis [29]. PGC1α is induced in skeletal muscle of both mice and humans following both short bouts and endurance exerciseand stimulates myokines, such as irisin, cleaved from FNDC5 [28], and meteorin-like [30] which can increase beige induction and thermogenesis.Lipolysis regulation in exercise is controlled by cardiac natriuretic peptides [32-34], which induce UCP1 expression in both brown and beige adipose tissue [35], and lactate, a byproduct of anaerobic exercise, induces browning [36]. While the exact mechanisms and the evolutionary advantage remainto be fully elucidated, it has been suggested that myokines, like irisin, are released proportional to shivering intensity and, therefore,lead to BAT stimulation where continuation of shivering thermogenesis would otherwise lead to inefficient heat production and loss of coordination [37]. Further work would be required to assess whether male and female participants respond differently to either or both components of the stimulation experienced in this study and how the response is moderated by previous exposure to cold-water swimming. The rise in BAT temperature probably reflects an increase in activity of both the sympathetic nervous system [38] and adrenal cortex [20, 39-41] due to both the effect of exercise and the cold exposure, although this did not impact on the cardiovascular system at the time of measurement. That we were able to record a clear increase in relative BAT temperature in all participants emphasizes the vital role of this organ in ensuring effective adaptation to acute cold-water exposure. The clear increase in relative BAT temperature in all participants implies a key role for this organ in adaptation to acute cold-water exposure. In conclusion, our findings offer the potential of an alternative strategy to activating BAT in adult humans which has previously been confined to prolonged daily exposure to a cold chamber [42-47] and paves the way for future studies in which both acute and chronic changes in BAT temperature can be assessed using thermal imaging. These techniques could be combined with assessments of metabolic and endocrine adaptations and undertaken in adults of normal and raised BMI in both the fasted and fed states.

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Disclosure

The authors declare no conflicts of interest.

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3.4 Hypothyroidism

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Brown Adipose Tissue Response to Cold Stimulation Is Reduced in Girls With Autoimmune Hypothyroidism

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Objective: The interaction between thyroid status and brown adipose tissue (BAT) activation is complex. We assessed the effect of autoimmune hypothyroidism (ATD) in female children on BAT activation, measured using infrared thermography.

Design: Twenty-six female participants (14 with ATD and 12 healthy controls) between 5 and 17 years of age attended a single study session. Thermal images were taken of the supraclavicular region before, and after, the introduction of a cool stimulus.

Results: Participants with ATD had lower resting (hypothyroid, 34.9 ± 0.7°C; control, 35.4 ± 0.5°C; $P = 0.03$) and stimulated (hypothyroid, 35.0 ± 0.6°C; control, 35.5 ± 0.5°C; $P = 0.04$) supraclavicular temperatures compared with controls, but there was no difference between groups in the temperature increase with stimulation. BAT activation, calculated as the relative temperature change comparing the supraclavicular temperature to a sternal reference region, was reduced in participants with ATD (hypothyroid, 0.1 ± 0.1°C; control, 0.2 ± 0.2°C; $P = 0.04$). Children with ATD were frequently biochemically euthyroid due to replacement therapy, but, despite this, increased relative supraclavicular temperature was closely associated with increased TSH ($r = 0.7$, $P = 0.01$) concentrations.

Conclusions: Girls with ATD had an attenuated thermogenic response to cold stimulation compared with healthy controls, but, contrary to expectation, those with suboptimal biochemical control (with higher TSH) showed increased BAT activation. This suggests that the underlying disease process may have a negative effect on BAT response, but high levels of TSH can mitigate, and even stimulate, BAT activity. In summary, thyroid status is a complex determinant of BAT activity in girls with ATD.

Freeform/Key Words: autoimmune, brown adipose tissue, cold-induced thermogenesis, hypothyroidism, infrared thermography, thyroid hormones

Abbreviations: ATD, autoimmune hypothyroidism; BAT, brown adipose tissue; BMI, body mass index; IQR, interquartile range; lnTSH, natural log–transformed TSH; PET, positron emission tomography; ROI, region of interest; SDS, SD score; SNS, sympathetic nervous system; TPO, thyroid peroxidase; T_{Rel}, relative supraclavicular temperature; T_{SCV}, supraclavicular temperature; UCP1, uncoupling protein 1; ΔT, change in temperature.

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Since its rediscovery in adult humans in 2009 [1–4], interest in brown adipose tissue (BAT) has increased steadily. BAT has an integral role in adaptive thermogenesis due to its capacity to rapidly generate significant quantities of heat from fatty acids and glucose, facilitated by uncoupling protein 1 (UCP1) in the mitochondrial membrane, allowing the disassociation of ATP production from mitochondrial respiration [5–9]. As heat is eventually lost from the body, this represents a net loss of energy and has the potential to contribute to body weight management. Despite promising results in rodents demonstrating weight loss, improved metabolic profiles, and greater insulin sensitivity following BAT stimulation [10–15], translation to human studies has been slow [16]. Human studies have been limited by high ionizing radiation associated with positron emission tomography (PET)–CT, considered to be the gold standard for imaging BAT. Thermal imaging has been established as a valid alternative [17–20].

As a major regulator of energy expenditure, the thyroid axis can modulate the heat-generating capability of BAT [21, 22]. Thyroid hormones cross the blood–brain barrier [23] and act on the hypothalamus to increase sympathetic nervous system (SNS) activation [24]. Under SNS control, brown adipocytes express elevated iodothyronine deiodinase 2, which converts intracytoplasmic free T4, taken up from the systemic circulation, into its metabolically active form, T3 [25, 26], causing a localized intracellular hyperthyroid environment. Translocation and binding of T3 to its intranuclear thyroid hormone receptor-β stimulates UCP1 transcription and translation [27], leading to heat generation. Reduced thyroid hormone concentrations may, therefore, affect BAT activity directly or by reducing SNS activation centrally. Despite this defined mechanistic pathway, *in vivo* studies in humans are limited and conflicting. In healthy volunteers, BAT activation is not associated with serum thyroid hormone concentrations [28, 29] but is with higher TSH concentrations [29]. BAT activity is increased in patients with hyperthyroidism and returns to normal after treatment [30]. In patients with hypothyroidism, BAT remains present, and indeed may become markedly hypertrophic in the absence of replacement therapy [31]. However, it is not clear whether treatment with T4 increases [32] or decreases [33] BAT activity, although a recent small study in healthy adults demonstrated a negative correlation between plasma free T4 and BAT volume [34].

Thyroid hormones are essential for brain and physical development in early life [35, 36] and continue to be critical through childhood when BAT activity is also increased [37, 38]. Adiposity patterns developed in this period can predict later obesity and metabolic health [39–43]. The influence of thyroid hormones on BAT activity, however, has not been examined in otherwise healthy children. Despite many patients achieving biochemical euthyroidaemia, physiological diurnal variation in TSH and thyroid hormone profiles is not achieved with hormone replacement therapy [44, 45].

Pediatric patients with hypothyroidism, for the most part, either have congenital hypothyroidism or autoimmune hypothyroidism, with the latter being more common in girls than boys. BAT is known to vary between sexes [46, 47], and so, to reduce heterogeneity, we compared girls with a diagnosis of autoimmune hypothyroidism (ATD) who, we hypothesized, would show reduced BAT activation in response to a cool stimulus compared with healthy age and sex-matched controls. We further hypothesized that those in the hypothyroid group who were relatively biochemically hypothyroid would have lower BAT activation than those who were relatively biochemically hyperthyroid.

1. Materials and Methods

A. Participants

To determine the effect of ATD on the response of BAT to a cool stimulus, female children and adolescents (5 to 17 years of age) with a diagnosis of ATD [defined as a TSH level >10 mU/L and anti–thyroid peroxidase (TPO) antibodies levels of >60 IU/L at diagnosis] and no

associated major disease (n = 14) were compared with healthy, age-matched controls (n = 12). All participants successfully completed the study protocol.

Participants with ATD were recruited from the pediatric endocrinology clinic of Nottingham University Hospital NHS Trust (Nottingham, UK). Control participants were either healthy siblings of participants with ATD or were attending the pediatric ear, nose, and throat clinic at the Nottingham University Hospitals NHS Trust for unrelated simple surgical procedures (such as grommet insertion or tonsillectomy). The study was approved by the Nottingham-2 NHS Research Ethics Committee (13/EM/0102) and performed in accordance with the Declaration of Helsinki. Written, informed consent was provided prior to participation from the child or her parent or legal guardian as appropriate. If consent was provided by the parent or legal guardian, the child was invited to provide written assent.

B. Study Sessions

Participants attended a 1-hour study session undertaken in the Academic Child Health Human Physiology laboratory in the Queen's Medical Centre campus of the University of Nottingham. Following informed consent, participants were required to wear a standard light cotton vest (0.06 Clo). A targeted medical history, current medications, and details of the last meal and physical activity during the preceding 24 hours were obtained from the child or her parent or caregiver. Basic anthropometric measurements of height and weight were made using a stadiometer (Seca, Hamburg, Germany) and class III digital weighing scales (Seca 899, Seca), respectively. The child or young person then sat upright, directly opposite a thermal imaging camera (FLIR B425, FLIR Systems, Danderyd, Sweden), which was level with her larynx [48] at a distance of ~1 m to ensure the shoulders remained within the frame while optimizing the proportion of the frame occupied by the region of interest (ROI). Skin temperature probes (M1024254, GE Healthcare, Fairfield, CT) were attached to the dorsum of the left hand and 2 cm below the midpoint of the left clavicle. Heart rate was recorded using pulse oximetry (Radical 7 pulse oximeter, Massimo, Irvine, CA) connected to the index finger of the left hand. The right hand was placed in an empty 5-L receptacle. Following an acclimatization period of at least 20 minutes, baseline images were acquired every 20 seconds for 1 minute followed by the introduction of cool water [median, 18.5°C; interquartile range (IQR), 18.0° to 18.9°C] into the receptacle to cover the right hand of the participant to the level of the distal forearm. A further 5 minutes of images were acquired at the same rate of three per minute. Children were free to stop at any time, but there were no reports of discomfort during the study imaging protocol and all sessions were completed.

C. Image Analysis

Thermal images saved in FLIR's proprietary JPEG format were converted to an openly accessible 16-bit portable network graphics format [18] and the radiometric data were converted to temperature data within MATLAB (2017b, Mathworks, Natick, MA) using a script adapted from Tattersall [49], producing temperature data identical to those produced by FLIR software (ThermaCAM Researcher Pro 2.10; data not shown).

Data were analyzed within the MATLAB programming environment, as previously described [18]. In brief, the image was displayed on a graphical user interface allowing the user to identify five points representing the apices of the supraclavicular ROIs [18, 50]. The outline of the ROI was then calculated as (i) straight lines from the neck and shoulder apices to the central apex and (ii) the shoulder contour between the shoulder and neck apices. This method allowed the accurate, efficient, and reproducible identification of the ROIs (data not shown). Temperature points within the ROIs were then analyzed to identify the hottest 10%, corresponding to BAT [18]. The supraclavicular temperature (T_{SCV}) was defined as the median temperature of the hotspot (equivalent to the 95th percentile of the ROI). Additionally, the median temperature of a circular reference region on the sternum, directly inferior to the central apex, with a diameter of 10 pixels was calculated.

D. Hormone Analysis

Plasma thyroid hormones and TSH were measured using Siemens ADVIA Centaur immunoassay systems with the following kits: FT3 assay no. 03154228 (119781) (reference ranges: 2 to 12 years, 5.1 to 7.4 pmol/L; 13 to 20 years, 4.7 to 7.2 pmol/L; intra-assay coefficient 2.6%), FT4 assay no. 06490106 (reference ranges: 2 to 12 years, 11.1 to 18.1 pmol/L; 13 to 20 years, 10.7 to 18.4 pmol/L; interassay coefficient 2.9%; intra-assay coefficient 2.7%), and TSH3-Ultra assay no. 06491080 (reference ranges: 2 to 12 years, 0.67 to 4.16 mU/L; 13 to 20 years, 0.48 to 4.17 mU/L; interassay coefficient 2.5%, intra-assay coefficient 2.3%). Anti-TPO antibodies were measured with the kit no. 10630887 (interassay coefficient 3.1%, intra-assay coefficient 4.1%).

E. Dietary and Physical Activity Analysis

Nutritional data were analyzed for nutrient composition (energy, carbohydrate, fat, and protein content) using online nutritional software (Cronometer, Revelstoke, BC, Canada; https://cronometer.com/).

Participants were asked to rate their involvement in a number of activities in three recent time periods (before school, during school, and after school) as either "None," "A little," or "A lot" using a standardized questionnaire [51]. Activities were classified as physical (such as running) or sedentary (such as watching television), and the number of activities in each category was summed.

F. Statistical Analysis

Anthropometric data [height, weight, and body mass index (BMI)] were analyzed both as absolute values and as age- and sex-normalized SD scores (SDSs). TSH results were natural log transformed (lnTSH) for further analysis to reduce data skew (Fig. 1A and 1B).

The relative supraclavicular temperature (T_{Rel}) was calculated by the difference between the supraclavicular and reference-region temperature ($T_{Rel} = T_{SCV} - T_{Ref}$). Resting temperatures were defined as the mean of the images in the minute prior to the introduction of the cool stimulus. A rolling average of 1-minute duration was calculated for the stimulation period, and the stimulated temperatures were defined as the maximum rolling average temperature during stimulation. Change in temperature (ΔT) was the difference between resting and stimulated temperatures. Summary measures are presented as the mean of each group with the difference and 95% CI of the difference unless otherwise stated. Independent Student *t* tests were used to compare BAT activation, as the change in relative temperature in response to the cool stimulus (ΔT_{Rel}), between the groups. Percentages were compared using a χ^2 test. Adjusted *P* values were calculated using linear regression. Statistical analysis was carried out using *R: A Language and Environment for Statistical Computing*, version 3.4.3 (R Core Team).

2. Results

There was no significant difference between participants in the ATD group and the control group in terms of baseline characteristics, although there was a trend toward participants in the ATD group being shorter for their age, as previously reported [52], and having a higher BMI (Table 1). Four (29%) of the participants were newly diagnosed (<1 month) and/or had not yet started treatment, and 10 (71%) had an existing diagnosis. The median (IQR) length of diagnosis was 61.5 (4.4 to 133) weeks and the age at diagnosis was 12 (11.2 to 13.8) years, which reflects the typical age of onset of ATD [53]. All children in the study group were treated with, or due to start, levothyroxine [median (IQR) dose: 50 (50 to 100) μg, 1.3 (0.7 to 1.7) μg/kg], and most children did not have significantly abnormal TSH, free T3, or free T4 on the day of testing (Fig. 1C). The median (IQR) TSH was 4.1 mU/L (3.5 to 15.7; normal range, 0.3 to

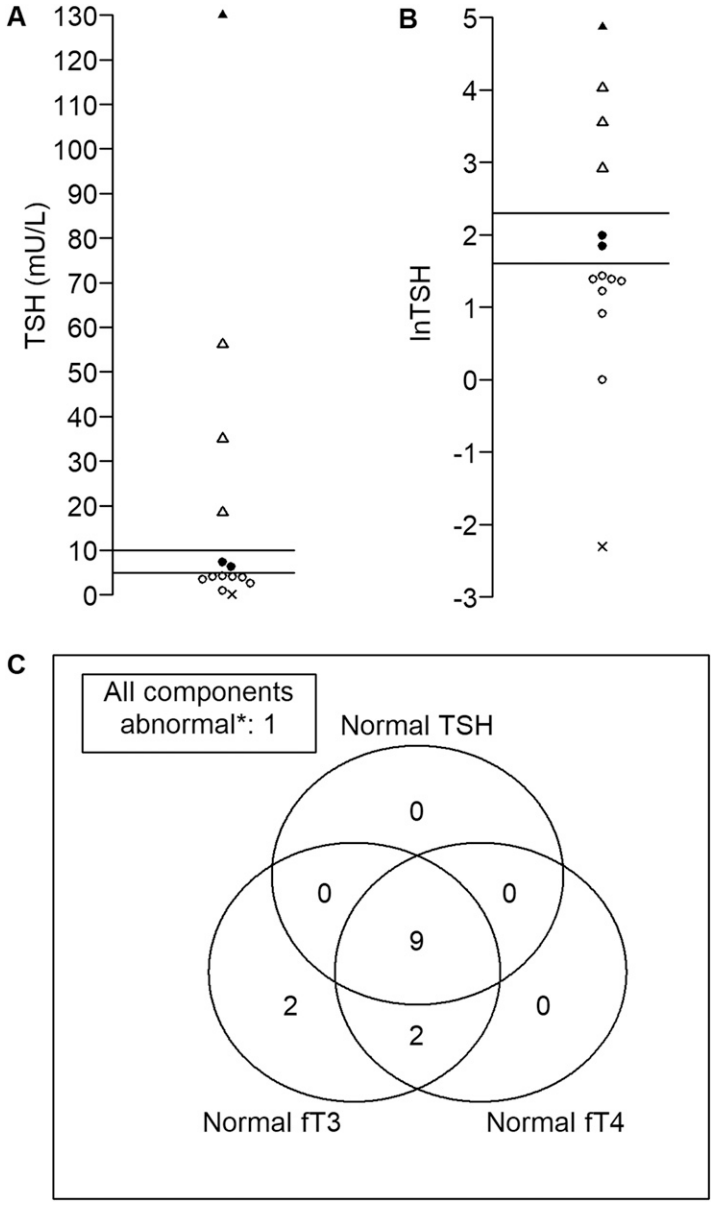

Figure 1. (A) TSH concentrations in participants with ATD. (B) Natural lnTSH concentrations in participants with ATD. ×, low; ○, normal; ●, borderline high; △, high; ▲, very high. (C) Venn diagram results of thyroid function tests of ATD group participants. In two cases where only a TSH was available (both in the normal range), the T3 and T4 were presumed to be normal; in three further cases where only a T3 result was not available, the T3 was presumed to be in the same category as the T4 result (one normal with a normal T4 and TSH, one normal with a normal T4 and high TSH, *one low with a low T4 and very high TSH); and two cases with borderline TSH concentration (5.6 to 10.0 mU/L) were classified as normal as both T3 and T4 levels were normal.

5.5), free T4 was 15.9 pmol/L (11.2 to 17.5; normal range, 10.0 to 19.8), and free T3 was 5.6 pmol/L (5.0 to 5.8; normal range, 3.5 to 6.7). Anti-TPO antibodies were raised in all patients at diagnosis (>1300 IU/mL in nine participants, 90.2 to 273.7 IU/mL in the other five participants).

There was no difference in the study conditions between the groups, including stimulation temperature, outside temperature, room temperature, time since the last meal, or in the number of physical activity sessions or sedentary sessions (Table 2). Energy and carbohydrate

Table 1. Characteristics of Participants

	ATD	Control	Difference (95% CI)	*P*
n	14	12		
Age, y	13.20	11.48	−1.72 (−3.88 to 0.44)	0.11
Height, cm	152.1	150.6	−1.4 (−14.8 to 12.0)	0.83
Height SDS	−0.33	0.71	1.03 (−0.01 to 2.08)	0.05
Weight, kg	55.5	47.2	−8.3 (−21.4 to 4.8)	0.20
Weight SDS	0.94	0.83	−0.11 (−1.09 to 0.86)	0.82
BMI, kg/m^2	23.7	20.0	−3.6 (−7.3 to 0.0)	0.05
BMI SDS	1.26	0.65	−0.61 (−1.54 to 0.33)	0.19
Core temperature, °C	36.8	36.8	0 (−0.4 to 0.4)	0.98

P values are results of *t* tests.

content of the last meal was higher in the control group, but this did not reach statistical significance. Protein content was similar but fat content trended toward being significantly higher in the control group ($P < 0.1$; Table 2). Heart rate was similar between the groups and did not change significantly following stimulation (Table 3).

Absolute T_{SCV} was lower in participants with ATD than in control participants in both the resting and stimulated state. There was less separation between the groups in resting and stimulated T_{Rel} than T_{SCV} and the difference was not significant. Age, BMI and BMI SDS, and weight and weight SDS were negatively associated with T_{SCV}, but not T_{Rel}, ΔT_{SCV}, or ΔT_{Rel}, and they were closely correlated with each other (Fig. 2). Neither height nor height SDS was associated with T_{SCV} or T_{Rel}. Because BMI SDS correlated with T_{SCV}, trended toward being significantly different between the groups, and there is a known relationship between BMI and BAT activity [4, 54–57], a *P* value adjusted for BMI SDS was calculated using linear regression (Table 4).

The mean response to cold water stimulation (mean ΔT_{SCV}) was 0.15°C (95% CI, 0.12° to 0.18°; $P < 0.001$). ΔT_{SCV} was similar between groups, but the relative response to cold water stimulation (ΔT_{Rel}) was significantly reduced in participants with ATD compared with healthy controls (Table 4). Although the ATD group also had a lower resting and stimulated T_{SCV}, after controlling for BMI SDS, the difference was no longer statistically significant (resting T_{SCV}, $F_{2,23} = 6.5$, $P = 0.10$; stimulated T_{SCV}, $F_{2,23} = 7.1$, $P = 0.12$). There was no difference in the resting or stimulated T_{Rel} between the groups, but there was a significant positive correlation in the ATD group with lnTSH (Fig. 3; resting T_{Rel}, $r = 0.7$, $P = 0.009$; stimulated T_{Rel}, $r = 0.7$, $P = 0.01$) and negative correlation with T4 (resting T_{Rel}, $r = -0.6$, $P = 0.04$; stimulated T_{Rel}, $r = -0.6$, $P = 0.04$), with a similar pattern seen at all time points

Table 2. Study Conditions of Each Group

	ATD	Control	Difference (95% CI)	*P*
Room temperature, °C	24.5	24.5	−0.0 (−1.3 to 1.2)	0.98
Outdoor temperature, °C	15.2	15.7	0.5 (−2.2 to 3.2)	0.71
Water (stimulus) temperature, °C	18.3	18.5	0.2 (−0.5 to 0.9)	0.56
Time since last meal, h	5.5	4.4	−1.2 (−6.1 to 3.7)	0.63
Energy of last meal, kcal	304	401	97 (−41 to 236)	0.16
Carbohydrate content of last meal, g	41	52	11 (−7 to 29)	0.22
Fat content of last meal, g	9	14	6	0.06[a]
Protein content of last meal, g	9	11	1	0.62[a]
Number of physical activities	10.4	10.8	0.5 (−4 to 4.9)	0.83
Number of sedentary activities	5.4	5.7	0.3 (−1.7 to 2.3)	0.75

Values are means, and *P* values are the results of *t* tests (except as noted).
[a]Value is the median, and *P* value is the result of a Mann–Whitney test.

Table 3. Summary of Heart Rate by Group Before, and During, Cold Stimulation

	ATD	Control	Difference (95% CI)	*P*
Resting HR, bpm	77	83	7 (−2 to 15)	0.11
Stimulated HR, bpm	79	87	8 (−2 to 17)	0.13
Difference, bpm (95% CI)	2 (−12 to 7)	3 (−12 to 5)		
P	0.62	0.41		

P values are results of *t* tests.
Abbreviations: bpm, beats per minute; HR, heart rate.

(data not shown). In the nine participants for whom data were available, there was no correlation between T3 and resting, stimulated, or change in T_{SCV}.

To further define the relationship between thyroid function and T_{Rel}, participants with ATD were categorized as either biochemically hypothyroid or biochemically euthyroid/hyperthyroid. A TSH cutoff of >10 mU/L was used to define the biochemically hypothyroid group. Compared with girls who were biochemically euthyroid or hyperthyroid (n = 10), biochemically hypothyroid girls (n = 4) had a significantly greater resting T_{Rel} [high TSH group, 1.7°C; low TSH group, 2.5°C; difference, 0.8°C (95% CI, −1.6° to 0.0°); $P = 0.04$; Fig. 3C] and a trend toward a greater stimulated T_{Rel} [high TSH group, 2.6°C; low TSH group, 1.8°C, difference 0.8°C (95% CI, 0.0° to 1.5°), $P = 0.05$]. There was no difference in change in temperature (ΔT_{Rel}) or in T_{SCV}.

Post hoc analysis did not show any meaningful association between T_{SCV} or T_{Rel} and sedentary behavior, physical activity, or nutritional composition of the last meal (energy, carbohydrate, fat, and protein content), once removal of a single influential outlier in sedentary behavior was excluded and fat content was corrected for BMI SDS. Increased carbohydrate content in the preceding meal was associated with lower ΔT_{Rel}, but visual inspection of the results failed to indicate a valid relationship.

3. Discussion

We demonstrate that female children and adolescents with ATD have a reduced thermogenic response to cold stimulation, as measured by the change in relative supraclavicular temperature (ΔT_{Rel}), which has been shown to be closely correlated with BAT activity measured using fludeoxyglucose (^{18}F) PET-CT [17–20]. However, contrary to expectation, those children with ATD who were currently biochemically hypothyroid (defined as TSH >10 mU/L) demonstrated greater resting and stimulated T_{Rel} than did their biochemically euthyroid counterparts, and a strong positive correlation was seen between TSH and BAT thermogenesis in the ATD group, indicated by resting and stimulated T_{Rel}. These apparently contradictory findings may help to explain the contrary and conflicting information previously published on this subject [28–30, 32–34]. Unfortunately, plasma TSH and thyroid hormone levels were not available for the control group, as this would have helped delineate further between the effect of the underlying disease and the effect of the TSH *per se*. However, in the subset of the group with ATD who did not have raised TSH, the relative supraclavicular temperature closely mirrors the distribution seen in a healthy population, indicating that the raised T_{Rel} is a consequence of the biochemical pathology, rather than the underlying autoimmune process, and that, given appropriate replacement therapy, this difference can be moderated. That the change in T_{Rel} in response to cold stimulation remains different between the study group and healthy controls suggests that a subtler difference in the thermogenic response of BAT remains, potentially independent of thyroid hormone levels.

BAT has been shown to interact with immunomodulation, potentially helping to identify self and prevent autoimmunity developing. Adipectomy of the intrascapular BAT in rats increases autoimmune processes [58, 59] and, in humans, lipodystrophy is associated with autoimmune diseases [60, 61]. This raises the possibility that the reduced BAT

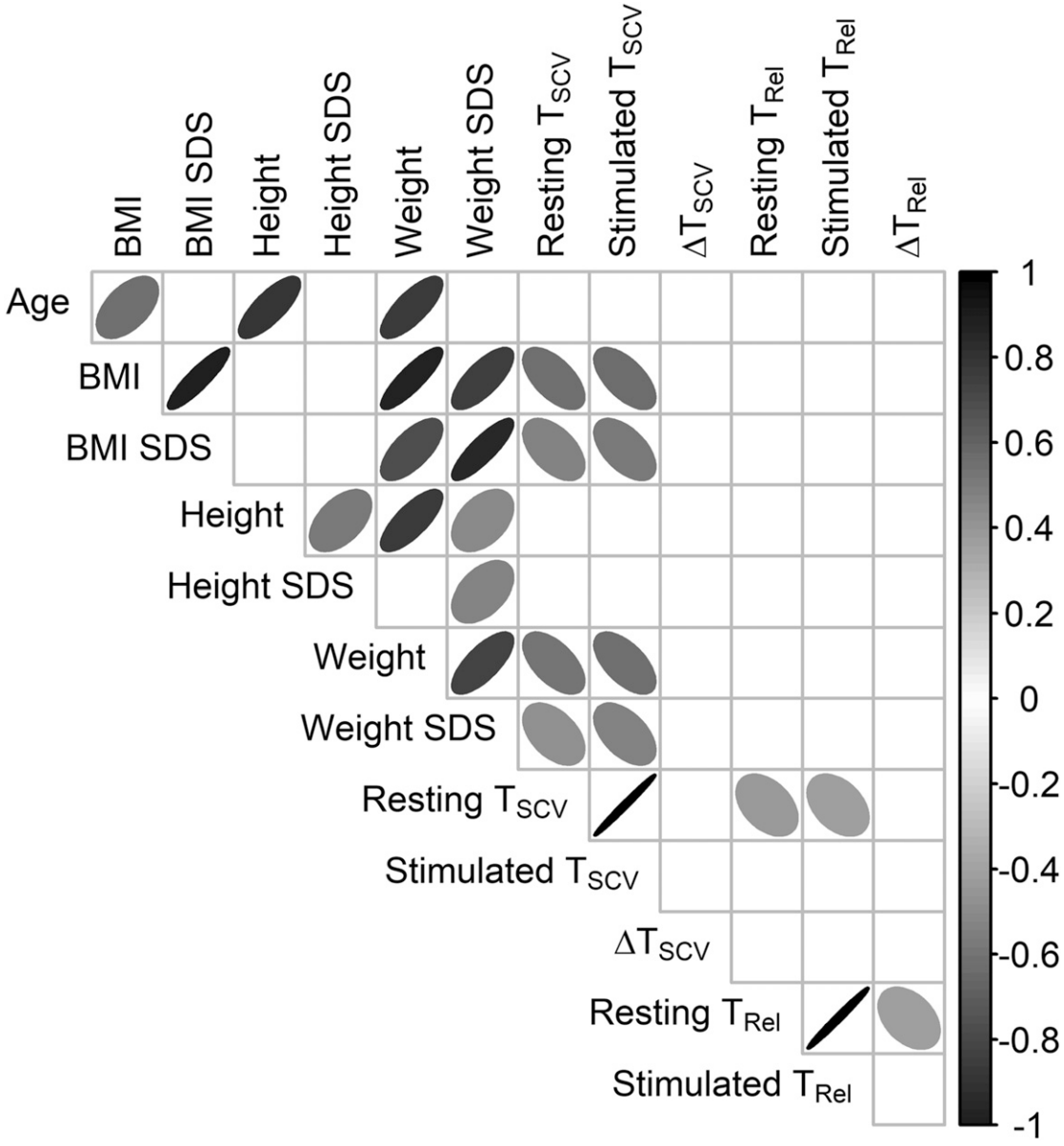

Figure 2. Correlogram of association between participant characteristics (age, BMI and BMI SDS, height and height SDS, and weight and weight SDS) and thermographic BAT measurements [resting, stimulated, and change (Δ) in T_{SCV} and resting, stimulated, and change in T_{Rel}]. Only significant ($P < 0.05$) associations are shown. Ellipse size and shading indicate the absolute value of the Pearson correlation coefficient ($r = 0$, white; $|r| = 1$, black); ellipse direction indicates the sign of the coefficient [down (\), negative; up (/), positive].

thermogenesis seen is, in fact, due to reduced BAT activity predisposing to autoimmune disease, rather than being a consequence of it, and that those with autoimmune disease may have less BAT prior to the onset of the later autoimmune pathology. However, there is no direct evidence for this in humans, and the animal models of near-total adipectomy represent a much greater insult than the difference that might be expected to occur between individuals naturally. The effects, if present, would therefore probably be subtle and likely to require larger studies to be detected.

Alternatively, there may be an autoimmune process reducing BAT thermogenesis directly. Anti-TPO antibodies have little cross-reactivity outside of the thyroid gland, except for breast tissue [62, 63] and possibly myeloperoxidase [64], so it is unlikely that they directly affect

Table 4. Absolute and Relative Supraclavicular BAT Temperatures (°C) of Each Group

	ATD	Control	Difference (95% CI)	*P*	Difference (Adjusted)	*P* (Adjusted)
Resting T_{SCV}	34.9	35.4	0.5 (0.1 to 1.0)	0.03	0.4	0.10
Resting T_{Rel}	1.9	1.6	−0.4 (−0.9 to 0.2)	0.20	−0.3	0.27
Stimulated T_{SCV}	35.0	35.5	0.5 (0.0 to 0.9)	0.04	0.3	0.13
Stimulated T_{Rel}	2.1	1.8	−0.3 (−0.8 to 0.3)	0.33	−0.2	0.42
ΔT_{SCV}	0.2	0.1	−0.0 (−0.1 to 0.0)	0.29	−0.1	0.20
ΔT_{Rel}	0.1	0.2	0.1 (0.0 to 0.2)	0.04	0.1	0.03

P values are results of *t* tests. Adjusted values are the result of a linear model adjusting for BMI SDS.

BAT. However, the presence of one autoimmune antibody increases the likelihood of the presence of another [65–69]. As only anti-TPO antibodies were measured in the study group, whether anti–TSH receptor antibodies or other antibodies were present is not known, and future research may want to consider testing a wider panel of autoantibodies to explore this further.

An alternative explanation may lie in the lack of physiological diurnal variation in thyroid hormones observed in patients on mono T4 replacement therapy. In the absence of thyroid disease, TSH levels are lower in the day, with a nadir in the late afternoon (between 3:00 and 5:00 PM) and 140% higher at night, peaking at 11:00 PM in children (and 2:00 to 4:00 AM in adults), along with a shorter 30-minute pulsatile cycle overlying the diurnal rhythm [44, 45]. There is a mirroring, but subtler, rise in both T4 and T3 levels, which increase ~7% and 15%, respectively, thought to be in response to TSH pulses, but also a rise in the morning independent of TSH, resulting in the highest levels in the morning and lowest in the late afternoon, along with a similar overlying short pulsatile cycle [44, 45]. Replacement therapy is typically (and for all participants in the study group) with levothyroxine (T4), taken once a day and usually in the morning. On replacement therapy, the TSH variation remains but the T3 and T4 levels rise to a maximum 3 hours after ingestion, in the middle of the day, before falling until the following dose the next morning [70], and there is no short pulsatile cycle overlying the diurnal cycle. BAT is known to exhibit a circadian rhythm [20] and, given the critical role of thyroid hormones in the cellular biochemistry of the brown adipocyte, the diurnal thyroid hormone variation may be important to the proper function of BAT and its absence may diminish the ability of BAT to respond maximally to a cold challenge. Additionally, levothyroxine monotherapy does not always fully resolve symptoms of hypothyroidism, and quality of life scores remain below those of healthy controls [71, 72], possibly due to a lack of euthyroid status at the tissue level [73–75]. A TSH in the normal range may be insufficient to drive the local conversion to T3 within the brown adipocyte, where T3 is not being produced by the thyroid gland (normally thought to account for 20% of circulating levels [76]).

The TSH receptor, previously thought only to be expressed by thyroid follicular cells, has now been shown to be expressed on many other extrathyroidal cells, including BAT [77–79], at least in small mammals. Furthermore, the insertion of an activating TSH receptor mutation to human orbital preadipocytes increases UCP1 gene expression [80]. Our results suggest a similar potential mechanism for activation of classical BAT depots *in vivo* in humans, but an indirect mechanism, either due to increased sensitivity to cold or by changes in plasma T3 and T4, should also be considered. Additionally, changes in thyroid hormone concentrations may affect BAT directly or via a central effect on the SNS, and the response to thyroid replacement therapy can vary between tissues [81], raising the possibility that the tissue-level thyroid status was not fully reflected by serum TSH concentrations, despite the buffering action of deiodinases.

Even in the presence of frank biochemical hypothyroidism, our results would suggest that TSH is able to exert a marked thermogenic influence on BAT, either directly or indirectly, raising the resting activity level while still allowing a similar response to the cold challenge,

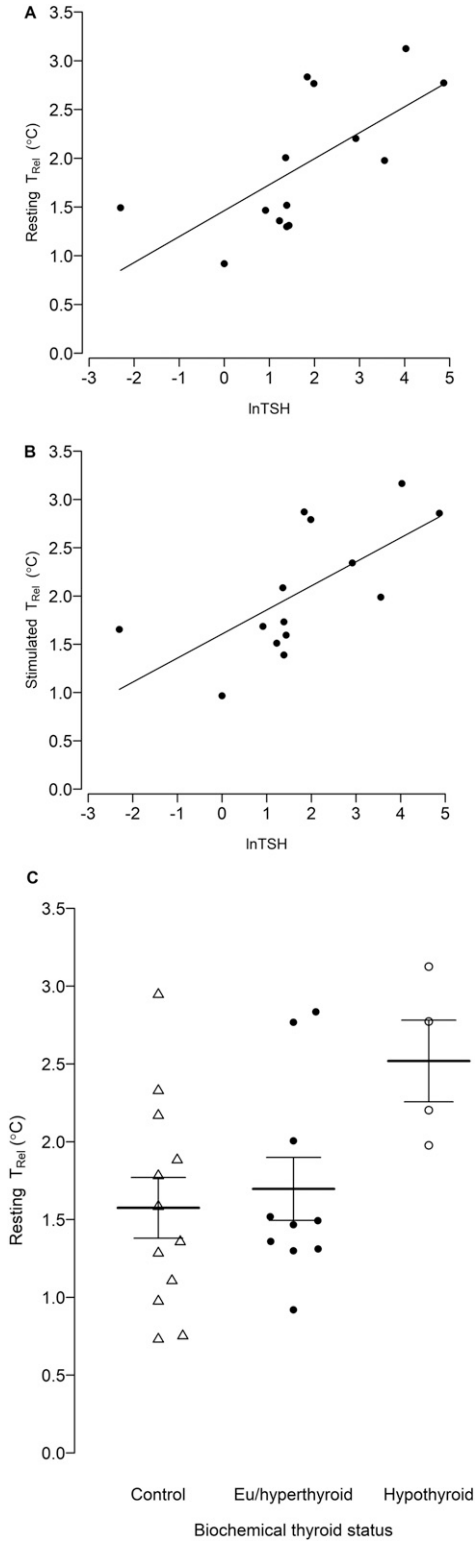

Figure 3. Association between lnTSH concentration of participants in the ATD group and (A) resting T_{Rel} (r = 0.7, P = 0.01) and (B) stimulated T_{Rel} (r = 0.7, P = 0.01). (C) Resting T_{Rel} of girls in the control group (△) compared with those with ATD who were biochemically euthyroid/hyperthyroid (TSH ≤10 mU/L, ●) and those who were biochemically hypothyroid (TSH >10 mU/L, ○). Bars are mean ± SEM.

even in the near absence of thyroid hormones. Via upregulation of iodothyronine deiodinase 2, the relatively abundant T4 can be converted intracellularly to the biologically active T3, potentially maintaining intracellular concentrations. This points to the critical importance of the BAT for survival.

Clearly, the relationship between thyroid status and BAT activity is complex, but the close association between TSH with both resting and stimulated relative BAT temperatures further adds to the concept that thyroid status is an important determinant of BAT activity in humans [22].

To further elucidate this, future studies should consider longitudinal repeat imaging of participants with a new diagnosis of hypothyroidism to assess the changes in BAT activity as they return a biochemical euthyroid status following initiation of thyroid replacement therapy.

Although the age difference was not statistically significant, the group participants with ATD were slightly older than those in the control group. Given the age of the participants, it is likely that there is a degree of heterogeneity in their pubertal status and, in that context, the age difference between the groups may have had an influence on the results. In our study setting, assessment of pubertal stage was not considered appropriate, but the lack of data is a potential weakness and prevents any adjustment for pubertal status. However, although sex hormones appear to affect BAT activity in rodents [82] via FSH [83], it is not known whether human BAT expresses FSH receptors in childhood. Additionally, we did not show any meaningful relationship between dietary intake immediately preceding imaging, or recent physical activity levels, and thermogenic measurements. However, this was not the focus of this study nor was it designed to rigorously investigate these variables, so caution should be applied before drawing any firm conclusions from the lack of association here.

We show that BMI and BMI SDS, weight and weight SDS, and age are all negatively associated with T_{SCV} (resting, maximal, and at all time points). There was a high degree of intercorrelation between these variables, and the size of our study means that it is not possible to distinguish between the relative effects of each variable. However, all of these variables potentially have a common factor in increasing the insulation between the superficial skin and BAT depot, either by a relative abundance of adipose tissue or by simply an increased size, suggesting this as a mechanism for reduced skin temperature rather than necessarily reduced BAT activity. Consistent with this assertion, when T_{SCV} is compared with a sternal reference point, the association between these variables is no longer significant, emphasizing the suitability of T_{Rel} to control for potential confounders. In contrast, height and height SDS do not correlate with any outcome measure of IR thermography and are also less likely to be associated with increased insulation between BAT and the skin surface, further reinforcing the proposed mechanism. Also, note that despite thermal imaging producing a two-dimensional image, as opposed to the three-dimensional image of PET-CT, there is a close relationship between each different measure of BAT function [18].

In summary, BAT activation was reduced in girls with a diagnosis of ATD compared with healthy controls. The unexpected finding of increased relative BAT temperature in subjects with suboptimal biochemical control now requires confirmation in larger studies.

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Author Contributions: J.M.L. conceived and designed the study, undertook the acquisition, analysis, and interpretation of the data, and drafted, revised, and approved this work. D.E.M. designed the analysis methods used and revised and approved this work. V.A. and E.F. substantially contributed to the acquisition of data and revised and approved this work. J.J.M. contributed to the data analysis and revised and approved this work. L.J.R. co-designed the study, contributed to the analysis methods used, and revised and approved this work. T.R. and L.D. contributed to the study design and revised and approved this work. M.E.S. co-designed the study, interpreted the data, and revised and approved this work. H.B. conceived and co-designed the study, interpreted the data, and revised and approved this work.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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3.5 Type 1 Diabetes

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Reduced Brown Adipose Tissue-Associated Skin Temperature Following Cold Stimulation in Children and Adolescents with Type 1 Diabetes

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Abstract

Brown adipose tissue (BAT) is essential to maintain body temperature. Its ability to convert chemical energy in glucose and free fatty acids to heat is conferred by a unique protein, UCP-1. BAT activity is greatest in children and adolescents, declining through adulthood. Blood glucose concentrations outside the normal non-diabetic range are common in type 1 diabetes and hyperglycaemia leads to insulin resistance in muscle and white adipose tissue, but whether this applies to BAT, is not known. To investigate the effect of type 1 diabetes on BAT activity, we measured the supraclavicular temperature of 20 children with type 1 diabetes and compared them to 20 age-matched controls, using infrared thermography. The diabetes group had lower stimulated supraclavicular temperatures (diabetes group: 35.03 (34.76-35.30)°C; control group: 35.42 (35.16-35.69)°C; p=0.037) and a reduced response in relative temperature following cold stimulation, after adjusting for BMI (diabetes group: 0.11 (0.03-0.18)°C; control group: 0.22 (0.15-0.29)°C; p = 0.034). In the diabetes group, there was no association between glycaemic measures and supraclavicular temperatures, but the method of insulin delivery may significantly affect the change in supraclavicular temperature with stimulation (injections: 0.01 (-0.07-0.09)°C; pump: 0.15 (0.04-0.26)°C; p=0.028). While further work is needed to better understand the glucose-insulin-BAT relationship, one possible explanation for the reduced supraclavicular temperature is that exogenous, unlike endogenous, insulin, is not suppressed by the activity of the sympathetic nervous system, preventing lipolysis-driven activation of BAT.

Keywords: Brown adipose tissue; brown fat; cold-induced thermogenesis; thermal imaging; infrared thermography; type 1 diabetes mellitus; insulin-dependent diabetes; cold stimulation; cold challenge.

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H.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

We confirm that there is no conflict of interest exists for the authors.

Introduction

Brown adipose tissue is a key component of adaptive thermogenesis, the ability to produce heat in response to an environmental challenge.[1] Mitochondrial respiration results in the production of adenosine triphosphate but, in the presence of proton leak, this energy is instead dissipated as heat.[2] Brown adipocytes contain a unique protein, uncoupling protein (UCP)-1, which drives proton leak, increasing the conversion of chemical energy to heat which is dissipated throughout the body and eventually lost to the environment.[3] UCP-1 activation is stimulated by raised intracellular concentrations of free fatty acids from sympathetic-nervous system (SNS)-induced lipolysis by a cyclic-AMP mediated mechanism.[4]

Efforts to increase energy expenditure, as a way of reducing obesity, typically focus on increasing voluntary activity through exercise. The difficulty in making permanent and sustainable changes means long term success using exercise, with or without dietary interventions in parallel, is limited.[5,6] Energy excess leading to obesity has become a major problem[7] even in children and adolescents,[8,9] in whom BAT is more abundant and the potential for intervention greater.[10-12] Despite this, relatively few studies have considered children, partially due to the high ionising radiation exposure of positron emission tomography-computed tomography (PET-CT) that is typically used to measure BAT activity and is, therefore, not ethical for use in healthy subjects.

BAT is sensitive to thermal stimuli, mainly through SNS stimulation,[13,14] although other direct mechanisms may also exist.[15] Following cold stimulation, increased SNS activity promotes BAT glucose uptake,[16] lipolysis and, consequently, heat production in BAT.[17,18] SNS activation also reduces pancreatic insulin secretion[19,20] and increases both glycolysis and lipolysis resulting in increased circulating glucose and fatty acids available for uptake into brown adipocytes.[21] The thermogenic response of BAT to cold stimulation has allowed us to develop a non-invasive method of measuring BAT activation using infrared thermography (IRT)[22-25] which has been shown to correlate closely with fludeoxyglucose-F-18 (^{18}F-FDG) uptake on PET-CT.[26-29] Our development of IRT analysis, and its validation as a reproducible method of measuring BAT activation, has permitted the inclusion of healthy children and other vulnerable groups,

allowing novel insights into previously excluded populations.

Hyperglycaemia due to absolute hypoinsulinaemia in type 1 diabetes contrasts with peripheral insulin resistance leading to hyperglycaemia despite hyperinsulinaemia in type 2 diabetes. People with type 1 diabetes require the administration of exogenous insulin therapy, either using regular subcutaneous injections or by continuous subcutaneous infusion via an insulin pump (CSII). However, glycaemic excursions above the non-diabetic upper limit of normal are commonplace, even with the modern intensive-insulin therapy,[30,31] and patients with type 1 diabetes maintain a mild overall hypoinsulinaemia in order to avoid recurrent hypoglycaemia – although both of these premises are being challenged by the current development of artificial insulin delivery systems. Hyperglycaemia increases peripheral insulin resistance[32,33] but the effect of chronic (treated) type 1 diabetes on BAT has not, however, been previously studied. This relative hypoinsulinaemia may be sufficient to permit the return, or persistence, of peripheral insulin resistance, as seen previously.[32] We, therefore, hypothesised that the thermogenic response of BAT to cold stimulation would be blunted in children and adolescents with type 1 diabetes compared to healthy matched controls.

Materials and methods

Participants and facilities

Twenty-one children and adolescents (aged between 5 and 18 years old) with type 1 diabetes mellitus (diabetes) and 20 healthy controls attended a single one-hour study session at the Academic Child Health Human Physiology Laboratory in the Queen's Medical Centre campus of the University of Nottingham. One participant in the diabetes group was excluded as, although they had an antibody-positive diagnosis of type 1 diabetes, in addition, they had markers of insulin resistance (requiring metformin and high insulin doses) and were a significant outlier in their infrared thermographic outputs as well. The Academic Child Health Human Physiology Laboratory is a temperature-controlled facility and ambient temperature was maintained at 23-24°C during study sessions. Where possible, all heating and cooling systems were inactivated prior to the start of the study session. If heating or cooling was essential to maintain an ambient temperature in the target range throughout the study session, patients

and equipment were shielded from airflow or direct heat during imaging.

The children with diabetes were recruited from the Nottingham University Hospital paediatric diabetes clinic. Healthy controls either were siblings of participants with diabetes or were recruited from patients attending the Nottingham University Hospital paediatric otorhinolaryngology clinic for simple surgical procedures (such as grommet insertion or tonsillectomy). Participants were asked to avoid caffeine, alcohol, recreational drugs and strenuous exercise on the day of the study but were otherwise asked to eat and drink normally and to take all regular medications.

The study was approved by the Nottingham-2 NHS Research Ethics Committee (13/EM/0102) and performed in accordance with the declaration of Helsinki. Written, informed consent was provided prior to participation from the child or their parent or legal guardian as appropriate. If consent was provided by the parent or legal guardian, the child was invited to provide written assent if they wished and were able to. All children gave verbal assent to take part.

Study sessions

As previously described,[34] participants wore a standard light cotton vest (0.06 Clo) and acclimatised to the room. A targeted medical history was taken and basic anthropometric measurements of height and weight were made. Participants were asked about their recent activities using a validated questionnaire[34,35] and the timing and content of their last meal, which was analysed as previously described.[34] In addition, participants with diabetes were asked about the timing and amount of their last insulin bolus and the total daily dose of insulin for the previous three days. Participants were then sat upright opposite an infrared camera (FLIR B425, FLIR Systems, AB, Danderyd, Sweden). Pulse rate was recorded continuously during imaging using pulse oximetry (Radical 7 Pulse Oximeter, Massimo, Irvine, CA, USA) connected to the index finger of the left hand and the tympanic temperature was recorded at the start of imaging. Thermally reflective skin markers were placed at the apices of the regions of interest (Figure 1) to enable accurate and reproducible identification of surface anatomy during image analysis.

Infrared thermographic images of the supraclavicular region were acquired (at

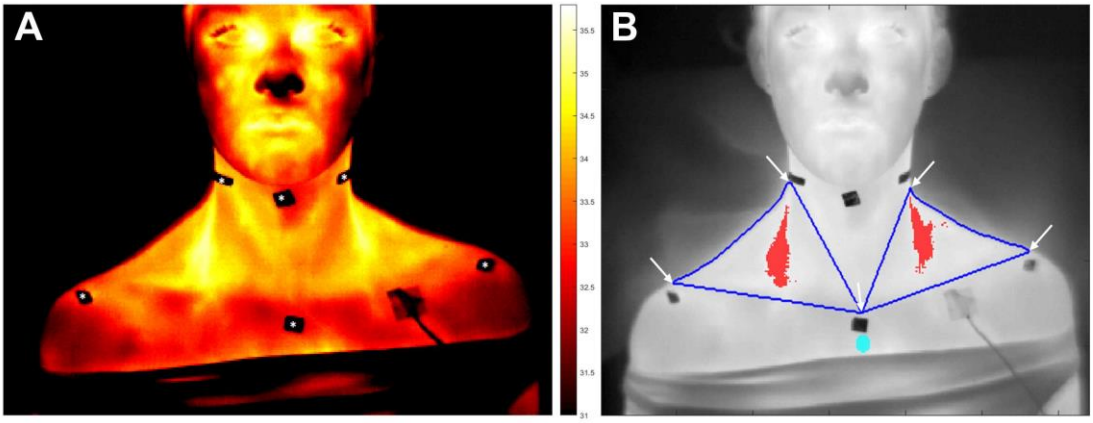

Figure 1: (A) Original thermogram. Visible are six thermally-reflective markers (white asterisk) placed just outside the ROI to mark its apices. Markers identify the acromioclavicular process (lower left and right), the clavicular notch (lower centre), the thyroid cartilage (upper centre) and the sternocleidomastoid at the point it crosses the contour of the neck (upper left and right). The scale is in degrees Celsius. (B) Thermograph after analysis. The contour of the computer-defined ROI is shown in dark blue, with warmest pixels (top 10%) in red and reference region in cyan. Apices shown by white arrows.

a rate of four per minute) to assess skin temperature overlying BAT first in the rested state and then during cold stimulation. Skin temperatures of the dorsum of left (non-cooled) hand and left subclavicular region were measured using skin temperature probes and were recorded every minute. To optimise acclimatisation and resting measurements, stimulation started either when the skin temperatures were unchanged for three consecutive readings or after 10 minutes of acclimatisation, whichever occurred first.

Cold stimulation was applied to the participant's right arm using a cooling blanket (Blanketrol® II, Cincinnati Sub-Zero Products, Cincinnati, OH, USA). Cooling was continued for 5 minutes at 15°C. Shivering was assessed by participant report and researcher observation and participants were asked to report any pain. No shivering was observed and no participant reported pain or shivering.

After imaging, participants with diabetes were asked to check a blood glucose level using their own glucometer or, if they did not have their own available, the study glucometer. GHb was measured as part of routine clinical care (Afinion™, Alere Inc, Waltham, MA, USA) on the same day as the study visit.

Image analysis

Images were analysed as previously described[25,26,34,36] but with the additional use of reflective markers to improve identification of anatomical landmarks. In previous analyses, although the anatomical landmarks necessary to identify the apices of the ROIs were easily identified on a thermal image in

the majority of participants, in some cases, it was less obvious and there remained the possibility of variation in the placement of apices between observers. To minimise this, we have developed thermally-reflective surface markers. These were placed on the participant prior to imaging using anatomical surface landmarks (Figure 1A). Markers were positioned to lie just outside of the ROI and were either square (1 by 1 cm, upper centre and three lower markers) or rectangular (0.5 by 2 cm, upper left and right markers) to ensure they were visible without being obtrusive and overlying other important areas. Introduction of the markers meant surface landmarks could be easily identified during image analysis, reducing variability in manual apex placement.

The primary measure was the median value of the warmest 10% of pixels in the ROI,[26] equivalent to the 95th percentile (T_{SCV}). A reference region on the sternum was defined as a circular area 10 pixels in diameter immediately below the central marker and the median value calculated (T_{Ref}).

Statistical analysis

Resting T_{SCV} was defined as the mean T_{SCV} of the minute before the start of stimulation. The mean T_{SCV} was calculated for each minute of stimulation and the stimulated T_{SCV} was defined as the maximum mean T_{SCV} during stimulation. The primary outcome was the difference between resting T_{SCV} and stimulated T_{SCV} (ΔT_{SCV}). We have recently shown that the supraclavicular temperature relative ($T_{Rel} = T_{SCV} - T_{Ref}$) to a sternal reference point is closely associated with BAT activity on PET-CT.[26] Relative supraclavicular BAT temperatures were similarly calculated (resting T_{Rel}, stimulated T_{Rel} and ΔT_{Rel} respectively).

Secondary analyses were undertaken to analyse associations between supraclavicular temperatures and diabetes-related variables such as GHb, blood glucose level after imaging (BGL), insulin regimen and total daily insulin dose (TDD, an average of the previous three days) and insulin on boad (IOB). IOB was calculated assuming a bilinear distribution of insulin activity with a peak at 75 minutes after administration and a duration of activity of 3 hours. Nutritional composition of the last meal and sedentary and physical activity scores were analysed for association with supraclavicular temperatures and diabetes-related variables.

Statistical analysis was carried out using

R: A Language and Environment for Statistical Computing, version 3.4.3 (R Core Team). Normality was assessed by the Shapiro-Wilk test in combination with a visual inspection of the data distribution. Differences between groups were assessed using the Student's unpaired T-test or the Mann Whitney U test, as appropriate, for continuous variables and the Chi-squared test for percentages. Since there is a known relationship between BMI and BAT activity,[18,23,37-39] and for consistency with previously published results, a post-hoc, analysis with ANCOVAs adjusted for the effect of BMI SDS on supraclavicular temperatures. Pearson correlation coefficients were calculated for associations between variables. An ANOVA was used to determine the effect of insulin regimen on ΔT_{SCV} after controlling for outdoor temperature.

A sample size of 34 participants (17 in the control group and 17 in the diabetes group) was required to detect a 25% difference in ΔT_{SCV} based on previously published data from healthy children[23] ($\sigma = 0.07$, power = 0.8; $\alpha = 0.05$). Results are given as mean ± SD or mean (95% confidence interval) unless otherwise stated.

Results

The groups were well matched and there was no difference between the characteristics of the participants or the study environment (Table 1). Overall, there was a small but significant response to cooling, in both the absolute and relative supraclavicular response to cooling (ΔT_{SCV}: 0.10 (0.05-0.15) °C, $p < 0.001$; ΔT_{Rel}: 0.16 (0.11-0.22) °C, $p < 0.001$). 35 participants (18 in the control group and 17 in the diabetes group) had a positive response to cooling ($\Delta T_{Rel} > 0$). Children and adolescents with diabetes had a significantly lower ΔT_{Rel} when adjusted for BMI SDS with a trend towards a difference in the unadjusted data (Table 2). There was no difference in ΔT_{SCV} between the groups.

Table 1 Characteristics of participants and study conditions for each group.

	Diabetes group (n = 21)	Control group (n = 20)	p
Age (years)	12.3 ± 2.6	11.1 ± 3.0	0.16
[1]Gender (% female)	38	50	0.75
Height (cm)	153.8 ± 17.7	146.5 ± 18.5	0.21
Height SDS	0.3 ± 0.9	0.4 ± 0.9	0.65
Weight (kg)	46.3 ± 14.5	41 ± 16.8	0.29
Weight SDS	0.3 ± 1.0	0.3 ± 1.2	0.88
BMI (kg/m^2)	19.0 ± 3.1	18.2 ± 3.6	0.45
BMI SDS	0.2 ± 1.1	0.1 ± 1.3	0.64
Core temp (°C)	37.1 ± 0.3	37.1 ± 0.4	0.80
Room temp (°C)	23.6 ± 0.8	23.3 ± 0.8	0.27
Outdoor temp (°C)	9.6 ± 5.8	7.7 ± 4.4	0.25
Water (stimulus) temp (°C)	15.1 ± 0.2	15.0 ± 0.0	0.33
[2]Time since last meal (hours)	2.1 (1.7-3.5)	2.7 (1.5-3.6)	0.96
Imaging started (24 hour clock ± mins)	13:52 ± 24	14:15 ± 80	0.37

Data are presented as means ± SD or [2]median (IQR). p values are a result of a T-test, [1]Chi-squared test or [2]Mann-Whitney U test. BMI: body-mass index; SDS: standard-deviation score; T: temp: temperature.

Table 2 Absolute and relative supraclavicular BAT temperatures of each group.

	Diabetes group	Control group	p
Resting T_{SCV}	34.97 (34.69-35.25)	35.29 (35.03-35.55)	0.090
Resting T_{Rel}	1.70 (1.51-1.89)	1.92 (1.67-2.16)	0.16
Stimulated T_{SCV}	35.03 (34.76-35.30)	35.42 (35.16-35.69)	0.037
Stimulated T_{Rel}	1.81 (1.62-2.00)	2.13 (1.90-2.36)	0.033
ΔT_{SCV}	0.06 (-0.01-0.13)	0.14 (0.07-0.20)	0.10
ΔT_{Rel}	0.11 (0.04 -0.19)	0.21 (0.14-0.29)	0.057
ΔT_{Rel} (adjusted for BMI)	0.11 (0.03 -0.18)	0.22 (0.15-0.29)	0.034

Data are means (95% confidence interval) in degrees centigrade. p values are the results of T-tests or (for adjusted ΔT_{Rel}) ANCOVA. T_{SCV}: Supraclavicular temperature; T_{Rel}: Relative supraclavicular temperature; ΔT: Change in temperature (Stimulated minus Resting).

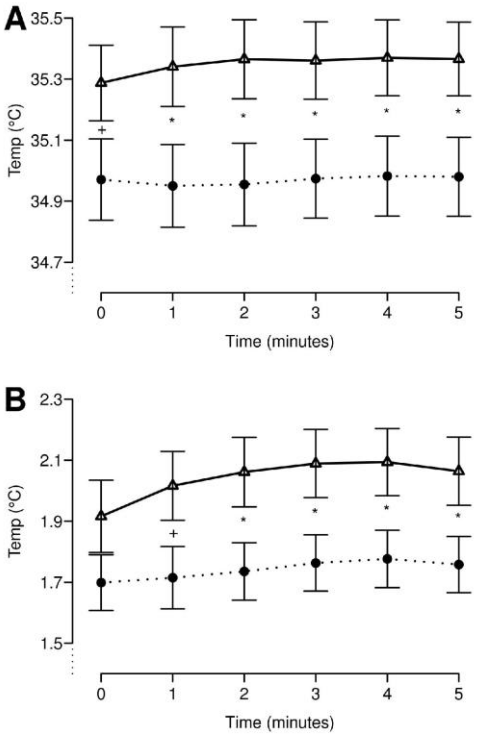

Figure 2: Times series of (A) absolute supraclavicular temperature and (B) relative supraclavicular temperature for the minute prior to stimulation (0 minutes) and the five minutes of stimulation. Each point comprises of the mean of the four readings taken during that minute. Diabetes group: n = 20; Control group: n = 20. Data presented as mean ± SEM. Diabetes group: closed circles, dashed line; Control group: open triangles, solid line. *0.01<p≤0.05, +0.05<p≤0.1 (between-group comparisons).

Participants with diabetes had a lower stimulated T_{SCV} and T_{Rel} and a trend towards lower resting T_{SCV} but not T_{Rel} (Figure 2).

Consistent with previous results,[34] age, height, weight/weight SDS and BMI/BMI SDS were all associated with lower absolute T_{SCV} (resting, stimulated and at all time points), height SDS was associated with resting, but not stimulated, T_{SCV} (Supplementary material 1). These inter-correlated associations are largely mitigated by using T_{Rel} (Figure 3A).

There was no significant difference in pulse rate (beats per minute: bpm) between the children with diabetes (D) and the control group (C) at rest (D: 86±12 bpm; C: 81±10 bpm; p = 0.11) or during stimulation (D: 87±13 bpm; C: 82±11 bpm; p = 0.19) and no significant change in pulse rate with stimulation in either group (D: p = 0.88; C: p = 0.70).

All patients with diabetes were on intensive insulin therapy (CSII, n = 7; MDI, n = 13). Most patients were relatively close to diagnosis (11 were within 2 years; median (range) 1.8 (0.1 to 15.5) years). The median (IQR) GHb in participants with diabetes was 55 (45-62) mmol/mol and their median (IQR) BGL post-imaging was 8.9 (5.6-12.2) mmol/L (Figure 4). In the UK, the current GHb target is 48 mmol/mol[40] and

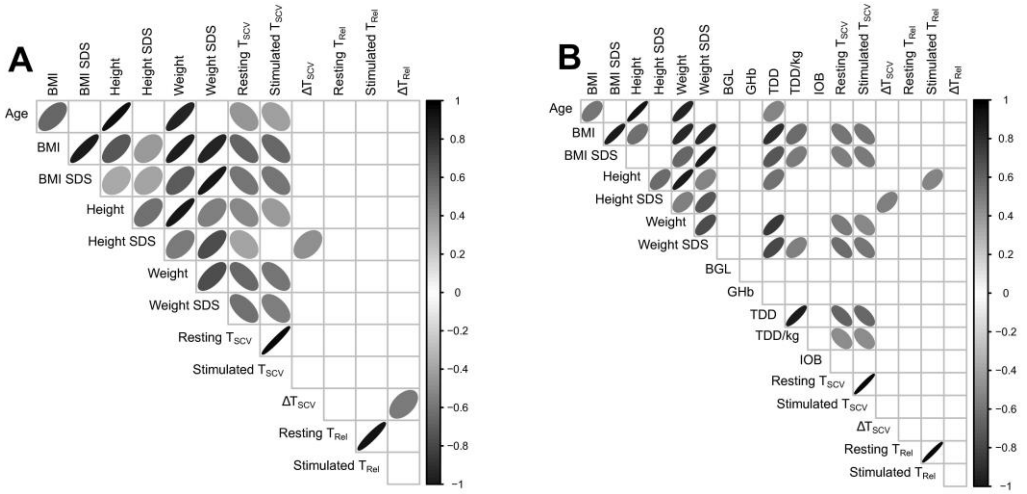

Figure 3: Correlogram showing associations between intrinsic factors and results of infrared thermography for (A) all participants and (B) participants in the diabetic group, including associations diabetes-related data. BGL: blood glucose level; SDS: standard deviation score; TDD: total daily dose of insulin; T_{SCV}: supraclavicular temperature; T_{Rel}: relative supraclavicular temperature; ΔT: change in temperature.

the national average in children is 64 mmol/mol.[41] There was no association with either GHb, BGL or IOB and IRT results (Figure 3B) with the an inverse association between IOB and resting T_{SCV} just failing to reach significance ($r = -0.45$, $p = 0.05$). Greater total daily insulin dose (TDD) was associated with reduced resting and stimulated, but not change in, T_{SCV} but was not associated with measurements of T_{Rel}. A similar pattern was seen TDD per kilogram bodyweight (Figure 5).

Participants on CSII had a greater response to stimulation than those on MDI (ΔT_{SCV}: MDI 0.01 (-0.07-0.09)°C; CSII 0.15 (0.04-0.26)°C; $p = 0.028$) but there was no difference in ΔT_{Rel}. Post-hoc analysis showed that there was no difference in GHb, post-imaging BGL or intrinsic characteristics between participants on MDI and those on CSII (Supplementary material 2) but identified that the outdoor temperature was, by chance, lower on the study days of patients on CSII (ΔT_{SCV}: MDI 11.2 ± 6.2°C; CSII 6.6 ± 3.5°C; $p = 0.046$). After adjusting for outdoor temperature, ΔT_{SCV} trended towards significance ($p = 0.059$).

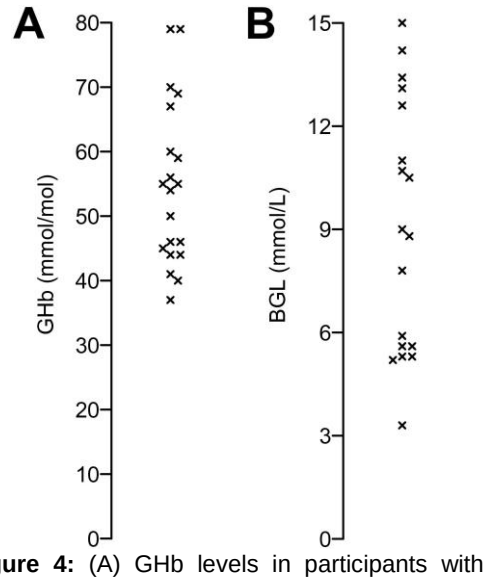

Figure 4: (A) GHb levels in participants with diabetes (B) Blood glucose levels at the end of the study session in participants with diabetes. BGL: blood sugar level

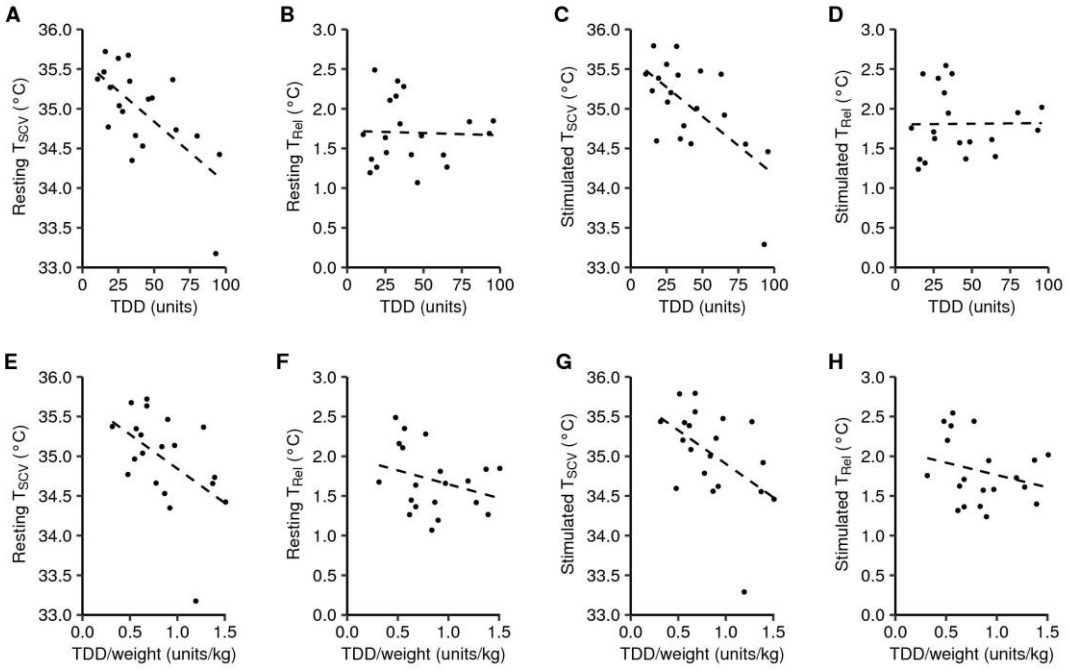

Figure 5: Relationship between (A)-(D) total daily dose of insulin (IU) or (E)-(H) total daily dose of insulin per kilogram body weight (IU/kg) and (A)(E) resting supraclavicular temperature, (B)(F) resting relative supraclavicular temperature, (C)(G) stimulated supraclavicular temperature and (D)(H) stimulated supraclavicular temperature. (A) r = -0.66, p<0.001; (B) r = -0.03 p>0.1; (C) r = -0.64, p<0.01; (D) r = 0.01, p>0.1; (E) r = -0.49, p<0.05; (F) r = -0.29, p>0.1; (G) r = -0.49, p<0.05; (H) r = -0.26, p>0.1. TDD: total daily dose of insulin; T_{SCV}: supraclavicular temperature; T_{Rel}: relative supraclavicular temperature.

There was no difference in nutritional components of the previous meal (energy, carbohydrate, protein or fat content) between the groups (data not shown). Increased ΔT_{Rel} (r = 0.38, p = 0.01) was associated with greater protein intake and, in the control group – but not in the diabetes group – energy intake (r = 0.46, p = 0.04). There was no difference in the reported physical activity scores between the groups but participants in the diabetes group were more likely to have increased reported sedentary behaviours (control group: 5.2 ± 1.8, diabetes group 6.5 ± 2.1, p = 0.04). There was no association between either physical activity scores or sedentary behaviour scores and IRT measures in either group or in the overall cohort. Increasing age was associated with reducing physical activity score (r = -0.45, p = 0.003) and increasing sedentary behaviour score, although the latter was not significant. In addition, being obese or overweight (BMI > 2) was associated with increased reported sedentary behaviours compared to normal weight participants (normal weight: 5.2 ± 1.8, overweight/obese: 7.0 ± 2.1, p = 0.02).

Discussion

Children and adolescents with diabetes showed reduced maximal supraclavicular skin temperature following cold stimulation both in

absolute terms and when compared to the sternal reference point. Since the resting temperature was also reduced, the change in temperature (ΔT) did not reach the 95% significance level. In order to partially mitigate the wide range of ages and mix of genders, ΔT_{Rel} was adjusted for the effect of BMI which varies by age and gender. After BMI was accounted for, the relative change in supraclavicular temperature (ΔT_{Rel}) was significantly reduced in children and adolescents with diabetes.

There was no evidence that poorer chronic glycaemic control (i.e. higher GHb) or current glycaemic status (i.e. higher BGL) was associated with reduced BAT response, similar to reports in adults with type 1 diabetes.[42] Consistent with this, although the participant excluded due to evidence of significant insulin resistance had low resting and stimulated T_{SCV} and T_{Rel} (data not shown), they maintained a response to cold stimulation just within the 95% confidence interval for the group (ΔT_{Rel}: 0.04°C) despite a high BMI, known to be associated with reduced BAT.[23,43,44] The lack of evidence of an association with glycaemic control contrasted with our initial hypothesis of peripheral insulin resistance secondary to mild hyperglycaemia causing blunting of the BAT response. Alternatively, hyperglycaemia might have been expected to increase BAT GLUT-1 expression and basal glucose uptake, therefore, potentially raising resting SCV temperature, which would have been consistent with reports that people with type 1 diabetes have increased energy expenditure, particularly when hypoinsulinaemic.[45,46] Although the difference was not significant, the direction was towards a lower supraclavicular temperature in the diabetes group, not a higher one that this would predict. Caution must be exercised when interpreting resting supraclavicular temperature as it is potentially more susceptible to influence from other factors[25,36,47] than a change in temperature (ΔT), which effectively uses the participant as their own control. However, our groups were well matched for both intrinsic and extrinsic factors (Table 1) improving the validity of the comparison. While a higher total daily dose of insulin per kilogram body weight, an indicator of insulin resistance, was associated with lower absolute supraclavicular temperature, there was no association with relative supraclavicular temperature, indicating that the association may be due to a confounding factor, possibly related to

body size. Further post-hoc adjustment for confounding factors was not possible due to the sample size in the study.

Normal weight participants reported fewer episodes of sedentary behaviours compared to overweight/obese participants but there was no evidence of an effect of physical activity levels on SCV temperatures. We found a significant association between the relative response to stimulation and recent protein intake and, only in the control group, energy content but not other meal components. While this may hint at a component of diet-induced thermogenesis,[48-50] the findings are not significant once corrected for multiple comparisons and are not consistent with our previous results.[34]

One limitation of this study is that the range of ages of the population will cause heterogeneity in the pubertal staging of the participants, which may influence BAT activity.[43,51,52] It was not appropriate with our ethical approval to formally assess puberty staging. Our cohorts were well matched for age and children with diabetes have a normal timing of puberty,[53,54] but a difference in pubertal staging between the groups cannot be excluded or adjusted for.

The second limitation of this study is that the changes in supraclavicular temperature were smaller than expected from previous results,[22,23] increasing the sample size that would have been needed to detect a difference. By making some mitigation for age, size by post-hoc adjustment for BMI, the power to detect differences in the groups was increased. However, a larger study would be beneficial to confirm whether the results of our study are indicative of a true difference in the groups and to allow additional variables to be included in models. However, the small numbers of participants means results of the secondary and post-hoc analysis which the study was not powered to detect differences in should be interpreted cautiously and need further work to confirm or refute these findings.

Finally, due to the age and medical background of the participants, it was necessary to optimise the study design to ensure it was ethical and acceptable to potential participants which included not undertaking invasive elements, such as repeat blood tests. As a result, it is not possible to comment about how a cold stimulus affects the various metabolic pathways and whether this differs at different body sites or between the groups.

Since we could not show a link between acute (blood glucose level) or chronic

(GHb) glucose control, alternative explanations need to be considered that fit better with the evidence. In healthy controls, cold stimulation increases SNS activation[16,20] which, as well as directly acting on brown adipocytes to increase glucose uptake, increase lipolysis and stimulate heat production, also suppresses pancreatic insulin secretion and increases hepatic glycolysis,[19,20,55] and cold exposure decreases plasma insulin concentrations.[16] An alternative to the insulin-resistance hypothesis is that SNS-mediated lipolysis is reduced in type 1 diabetes as insulin is not under endogenous control and so circulating levels cannot reduce in response to SNS activation. Indeed, the association between resting T_{SCV} and IOB trended towards significance. Since insulin is a potent inhibitor of lipolysis, which is essential for UCP1 upregulation and activation, BAT resting activity and functional response to stimulation may be diminished, even in the presence of insulin-stimulated glucose uptake. Glucose uptake does not always reflect BAT function,[56] suggesting caution of the over-reliance on 18F-FDG PET-CT as the primary imaging technique for BAT studies. This alternative hypothesis would be supported by the suggestion in our results that the method of insulin may be important (MDI vs CSII). MDI-users typically give a single daily basal insulin injection which provides a near-steady release from a subcutaneous depot throughout the day in contrast to CSII-users who can vary their basal insulin dose across the day and may, therefore, be able to respond to diurnal variations in BAT activity[57-60] if this affects their glucose levels and insulin requirements. Further work could address this hypothesis directly by actively controlling IOB prior to, or during, imaging sessions.

The effects of insulin in type 1 diabetes are further complicated by the administration of exogenous insulin by the subcutaneous route. Endogenous insulin released by the pancreas results in the highest concentrations of insulin in the portal circulation and lower peripheral levels. In contrast, subcutaneous administration results in relative peripheral hyperinsulinaemia and hepatic hypoinsulinaemia compared to the physiological non-diabetic state, which may reduce peripheral insulin-mediated glucose uptake and increase hepatic glucose production.

The potential for BAT to have a substantial impact on glucose homeostasis is highlighted by improved diet-induced thermogenesis and post-prandial insulin sensitivity following

prolonged cold exposure,[61] improved glycaemic control following BAT stimulation with FGF-21[62] and even the reversal of type 1 diabetes by BAT transplant in mouse models.[63,64] Since increased BAT activity has the potential to improve glycaemic control, it is feasible that patients with type 1 diabetes with lower BAT activity may find good control harder to achieve, as well as poor diabetic control diminishing BAT response. Further work is required to look at the direct effect of insulin and insulin-resistance on BAT activity and function in healthy controls, patients with type 1 diabetes and obese individuals which might allow tailored interventions, such as prolonged mild cold stimulation, to be directed at improving long-term glycaemic control.

In summary, we demonstrate that change in adjusted relative supraclavicular temperature in response to cold stimulation is reduced in children and adolescents with type 1 diabetes compared to healthy controls, they have a lower stimulated temperature and there is no evidence that poorer glycaemic control is associated with adverse BAT response. A possible explanation for the difference between children with diabetes and healthy controls is the inability of exogenous insulin to respond to SNS activity.

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Author contributions

J.M.L. conceived and designed the study, undertook the acquisition, analysis and interpretation of the data, and drafted, revised and approved this work.

D.E.M. designed the analysis methods used and revised and approved this work.

L.R. designed the study, contributed to the analysis methods used and revised and approved this work.

T.R contributed to the design of the study and revised and approved this work.

L.D contributed to the design of the study and revised and approved this work.

M.E.S. co-designed the study, interpreted the data, revised and approved this work.

H.B. conceived and co-designed the study, interpreted the data, revised and approved this work.

Declaration of interest

The authors have nothing to declare.

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Data availability

The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request. Original images are not available to preserve the confidentiality of participants.

Resource availability

The software used for infrared thermographic analysis in the current study is available from the corresponding author upon reasonable request.

Section 4 Conclusions

4.1 Validation of infrared thermography for BAT analysis

The heat-generating properties of BAT, combined with the superficial location of the supraclavicular depot, have made it uniquely amenable to measurement with infrared thermography. Although our group, and others, have previously demonstrated the potential of this imaging modality, I have, for the first time, clearly shown a close association between measurements of BAT activity made using ^{18}F-FDG PET-CT and using infrared thermography, in particular by comparing the supraclavicular temperature to a reference point ($T_{Rel} = T_{SCV} - T_{Ref}$) and looking at the change over time (ΔT_{Rel}). Given that the two techniques are measuring fundamentally different aspects of the BAT profile (with PET-CT measuring a putative input of glucose uptake whereas IRT is measuring the output of heat production), a closer association would not necessarily be expected.

The use of the reference region has been shown to be a simple way to mitigate the effect of overall skin changes. Reference skin temperature can be obtained by alternative methods, such as the use of multiple thermocouples (Liu et al. 2011). However, the use of the sternal reference region has the advantage of being able to be applied flexibly and retrospectively to any appropriately framed thermal image and can be integrated into the same image analysis process.

While BAT activation is associated with an increase in the temperature of the overlying skin, many other components can influence skin temperature such as the thickness of the subcutaneous adipose tissue and skin perfusion. There are also several non-physiological factors which can also influence results such as camera position, presence of radiant heat sources or air movement. Where these factors are not appropriately controlled for in the

study design and execution, subtle temperature changes may be lost to the increase in experimental noise.

This goes some way to explaining why, despite the demonstration of close association with PET-CT, infrared thermography is not yet fully accepted (Gatidis et al. 2016) with some studies questioning its validity. A recent review, for instance, scored studies against the PEDro Scale for clinical trials, even though most of the papers included were not randomised control trials (Jimenez-Pavon et al. 2019). Nevertheless, given the increasingly widespread use of infrared thermography in the study of BAT activity, a single-blinded randomised control trial comparing infrared thermography results of cooled and non-cooled participants would now be timely.

4.2 Development of image analysis methodology

The previous methods for image analysis were either very time-intensive or used crude regular polygons to approximate the ROI. In collaboration with a colleague in the University of Nottingham's Faculty of Engineering, I was able to develop an application to semi-automate the image analysis process resulting in an 86% increase in speed without any increase in variation on repeated analysis (Section 3.1 above). I have also developed thermal skin-markers (Section 3.5 above) to further improve the accuracy and reproducibility of results. While these were initially developed to allow users to identify the key anatomical landmarks rapidly and reliably during image analysis, they have also allowed us to continue the automation of image analysis. Future work will look to continue the automation of images, using skin markers. The changes in skin temperatures being looked for are subtle and close to the limits of the accuracy of modern cameras. Each image has a degree of random noise within the signal and averaging across multiple images would reduce the noise, in a similar way to the methods used to create MRI and CT images. A combination of new technology (such as the ready availability of affordable

cameras with IRT video capability and improved sensitivity) alongside the automation of image analysis could lead to substantial further developments in the methodology of IRT of BAT and further work has been started to explore this potential in more detail.

4.3 BAT activation under extreme stimulation

Having established IRT as a validated method, I have demonstrated that, in the extreme conditions of cold stimulation with exercise, a major BAT response can be seen, resulting in the relative sparing of the supraclavicular region. Cool exposure is the classical method of BAT stimulation (Smith and Roberts 1964; Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Cypess et al. 2012) but the effect of exercise is less well established (Aldiss et al. 2018; Sanchez-Delgado et al. 2015; De Matteis et al. 2013). It was not possible from these results to distinguish the effect of each component separately or even whether exercise had a net positive or negative effect on cold stimulation alone. In addition, the severity of cold exposure was likely to have instigated some element of shivering thermogenesis as well as non-shivering thermogenesis. Further work is, therefore, necessary to establish the relative contributions of these two factors.

This study additionally highlighted the ability of infrared thermography to be used away from the laboratory. While this inevitably required some compromise in study conditions (such as the degree of thermal stability of the room), the ability to undertake studies in a real-world setting is valuable and in stark contrast to other imaging techniques, such as PET-CT. This is consistent with previous results from our group of children in the school (Robinson et al. 2014) or home (Symonds et al. 2012) environment.

4.4 BAT activity in children with hypothyroidism

While thyroid hormones, particularly local T3 levels, are known to be required for the proper functioning of BAT their effect, and that of TSH is complex (Bianco and McAninch

2013; Cannon and Nedergaard 2010). Whether varying any one of TSH, T3 or T4 will increase or decrease BAT activity is likely to depend on the starting concentration of the one being varied, the levels of the other two, the underlying disease model being used and the method of measurement – and these factors may be moderated and buffered by intracellular DIO2 expression. As a result, studies come to different conclusions.

Due to ethical considerations, thyroid function test results were only available in our study in the group with hypothyroidism and it would be valuable to understand the effect of TSH and thyroid hormone concentrations within a healthy group of individuals with physiological thyroid levels and diurnal variations maintained and, because of the preponderance of autoimmune conditions in females, we focused on this group so cannot be sure that our results would extrapolate to males or other age groups.

Participants with autoimmune hypothyroidism in our study had a lower resting and stimulated supraclavicular skin temperature. These differences were probably due to differences between the groups as they were no longer significant once corrected for BMI and the change in temperature (ΔT_{SCV}) as well as measures of relative supraclavicular skin temperatures (T_{Rel}) – which all correct for interparticipant differences such as size and adiposity – were not different between the groups. However, within the hypothyroid group, higher TSH levels (indicating under treatment of hypothyroidism) were associated with higher resting and stimulated T_{Rel}. Further work should seek to understand this relationship more carefully by measurement of thyroid function tests in a population without underlying thyroid disease and by longitudinal repeat measurements in patients newly diagnosed with hypothyroidism as biochemical control is restored.

4.5 BAT activity in children with type 1 diabetes

The association between glucose and BAT is well known due to the prominent use of ^{18}F-FDG PET-CT, particularly in humans, to measure BAT. In our group of children and young people with type 1 diabetes, there was a lower absolute and relative supraclavicular temperature. The relatively small numbers and the heterogeneity of the group reduced the power of the study to detect differences and this was partially mitigated by controlling for BMI, revealing a difference in the change in relative supraclavicular temperature in response to cold stimulation. No association was found with glycaemic control either at the time of imaging (blood glucose level) or over the preceding weeks (glycosylated haemoglobin) in the diabetes group. Further work could expand on these findings by focusing on a more homogenous group, particularly in terms of pubertal status – or by increasing the study size to allow this to be included in the models as a variable – to establish whether exogenous insulin materially affects BAT responses. Furthermore, an interventional study design (for instance, utilising concurrent glucose and insulin infusions) could establish a causative direction of the presented results.

4.6 Future development of infrared thermography

Infrared thermography is increasingly accepted as an important method for measuring BAT activation. However, key questions about its validity and implementation continue to be legitimately asked (Gatidis et al. 2016; Moonen, Nascimento, and van Marken Lichtenbelt 2019; Jimenez-Pavon et al. 2019) some of which are developed in the current body of work and some of which will require further work.

Future work focused on children needs to better account for pubertal status either by focusing on participants at the same stage (e.g. pre-pubertal) or by measuring and correcting for it, with study sizes calculated to allow for this homogeneity.

To date, thermal imaging studies have used a wide range of stimulation protocols (Moonen, Nascimento, and van Marken Lichtenbelt 2019; Jimenez-Pavon et al. 2019). Where the stimulus itself is being tested (Dulloo et al. 2000; Velickovic et al. 2019; Yoneshiro et al. 2012), this may be appropriate but where cooling is used to measure differences in supraclavicular temperatures between groups, consistency would allow better comparison between studies. Variations in the precise definitions used for the region of interest and the point measurement of that region of interest may contribute to the lack of positive results in some studies (Martinez-Tellez et al. 2019). As such, uniform protocols also need to be established and universally adopted to allow results to be quality controlled and results compared between groups. However, at apparent tension with this, methods are being rapidly developed – including locally with plans to fully automate the analysis process. Protocols, therefore, need to establish core principles (such as acclimatisation standards, definitions of regions of interest and which percentile is used as the point measure) while not preventing innovation from continuing. Within the use of IRT in sports medicine, a Delphi consensus statement has been produced to outline best practice (Moreira et al. 2017) and an equivalent adapted version for use in BAT studies would be a useful development.

Gatidis et al. (2016) found that a single-point unstimulated thermal image correlated with BAT on thermoneutral PET/CT, undertaken for clinical use, but that significance is lost when correcting for subcutaneous adipose tissue or BMI. Imaging at thermoneutral conditions underestimates BAT prevalence (Lee et al. 2011) and I have shown that IRT outcomes are most appropriately measured using the participant as their own control by use of comparison of supraclavicular temperature to a reference point and by measuring the change from (rested) baseline temperature (Section 3.2). The relevance of subcutaneous adipose tissue as a confounder, along with the potential heat-generating

properties of other local tissues, remains an area of active debate (Moonen, Nascimento, and van Marken Lichtenbelt 2019; Jimenez-Pavon et al. 2019). While the net effect, as least in lean males, results in a close correlation between results from PET/CT and IRT (Section 3.2), further work considering the interaction of other heat generation (muscle), insulation (adipose), and transportation (vasculature) is needed and future work should utilise a multi-faceted approach using *in-silico* modelling, complex phantoms and *in-vivo* work. The same studies may also seek to establish whether surrogate markers, such as skin-thickness (measured using callipers) or more general adiposity measures (such as BMI, or waist or hip circumference), can be reliably used to adjust supraclavicular skin measurements for the insulative effects of subcutaneous adipose tissue thickness (measured on PET-CT).

Jimenez-Pavon et al. (2019) criticise much of the work in the field of measurement of BAT by IRT for its low methodological quality but use a scale designed to assess clinical trials of physiotherapy which scores against criteria not relevant to many of the studies in question. However, the lack of randomisation and blinding of assessors is a source of potential bias. As infrared thermography is being increasingly used in the field of BAT research, findings from some of the early exploratory work must be tested robustly using a design to address these current gaps. As such, a sufficiently large, randomised control trial of stimulated and non-stimulated participants by a group with experience in undertaking studies using infrared thermography in human volunteers is now timely.

Section 5 Appendices

5.1 Brown fat imaging using thermography (BITS) — Study Documentation

5.1.1 Research Ethics Committee Approval

University of Nottingham
UK | CHINA | MALAYSIA

Email: FMHS-ResearchEthics@nottingham.ac.uk

Faculty of Medicine & Health Sciences
Research Ethics Committee
c/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
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24 August 2017

Dr James Law
Clinical Lecturer in Child Health
Division of Child Health, Obstetrics & Gynaecology
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Dear Dr Law

Ethics Reference No: : D19062014 **– please always quote**	
Study Title: A study of brown adipose tissue activation in healthy volunteers using thermal imaging.	
Chief Investigator/Supervisor: Professor Helen Budge, Professor of Neonatal Medicine, Division of Child Health	
Co Investigators/supervisors: Professor Michael Symonds, Professor of Developmental Physiology, Dr James Law, Clinical Lecturer, Division of Child Health.	
Other Key Investigators/student: Medical students conducting BMedSci project, NIHR Academic Clinical Fellows/NIHR Academic Foundation Programme doctors, Post-doctorate researchers and Doctorate students registered with the University of Nottingham - to be assigned.	
Type of Study: Student project	
Proposed Start Date: 30/06/2014	**Proposed End Date:** 30.06.2020 - 6 years
No of Subjects: 10-30 per group	**Age:** 16+years
School: School of Medicine	

Thank you for notifying the Committee of Amendment no 3: 24 July 2017 as follows:

- Addition of measurements of whole body composition and autonomic nervous system activation.
- Extension of Study End date to 30.06.2020

and the following documents were received:

- FMHS REC Notice of Amendment, Application form and supporting documents version 1.2: 24.07.17

These have been reviewed and are satisfactory and the study amendment no 3: 24 July 2017 has been given a favourable opinion.

A favourable opinion is given on the understanding that the conditions set out below are followed:

1. You should follow the protocol agreed and inform the Committee of any changes using a notification of amendment form (please request a form).
2. You must notify the Chair of any serious or unexpected event.
3. An End of Project Progress Report is completed and returned when the study has finished (please request a form).

Yours sincerely

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

5.1.2 Protocol

A study of brown adipose tissue activation in healthy volunteers using thermal imaging

Version:	Version 1.2 24th July 2017
Short title:	Brown fat imaging using thermography study (BITS)
REC reference:	D19062014 SoM Child
Trial sponsor:	University of Nottingham
Funding source:	University of Nottingham

STUDY PERSONNEL AND CONTACT DETAILS

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Abbreviations

AE	Adverse Event
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CI	Chief Investigator
GCP	Good Clinical Practice
JPEG	Joint Photographic Experts Group (a standard format for electronic pictures)
PIS	Participant Information Sheet
REC	Research Ethics Committee
R&D	Research and Development Department
UCP1	Uncoupling Protein 1

BACKGROUND

Brown adipose tissue (BAT) is a highly metabolically active tissue, with a basal oxygen consumption rate many times that of white adipose tissue (WAT) (1). BAT is essential to the generation of heat in non-shivering thermogenesis, a key component of the early adaptation of the newborn in their transition from the warm intrauterine environment to the colder extrauterine one. BAT achieves its heat-generating properties via the expression of the uncoupling protein 1 (UCP1) which is unique to this tissue. UCP1 uncouples adenosine triphosphate (ATP) production at the mitochondrial membrane from the oxidation of glucose. Instead, the energy is dissipated as heat. This function is in stark contrast to that of white adipose tissue which primarily stores energy as fat.

In adult humans, the main depot of BAT is located in the supraclavicular region (2, 3). Previous attempts to study BAT have relied on invasive or expensive techniques. The main methods have been post-mortem studies, tissue biopsy and positron emission tomography with F-18 fluorodeoxyglucose (FDG-PET), single-photon emission tomography (SPECT) scanning with radioactive tracers such as I-123-meta-iodobenzylguanidine or Tc-99m-tetrofosmin. Therefore, studies have been limited in the number of participants studied and it has been difficult to justify studying volunteers in whom such investigations are not clinically indicated, given the degree of radiation exposure. A new technique has been developed which uses non-invasive infrared thermography (thermal imaging) to measure the temperature change in the specific area of the neck overlying BAT and data validating this technique has been recently published(4). Infrared thermography overcomes the problems of studying BAT in healthy volunteers as it is completely non-invasive, acceptable to volunteer subjects, cost-effective, simple and quick. Repeated measurements are also acceptable.

We have previously shown that BAT activation occurs in children following immersion of a hand in cool (~15-20°C) tap water (4) (Nottingham Medical School Ethics Reference Number Q/09/2010). However, it is well documented that not all adults have

demonstrable active BAT (2). There are a number of proposed factors which may influence BAT activation, and the relative importance and effect of these factors remains unclear. Factors such as age, BMI, race and gender are known to affect BAT activity (5-8) and their effect on BAT activation measured using thermal imaging has been studied a little in children (4, 9) but not at all in adults. For other factors there is some data from animal models, indicating that lactation affects adiposity (10) and BAT activity (11), and that BAT is activated by calorie intake from a single meal (12) but human data is scarce or absent (13). BAT is also activated by cold but emerging evidence suggests a more intricate relationship with the seasons and weather, including day length, temperature and season (14-16). This data is largely observational comparing separate cohorts. In order to explore the factors responsible for BAT activation more directly, we will investigate the effect on BAT activation of intrinsic factors such as age, body composition, autonomic nervous system activation, race and gender and extrinsic factors such as the stimulus used and the environmental conditions (e.g. room temperature, outdoor temperature, and season). With the exception of breastfeeding, the stimuli proposed in this study have been previously approved by the Nottingham Medical School Ethics Committee (Reference D 05 2011).

STUDY OBJECTIVES AND PURPOSE

Purpose

This is a study to look at factors affecting BAT activation measured using thermal imaging.

Primary outcome

The primary outcome will be brown adipose tissue activation, calculated by measuring the change from baseline in the median temperature of the upper percentiles of temperature in the region of interest in the front of the neck using a thermal imaging camera.

Secondary objectives

The secondary outcomes include:

- associated changes in physiological measurements such as heart rate, skin perfusion, resting energy expenditure, or skin temperature
- effect of influencing factors such as season, gender, age, body composition, sympathetic nervous system activation and environmental temperature.
- effect of potential stimulation including changes in room temperature, cooling, eating, mental challenges (such as arithmetic) or the act of breastfeeding.

STUDY DESIGN

The study will be conducted using healthy volunteers and carried out within the campuses of the University of Nottingham or at convenient sites for study participants such as schools and sports centres (with permission from relevant authorities). Participants will be recruited by word of mouth and posters/flyers.

Each participant will be required to attend at least one study session but will be invited to provide consent to be contacted at a later date and asked if they wish to attend a further session in order to look at intra-participant variation in BAT activity, for instance associated with changes in season.

At each study session, each participant will be exposed to a single BAT stimulus or will be assigned to a control group. The effect of stimuli will be ascertained by comparing the activation of different groups. Participants may attend more than one session and the statistical analysis will allow for paired values between the groups. Associations with physiological parameters and influencing factors will be investigated by analysing the data using appropriate statistical modelling.

Participants who give informed consent will have basic anthropometric and physiological measurements taken (such as height, weight, body composition, heart rate, autonomic nervous system activation, skin temperatures and core (tympanic) temperature) and be asked to provide personal information relevant to the primary and secondary objectives

(including age, gender and race). A series of baseline (unstimulated) images will be acquired. The BAT stimulus will then be introduced unless they are assigned to the control group. In either case, a further series of thermal images will be acquired.

In order to standardise the effect of the stimulus, the volunteer may be asked to arrive having only taken clear fluids from midnight. In this case, volunteers will be allowed to freely drink water or sugar-free fruit squash but will be asked to refrain from drinks containing caffeine or calories. Where abstinence is required, imaging will be conducted in the morning and will be finished before midday. No volunteer will be asked to undertake any dietary manipulation prior to written informed consent being obtained. Only healthy adult volunteers (over 16 years old) without relevant medical problems, such as diabetes, will be eligible to take part in studies where they are required to make changes to their usual dietary intake.

This non-invasive study will be undertaken by appropriately trained investigators who are members of staff or students at the University of Nottingham. Any investigator who is not medically qualified will first attend a number of study sessions with a medically qualified investigator to become familiar with the techniques and will only undertake sessions using the same stimulus and in the same environment as that in which they have observed. Where a cooling stimulus is being used, the investigator will be familiar with the signs and symptoms of hypothermia, know the initial management, and will have access to a medically qualified investigator, if not medically qualified themselves.

The pixels of a thermal image contain data about the temperature recorded at that point (in contrast to the pixels of a photographic image which contain colour data). On each thermal image, the region of interest will be defined and the pixel data for the region of interest will be analysed to determine the median temperature of the upper percentiles.

Duration of the study and participant involvement

- Recruitment to the study will commence on the first University working day following notification of Ethics Committee Approval and conclude 5 years and 364 days from the study's start, giving a total duration of 6 years.
- The end of the study will be following the last session on the last participant expected to be on or before end July 2020.
- Each participant will be required to attend at least one session lasting not more than two hours.
- Participants will be invited to give their consent for the investigators to contact them in the future and invite them for further imaging, but this will not be essential for participation.
- Participants' fully anonymised data will remain on a database of thermal imaging participants. This will be stored securely by the University of Nottingham in order to allow comparisons to be made with future subjects and studies.
- Participants may withdraw from the study at any time and without giving reason. Participants will be made aware (via the information sheets and consent form), that should they withdraw from the study the data collected to date cannot be erased and will be used in the final analyses, although no further data will be collected.

Eligibility criteria

- Volunteers will be composed of healthy adults and children more than five years old.
- Participants with scar tissue affecting the area of interest or medical conditions and medications likely to affect BAT activity, as determined by the investigators, will be excluded.
- Participants with an allergy or dietary restriction to a proposed stimulus will be excluded.

Recruitment

- The study will be carried out in the on the campuses of the University of Nottingham or at sites convenient to study participants such as schools and sports centres.

- Participants will be recruited by posters/flyers and by word of mouth. Potential participants will express their interest by contacting the researchers either by telephone or email. When contact is made, participants will be provided with a copy of the participant information sheet (PIS) by email or post. They will have the PIS for at least 24 hours prior to providing informed consent to participate in the study session.
- Where the participant is under 16 and is not deemed to be Gillick competent by the Investigator, informed consent will be sought from their parent or legal guardian. The child's verbal assent will also be checked on the study day.

Description of interventions

Non-invasive anthropometric and physiological measurements will be taken such as height, weight, heart rate and core (tympanic) temperature, using standard equipment which is commercially available for these purposes and conforms to necessary ISO standards where applicable. Body composition and autonomic nervous system activation will be calculated using measures of bioimpedance (BIA-ACC, Biotekna, Venice, Italy) and photoplethysmography (PPG). Participants will be asked to provide personal information relevant to the primary and secondary objectives such as age, gender and race. Participants will be asked whether they take any medications and whether they have any medical conditions, as medications such as beta-blockers and conditions such as hyperthyroidism, may affect brown adipose tissue function.

Participants will be positioned facing a thermal imaging camera to allow a series of baseline (unstimulated) images to be acquired prior to the introduction of a BAT stimulus. A further series of thermal images will then be acquired. During image acquisition, typically several images will be taken each minute for up to two hours. Participants may also be assigned to a control group who would have the same images acquired but would not be exposed to a stimulus.

Brown adipose tissue will be activated by:

- applying a cooling stimulus.

Our previous cooling techniques include: cooling of a peripheral region of the body for instance by immersion of their hand in cool tap water (typically 15-20°C) or application of a commercially available cooling mat, whole-body cooling, for instance, using a cooling mattress or bodysuit, or manipulation of the room temperature to slightly below thermoneutrality (at temperatures from 17°C at which the volunteer is still comfortable but not shivering). Shivering will be avoided as it may be uncomfortable and undesirable (activating an alternative pathway of thermogenesis). Core body temperature will be frequently measured throughout any period of cooling to ensure it remains above 35.0°C. In order to help ensure hypothermia (defined as a core body temperature of less than 35.0°C) is not induced, if a core body temperature less than 35.3°C is recorded, the study session will be terminated and cooling stimulus removed.

- consuming standard food and drink such as water, milk, a snack or a small meal. Any dietary restrictions including allergies will be ascertained prior to the study commencing. Where exposure to a dietary restriction cannot reasonably be excluded, the study will not continue.
- mental exercise such as arithmetic tests.
- breastfeeding.

  A cohort of women who are currently breastfeeding will be imaged during breastfeeding to assess the response of BAT. Sufficient privacy will be provided and images will be taken by a female member of the research team. Images will be focused on the head and neck. Neither the infant nor the nipples and areolar of the breast will be visible on any images for analysis.

STATISTICS

Methods

Thermal images will be 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 will be displayed as coloured isotherms.

The change in median temperature of the region of activity between the baseline and stimulated images will be calculated (ΔT). The normality of the data will be tested. Comparisons between groups experiencing different BAT stimuli will be tested using analysis of variance (if data have a parametric distribution) or Mann-Whitney U test (if data have a non-parametric distribution). Associations with physical parameters and influencing factors will be investigated using statistical modelling. Statistical advice will also be sought from an experienced statistician from the University of Nottingham if required.

Sample size and 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. For instance, using previously published data in children (4) with a mean BAT activation of 0.37°C and an estimated standard deviation 0.368, for an expected biologically significant change of 0.3°C, 25 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 (17). In adults with an estimated standard deviation of 0.212 (4), 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 (17).

In order to allow for any dropout or potential technical problems with images, we will aim to recruit 20% more than the minimum number required to give an 80% power (rounded up to the nearest whole number).

STUDY MANAGEMENT

The Chief Investigator will have overall responsibility for the study and shall oversee all study management. The data custodian will be the Chief Investigator.

Criteria for terminating the study

Previous studies conducted by the research group have not shown any safety concerns with the study regimen in healthy volunteers. If safety concerns become evident, then the study would be terminated.

Data Management

The data will be collected by a member of the research team and stored by the research team securely at the University of Nottingham during the data collection period, and then by the Chief Investigator, or their nominated replacement, for seven years following the last publication. During the study, the research team will meet in alternate weeks.

ADVERSE EVENTS

No adverse events are expected as a result of participation in this study. Any event which causes a study session to be terminated prematurely will be recorded and the Chief Investigator will be informed.

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5.1.3 Participant information Leaflet (children)

What do you have to do?

If you think you will want to take part in the study, you should avoid tea, coffee or cola the day you come to see us but you can otherwise eat and drink normally. It would also help if you can avoid running around for one hour before you see us. We don't mind what you do after the pictures have been taken.

If it is okay with you, we may contact you another time to ask if you would like to take part again.

You can change your mind at any time. You do not

What if you want to stop doing the study?

have to tell anyone why. No-one will mind. We will still need to use the data we already have. No-one will be able to tell that you have taken part.

What if there is a problem?

If you have a problem or worry, let your parent know or speak to one of the people helping to run the study.

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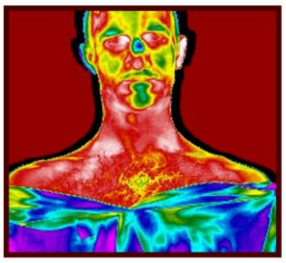
Study Information For Children



The
B rown fat
I maging using
T hermography (heat)
S tudy

BITS
Study Volunteers Wanted

01001010
01100001
01101101
01100101
01110011

What is this study about?

We would like you to take part in our study. We want you to know why we are doing this work. We will tell you what you would need to do. We will go through this sheet with you. We will answer any questions you have.

You can talk to your parents about the study. Please ask any questions you think of.

What is the purpose of the study?

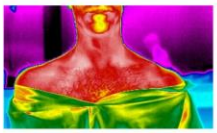
We want to learn more about a special part of your body. It is called brown fat and it works to keep us warm. Brown fat may help to stop people getting fatter. We are interested in what things turn it on and off.

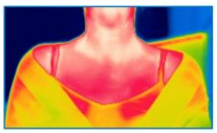
Why have you been invited?

You have been invited because you have expressed an interest in taking part.

What do the pictures look like?

The part of the body we want to see is mainly in the neck. We can see it using a special camera which can "see" heat.





The heat camera takes the heat images in a similar way to a normal digital camera but shows hot and cold rather than details of the way we look. The pictures are centred on the neck.

What does it involve?

You will need to wear a vest top. We will record how fast your heart is going. We will put a button on your skin that tells us how warm your skin is.

We would then like to take some heat pictures and ask you to do something which will turn on the brown fat. This may be putting your hand in cool water, or drinking for instance.

Do you have to take part?

You do not have to take part. It is up to you. If you do not want to take part, just tell your parent or one of the doctors or nurses looking after you.

5.1.4 Participant information Leaflet (adults)

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Will taking part be kept confidential?

We will follow ethical and legal practice and all information which is collected about you during the course of the research will be kept strictly confidential. The data will be stored in a secure and locked office, and on a password protected database. Any information that leave the research unit will have your name and address removed so that you cannot be recognised from it. Only members of the research team and regulatory authorities will have access to your data.

What will happen to the results?

The results of the study will be published in academic journals so that others can see them. You will not be identified in anyway.

Who is funding the research?

This study is being organised by, and has been funded by, the University of Nottingham.

BITS Information Leaflet FV1.2 01.10.15

What if there is a problem and I want to complain?

If you have a concern about any aspect of this study, you should ask to speak to the researcher (Dr Law) who will do his best to answer your questions, or you can speak to the Chief Investigator, Prof Helen Budge on 0115 8230611. If you are still unhappy, you should then contact the Ethics Committee Secretary, Mrs Sabir on 0115 8231063 or louise.sabir@nottingham.ac.uk

Contact:

If you would like more information, please contact:

James Law

either by

telephone: 0115 8230618

or by email:

james.law@nottingham.ac.uk

Thank you for taking the time to read this leaflet

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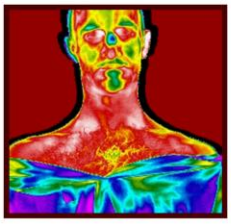
BITS
Study Information



The
Brown fat
Imaging using
Thermography (heat)
Study

BITS Study Volunteers Wanted

01001010
01100001
01101101
01100101
01110011

What is this study about?

We would like to invite you to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part or not. You may keep this leaflet.

What is the purpose of the study?

The study aims to increase our knowledge about brown fat. This is a special tissue in the body which produces heat and may help people avoid becoming overweight. We are interested in how different factors may affect the activity of this tissue. These include factors to do with you (like your age and gender) and factors to do with your environment (such as the temperature).

Why have you been invited?

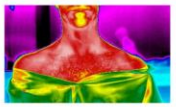
You have been invited because you have expressed an interest in taking part.

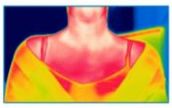
What does the study involve?

We would like you to meet with you on a single occasion initially. When we meet you, we will confirm that you still want to take part and ask you to sign a consent form. You will be given a copy to keep. You will be asked if you would be happy for us to contact you again in the future. We would then like to take some basic details like your date of birth, height and weight followed by a series of thermal (heat) images over up to 2 hours. Part way through this time we will ask you to do something which will stimulate the brown fat. This may be putting your hand in cool water, or drinking something for instance. You will know exactly what you will be asked to do before we start. We may also ask to measure things like your heart rate and skin temperature during this time. If you think you will want to take part in the study, you should avoid caffeine, alcohol or recreational drugs from midnight and we would ask you are as sedentary as possible for one hour prior to the imaging.

What do the pictures look like?

The heat camera takes the heat images in a similar way to a normal digital camera but shows hot and cold rather than details of the way we look. The pictures are centred on the neck.





What do you have to do?

Before the study session, we ask that you refrain from caffeine, alcohol, or recreational drugs from midnight if you wish to take part. You may also be asked not to eat or drink anything except water and squash from midnight. There are no restrictions about what you can do after the imaging has been done.
If you give us permission we may contact you in the future to ask if you would like to take part in further imaging or brown fat studies.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Any information already collected will be used in the final analysis but no further information will be collected.

What are the possible disadvantages and risks of taking part?

We do not expect there to be any risk in taking part. You will be able to take any usual medications.
If you have a significant medical condition or are taking certain medications, you may not be able to take part in the study to avoid it affecting the results. The investigator will discuss this with you when you speak to them.

5.1.5 Consent form (children without capacity)

University of Nottingham,
School of Medicine,
Division of Child Health, Obstetrics & Gynaecology

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Healthy Volunteer's Consent Form for children without capacity
Final version 2.0: 8th July 2014

BROWN FAT IMAGING USING THERMOGRAPHY STUDY (BITS)

Principal Investigator: Professor Helen Budge
Co-Investigators: Dr James Law, Professor Michael Symonds

REC ref: D19062014 SoM Child

Name of Participant:_____

Study number:_____

Please initial box

1. I confirm that I have read and understand the information sheet version number 1.1 dated 8th July 2014 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my child's participation is voluntary and that I am free to withdraw them at any time, without giving any reason. I understand that should I withdraw them then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐
3. I understand that data collected in the study may be looked at by responsible individuals from the University of Nottingham and the research group where it is relevant to my child taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my child's personal details will be kept confidential. ☐
4. I understand that information about my child recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published. ☐
5. My child has not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation. ☐
6. **Optional:** I consent to being contacted in the future regarding further imaging or brown fat studies. ☐
7. **Optional: Consent for storage and possible use in future research** I agree that the data sets collected about my child can be stored by the above research team at the University of Nottingham for possible use in future studies (approved by an Ethics Committee). ☐
8. I understand that some of these studies may be carried out by researchers other than the current Research team. Any data used will be anonymised, and I will not be identified in any way. ☐
9. I confirm that I have disclosed relevant medical information before the study. ☐
10. I voluntarily agree for my child to take part in the above study. ☐

Name of Parent	Date	Signature
Name of Person taking consent	Date	Signature

2 copies: 1 for participant, 1 for the project notes.

5.1.6 Consent form (adults)

University of Nottingham,
School of Medicine,
Division of Child Health, Obstetrics & Gynaecology

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Healthy Volunteer's Consent Form
Final version 2.1: 1st October 2015

BROWN FAT IMAGING USING THERMOGRAPHY STUDY (BITS)

Principal Investigator: Professor Helen Budge
Co-Investigators: Dr James Law, Professor Michael Symonds

REC ref: D19062014 SoM Child

Name of Participant:_____

Study number:_____

Please initial box

1. I confirm that I have read and understand the information sheet version number 1.2 dated 1st October 2015 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐
3. I understand that data collected in the study may be looked at by responsible individuals from the University of Nottingham and the research group where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐
4. I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published. ☐
5. I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation. ☐
6. **Optional:** I consent to being contacted in the future regarding further imaging or brown fat studies. ☐
7. **Optional: Consent for storage and possible use in future research** I agree that the data sets collected about me can be stored by the above research team at the University of Nottingham for possible use in future studies (approved by an Ethics Committee). ☐
8. I understand that some of these studies may be carried out by researchers other than the current Research team. Any data used will be anonymised, and I will not be identified in any way. ☐
9. I understand that prior to attending the study session, I may need to refrain from eating and drinking anything from midnight except water and sugar-free squash. I do not know of any health reason why I should not fast in this manner. ☐
10. I confirm that I have disclosed relevant medical information before the study. ☐
11. I voluntarily agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

2 copies: 1 for participant, 1 for the project notes.

5.2 BRown Adipose tissue in children with Diabetes or Thyroid dysfunction (BRAIDT) — Study Documentation

5.2.1 Research Ethics Committee Approval

NHS
Health Research Authority
NRES Committee East Midlands - Nottingham 2
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

08 May 2013

Dr Helen Budge
Clinical Associate Professor in Neonatology
University of Nottingham
Child Health, Division of Human Development
School of Clinical Sciences, E Floor, East Block
Queen's Medical Centre Campus, Nottingham
NG7 2UH

Dear Dr Budge,

Study title:	**A case-control study of brown adipose activation in children with Type 1 diabetes mellitus, hypothyroidism or hyperthyroidism**
REC reference:	**13/EM/0102**
Protocol number:	**12086**
IRAS project ID:	**88229**

Thank you for your letter of 26 April 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Lynda McCormack , nrescommittee.eastmidlands-nottingham2@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the

study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity	Henderson Corporate	06 August 2012
Investigator CV	Dr Helen Budge	12 February 2013
Letter from Sponsor	Signed by Paul Cartledge	12 February 2013
Letter of invitation to participant	Parent Letter Version 1.0	10 February 2013
Other: Personal Information Form	1.0	10 February 2013
Other: Data Collection Form	1.0	10 February 2013
Other: Telephone Discussion Pro Forma	1.0	10 February 2013
Other: Parent Letter	Healthy Volunteer - Version 1.0	10 February 2013
Other: Letter from Funder	Email from Richard Berrington - Nottingham Hospitals Charity	11 February 2013

Participant Consent Form: Young person with capacity	2.0	25 April 2013
Participant Consent Form: Parent	2.0	25 April 2013
Participant Information Sheet: 5-10 year olds	2.0	25 April 2013
Participant Information Sheet: 5-10 year olds (siblings)	2.0	25 April 2013
Participant Information Sheet: 5-10 year olds (healthy volunteers)	2.0	25 April 2013
Participant Information Sheet: 11-15 year olds	2.0	25 April 2013
Participant Information Sheet: 11-15 year olds (healthy volunteers)	2.0	25 April 2013
Participant Information Sheet: 11-15 year olds (siblings)	2.0	25 April 2013
Participant Information Sheet: Parents of healthy volunteers	2.0	25 April 2013
Participant Information Sheet: Parents of siblings	2.0	25 April 2013
Participant Information Sheet: Parents	2.0	25 April 2013
Participant Information Sheet: 16 year olds	2.0	25 April 2013
Participant Information Sheet: 16 year olds (healthy volunteers)	2.0	25 April 2013
Participant Information Sheet: 16 year olds (siblings)	2.0	25 April 2013
Protocol	1.0	10 February 2013
Questionnaire: Physical Activity Questionnaire	2.0	25 April 2013
REC application	88229/41269 0/1/1	11 February 2013
Response to Request for Further Information		26 April 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/EM/0102	Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely,

Dr Martin Hewitt
Chair

Email: NRESCommittee.EastMidlands-Nottingham2@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Paul Cartledge*

Charlotte Davies, Nottingham University Hospitals NHS Trust

5.2.2 Protocol

A case-control study of brown adipose activation in children with Type 1 diabetes mellitus, hypothyroidism or hyperthyroidism

Final Version 4.0 10th May 2016

Short title: BRown fat Activation In Diabetic & Thyroid disorders in children

Acronym: BRAID-T

NRES reference: 13/EM/0102

Trial Sponsor: University of Nottingham

Sponsor reference: 12086

Funding Source: University of Nottingham

STUDY PERSONNEL AND CONTACT DETAILS

Sponsor: University of Nottingham

Contact name
Ms Angela Shone
Head of Research Grants and Contracts
Research and Graduate Services,
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Lenton Lane, Nottingham, NG7 2NR

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Study Coordinating Centre: Division of Academic Child Health,
University of Nottingham
Queens Medical Centre Campus, Derby Road,
Nottingham, NG7 2UH

SYNOPSIS

Title	A case-control study of brown adipose activation in children with Type 1 diabetes mellitus, hypothyroidism or hyperthyroidism
Short title	BRown fat Activation In Diabetic & Thyroid disorders in children
Acronym	BRAID-T
Chief Investigator	Professor Helen Budge (Professor of Neonatal Medicine)
Objectives	To determine whether BAT activation is altered in children with Type 1 diabetes, hypothyroidism or hyperthyroidism
Study Configuration	Single centre (Queen's Medical Centre) case-control study
Setting	Secondary care (paediatric clinics, general endocrinology clinics and paediatric pre-operative assessment clinics)
Sample size estimate	This is a pilot study to assess feasibility. Using previously published data [1] with a mean BAT activation of 0.37°C and standard deviation 0.368 in healthy children, for an expected biologically significant change of 0.3°C, 36 participants in the control group and 18 in each of the other three groups, would give 80% power to detect the difference with an α of 0.05 for a two-tailed t-test. As this visit requires a single visit, dropout is not anticipated. However, to allow for any potential technical problems with images, 40 participants will be recruited into the control group and 20 patients will be recruited in each of the other three groups. Addendum:

	Recent data suggests there is a seasonal effect of BAT activity [2]. In order to be able to allow for any variation due to the time of year, we would like to increase our numbers to 80 participants in the control group and 40 patients in each of the other group to allow recruitment across seasons.
Number of participants	There are 3 groups of 40 children (patients with diabetes, hypothyroidism, hyperthyroidism) and a control group of 80 children, making a total of 200 participants.
Eligibility criteria	Patients between 5 years and 16 years old with a diagnosis of Type 1 diabetes mellitus, hypothyroidism or hyperthyroidism, who are able to attend the study session. Healthy controls aged between 5 years and 16 years of age who are able to attend the study session. These subjects will be siblings of patients or patients attending for simple unrelated surgical procedures (e.g. grommet insertion).
Description of interventions	Patients and the control volunteers will be asked to sit facing a thermal imaging camera whilst non-invasive baseline measurements are taken. Brown adipose tissue activation will be stimulated by gentle cooling of their hand by a cooling blanket or immersion in cool tap water (typically 15-17°C) followed by 10 minutes of further imaging with the thermal camera. Height, weight, heart rate, skin temperature and tympanic membrane temperature will be measured non-invasively. Patients with diabetes will be asked to test their blood sugar level at the end of the period of imaging as they would routinely do more than once a day. Where specific consent is provided, participants will provide a sample of saliva taken for DNA. Patients and control subjects will also be asked to complete a questionnaire to assess their background levels of physical activity.
Duration of study	1st June 2013 - 1st June 2017

	Each participant initially required to attend a single study session of 1-hour duration. Patients with a new diagnosis of hypothyroidism or hyperthyroidism will be invited for a repeat study session at 6-12 months.
Outcome measures	The primary outcome will be: • The difference in supraclavicular BAT activation with stimulation between healthy controls and patients with Type 1 diabetes mellitus, between healthy controls and patients with a diagnosis of hypothyroidism and between healthy controls and patients with hyperthyroidism. Activation is measured as the change in mean temperature of the region of interest. Secondary outcomes are: • Difference in baseline BAT temperature between groups • Effect of blood sugar level on BAT activation in diabetic patients • Effect of HbA_{1C}, a measure of diabetic control, on BAT activation in diabetic patients • Effect of current thyroid status on BAT activation in patients with thyroid disorders • Relationship between BMI and BAT activation • Effect of physical activity levels on BAT activation • Effect of environmental temperature, day length and seasonality on BAT activation.
Statistical methods	Thermal images will be analysed to determine the mean temperature of the regions of activity, defined as the upper decile of temperature data in the region of interest (i.e. the anterior triangle of the neck). Visual representations of the data will be displayed as coloured isotherms (see Fig 1C, Page 22). The change in mean temperature of the region of activity between the baseline and 5 minute and 10 minutes measurements will be calculated (ΔT).

	Comparisons will be made between ΔT for the control group and diabetic patients, the control group and patients with hypothyroidism and the control group and patients with hyperthyroidism. The normality of the data will be tested. Comparisons will be made using analysis of variance (if data have a parametric distribution) or Mann-Whitney U test (if data have a non-parametric distribution). Statistical advice will also be sought from an experienced statistician from the University of Nottingham if required.

ABBREVIATIONS

AE	Adverse Event
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BSL	Blood Sugar Level
CI	Chief Investigator overall
CGM	Continuous Glucose Monitoring
DAP	Data Analysis Plan
DCF	Data Collection Form
DMC	Data Monitoring Committee
DoH	Department of Health
GCP	Good Clinical Practice
HbA_{1C}	Glycosylated Haemoglobin A – a measure of medium-term diabetic control
HT Act	Human Tissue Act
HTA	Human Tissue Authority
HSE	Health Survey for England
ICF	Informed Consent Form
JPEG	Joint Photographic Experts Group (a standard format for electronic pictures)
NHS	National Health Service
NRES	National Research Ethics Service
P/GIS	Parent / Guardian Information Sheet
PI	Principal Investigator at a local centre

PIS	Participant Information Sheet
REC	Research Ethics Committee
R&D	Research and Development department
SNS	Sympathetic Nervous System
TFT	Thyroid function tests
UCP1	Uncoupling Protein 1

TABLE OF CONTENTS

STUDY BACKGROUND INFORMATION AND RATIONALE

Brown adipose tissue (BAT) is a highly metabolically active tissue, generating approximately 300 Watts of heat per kilogram compared with 1 W/kg in other tissues [3]. It is essential to the generation of heat in non-shivering thermogenesis which is a key component of the early adaptation of the newborn in their transition from the warm intrauterine environment to the colder extrauterine one. BAT achieves its heat-generating properties via the expression of the protein UCP1 (uncoupling protein 1) which is unique to this tissue. This protein uncouples mitochondria from producing adenosine triphosphate (ATP) from the energy released from the oxidation of glucose. Instead, the energy is dissipated as heat. This function is in stark contrast to that of white adipose tissue which primarily stores energy as fat.

BAT shares a common embryological precursor with myocytes and they continue to share a number of features postnatally. They are both innervated by the sympathetic nervous system (SNS) and are stimulated by insulin, causing an increase in the uptake of glucose [4]. Glucose acts as the major substrate. In muscle cells, the chemical energy of glucose is converted to kinetic energy (often via storage as glycogen), whereas in BAT it is released as heat. Thyroxine is a key modulator of BAT causing upregulation of UCP1 expression resulting in a greater capacity for heat production.

In addition to the role of BAT in thermogenesis, it may be involved in energy homeostasis. BAT is activated soon after a meal [5] and is upregulated when nutritionally sub-optimal diets (a “cafeteria” diet) are provided [6] so that some obesogenic diets do not result in as much weight gain as would be expected from their calorific intake, possibly due to the dissipation of energy by BAT. In human studies, levels of BAT have been shown to be reduced in obese individuals [7, 8]. The relationship between BAT and obesity is one of significant interest since the UK has one of the fastest-growing rates of obesity in the world with a fourfold increase in the last 25 years. The latest data from the Health Survey for England [9] show that the prevalence of obesity rose from 11 to 17% among boys and from 12% to 15% among girls between 1995 and 2010. In 2010, 30% of children aged between 2 and 15 years old were classified as being overweight or obese.

Although the role of BAT in thermogenesis has been explored since the 1960s [10] and it is well described in the newborn infant, it was previously thought to be rapidly lost in childhood [11, 12]. Interest in its roles outside the newborn period has been renewed as it has recently been shown to be present in adults [8, 13-16] and children [1]. These studies have shown that, in adult humans, the main collection of BAT is located in the supraclavicular region. A

volume of 40-50g would contribute 20% of the body's energy expenditure, representing a significant clinical proportion. Estimations of the average weight of BAT present in humans vary between studies [8, 16] and there is variation between individuals. However, it is likely that much of this variation is due to the underestimation of actual activity in studies utilising retrospective analysis of PET-CT scans performed in a thermoneutral environment.

This study aims to build on what is already known about BAT by exploring the association between BAT activation and conditions where physiological control of key BAT controllers is lost, namely Type 1 diabetes mellitus (insufficient production of insulin) and thyroid dysfunction (over- or under-production of thyroxine).

As mentioned above, the key substrate for BAT is glucose, and insulin increases the uptake of glucose by BAT significantly [4]. Conversely, BAT is a key component of the body's glucose homeostasis mechanisms and its ability to regulate energy expenditure. Indeed, in a mouse model of Type 1 diabetes, BAT transplant was shown to restore glycaemic control without the need for exogenous insulin [17].

Type 1 diabetes is a condition of insufficient insulin production due to destruction of the beta-cells in the pancreas which secrete it. The lack of insulin causes hyperglycaemia (high blood sugars) which requires lifelong treatment with insulin injected into the skin. In a healthy individual without diabetes, there is tight control of blood glucose levels by variation in the amount of insulin secreted. The aim of diabetes management is to match this physiological control as closely as possible but this is never fully achievable. As a result, patients with Type 1 diabetes tend to have higher blood sugar levels than healthy controls but the suboptimal availability of insulin to cells means that they are unable to utilise the glucose efficiently. Type 1 diabetes affects 186 children under 18 in every 100 000 in the UK [18] and the management of diabetes is estimated to cost the NHS over £2bn/year [19].

The association of changes in BAT activation with this disease process has not previously been investigated in humans. We hypothesise that BAT of Type 1 diabetic patients is functionally different from that of healthy controls and will show less activation.

Although BAT is primarily activated by the SNS, thyroxine acts as a key modulator of this activation. The active form of the thyroid hormone (T3) is formed by the deiodination of the inactive form, thyroxine (T4). T4 is activated locally within the brown adipocyte via type 2 iodothyronine deiodinase (DIO2). SNS stimulation causes an increase in DIO2 activity with resulting high local concentrations of T3. These high levels saturate the cell's thyroid hormone receptors which, in turn, causes an increase in UCP1 expression [20]. Animal

models of hypothyroidism show a marked reduction in the ability to respond to cold stress by increasing non-shivering thermogenesis and removal of the ability of the cell to convert T4 to T3 results in a markedly detrimental effect on the function of the BAT [20]. We, therefore, plan to study conditions affecting an individual's thyroid status, i.e. hypothyroidism and hyperthyroidism.

Congenital hypothyroidism affects 1 in 4000 children in the UK [21]. Treatment is lifelong and consists of replacement thyroid hormone (thyroxine). The incidence of hyperthyroidism in children is approximately 0.26-0.44% [22] and current management is to render the patient hypothyroid and then replace hormone levels with thyroxine. Given the role of thyroid hormones in BAT activation, we would expect BAT to show less sensitivity to stimulation in patients with low thyroid levels than in healthy controls. In patients with hyperthyroidism, we would expect BAT to show increased activation.

Previous attempts to study BAT have relied on expensive and invasive techniques. The main methods have been positron emission tomography with F-18 fluorodeoxyglucose (FDG-PET), single-photon emission tomography (SPECT) scanning with tracers such as I-123-meta-iodobenzylguanidine or Tc-99m-tetrofosmin and/or tissue biopsy. Studies have therefore been limited in the number of participants studied and it has been difficult to justify studying volunteers in whom such investigations are not clinically indicated, given the large amounts of radiation exposure for children which are required [1]. For this reason, there are no studies to date validating these techniques in children. A new technique has been developed which uses non-invasive infrared thermography (thermal imaging) to measure the temperature change in the specific area of the neck overlying BAT and data validating this technique has been recently published [1]. Infrared thermography overcomes the problems of studying BAT in children as it is completely non-invasive, acceptable to volunteer subjects, cost-effective, simple and quick.

Utilising this validated non-invasive technique in the proposed study will help us to understand whether these endocrine conditions are associated with changes in BAT activation. Building on the proposed body of work, future techniques to manipulate brown adipose tissue activation may be able to be developed which benefit patients with endocrine disorders as well as offer new insights into weight control in the prevention of obesity.

As thermogenesis in brown adipose tissue is likely to be controlled by the combination of environmental and genetic factors, we will collect salivary DNA samples from participants who consent to such analyses. These will be analysed for genes which control BAT activation, such as UCP1. Salivary samples will be stored in the HTA compliant facility at

the University of Nottingham. All samples will be processed in accordance with the Human Tissue Authority codes of practice.

This will form an optional part of the study and those who choose not to consent will still be able to participate in the rest of the study. For those who consent to the collection of saliva for DNA analyses, all procedures will be conducted in line with Human Tissue Act (HTA) guidance on DNA storage for research [23, 24]. Samples will not be available for commercial use, there is no intention to export samples abroad and this will be explained to participants. Parents can ask for the sample to be destroyed at any time. Once participants reach 16 years of age, they will be contacted and given the option for their DNA to be destroyed. In the case that the young person cannot be contacted within 8 weeks of their 16th birthday to ascertain continuing consent, the DNA sample will be destroyed.

STUDY OBJECTIVES AND PURPOSE

PURPOSE

This is a pilot study to look at differences in brown adipose tissue activation in patients with diabetes or thyroid dysfunction.

PRIMARY OBJECTIVE

The primary objective is to test the hypotheses that the activation of brown adipose tissue following a moderate cool stimulus will be:

- reduced in patients with diabetes or hypothyroidism compared to that of healthy controls and
- increased in patients with hyperthyroidism compared to healthy controls.

Brown adipose tissue activation will be calculated by measuring the increase in the mean temperature of the upper decile of temperature data in the anterior triangle of the neck using a thermal imager calibrated to detect brown adipose tissue activation.

SECONDARY OBJECTIVES

Secondary objectives are to investigate the:

- difference in baseline BAT temperature between groups
- effect of blood sugar concentration on BAT activation in diabetics
- effect of HbA_{1C}, a measure of diabetic control, on BAT activation in diabetics

- effect of current thyroid status on BAT activation in patients with thyroid disorders
- relationship between BMI and BAT activation
- effect of physical activity on BAT activation
- effect of environmental temperature, day length and seasonality on BAT activation.

STUDY DESIGN

STUDY CONFIGURATION

This is a single-centre case-control observational study. There will be four arms to the study:

1. Patients with diabetes
2. Patients with hypothyroidism
3. Patients with hyperthyroidism
4. Healthy controls.

The cases will be recruited from the population of patients with Type 1 diabetes mellitus attending the paediatric diabetic clinic at Nottingham University Hospital NHS Trust's Queen's Medical Centre Campus, Nottingham, and patients with hypothyroidism or hyperthyroidism attending the general paediatric endocrinology clinic or admitted as an inpatient at the same hospital. Patients with dual diagnoses of diabetes and thyroid dysfunction will be excluded as it will not be possible to calculate the significance of a dual diagnosis using this sample size.

Siblings of patients in the study will be invited to take part in the study in the control subject group. Where the patients do not have a sibling, the participant does not wish for their sibling to be asked or the sibling does not wish to participate, volunteers will be recruited from patients attending for consideration of elective "simple" unrelated surgical procedures (such as grommet insertion or adenoidectomy).

The study will be conducted as a single visit at a suitably convenient time for the participants. Where possible, this will be timed to coincide with a routine visit to the hospital, e.g. outpatient clinic attendance.

STUDY MANAGEMENT

The Chief Investigator has overall responsibility for the study and shall oversee all study management.

The data custodian will be the Chief Investigator.

DURATION OF THE STUDY AND PARTICIPANT INVOLVEMENT

Recruitment to the study will commence on 1st June 2013 and conclude on 1st June 2017, giving a total duration of 4 years.

Each participant will be required to attend a single session lasting one hour, except for patients with a new diagnosis of hypothyroidism or hyperthyroidism who will be invited to participate in a second session of imaging at 6-12 months. No participant will be required to make any changes to their usual testing regimen or medication. There are no plans to follow up the participants except for patients with thyroid dysfunction.

End of the Study

The end of the study will be following the last procedure on the last participant and their salivary sample (if provided), expected to be on or before 1st June 2017.

SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

The study will be carried out at the Nottingham University Hospital NHS Trust's Queen's Medical Centre Campus. The Nottingham Children's Hospital, which is based at the Queen's Medical Centre, provides services to the East Midlands area. It is the regional centre for paediatric endocrinology including over 410 children with diabetes and thyroid disorders.

The facilities and equipment required for the conduct of the study are most readily accessible at Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus including the thermal imaging device and access to an area with a controlled and consistent ambient temperature.

This study focuses exclusively on children. Childhood is the period when the risk factors for obesity are established and the period when BAT is known to be most active. By focusing on this group, we would expect to see a contrast between the cases and controls which

would allow any differences to be identified. Childhood is also the typical period for presentation of congenital hypothyroidism and Type 1 diabetes.

Participants will be recruited from:

- Patients with Type 1 diabetes mellitus – Paediatric Diabetes Outpatient Clinic at Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus
- Patients with thyroid dysfunction – General Paediatric Endocrine Outpatient Clinic and Inpatient Wards at Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus
- Healthy control volunteers – siblings of cases or patients awaiting consideration of "simple" unrelated surgical procedures (e.g. adenoidectomy, grommet insertion) at Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus

The method of recruitment outlined below is consistent with an approach which has previously received a favourable opinion from the REC in Nottingham (e.g. ADDRESS-2, NRES reference 10/H0505/85) and which has received positive feedback from service users.

Potential participants with thyroid dysfunction or diabetes

Patients with thyroid dysfunction or diabetes generally attend outpatient clinic every 3-6 months. It will therefore be prohibitive to wait until patients attend clinic before they are approached by a member of their team and thereafter time the study protocol to coincide with their next clinic appointment in order to avoid additional trips to hospital and minimise disruption. Therefore, the initial approach will be via a letter sent from a member of the patient's usual care team (which may include the Investigator) to the parents or legal guardian of all patients fulfilling the entry criteria. This letter will inform them about the study and include the appropriate Patient Information Sheets.

Parents or legal guardians of patients who are admitted to hospital with a new diagnosis of hypo- or hyperthyroidism will be informed about the study by a member of their usual care team and given the appropriate Patient Information Sheets. The initiation of medical treatment in patients with thyroid dysfunction often restores euthyroidism relatively quickly. We believe there will still be a difference in BAT activation after this time, but any

difference is likely to be greatest soon after diagnosis when thyroid status is most abnormal.

If the patient is due to attend the outpatient clinic, a member of the care team will follow this with a telephone discussion with a person with parental responsibility and (where appropriate) the potential participant, to discuss the study further and ascertain their interest in participating. If the patient is an inpatient, this discussion will take place face-to-face and will follow the same format. If they express a positive interest, the Investigator, or member of the team, will go on to conduct a structured discussion with the person with parental responsibility and (where appropriate) participant of all aspects pertaining to participation in the study, including an assessment of eligibility against the inclusion and exclusion criteria. During this discussion, they will have the opportunity to understand the objectives, the risks and inconveniences of the study and the conditions under which it is conducted. It will be explained that entry into the study is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time without any detriment. It will be explained that, in the event of their withdrawal, the data collected so far cannot be erased and will be used in the final analyses. Details of the discussion will be recorded on the Telephone Discussion Pro-forma enclosed.

In order to allow those receiving the initial letter to express a wish to decline any further communication from the study team, a phone number will be provided on the initial letter which will allow a message to be left to this effect on an answering machine so that no direct contact with the research team is required. Potential participants will be informed that if they would like someone to call them back, a message to that effect can also be left.

If the participant or person with parental responsibility expresses a preference during a telephone call to discuss the study face-to-face, a meeting will be arranged with the Investigator or their nominee in the Division of Academic Child Health of Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus.

The study date will be a minimum of 24 hours after the initial discussion and where possible, the study date will be timed to coincide with a clinic visit or hospital admission. Where this is not possible, an alternative date will be offered and reasonable travel costs will be reimbursed. When the participant and the person with parental responsibility attend the study session, they will be given a further opportunity to discuss the study and

to ask questions. Those who wish to participate in the study will be asked for written consent prior to the study session commencing.

If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the study, the participant information sheets, and consent forms, but the consent forms and information sheets will not be available printed in other languages.

Potential participants who are siblings of patients

If at the end of the initial discussion, the person with parental responsibility and the patient express an interest in attending a study date, they will be asked whether the patient has a sibling who might like to take part as a "healthy volunteer". If this is the case, and the patient is agreeable with their sibling taking part, the appropriate Study Leaflets for Siblings will be posted and a suitable time and date arranged to discuss the sibling's participation. These details will be recorded on the Telephone Discussion Pro-forma.

If the sibling and person with parental responsibility express a positive interest, the Investigator or member of the team will go on to conduct a further telephone discussion in the same manner as detailed above. If the sibling and their parent agree to participate in the study, the study date will be offered at a convenient time. Siblings who consent to be part of the control group will be offered a study date to coincide with the clinic date for their sibling where possible. If this is not convenient, an alternative appointment will be offered.

Potential participants who are patients awaiting simple unrelated surgery

Where patients awaiting simple unrelated surgery are approached to form part of the control group, the initial approach will be from a member of the patient's care team. This will either be via a letter sent to the parents or legal guardian of patients fulfilling the entry criteria or directly by a member of the patient's care team at a clinic appointment. If they express an interest, the Investigator or their nominee will follow this up with a phone call or face-to-face meeting to discuss the study further. If the initial approach has been by letter, interest will be ascertained at the start of the contact. If they express a positive interest, the Investigator or member of the team will go on to conduct the discussion as detailed above. If the patient and their parent agree to participate in the study, the study date will be offered to coincide with a further clinical visit as detailed above.

Inclusion criteria

Cases:

- Aged between 5 years and 16 years old and
- Diagnosis of Type 1 diabetes mellitus or hypothyroidism or hyperthyroidism

Controls:

- Aged between 5 years and 16 years old and
- Sibling of a case or awaiting simple surgery (e.g. adenoidectomy, grommet insertion)

Exclusion criteria

- Dual diagnosis of diabetes mellitus and thyroid dysfunction
- Unable to attend the study session
- Unable to comply with study requirements (cool water and briefly sitting still)
- Carer or parent unable to give informed consent
- Previous or current head and neck malignancy
- Previous neck surgery
- Another endocrinological diagnosis (e.g. Growth Hormone Deficiency, Hypopituitary, Addison's Disease)
- Current participation in another study
- Currently requiring acute admission to hospital (e.g. due to Diabetic Ketoacidosis) unless due to thyroid status
- Consumption of caffeine, alcohol or recreational drugs since midnight before attending the study session
- Diagnosis of diabetes mellitus, hypothyroidism or hyperthyroidism in controls

Expected duration of participant participation

Following informed consent, each participant will be required to attend a single session lasting one hour. Patients with a new diagnosis of thyroid dysfunction will be offered the opportunity to take part in a further study session at 6-12 months. There are no other plans to follow up the participants.

Participant Withdrawal

Participants may withdraw from the study at any time and without giving a reason. Their participation may be suspended by the Investigator if it is felt that they are unable to fully comply with the study protocol e.g. if they are unable to sit still or keep their hand immersed in the water for the required time. In this eventuality, they will be thanked for their participation but their study participation will be discontinued. Following a discussion with the participant and their parent/carer, the Investigator will decide whether the participant should be withdrawn or a further attempt be made at another date.

Participants will be made aware (via the information sheet and consent form) that should they withdraw the data collected to date cannot be erased and will be used in the final analyses.

The reason for discontinuation or withdrawal will be documented at the time of the decision. No further information will be collected once the decision has been made to withdraw the participant from the study.

Withdrawn participants will be replaced where possible.

Participants may be withdrawn from the study either at their own request or at the discretion of the Investigator. The participants will be made aware that this will not affect their future care.

Informed consent

Young Persons (16 years or older)

The Investigator will explain the details of the study and provide a Study Leaflet for 16-Year-Olds, ensuring that the participant has sufficient time to consider participating or not. The parent, if present, will be offered a Study Leaflet for Parents. The Investigator will answer any questions that the Young Person and their parent or legal guardian have concerning study participation. Informed consent will be collected from the Young Person before they undergo any interventions (including physical examination and history taking) related to the study.

Where participants are deemed to have capacity to consent to take part in the study, they will be invited to do so. In this case, written informed consent will be received and this will be recorded on a “Consent Form for Young Person with Capacity”. This form will be signed and dated by the Young Person and the Investigator before they enter the study. If

the Young Person also indicates their written assent to providing a DNA sample, a parent or legal guardian will be required to provide written consent to this in accordance with the Human Tissue Act which requires parent/guardian consent for all participants below 18 years of age, and this will be recorded on the same form.

Child Participants

All other participants will have written informed consent provided by a parent or legal guardian. The Consent Form will be signed and dated by the parent or legal guardian before the participant enters the study. The Investigator will explain the details of the study and provide a Parent Information Sheet and a Child Information Sheet, ensuring that the participant and their parent or legal guardian has sufficient time to consider participating or not. The Investigator will answer any questions that the participant and their parent or legal guardian has concerning study participation. Informed consent will be collected from the parent or legal guardian of each participant before they undergo any interventions (including physical examination and history taking) related to the study. The child will give their assent on the consent form if they wish. If they do not wish to provide written assent, but indicate their assent by their behaviour, this will be recorded on the Consent Form. In the event of any conflict between the parent and the child, the child will not enter the study.

In all cases, one copy of the consent form will be kept by the participant's parent or legal guardian, one will be kept by the Investigator, and a third will be retained in the patient's hospital records. Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the study, continuing consent will be obtained using an amended Consent Form which will be signed by the participant or their parent or legal guardian as appropriate.

STUDY REGIMEN

Following recruitment, the participant will be invited to attend a study session. This will last for one hour and will be carried out on the same day as the participant is due to attend the hospital for an outpatient clinic appointment. The only foreseeable reason for the session lasting longer than one hour would be if there is a technical problem with the thermal imaging equipment. In this case, the participant will be given the option to continue to participate, return on another date, or to withdraw from the study.

No changes will be made to any treatment and no medication will be a reason for exclusion from the study unless this prevents them from attending a study session. All current medication will be documented on the Data Collection Form.

In order for the participant to be eligible for the study, they will also not have consumed any caffeine, alcohol or recreational drugs since midnight prior to attending the study session. They will be asked to have remained as sedentary as possible for one hour prior to the study.

The study session will take place in Academic Child Health situated in the Nottingham Children's Hospital of Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus. Temperature control in the study room allows the environmental temperature to be set at a standard comfortable room temperature. Previous studies have shown that the supraclavicular increase in temperature only occurs when subjects are maintained in an environmental temperature of 19-20°C compared to a warm room of 25-26°C [1].

Before commencement of the study, the Investigator will be trained in the accurate measurement of height and weight in children by the senior nursing staff of the children's growth clinic at Nottingham University Hospitals NHS Trust's Queen's Medical Centre Campus. In light indoor clothing without shoes and with the child standing upright with bare feet and his/her back in contact with the stadiometer, height will be measured with a stadiometer using the stretch stature method in which the height is given as the maximum distance from the floor to the highest point on the skull when the head is positioned so that an imaginary line joining the eyes to the tragion is at a right angle to the long axis of the body (the Frankfort plane). The child will be asked to take and hold a deep breath whilst the Investigator places the headboard onto the top of the skull, whilst ensuring that the child's feet are flat onto the ground. The measurement will be recorded to the nearest 0.1 cm at the end of the child's inspiration and will be repeated three times for reproducibility. The child will be weighed wearing light indoor clothing without shoes. The weighing scales will be zeroed and the weight measurements (repeated three times for reproducibility) taken with the child standing on the centre of scales and without support.

A targeted medical history will be obtained including a history of asthma, heart disease or neurological symptoms and recorded on the Data Collection Form along with details of any medications. Details of the last meal (time and content) will also be recorded on the Data Collection Form. In order to standardise the information provided, participants will be asked to indicate the image which most closely represents their meal using the Carbs & Cals & Protein & Fat book [25]. This is a picture book which helps determine the nutritional content of a meal.

The participant will then be seated in an upright posture with arms adducted and with head, neck and upper shoulders unclothed in order that the thermal imaging camera can detect

heat from the neck and supraclavicular area. This will typically be possible by providing a vest-style summer T-Shirt (Figure 1A) to wear and a private environment in which to change into this. An additional wrap will be provided for modesty (Figure 1B). Children will replace their own clothes immediately after imaging.

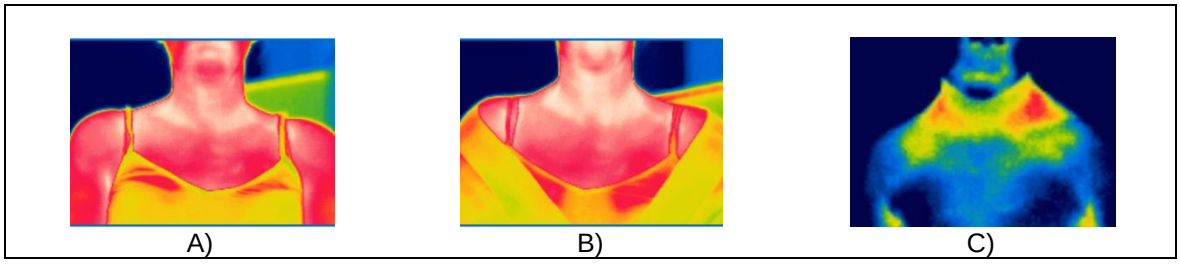

Figure 1. Thermal images of A) child wearing a vest-style T-Shirt B) with additional wrap C) Processed thermal image showing active BAT in the supraclavicular region of the neck displayed as coloured isotherms. Images provided by L. Elvidge (TIGER Study [26])

Participants will be sat in the centre of the room away from all heat-emitting objects and 1.0m away from a thermal imaging camera (FLIR b60 2.3 Megapixel Infrared Camera, FLIR Systems AB, Danderyd, Sweden) fixed on a tripod at an initial set distance of 1.0m from the floor. For smaller patients, the camera may be moved closer to ensure the shoulders fill approximately two-thirds of the image frame in order to maximise the number of pixels in the region of interest.

This positioning will ensure comfort as well as the optimum position for visualisation of the supraclavicular BAT depots. The participant's tympanic temperature will be measured using a tympanic thermometer (such as the Braun ThermoScan® IRT4520, Kaz Europe SA, Lausanne, Switzerland). An iButton® (such as model DS1921H-F50 Maxim, Sunnyvale, CA, USA) will be used measure skin temperature and a pulse oximeter (such as CMS50QA Pulse Oximeter, Contec Medical System Co. Ltd, Qinhuangdao, China) will be used to measure the participant's pulse throughout.

Once the participant is relaxed and sitting comfortably, they will be asked to remain as still as possible for up to 10 minutes in order to reduce baseline BAT activity. Following the period of acclimatisation, initial thermal image**s** will be taken to provide baseline data. Remaining seated and still, one hand will be gently cooled using either a cooling blanket or by immersion in cool tap water typically at 15-17°C. Previous work has shown that cool tap water will activate BAT, does not cause shivering or discomfort, and that consistent images are obtained [1, 26].

A series of images will be taken over 10 minutes following immersion of a hand. Our experience is that this period is well tolerated. Once the thermal images have been

recorded, the cooling stimulus will be removed. The thermal imaging recordings will be retained in the study archives; personal identifiers will be removed and each image will be labelled with the participant's study identification number, their date of birth, the image sequence number and the date that the image was taken.

Patients with diabetes will be asked to check their blood sugar level at the end of the period of imaging and this will be recorded on the Data Collection Form. This will serve as a check to ensure that normoglycaemia has been maintained and will allow the analysis of any association of blood sugar level with BAT activation. This is a minimally invasive procedure and is one which diabetic patients would expect to perform routinely more than once a day. Blood sugar levels will be measured using a glucometer and reagent strips (such as Accu-Check® Aviva Blood Glucose Meter System & Test Strips, Roche, Basel, Switzerland). If the patient has a continuous glucose monitoring (CGM) device, a reading will be taken from this and the Data Collection Form annotated.

If consent has been given, a salivary sample of DNA will be taken and stored at an HTA compliant facility at the University of Nottingham. The salivary sample will be obtained using a standard salivary DNA sampling kit (Oragene®•DISCOVER from DNA Genotek, Ontario, Canada). This is a non-invasive non-painful collection which involves spitting into a receptacle. If the participant's parent/ legal guardian do not consent and the child participant does not assent to the procedure, no sample will be taken and no further attempt to take a sample will be made.

Finally, the participants will be invited to complete a validated questionnaire to assess their recent level of physical activity [27-29] as BAT activation and physical activity are likely interrelated components of energy balance.

Patients with thyroid dysfunction will be given the opportunity to be involved in further imaging at 6-12 months. All other participants will attend one study session and no further active involvement will be required.

Consent to access the participant's medical records will be obtained as part of the consent process and detailed on the consent form. Relevant medical and demographic will be collected from the patient or their carer and their medical records and recorded on a Data Collection Form and Personal Information Form.

Data collected on the Personal Information Form will consist of:

- Study ID

- Name and NHS number
- Date of birth

Data from the study session will be collected on the Data Collection Form and will consist of:

- Height and weight on day of study
- Gender
- Core body temperature
- Skin temperature and heart rate at baseline and one-minute intervals up to 5 minutes.

Other data, also collected on the Data Collection Form, will consist of:

- Study ID & Study group
- Date of birth
- Date of visit
- Targeted medical history
- Current medication including times and doses - If on insulin a) the types of insulin used, b) the regimen and c) the time and amount of the last dose of insulin of each type
- Time and description of the last meal
- In patients with diabetes, their HbA_{1C} result closest to the study date
    - HbA_{1C} is a measure of a patient's medium-term diabetic control. This blood test is performed as part of the patient's clinical care and no additional procedure or sample is required. Results will be taken from NotIS (Nottingham University Hospitals NHS Trust's Information System) by a study Investigator.
- In patients with diabetes, blood sugar level following imaging
    - Blood sugar level monitoring is a routine part of the management of diabetes and patients with diabetes would be expected to test their blood sugar levels more than once a day every day.
- In patients with thyroid disorders, the result of the Thyroid Function Tests (TFTs) closest to study date
    - This blood test is performed as part of the patient's clinical care and no additional procedure or sample is required. Results will be taken from NotIS

(Nottingham University Hospitals NHS Trust electronic results system) by a study Investigator.

Criteria for terminating the study

Previous studies conducted by the research group have not shown any safety concerns with the study regimen in healthy volunteers. If safety concerns become evident, then the study would be terminated.

TRANSPORT AND STORAGE OF THE TISSUES

The salivary sample will be obtained using a standard salivary DNA sampling kit (Oragene®•DISCOVER from DNA Genotek, Ontario, Canada). This is a non-invasive non-painful collection which involves spitting into a receptacle. Samples will be stored in a linked anonymised format at the University of Nottingham and labelled using a combination of the participant's Study Identification Number and date of birth and the date that the sample was provided. This will permit accurate linkage to clinical data and the consent form.

Samples will be transported and stored in the receptacle pending DNA extraction. Prior to DNA extraction samples will be stored in an HTA compliant facility in the University of Nottingham. Saliva samples with be stored in aliquots at -80°C. The analysis of samples will take place at the University of Nottingham within the Division of Academic Child Health.

The master database will be held by the University of Nottingham in a password encrypted file.

LABORATORY ANALYSES

All laboratory analysis will take place in the laboratories of the Division of Academic Child Health at the University of Nottingham.

DNA will be extracted from the saliva samples and will be analysed for genes involved in the control of brown fat and for methylation. These analyses will be used to look for the correlation between epigenetic factors and BAT activation. Provision of a DNA sample is optional and this is clearly stated in the Patient Information Sheets and the Consent Form.

All procedures will be performed by appropriately trained members of the Division of Academic Child Health. The laboratories are operated according to University of Nottingham Health & Safety guidance and are supervised by University laboratory staff of

the Division of Academic Child Health who are line managed within the School of Clinical Sciences.

STATISTICS

Methods

The thermal imaging camera saves the sequentially labelled images in the Joint Photographic Experts Group (JPEG) format, with encoded radiometric metadata. Thermal images will be converted to a format which is able to be imported into Matlab (The MathWorks, Inc., Natick, MA, USA). Radiometric temperature data from the defined area of interest in the neck will be analysed using a custom-written Matlab code. The algorithm calculates the change in median temperature between regions of thermal activity. To define regions of thermal activity, temperature data will be ordered by $T_1, T_2, ..., T_n$ as $T_1' \leq T_2' \leq ... \leq T_n'$, i.e. in ascending order, where T is the temperature detected by the thermal imaging camera sensor array (˚C). Then $\overline{T}' = \sum_{i=n-m+1}^{n} T_i' / m$ will be calculated, where *m* is the size, and $\overline{T}'$ is the mean of the upper 10th percentile of temperatures in the region of interest. The variance of repeated values and the difference between $\overline{T}'$ at the five-minute post cool challenge endpoint and the prechallenge $\overline{T}'$, $\Delta\overline{T}'$ (°C), will, therefore, be calculated. Visual representations of the thermal data will be displayed as false-coloured thermal images and grey-scale thermal images with the upper percentile of pixels in the ROI highlighted.

Preliminary analysis of thermal images shows a degree of random variation overlying the trends in temperature change. In order to quantify the underlying trend more precisely, we would like to increase the frequency of images taken during cold exposure and analyse the results using a rolling average (moving average).

Data will be subjected to Kolmorgov-Smirnov analysis to determine normality. Activation and baseline temperature of BAT in each patient group, assessed on thermal imaging, will be compared to that in controls using analysis of variance (if data have a parametric distribution) or by Mann-Whitney U test (if data have a non-parametric distribution). The association of BAT activation with BMI, physical activity, blood sugar levels, HbA_{1C} and thyroid function will be undertaken using Pearson's correlation (if data have a parametric distribution) or by Spearman's rank correlation (if data have a non-parametric distribution). All statistics will be performed using PASW (formerly known as SPSS: v 17.02, IBM, Chicago, USA) software for Windows or R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria). Statistical advice will

also be sought from an experienced statistician from the University of Nottingham if required.

Sample size and justification

Using previously published data [1] with a mean BAT activation of 0.37°C and standard deviation 0.368 in healthy children, for an expected biologically significant change of 0.3°C, 36 participants in the control group and 18 in each of the other three groups, would give 80% power to detect the difference with a significance level of 5% for a two-tailed t-test [30].

As this visit requires a single visit, dropout is not anticipated. However, to allow for any potential technical problems with images, we intended to recruit 40 participants into the control group and 20 patients will be recruited in each of the other three groups.

Recent data suggests there is a seasonal effect of BAT activity. This has been shown in PET-CT scans, undertaken for another reason [2]. We would like to show whether the same difference is found in children using thermal imaging. In order to be able to allow for any variation in BAT activity due to the time of year, we would like to increase our numbers to 80 participants in the control group and 40 patients in each of the other group in order to allow recruitment across seasons.

ADVERSE EVENTS

No adverse events are expected as a result of participation in this study. In everyday life, patients with diabetes may occasionally experience episodes of hypoglycaemia and it is possible that such an episode might coincide with the study visit. If the patient, parent or medical person in attendance thinks that the child is exhibiting signs of hypoglycaemia, they will be asked to check their blood sugar. If the patient is hypoglycaemic on testing, they will take their usual hypoglycaemia oral glucose treatment as long as they are conscious and able to safely do so. This is usually a sweet drink (such as 50ml Lucozade™) or sugar tablets (such as Lucozade Sport™ glucose tablets) followed by a longer-acting carbohydrate (such as a biscuit or a piece of toast). Lucozade™, sugar tablets and biscuits will be available in the study room.

The researchers will be medically trained and able to recognise the symptoms and initiate appropriate treatment. If the patient is unconscious or unable to safely take an oral treatment, or in the event of any further concern, the QMC Campus Paediatric Medical Team is resident at QMC and always available to offer medical assistance. If any patient

with diabetes suffers an episode of hypoglycaemia, this will be recorded and noted as an adverse event.

ETHICAL AND REGULATORY ASPECTS

No ethical concerns are expected to be raised by this study. No treatment is being offered. Participants will be made aware both that entry into this study will not directly affect their medical care, but also that entry into the study would not be expected to have any detrimental effect.

A targeted medical history will be taken and any sensitive or personal information disclosed during that process will be treated as confidential and not disclosed, except where there is a professional or statutory duty to do so. This event is not considered to be likely but, if it were to occur, the participant and, where appropriate, their parent or legal guardian would be informed before disclosure unless to do so would be inappropriate or put the child at risk.

ETHICS COMMITTEE AND REGULATORY APPROVALS

The study will not be initiated before the protocol, consent forms and participant information sheets have received approval / favourable opinion from the Research Ethics Committee (REC), and the respective National Health Service (NHS) Research & Development (R&D) department. Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant and GP information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the REC are notified as soon as possible and approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately and the REC will be informed.

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996, the principles of Good Clinical Practice, and the Department of Health Research Governance Framework for Health and Social Care, 2005.

INFORMED CONSENT AND PARTICIPANT INFORMATION

The process for obtaining participant informed consent or assent and parent/guardian informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The Investigator or their nominee and the participant or other legally authorised representative shall both sign and date the Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Study records. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the study.

The decision regarding participation in the study is entirely voluntary. The Investigator or their nominee shall emphasise to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No study-specific interventions will be done before informed consent has been obtained.

The Investigator will inform the participant of any relevant information that becomes available during the course of the study and will discuss with them, whether they wish to continue with the study. If applicable, they will be asked to sign revised consent forms.

If the Consent Form is amended during the study, the Investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Consent Form by the REC and use of the amended form (including for ongoing participants).

RECORDS

Data Collection Forms

Each participant will be assigned a study identity code number, for use on Data Collection Forms, other study documents and the electronic database. The documents and database will also use their date of birth (dd/mm/yy).

Data Collection Forms will be treated as confidential documents and held securely in accordance with regulations. The Investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Study Number, to permit the identification of all participants enrolled in the study, in case additional follow-up is required.

Data Collection Forms shall be restricted to those personnel approved by the Chief Investigator and recorded as such in the study records.

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief Investigator shall sign a declaration ensuring accuracy of data recorded in the Data Collection Forms.

Source documents

Source documents will be filed at the Investigator's site at the University of Nottingham and may include but are not limited to, consent forms, biological data, and laboratory results. Only study staff shall have access to study documentation other than the regulatory requirements listed below. A copy of the consent forms will be stored in the patient's clinical medical case records at Nottingham University Hospitals NHS Trust.

Direct access will be permitted when required to all source documents and other study documentation e.g. signed consent forms, for the purpose of study monitoring and audit and other lawful regulatory inspection by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (e.g., DH, Human Tissue Authority and the REC).

Each participant will be assigned a study identity code number (the Participant Study Number) for use in the electronic database. The database will also use their date of birth. The Investigator will make a separate confidential log of the participant's name, date of birth, NHS number and Participant Study Number, to permit identification of all participants enrolled in the study. The database and the log will be handled as confidential documents and held securely in accordance with regulations. All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated and the correction inserted, initialled and dated. The Investigator and Chief Investigator will sign a declaration ensuring accuracy of data recorded. Other than the regulatory requirements listed above, only the Investigator and Chief Investigator will have access to study documentation.

All study staff and Investigators will endeavour to protect the rights of the study's participants to privacy and informed consent and will adhere to the Data Protection Act, 1998. Individual participant information obtained as a result of this study will be considered confidential and disclosure to third parties will be prohibited with the exception noted below. Participant confidentiality will be further ensured by utilising identification code numbers.

Computer held data including the study database will be held securely and password protected. All data will be stored on a secure web server. Access will be restricted by user identifiers and passwords (encrypted using a one-way encryption method). Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

Sample Labelling

Samples will be labelled with the participant's assigned study identification number and their date of birth.

Direct access to source data/documents

Study documents, including progress notes and copies of laboratory and medical test results, shall be made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for clinical study participants and study staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but study participants may have recourse through the NHS complaints procedures.

The University of Nottingham as research Sponsor indemnifies its staff, research participants and research protocols with both public liability insurance and clinical trials insurance. These policies include provision for indemnity in the event of a successful litigious claim for proven non-negligent harm.

STUDY CONDUCT

Study conduct will be subject to systems audit for inclusion of essential documents, permissions to conduct the study, curricula vitae of study staff and training received, local document control procedures, consent procedures and recruitment logs, adherence to procedures defined in the protocol (e.g. inclusion/exclusion criteria, timeliness of visits), accountability of study materials and equipment calibration logs.

The Study Coordinator, or where required, a nominated designee of the Sponsor, shall carry out a site systems audit at least yearly and an audit report shall be made.

STUDY DATA

Monitoring of study data shall include confirmation of informed consent, source data verification, data storage and data transfer procedures, local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Study Coordinator, or where required, a nominated designee of the Sponsor, shall carry out monitoring of study data as an ongoing activity.

Entries on Data Collection Forms will be verified by inspection against the source data. A sample of Data Collection Forms (10% or as per the study risk assessment) will be checked on a regular basis for verification of all entries made. In addition, the subsequent capture of the data on the study database will be checked. Where corrections are required these will carry a full audit trail and justification.

Study data and evidence of monitoring and systems audits will be made available for inspection by the REC as required.

RECORD RETENTION AND ARCHIVING

In compliance with the International Conference on Harmonisation Good Clinical Practice guidelines, regulations and in accordance with the University of Nottingham Code of Research Conduct and Research Ethics, the Chief Investigator will maintain all records and documents regarding the conduct of the study. These will be retained for at least 7 years or for longer if required. If the responsible Investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.

The study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all study databases and associated meta-data encryption codes.

Addendum:

In addition, fully anonymised data will be collected and stored on a database of brown adipose tissue thermal imaging. This data will include key demographic and medical details (such as gender, age, medications, major medical diagnoses) and study details (such as date and time, room temperature, outside temperature, water temperature) as well as the results. By pooling results, future hypotheses and research questions may be able to be investigated with greater power without exposing volunteers to further investigations. Permission for retention of this data will be given as an optional component of the consent form.

DISCONTINUATION OF THE STUDY BY THE SPONSOR

The Sponsor reserves the right to discontinue this study at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice as appropriate in making this decision.

STATEMENT OF CONFIDENTIALITY

Individual participant medical or personal information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above.

Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.

Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

Data generated as a result of this study will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

The study results will be analysed, written as a scientific manuscript and offered for peer-reviewed publication in medical academic literature. Participants will not be identified in any publications. The intention would be to have publication accepted before March 2017. Information about the outcomes of the study will also be available to participants, parents, guardians and interested parties through the University of Nottingham Academic Child Health website.

USER AND PUBLIC INVOLVEMENT

The study has been discussed with patients attending a diabetic clinic and with a local diabetic support group consisting of carers and diabetic children. These two groups have commented on the design of the study, its acceptability and offered feedback on the wording of the leaflets.

STUDY FINANCES

Funding source

This study is funded by the University of Nottingham.

Participant stipends and payments

Participants will not be paid to participate in the study but will be offered a £20 gift voucher as a thank you for their involvement. Any participant who enters the study but later withdraws, or is withdrawn, will still be offered the voucher. Travel expenses will be offered for any hospital visits in excess of usual care.

SIGNATURE PAGES

Signatories to Protocol:

Chief Investigator: (name) _____

Signature: _____

Date: _____

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5.2.3 Invitation letters

5.2.3.1 Letter to potential diabetes or thyroid group participants

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Children's Hospital,
Queen's Medical Centre,
Derby Road,
Nottingham, NG7 2UH
0115 9249924

[insert date]

Dear [insert parent's name]

I am writing to invite [insert child's name] to participate in a study that a team at the University of Nottingham, together with Nottingham University Hospital's NHS Trust, are carrying out to learn more about a tissue called brown fat. One of the groups being looked at is children with [insert condition of diabetes, underactive thyroid, overactive thyroid] and this is the reason that you are being contacted.

The study involves a short visit where [insert child's name] would be asked to sit in front of a thermal (heat) imaging camera which would take a few pictures of their neck over about 10 minutes. This visit would be on the same day as you are visiting the hospital for another reason wherever possible. We are expecting to see you in clinic in the next few weeks which is why we are sending you this letter now.

If you think you would be interested in learning more about this study please take a few moments to look at the leaflets that are enclosed with this letter. There is one for you and one for [insert child's name].

We will try and phone you over the next few days to see if you would be interested in taking part. Unfortunately due to the time it takes, it is usually only possible to offer this opportunity to four patients per clinic. So we do not disturb you unnecessarily, once we have filled the available study slots, we will not continue making phone calls. If you would like to take part but do not hear from us, please contact the research team directly – their details are on the back of the leaflet.

We would also like to invite children without diabetes or thyroid problems to be part of the comparison group. If [insert child's name] has a sibling and they might be interested in taking part, we can discuss this further when we speak to you.

If you want to tell us not to phone you, you can phone 0115 8230613 and leave a message on the answer machine. You will not need to speak to anyone directly. Alternatively, if you would like someone to call you back, please leave your name and number and a member of the research team will get back to you.

If you have any questions, please contact a member of the research team using the details on the leaflets.

Yours sincerely

[Insert Consultant]

BRAID-T Approach Letter Final version 4.0 10/05/16

5.2.3.2 Letter to potential control group participants

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Children's Hospital,
Queen's Medical Centre,
Derby Road,
Nottingham, NG7 2UH
0115 9249924

[insert date]

Dear [insert parent's name]

I am writing to invite [insert child's name] to participate in a study that a team at the University of Nottingham, together with Nottingham University Hospital's NHS Trust, are carrying out to learn more about a tissue called brown fat. We are looking at children with diabetes and thyroid problems and comparing them to healthy children without these conditions.

I am writing to you to see if you would be interested in [insert child's name] taking part as one of the healthy children.

The study involves a short visit where [insert child's name] would be asked to sit in front of a thermal (heat) imaging camera which would take a few pictures of their neck over about 10 minutes. This visit would be on the same day as you are visiting the hospital for another reason wherever possible. We are expecting to see you in clinic in the next few weeks which is why we are sending you this letter now.

If you think you would be interested in learning more about this study, please take a few moments to look at the leaflets that are enclosed with this letter. There is one for you and one for [insert child's name].

We will try and phone you over the next few days to see if you would be interested in taking part. Unfortunately due to the time it takes, it is usually only possible to offer this opportunity to four patients per clinic. So we do not disturb you unnecessarily, once we have filled the available study slots, we will not continue making phone calls. If you would like to take part but do not hear from us, please contact the research team directly – their details are on the back of the leaflet.

If you want to tell us not to phone you, you can phone 0115 8230613 and leave a message on the answer machine. You will not need to speak to anyone directly. Alternatively, if you would like someone to call you back, please leave your name and number and a member of the research team will get back to you.

If you have any questions, please contact a member of the research team using the details on the leaflets.

Yours sincerely

[Insert Consultant]

BRAID-T Approach Letter (Healthy Volunteers) Final version 4.0 10/05/16

5.2.4 Data Collection forms

5.2.4.1 Personal Information Form

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

BRown fat Activation In Diabetic & Thyroid disorders in children (BRAID-T)
PERSONAL INFORMATION FORM
Final Version 1.0 10th February 2013

Study ID			

First name	
Middle name(s)	
Surname	

Date of Birth	D	D	M	M	Y	Y

NHS number										

THIS FORM IS TO BE FILED IN A LOCKED CABINET IN THE INVESTIGATORS OFFICE

5.2.4.2 Telephone Interview Pro-forma

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

BRown fat Activation In Diabetic & Thyroid disorders in children (BRAID-T)
TELEPHONE DISCUSSION PRO-FORMA
Final Version 1.0 10th February 2013

Identification Number			

This identification number should be used on the page 3 of this form to ensure clinical data remains anonymous. If the patient consents to participating in the study, this identification number will become the Study Identification Number.

Name of child	
Name of parent	
Phone number used	

1a	Introduce self	1b	Explain reason for calling	1c	Ask if appropriate time to talk	Y / N
					If no go to 2. If yes go to 3.	
2a	Ask if another time would be more convenient / offer face to face discussion	Y / N	2b	If no, thank them for their time. If yes, note time and date	Time and date to call back / face to face meeting *(delete as appropriate)*:	
3a	Ask if letter and information sheets received	Y / N	3b	If no, ask for address and record here; arrange to resend information. If yes, continue with telephone discussion	Address:	
4a	Ask if child is present	Y / N	4b	If child not present offer to call them to discuss the study separately.	Time, date and number:	

5a	If child is present ask if they would like to discuss together (if speaker phone or equivalent available) or separately.	Spoken to together	Spoken to separately	Child not spoken to	Proceed with discussion and explanation of study going through all information on the Information Sheet

THE FRONT PAGE OF THIS FORM IS TO BE SEPARATED AND FILED IN A LOCKED CABINET WITH THE PERSONAL INFORMATION FORMS

THIS PAGE HAS BEEN LEFT BLANK INTENTIONALLY

THE FRONT PAGE OF THIS FORM IS TO BE SEPARATED AND FILED IN A LOCKED CABINET WITH THE PERSONAL INFORMATION FORMS

Identification Number			

Check exclusion criteria:

6a	Dual diagnosis of T1DM & thyroid dysfunction (or single diagnosis in control group)	Y	N	6b	Other endocrine disorders, head or neck malignancy or neck surgery	Y	N	6c	Current participation in another study	Y	N

Explain that: *(Tick box)*

7a	Participation is voluntary	☐	7b	Treatment/care will not be affected by their decision	☐	7c	Can withdraw at any time. In that case, data collected so far cannot be erased and will be used in final analyses.	☐

8a	Ask if they wish to participate in study	Y	N	If no, thank them for their time. If yes, go to 8b			8b	Ask if child has a sibling*	Y	N
8c	Would child be happy for sibling to take part?*	Y	N	8d	Would sibling like to consider taking part as a healthy volunteer?*	Y	N	8e	Sibling meets inclusion criteria and none of exclusion criteria*	Y	N

* If the answer is "Yes" to each question, move to next. If the answer is "No" to any question, move to section 9. If answer is "Yes" to questions 8b-8e, take a further Telephone Discussion Pro-forma and record name of sibling and parent, phone number and address is the relevant spaces. Send "Healthy volunteer" PIL and arrange a suitable time to phone back and discuss. DO NOT record ANY person identifiable data on pages 2-3 of this Telephone Discussion Pro-froma.

9a	Ask if they wish to book a study date now	Y	N	Study time & date / time & date to phone back to book a date: *(delete as appropriate)*	If applicable, reason, if given, for not booking a date now (e.g. want to discuss with other parent)

Repeat that they can change their mind at any time and without giving a reason. ☐

Request that they let us know if they do change their mind so we can offer the date to someone else. ☐

Ask to refrain from alcohol, recreational drugs or caffeine for from midnight prior to attending for the study. ☐

Ask to avoid excessive activity for 1 hour prior to study. ☐

Thank them for their time and say that we look forward to seeing them on the [date].

THE BACK PAGE OF THIS FORM IS TO BE SEPARATED AND FILED IN A LOCKED CABINET AWAY FROM ANY FORMS CONTAINING PERSONAL IDENTIFIABLE DATA

Further Information

Please record any further relevant information noting the section number if relevant. This should include any pertinent points of the conversation which may include questions asked and brief description of the answer given. Detail of any follow up conversations can also be recorded here.

DO NOT RECORD ANY PERSON IDENTIFIABLE DATA HERE

THE BACK PAGE OF THIS FORM IS TO BE SEPARATED AND FILED IN A LOCKED CABINET AWAY FROM ANY FORMS CONTAINING PERSONAL IDENTIFIABLE DATA

5.2.4.3 Study session Data Collection Form

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

BRown fat Activation In Diabetic & Thyroid disorders in children (BRAID-T)
DATA COLLECTION FORM
Final Version 4.0 10th May 2016

Study ID				Date of Birth	D	D	M	M	Y	Y	Date of visit	D	D	M	M	Y	Y

Study group *(circle)*	Control (sibling)	Control (ENT)	Diabetes	Hypothyroid	Hyperthyroid

1. Checks

1a Consent form signed?	Y	N	1b Diagnosis of Type 1 Diabetes Mellitus?	Y	N	1c Diagnosis of hypothyroidism?	Y	N
1d Diagnosis of hyperthyroidism?	Y	N	1e Other endocrine diagnosis?	Y	N	1f Previous neck surgery?	Y	N
1g Previous head or neck malignancy?	Y	N	1h Current participation in another study?	Y	N	1g Abstained from alcohol, caffeine and recreational drugs since midnight?	Y	N

2. Anthropometric data

Height				.		cm	Weight				.			kg (avg of 3 measurements)

Gender	M	F

3. Past medical history

3a Diagnosis of asthma	Y	N	*If yes, give details below (including age of onset, current medication (name, dose, frequency), number of courses of antiobiotics & days of school missed in last 12 months, seasonal variation)*
3b Any heart conditions	Y	N	*If yes, give detail below (including diagnosis, symptoms, current medication (name, dose, frequency)*
3c History of fits, faints or funny turns	Y	N	*If yes, give detail below (including diagnosis, symptoms, current medication (name, dose, frequency)*

4. Last meal

Time	H H M M	*Description (including contents and approximate amount; refer to "Carbs & Cals" book where possible)*

5. Medication

5a Thyroid medication (go to 5b if not applicable): *if thyroid replacement medication, units will be micrograms*

Name	Dose	Units	Times *(to nearest hour using 24 hour clock, e.g. if given at 5pm, write "17")*
	.	mcg	H H H H H H H H
	.	mcg	H H H H H H H H
	.	mcg	H H H H H H H H

5b Insulin regimen (go to 5e if not applicable): *circle one option*

TDS	QDS	Continuous insulin infusion ("pump")
Other (give details): **Note here if carbohydrate counting**		

5c Insulin injection details (go to 5d if on continuous insulin infusion)

Name of insulin preparation	Dose	Times *(approx usual times to nearest hour using 24 hour clock, e.g. if given at 5pm, write "17")*	Last dose: Amount	Last dose: Time
	Units	H H H H H H H H	Units	H H
	Units	H H H H H H H H	Units	H H
	Units	H H H H H H H H	Units	H H

5d Continuous insulin infusion ("pump") details

Daily **basal** infusion Units Current basal rate . Units/hour

Total daily infusion (last 3 days) 1. Most recent Units 2. Previous Units 3. Least recent Units

Last Bolus . Units Time given H H M M

Patients should be able to use their pump to provide all of these readings.

5e Other medication: *Ask specifically about beta-blockers, salbutamol and steroids*

Name	Dose	Units	Times *(approx usual times to nearest hour using 24 hour clock, e.g. if given at 5pm, write "17")*
	.		H H H H H H H H
	.		H H H H H H H H
	.		H H H H H H H H

6. Measurements

					6b Cooling method:	✓		Temp			
6a Volunteer core temp			.		°C	Limb immersion				.		°C
6c Outside temperature*			.		°C	Cooling blanket				.		°C
6d Room temperature			.		°C	Other (please state):				.		°C

*As found on "http://www.metoffice.gov.uk/public/weather/observations/?tab=last24hours" for Lenton, Nottingham

6e-1	Time	Peripheral Skin Temp	Central Skin Temp	Heart Rate
Resting start	H H M M	. °C	. °C	bpm
1 min	H H M M	. °C	. °C	bpm
2 mins	H H M M	. °C	. °C	bpm
3 mins	H H M M	. °C	. °C	bpm
4 mins	H H M M	. °C	. °C	bpm
5 mins	H H M M	. °C	. °C	Bpm
6 min	H H M M	. °C	. °C	bpm
7 mins	H H M M	. °C	. °C	bpm
8 mins	H H M M	. °C	. °C	bpm
9 mins	H H M M	. °C	. °C	bpm
10 mins	H H M M	. °C	. °C	bpm

6e-2	Time	Peripheral Skin Temp	Central Skin Temp	Heart Rate
Stimulus start	H H M M	. °C	. °C	bpm
1 min	H H M M	. °C	. °C	bpm
2 mins	H H M M	. °C	. °C	bpm
3 mins	H H M M	. °C	. °C	bpm
4 mins	H H M M	. °C	. °C	bpm
5 mins	H H M M	. °C	. °C	bpm
6 min	H H M M	. °C	. °C	bpm
7 mins	H H M M	. °C	. °C	bpm
8 mins	H H M M	. °C	. °C	bpm
9 mins	H H M M	. °C	. °C	bpm
10 mins	H H M M	. °C	. °C	bpm

6f Session completed?	Y	N	*If no give details below (reason for stopping, discussion with child and parent/s, agreed plan)*

7. Post-imaging actions

Circle Y for Yes, or N for No.

7a Activity questionnaire completed?	Y	N

7b Consent for DNA analysis received?	Y	N

7c Saliva sample for DNA analysis obtained?	Y	N	N/A

7d Blood sugar level after imaging (patients with diabetes only; go to section 8 if not applicable):
Study glucometer should be used unless the patient has a continuous glucose monitoring device. If study device used write "study device" as device used. If patient's own equipment used write make + model

Time	H	H	M	M

Result			.		mmol/l

If less than 4mmol/l record action taken below (section 9)

Device used	

8. Blood results

8a HbA1C (go to 8b if not applicable):

Date of test	D	D	M	M

Result				mmol/ml

8b Thyroid Function Tests (go to section 9 if not applicable):

Date of test	D	D	M	M

TSH			.		mU/l

T3			.		pmol/l

T4			.		pmol/l

9. Further Information

Please record any further relevant information here including where there was insufficient space in the relevant section. Please note the section number if relevant.

5.2.4.4 Physical Activity Questionnaire

Study ID			

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Waking up Brown Fat
PHYSICAL ACTIVITY QUESTIONNAIRE
Final Version 3.0 14th April 2014

Section A. What did you do today before school?

Please circle to show whether you did the activity not at all **or** a little **or** a lot.

1. Bicycling		1 None	2 A little	3 A lot
2. Exercise: push-ups, sit-ups, weight training		1 None	2 A little	3 A lot
3. Climbing on playground equipment		1 None	2 A little	3 A lot
4. Team sports: football, netball		1 None	2 A little	3 A lot
5. Racket Sports: badminton, tennis		1 None	2 A little	3 A lot
6. Ball games: dodge ball, frisbee		1 None	2 A little	3 A lot
7. Games: chase, hopscotch		1 None	2 A little	3 A lot
8. Outdoor Play: climbing trees, hide & seek		1 None	2 A little	3 A lot
9. Swimming:		1 None	2 A little	3 A lot
10. Skipping		1 None	2 A little	3 A lot

Before school

Waking up Brown Fat
Physical Activity Questionnaire Final Version 3.0 14th April 2014

Page 1 of 6

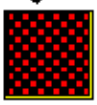
Study ID			

Section B. What activities did you do yesterday after school or the last day you were at school?

1. Bicycling		1 None	2 A little	3 A lot
2. Exercise: push-ups, sit-ups, weight training		1 None	2 A little	3 A lot
3. Climbing on playground equipment		1 None	2 A little	3 A lot
4. Team sports: football, netball		1 None	2 A little	3 A lot
5. Racket Sports: badminton, tennis		1 None	2 A little	3 A lot
6. Ball games: dodge ball, Frisbee		1 None	2 A little	3 A lot
7. Games: chase, hopscotch		1 None	2 A little	3 A lot
8. Outdoor Play: climbing trees, hide & seek		1 None	2 A little	3 A lot
9. Swimming:		1 None	2 A little	3 A lot
10. Skipping		1 None	2 A little	3 A lot
11. Dance		1 None	2 A little	3 A lot
12. Walking		1 None	2 A little	3 A lot

After school

Study ID			

13. Running		1 None	2 A little	3 A lot
14. Skateboarding / Skating		1 None	2 A little	3 A lot
15. Watch TV / DVDs		1 None	2 A little	3 A lot
16. Play Video Games		1 None	2 A little	3 A lot
17. Homework/Reading		1 None	2 A little	3 A lot
18. Play Board Games		1 None	2 A little	3 A lot
19. Housework		1 None	2 A little	3 A lot
20. Using phone		1 None	2 A little	3 A lot
21. On computer		1 None	2 A little	3 A lot
22. Arts and crafts		1 None	2 A little	3 A lot
20. Other activity _____	?	1 None	2 A little	3 A lot

After school

Study ID			

Section C. What activities did you do during school yesterday or the last day you were at school?

1. Exercise: push-ups, sit-ups, weights		1 None	2 A little	3 A lot
2. Gymnastics: bars, beams, trampoline		1 None	2 A little	3 A lot
3. Team sports: football, netball		1 None	2 A little	3 A lot
4. Racket Sports: badminton, tennis		1 None	2 A little	3 A lot
5. Ball playing: frisbee, catch		1 None	2 A little	3 A lot
6. Games: chase, tag, hopscotch		1 None	2 A little	3 A lot
9. Climbing on playground equipment		1 None	2 A little	3 A lot
10. Skipping		1 None	2 A little	3 A lot
11. Dancing		1 None	2 A little	3 A lot
12. Walking		1 None	2 A little	3 A lot
13. Running		1 None	2 A little	3 A lot
15. Other during school_____	?	1 None	2 A little	3 A lot

During school

Study ID			

Section D. Out of school clubs and activities

Are there any activities that you do most weeks? Things like swimming, cubs, brownies, dance, drama, football, ice skating.

Activity		I do this most weeks	How many times a week usually?
Swimming			
Team sports			
Martial arts (eg karate, judo)			
Dancing			
Drama or music			
Skating			
Racket sports			
Clubs (eg brownies or cubs)			
Other club or activity			

5.2.5 Consent forms

5.2.5.1 Consent form for parents of participants

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Study Number:			

Waking up Brown Fat

A case-control study of brown adipose activation in paediatric patients with Type 1 diabetes mellitus, hypothyroidism or hyperthyroidism

PARENT CONSENT FORM

Final Version 4.0 10th May 2016

Name of Researchers: Dr James Law, Dr Lindsay Robinson, Dr Tabitha Randell, Dr Louise Denvir, Mr Andrew Marshall, Professor Michael Symonds, Professor Helen Budge

Name of Participant (Child):	

Please initial box

1	I confirm that I have read and understand the study leaflet dated 10th May 2016 (version 4.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2	I understand that my child's participation is voluntary and that I am free to withdraw them at any time without giving any reason, and without my child's medical care or our legal rights being affected. I understand that, should I withdraw, the information collected so far cannot be erased and this information will be used in the study analysis.	☐
3	I understand that relevant sections of my child's medical record, including content stored electronically on Nottingham University Hospitals NHS Trust's Information Systems, will be looked at by members of the research team where it is relevant to my child taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my child's participation in this study. I understand that my child's personal details will be kept confidential.	☐
4	I agree for my child (named above) to take part in the above study.	☐
5	**OPTIONAL:** I agree to my child providing a sample of his/her DNA in the form of a salivary ("spit") sample. I understand that this will be stored by the University of Nottingham and may be analysed at a later date to look at genes associated with brown adipose tissue function.	☐

Page 1 of 2

Waking Up Brown Fat: Parent Consent form
Final Version 4.0 10th May 2016

6 **OPTIONAL:**

I agree to thermal images of me being used for illustrative purposes in academic publications including academic journals, conference posters and presentations and academic theses. ☐

7 **OPTIONAL:**

I agree for my child's study results and their anonymised information to be stored by the University of Nottingham on a database of thermal imaging. I understand that all data will be kept confidential and that my child will not be identifiable. ☐

_____	_____	_____
Name of person giving consent	Date	Signature
_____	_____	_____
Name of person receiving consent	Date	Signature

Section for children:

I agree to take part in this study

_____	_____	_____
Name of Child (for assent)	Date	Signature
_____	_____	_____
Witness to assent (if child does not wish to sign)	Date	Signature

1 for participant, 1 for research site file and 1 for the medical notes (if available)

5.2.5.2 Consent form for participants with capacity to consent

Nottingham University Hospitals NHS Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Study Number:			

Waking up Brown Fat

CONSENT FORM FOR YOUNG PERSON WITH CAPACITY

Final Version 4.0 10th May 2016

Name of Researchers: Dr James Law, Dr Lindsay Robinson, Dr Tabitha Randell, Dr Louise Denvir, Mr Andrew Marshall, Professor Michael Symonds, Professor Helen Budge

Name of Participant (Young Person):	

		Please initial box
1	I confirm that I have read and understand the study leaflet dated 10th May 2016 (version 4.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without my medical care or legal rights being affected. I understand that, should I withdraw, the information collected so far cannot be erased and this information will be used in the study analysis.	☐
3	I understand relevant sections of my medical record, including content stored electronically on Nottingham University Hospitals NHS Trust's Information Systems, will be looked at by members of the research team where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential.	☐
4	I agree to take part in the above study.	☐
5	**OPTIONAL:** I agree to have my study results and anonymised information stored by the University of Nottingham on a database of thermal imaging. I understand that all data will be kept confidential and that I will not be identifiable in anyway.	☐
6	**OPTIONAL:** I agree to thermal images of me being used for illustrative purposes in academic publications including academic journals, conference posters and presentations and academic theses.	☐

----------	----------	----------
Name of person giving consent	Date	Signature
----------	----------	----------
Name of person receiving consent	Date	Signature

7 **OPTIONAL:**

I agree to my child providing a sample of his/her DNA in the form of a salivary ("spit") sample. I understand that this will be stored and analysed to look for genes associated with brown adipose tissue function. ☐

_____	_____	_____
Name of person giving consent	Date	Signature
_____	_____	_____
Name of person receiving consent	Date	Signature
_____	_____	_____
Young person's assent	Date	Signature

1 for participant, 1 for research site file and 1 for the medical notes (if available)

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