Identification of factors that stimulate the brown adipose tissue in healthy adults and patients

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Abstract

Obesity is caused by excess energy intake and/or low energy expenditure which contributes to the onset of many diseases including cardiovascular disease, diabetes and cancer. For this reason, many studies are focusing on the prevention of obesity as it is a crucial public health issue. In mammals, adipose tissue is comprised of white and brown adipose tissue (BAT). Their function differs, as the main role of the white adipose tissue is to store excess energy, whereas BAT can rapidly produce heat when stimulated, thereby, increasing the energy expenditure. Consequently, enhancing BAT activation could be a potential target to prevent and/or reduce obesity.

Many researchers are now exploring ways to activate BAT in adults using expensive techniques that are not readily available for studying both patients and healthy volunteers. These methods, include positron emission tomography/computed tomography scanning and magnetic resonance imaging. However, the non-invasive method of infrared thermography (IRT) has been shown to quantify BAT activation in humans. This is achieved by measuring absolute or relative changes in the main BAT depot, the supraclavicular (SCR) depot.

Acute exposure to either cool water or air can activate BAT, though whether this can be achieved using a more routine intervention is currently unknown. In my first study, I hypothesised that daily cool showers could promote BAT function. Healthy male adults were recruited and baseline BAT temperature measured using IRT, as well

as salivary cortisol and a three day food and exercise diary were measured. Then, they were asked to take daily cool showers for three minutes for seven days and then to attend a second IRT session. Following seven days of cool showers, the maximum BAT temperature was decreased whereas the nearby reference temperature was increased. Neither salivary cortisol, resting metabolic rate, nor eating patterns were changed. These findings suggest an indirect change in BAT function.

Another factor that could potentially be used as a therapeutic compound is cannabidiol (CBD), which has been suggested to promote mitochondrial activity in vitro and in vivo and stimulate the expression of brown fat related genes in fat cell lines. It has yet to be determined whether it can promote BAT function in humans and I hypothesised that ingestion of CBD could stimulate BAT. In a second blinded randomised control study, healthy male lean and overweight (OW) adults were randomly allocated to receive either placebo or 582mg of CBD tablets, daily for a period of seven days. Prior and post oral ingestion, I measured BAT using IRT, on the first and last day of the tablets' administration. Neither acute nor chronic ingestion of CBD, however, had any notable effect on BAT in adult males, who were either OW or of normal body weight.

Prolactin receptors are expressed in brown adipocytes and it has been proposed that prolactin could regulate BAT function. Therefore, in a third study, I hypothesised that BAT function would be increased in patients with hyperprolactinaemia prior to individualised treatment with dopamine agonist tablets. Patients with high serum prolactin

concentration were recruited and BAT activity was measured by IRT prior and post treatment. I showed that the serum prolactin was inversely related to maximum BAT temperature prior to any treatment. Additionally, there was a trend for a reduced BAT temperature, whereas reference temperature was significantly decreased. Both these findings suggest that modulating prolactin secretion could potentially affect BAT stimulation.

In my fourth and final study, I explored the feasibility of the IRT application in a clinical setting, by assessing BAT in morbidly obese female patients, after their first clinical appointment. I demonstrated that the IRT technique is acceptable to the patients and body mass index (BMI) was inversely related to BAT activity.

In conclusion, in my thesis it is demonstrated that IRT can be used to assess BAT function in response to various stimuli in both healthy volunteers and patients.

Acknowledgments

Firstly, I am grateful to God for giving me the health, strength and patience to accomplish such great work. I also would like to thank the University of Nottingham for awarding me with the Vice-Chancellor's Scholarship for Research Excellence and for providing me with the opportunity to make my biggest dream come true and start my journey as a PhD candidate.

There is a list of names who helped me through this journey to accomplish the specific goal I set.

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The combinatory contribution of all these extraordinary people, made me realise that without them I would be like a car with missing wheels and my work wouldn't have been reached up to this level.

Declaration

The work in this thesis was performed within the Academic Division of Child Health, Obstetrics and Gynaecology at Nottingham University Hospital, Queens Medical Centre, Nottingham, the Medical School Building of the University of Nottingham in Derby and Royal Derby hospital between September 2015 and August 2019.

Unless otherwise stated, this thesis illustrates my own work completed under the supervision of Professor Michael E. Symonds and Professor Helen Budge.

This thesis is an accurate representation of the work performed and no other study reproducing this work, to my knowledge, has been carried out within the University of Nottingham.

Anastasia-Viktoria Lazaridi

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Oral presentations

Lazaridi, A. V., M. E. Symonds, H. Budge. Brown adipose tissue activity after exposure to cannabidiol in healthy males. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham; 2019 June 11; Nottingham, United Kingdom.

Lazaridi, A. V., M. E. Symonds, H. Budge. Metabolically active brown adipose tissue in healthy adults through mild cold water exposure. Postgraduate Oral Presentation Event, School of Medicine, University of Nottingham; 2017 October 27; Nottingham, United Kingdom.

Lazaridi, A. V., M. E. Symonds, H. Budge. The acclimatisation study. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham; 2017 May 30; Nottingham, United Kingdom.

Lazaridi, A. V., M. E. Symonds, H. Budge. The acclimatisation study: Study Design. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham; 2017 April 20; Nottingham, United Kingdom.

Lazaridi, A. V., M. E. Symonds, H. Budge. The temperature study. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham; 2016 September 27; Nottingham, United Kingdom.

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Lazaridi, A. V., M. E. Symonds, H. Budge. The effects of cool showers on brown adipose tissue in healthy adults: The CoR-BAT study. Study Design. Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham; 2016 April 26; Nottingham, United Kingdom.

Poster presentations

Lazaridi, A. V., M. E. Symonds, H. Budge. (2017). Acclimatisation periods in the in vivo analysis of human brown adipose tissue. Nottingham Paediatric Research Showcase Conference; 2017 June 14; Nottingham, United Kingdom.

Lazaridi, A. V., M. E. Symonds, H. Budge. (2016). Activating brown fat could reduce body weight. M&HS Faculty Postgraduate Research Forum, School of Medicine, University of Nottingham; 2016 June 26; Nottingham, United Kingdom.

Table of Contents

List of figures

List of tables

List of abbreviations

Abbreviation	Definition
^{123}I	Iodine radioisotope
^{123}I-MIBG	^{123}I-Metaiodobenzylguanidine
[$_{18}$F]-FDA	[$_{18}$F]-fluorodopamine
^{18}F-FDG	^{18}F-fludeoxyglucose
AC	Adenyl cyclase
acyl	Acyl group
acyl-CoA	Acetyl coenzyme A
ANOVA	Analysis of variance
ASCs	Adipose stromal cell
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CB_2	Cannabinoid type 2
CBD	Cannabidiol
CIT	Cold induced thermogenesis
CV	Coefficient of variation
CVDs	Cardiovascular diseases
DA	Dopamine agonists
DIT	Diet-induced thermogenesis
EE	Energy expenditure
FDG	Fluorodeoxyglucose
FFAs	Free fatty acids

FLIR	Forward-looking infrared
G	guanine nucleotide-binding
GP	General practitioner
Gs	Guanine nucleotide-binding proteins
H_2O	Water
HSL	Hormone-sensitive lipase
HTA	Human Tissue Act
IRT	Infrared thermography
ISF	Investigator site file
JPEG	Joint photographic experts group
kcals	Kilocalories
MANOVA	Multivariate analysis of variance
MatLab	matrix laboratory
MIBG	Metaiodobenzylguanidine
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
$Myf5^-$	myogenic factor 5-negative
$Myf5^+$	myogenic factor 5-positive
NCDs	Non-communicable diseases
NE	Norepinephrine or noradrenaline
NHS	National Health Service
NST	Non-shivering thermogenesis
NW	Normal weight
OW	Overweight
PA	Physical activity

PET/CT	Positron emission tomography/computed tomography
PIS	Participants information sheet
PKA	protein kinase A
PNG	Portable network graphics
PO_4	Phosphate
PRL	Prolactin
RCT	Randomised control trial
REC	Research ethics committee
Ref	Reference
RMR	Resting metabolic rate
ROI	Region of interest
SCR	Supraclavicular
SNS	sympathetic nervous system
SPECT	single-photon emission computerised tomography
Std Dev	Standard deviation
SUV	Standardised uptake value
T_3	triiodothyronine
T_{base}	Baseline temperature
$T_{baseSCR}$	Baseline supraclavicular temperature
TGs	Triglycerides
T_{max}	Maximum temperature ±20 seconds
T_{maxSCR}	Maximum supraclavicular temperature
$T_{postREF}$	Post-stimulation reference temperature
UCP-1	Uncoupling protein one
UK	United Kingdom

USA	United States of America
WAT	White adipose tissue
WHO	World Health Organization
ΔT	Change of the temperature
ΔT_{REL}	Relative temperature
$\Delta T_{REL\text{-}base}$	Baseline relative temperature
$\Delta T_{REL\text{-}max}$	Maximum relative temperature
ΔT_{SCR}	Change of the supraclavicular temperature

1 Introduction

1.1 Obesity and non-communicable diseases

1.1.1 Obesity

1.1.1.1 Definition and classification

Hippocrates, a Greek physician who lived in the 5th century B.C., known as the father of Western Medicine (Grammaticos and Diamantis 2008), noted that "Those who are constitutionally very fat are more apt to die quickly than those who are thin" (Coar 1982). Industrialisation had a dramatic effect upon the excessive amounts of recently introduced food consumed (Schmidhuber and Shetty 2005), leading to the onset of diet-related chronic diseases, which include non-communicable diseases (NCDs) (Cordain, Eaton et al. 2005). The World Health Organization (WHO), defines overweight (OW) and obesity as the storage of excess, redundant fat which can contribute to the development of adverse risk factor to adult health (WHO 2015a). Also, having a sedentary life can contribute to the storage of this redundant fat (Lazaridi 2012).

Measurements to classify people according to weight for height, such as bioelectrical impedance, skinfold thickness and dual-energy x-ray among others have been developed (Durnin and Rahaman 1967, Lukaski, Johnson et al. 1985, Svendsen, Haarbo et al. 1993). Though, from 1995, the most commonly used measure in adult studies where no such equipment can be used is the body mass index (BMI), which classifies a person according to the weight for height using the following formula:

$$BMI = \frac{weight\ (kg)}{height\ (m)^2}$$

BMI divides people into 4 main groups; underweight, normal weight (NW), OW and obese (WHO 1995), Table 1.1.

Table 1.1 International classification of body mass index in adults.

Classification*	BMI (kg/m²) Principal cut-off points
Underweight	<18.50
Normal weight	18.50 - 24.99
Overweight (or pre-obese)	≥25.00-24.99
Obese (Class I)	≥30.00-34.99
Obese (Class II)	35.00-39.99
Obese (Class III)	>40.00

*From (WHO 1995); BMI: Body mass index.

1.1.1.2 Prevalence

Since 1981, the prevalence of obesity has been dramatically increasing, with women having a higher BMI trend than men worldwide (Bhurosy and Jeewon 2014). Even though future estimates regarding obesity reveal an upward trend worldwide (Butland, Jebb et al. 2007, Finkelstein, Khavjou et al. 2012, OECD 2013), the prevalence of adult and child obesity has almost plateaued in developed, but not developing, countries (Ng, Fleming et al. 2014, Hruby and Hu 2015). From 1980 to 2014, the prevalence of obesity worldwide has been doubled, to 14% and 10% of women and men respectively (WHO 2015b). Looking at the OW population, worldwide, the number of OW people is increasing, reaching 40% for women and 38% for men, accounting for over one-third of the total population (WHO 2015c).

In the United Kingdom (UK) and Northern Ireland alone, from 1975 to 2016, the prevalence of obesity increased by 18.4%, i.e. from 9.4% to 27.8% for both sexes (5.9% rise from 2006 to 2016) (WHO 2017a) and the prevalence of OW adults increased by 23.6%, i.e. from 40.1% to 63.7% (5.3% rise from 2006 to 2016), accordingly (WHO 2017b). For the same period, a similar trend was observed in other countries such as Greece, where the prevalence of obesity surged from 9.8% to 24.9% (4.1% rise from 2006 to 2016) (WHO 2017a) and of OW by 21.8%, i.e. from 40.5% to 62.3% (4.4% rise from 2006 to 2016) (WHO 2017b).

1.1.1.3 Health impact and management

Being OW or obese can lead to a series of metabolic anomalies such as dyslipidaemia, insulin resistance and hypertension, causing the "metabolic syndrome" (Alberti, Zimmet et al. 2005). This syndrome can lead to the development of NCDs, such as cardiovascular diseases (CVDs) and diabetes (Poirier, Giles et al. 2006, Mottillo, Filion et al. 2010). Adverse outcomes of obesity can be minimised by following a healthy diet or increasing the physical activity (PA), e.g. active sports (Popkin 2006, Beaglehole, Bonita et al. 2011). Thus, energy expenditure (EE), which includes the calories that the body consumes in order to stay alive, described as resting metabolic rate (RMR), and the calories consumed through any PA, is crucial for weight management, which is achieved through having a stable energy balance (Roberts, Das et al. 2004). As technology is part of our everyday lives, eHealth interventions which use a combination of communication technologies such as mobile apps, websites, podcasts

and the internet to achieve behavioural change and wellbeing, have recently introduced as part of the obesity management for adults and children (Hutchesson, Rollo et al. 2015, Hammersley, Jones et al. 2016, Joiner, Nam et al. 2017, Cotie, Prince et al. 2018).

1.1.2 Non-communicable diseases

NCDs are chronic medical problems or diseases that are not transmitted between people. The main types of NCDs are:

1. CVDs
2. diabetes
3. cancer and
4. chronic respiratory diseases (WHO 2015d).

WHO has estimated that, between 2010 and 2020, NCDs will be responsible for 44 million of deaths globally, predicting an increase of up to 15%, worldwide (Mathers, Fat et al. 2008). According to the UK's profile for 2016, approximately 89% of the total population deaths (for both sexes) were caused by NCDs. The leading cause of these deaths was CVDs, accounting for one in four deaths (WHO 2018a).

Zsuzsanna Jakab, the WHO Regional Director for Europe, referred to NCDs as a 21st century "lifestyle epidemic" (Stachenko 2010). Since 2000, ischaemic heart disease and stroke were the main two causes of death worldwide (WHO 2018b). The main risk factors responsible for the global burden of CVDs, include dietary risks and high blood pressure. Other factors relating to obesity and CVDs include high BMI,

physical inactivity, high total cholesterol and high fasting glucose (Benziger, Roth et al. 2016).

1.2 The adipose tissue

In mammals, fat consists of two major functional types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT and BAT are generally located in different parts of the body, though they can also be found in same depots, and their size depends on various factors (Saely, Geiger et al. 2012). In addition, they have different actions and developmental origins. However, the cells of both of these tissues are called "adipocytes" because they are primarily made up of lipids and triglycerides (TGs) (Cinti 2006). The diversity between WAT and BAT may help to understand their differences.

1.2.1 Adipose tissue development

In humans, the development of WAT begins during the second trimester of pregnancy (c. 5 to 29 weeks gestation) until the creation of "definitive fat lobules" (Poissonnet, Burdi et al. 1984, Poissonnet, LaVelle et al. 1988). Even though the presence of BAT can be identified at birth, in childhood, adolescence and some adults (Cypess, Lehman et al. 2009, van Marken Lichtenbelt, Vanhommerig et al. 2009, Robinson, Ojha et al. 2014), its formation during embryogenesis is still not fully defined. Furthermore, adipogenesis is a process which takes place throughout life (Gesta, Tseng et al. 2007).

Differentiation is a process in which cells specialise to execute specific functions in the human body (Cristancho and Lazar 2011). Adipose

tissue cells originate from stem cells, which have a self-renewing ability through cell division and can differentiate into various multi-lineages (Wei, Yang et al. 2013). Mesenchymal stem cells (MSCs), the adult stem cells that are derived from bone marrow (Pittenger, Mackay et al. 1999), have the capacity to differentiate into chondrocytes, adipocytes, myoblasts and osteoblasts (Dominici, Le Blanc et al. 2006). MSCs are named after their ability to differentiate in the mesoderm, thus adipocytes, as well as myocytes, have a mesodermal origin (Dominici, Le Blanc et al. 2006, Enerback 2009).

Brown adipocytes can share the same lineage as myocytes; myogenic factor 5-positive ($Myf5^{+}$). $Myf5^{+}$ is a protein that plays a vital role during the regulation of myogenesis (Seale, Bjork et al. 2008). This myogenic signature is not seen in white adipocytes (Timmons, Wennmalm et al. 2007, Francetic and Li 2011). Instead, the origin of WAT is from the precursor myogenic factor 5-negative ($Myf5^{-}$) (Sanchez-Gurmaches, Hung et al. 2012). After MSCs have followed a specific lineage, which is either the adipocyte or the myogenic one, the committed white or brown pre-adipocytes take their final form as adipocytes (Cristancho and Lazar 2011), Figure 1.1.

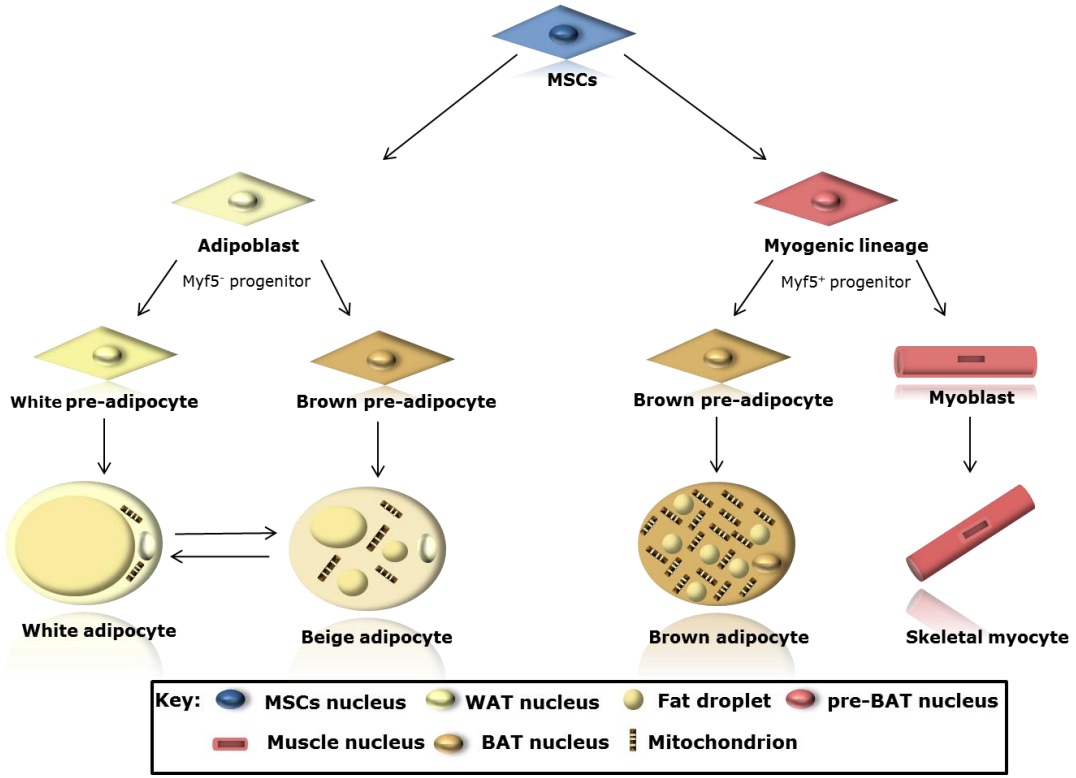


Figure 1.1 Adipocyte and myocyte differentiation.

MSCs can follow different lineages such as adipocyte and myocyte. White and beige adipocytes derive from the Myf5^{-} progenitor, whereas brown adipocytes come from the myogenic lineage deriving from Myf5^{+} progenitor, through developmental triggers such as bone morphogenetic proteins. MSCs: Mesenchymal stem cells, Myf5^{-}: Myogenic factor 5-negative, Myf5^{+}: Myogenic factor 5-positive, WAT: White adipose tissue, BAT: Brown adipose tissue.

1.3 White adipose tissue

It was thought that the primary role of WAT was the storage of energy, as TGs, in the body (Fonseca-Alaniz, Takada et al. 2006). However, the discovery of the hormone leptin secreted from WAT, almost 20 years ago, recognised WAT as an endocrine organ which produces and releases hormones, and not only a storage one (Halaas, Gajiwala et al. 1995, Trayhurn and Beattie 2001, Coelho, Oliveira et al. 2013, McGown, Birerdinc et al. 2014).

It is well described that WAT has an important role through the regulation of many physiological functions, such as immunity, lipid metabolism and angiogenesis amongst others (Fonseca-Alaniz, Takada et al. 2006, Bohler, Mokshagundam et al. 2010, Bourin, Bunnell et al. 2013, Corvera and Gealekman 2014, Das, Ma et al. 2015, Morrison 2016). Also, WAT can be considered as an organ that can regulate energy homeostasis due to its ability to secrete hormones that can regulate appetite, promoting satiety (Klok, Jakobsdottir et al. 2007), Figure 1.2.

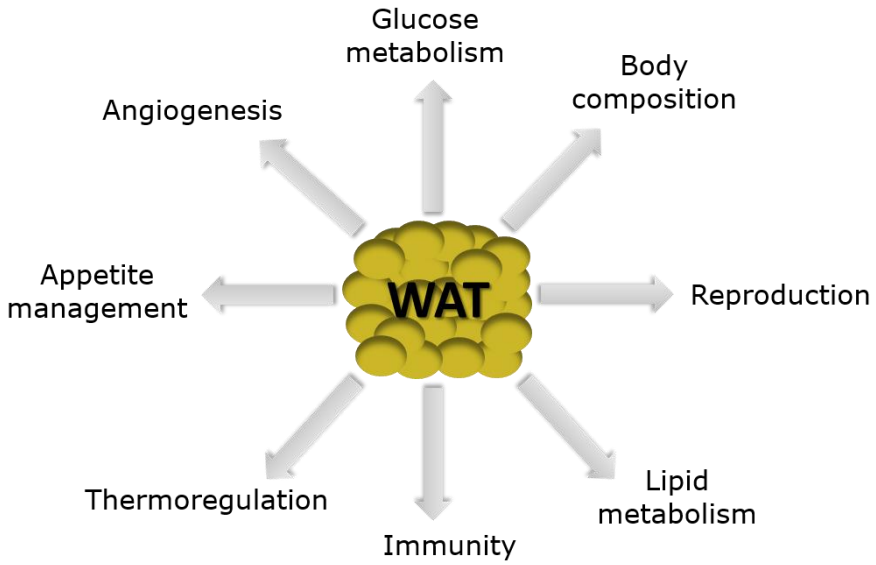


Figure 1.2 Some of the physiological functions in which white adipose tissue is involved.

White adipocytes have a primary role in the regulation of the lipid and glucose metabolism, immunity and thermoregulation, among other body functions. WAT: White adipose tissue.

The two major sites of WAT are the visceral and subcutaneous depots. The visceral depots are located around the internal organs, in the perirenal, retroperitoneal, omental and mesenteric areas. The subcutaneous depots are found under the skin, mainly in the gluteal, femoral and abdominal regions (Gesta, Tseng et al. 2007). WAT also has some smaller depots such as epicardial and intermuscular ones (Cinti 2006, Sacks and Fain 2007). The major depots exhibit considerable variation in their distribution, depending on sex, ethnicity and age (Lemieux, Prud'homme et al. 1993, Lear, Humphries et al. 2007, Kuk, Saunders et al. 2009).

Linked to cell morphology, the shape of white adipocytes, in general, is spherical but the size varies. This results from their morphology which includes unilocular lipid droplets that can occupy 95% of the cell volume. The diameter of adipocytes varies from 20 to 200 micrometres (Cinti 2006, Trujillo and Scherer 2006). Adipose tissue

also includes other types of cells such as preadipocytes, endothelial cells, some immune cells, referred as stromal vascular fraction cells, which have varying physiological functions (Yang, Galea et al. 2012, Welte 2015).

1.3.1 Browning of white adipose tissue

The "browning" or "beiging" of WAT is a response to various stimuli which results to the induction of the beige adipose tissue (Finlin, Memetimin et al. 2018). Therefore, during prolonged cold exposure (c. 4^{o}C), beige adipocytes in rodent periovarian WAT can express uncoupling protein one (UCP-1); a marker necessary for the non-shivering thermogenesis (NST) (Cousin, Cinti et al. 1992). A study in mice (Vitali, Murano et al. 2012), after ten days of cold exposure in 6^{o}C compared to those housed at 28^{o}C, revealed a browner colour in the subcutaneous depots, while WAT in that depot had almost vanished. More recently, an in vitro study revealed browning of adipogenic cultures after cold exposure (Velickovic, Leija et al. 2018). There are many other browning agents which have been confirmed to contribute to the browning of WAT in mice, including capsaicin (Baskaran, Krishnan et al. 2016), berberine (Zhang, Zhang et al. 2014), cinnamon (Kwan, Wu et al. 2017), melatonin (Jiménez-Aranda, Fernández-Vázquez et al. 2013) and exercise (Boström, Wu et al. 2012).

1.3.2 Beige adipose tissue

"Beige" (Ishibashi and Seale 2010) or "brite" (Petrovic, Walden et al. 2010) adipocytes is another category of adipose tissue detected in WAT. This fat is more similar to BAT (Figure 1.3), as it expresses UCP-1 and is comprised of mitochondria and multilocular lipid droplets (Cannon and Nedergaard 2004). Beige tissue shares the same embryonic precursors of WAT (Petrovic, Walden et al. 2010) and has a similar function to WAT and during a stimulus has a similar thermogenic effect as BAT (Petrovic, Walden et al. 2010, Wu, Cohen et al. 2013).

There are various theories of the development of the beige adipocytes which include the differentiation from white or brown adipocytes, the trans-differentiation from white adipocytes and the existence of a beige precursor (Elabd, Chiellini et al. 2009, Petrovic, Walden et al. 2010, Pisani, Djedaini et al. 2011).

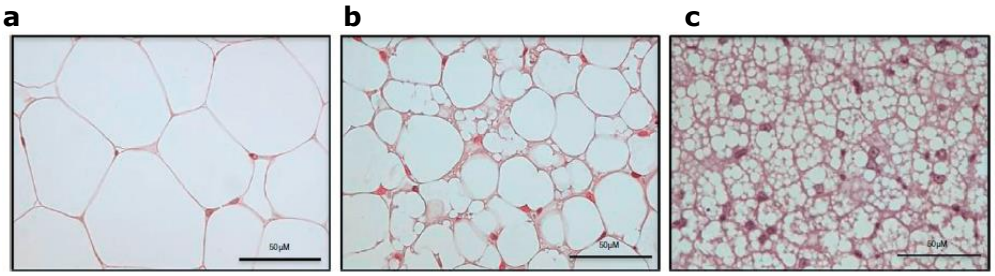


Figure 1.3 Histological images from white, beige and brown adipose tissues.

The white adipocytes (a) have unilocular lipid droplets, beige adipocytes (b) apart from the multilocular lipid droplets include a small amount of mitochondria whereas the brown adipose (b) tissue has a lot of mitochondria and a small amount of multilocular lipid droplets. Scale 50µM. From (Keipert and Jastroch 2014).

1.4 Brown adipose tissue

BAT is named for its brownish colour due to the large amounts of mitochondria inside the cell and from a high vascularisation (Cannon and Nedergaard 2004). The primary function of BAT, in mammals, is to produce heat instead of adenosine triphosphate (ATP), an energy carrying molecule, resulting in NST (Cannon and Nedergaard 2004). The main marker of BAT during stimulation is the UCP-1 which is found on the inner mitochondrial membrane and is responsible for the onset of NST (Heaton, Wagenvoord et al. 1978, Aquila, Link et al. 1985, Nedergaard, Golozoubova et al. 2001). The activation of BAT in mammals, including in neonates during birth, acts in response to cold exposure, producing heat in order to maintain body temperature (Heaton 1972). In rats, it has been estimated that only 40-50gr of activated BAT could be responsible for 20% of the total EE (Rothwell and Stock 1983). Furthermore, if full activation of BAT was possible, this could result in 4.1 kilograms of weight loss in humans over one year period (Virtanen, Lidell et al. 2009).

BAT may have several roles including:

- acting as an endocrine organ secreting "batokines"; factors that act either on the brown adipocytes or on other cells (Villarroya, Cereijo et al. 2013, Villarroya, Cereijo et al. 2017). For example, during NST, the enzyme type II thyroxine 5'-deiodinase in brown adipocytes is increased during cold exposure, enhancing the production of the active thyroid hormone triiodothyronine (T_3) (Silva and Larsen 1983).

- regulating glucose homeostasis, since BAT activated during cold exposure has the potential to clear significant amounts of glucose from the circulation (Orava, Nuutila et al. 2011). Approximately only 70gr of activated BAT, in humans, is needed for the clearance of up to 30gr of glucose from the circulation during a day (Chondronikola, Volpi et al. 2014). However, in obese people, there is a lower response of BAT during cold and insulin stimulation than in lean people (Orava, Nuutila et al. 2013).
- a vasoprotective role by the secretion of hydrogen peroxide, which reduces vessel contractions and, potentially, might be able to restore vascular function and prevent vessel-related complications in CVDs (Friederich-Persson, Nguyen Dinh Cat et al. 2017).

Historically, Conrad Gessner was the first to document BAT, in 1551, in the interscapular region of the marmot and described it as "neither fat nor flesh – but something in between" (Gessner 1551). Then, in 1902, Hatai identified BAT in the cervical region of human fetuses but not in adults, naming it "interscapular gland" (Hatai 1902). Heaton was the first one to map its distribution in humans, from birth up to 80 years of age. He concluded that the distribution declined after the first decade of life and also that the tissue was inactive during later years of life (Heaton 1972). Later, in 1979, Rothwell and Stock, whilst studying the diet induced thermogenesis (DIT) during the metabolism of food, suggested that BAT was involved, due to the similarity of DIT and NST, (Rothwell and Stock 1979), although this remains controversial (Kozak 2010). Even though many studies had already

revealed the location of BAT in the human body (Okuyama, Sakane et al. 2002, Cohade, Osman et al. 2003, Fukuchi, Ono et al. 2003, Okuyama, Ushijima et al. 2003), it was not until 2007 that BAT was shown to be metabolically active in adult humans (Nedergaard, Bengtsson et al. 2007).

1.4.1 Anatomic location

Heaton mapped 18 different areas where BAT was found in humans in a series of post-mortem examinations (Heaton 1972). However, at that time, technology was less sophisticated. After the development of Positron emission tomography/computed tomography (PET/CT) in 1993 (Townsend 2008), the anatomical distribution of BAT in humans was determined with more accuracy, Figure 1.4 and Table 1.2 (Sacks and Symonds 2013).

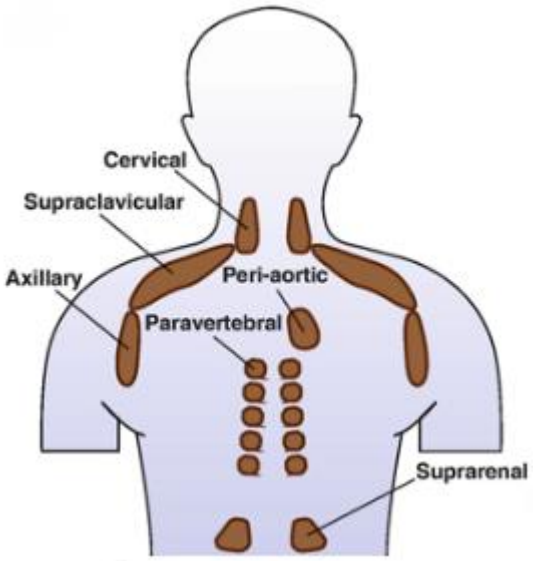


Figure 1.4 Location of brown adipose tissue in adults.

Visual location of the main depots of the brown adipose tissue in adults during cold stimulation. From (Jung, Sanchez-Gurmaches et al. 2018).

The region that BAT is most often detected in studies which use PET/CT scanning is in the supraclavicular (SCR) area (Au-Yong, Thorn et al. 2009, Drubach, Palmer et al. 2011, Lee, Ho et al. 2011).

Table 1.2 Anatomic locations of brown adipose tissue in humans.

	Anatomic locations of BAT*
Visceral brown adipose tissue	Perivascular: aorta, common carotid artery, brachiocephalic artery, paracardial mediastinal fat, epicardial coronary artery and cardiac veins, internal mammary artery, and intercostal artery and vein
	Periviscus: heart, trachea and major bronchi at hila, oesophagus, greater omentum, and transverse mesocolon
	Around solid organs: thoracic paravertebral, pancreas, kidney, adrenal, liver, and hilum of the spleen
Subcutaneous brown adipose tissue	Between anterior neck muscles and supraclavicular fossa
	Under the clavicles
	Axilla
	Anterior abdominal wall
	Inguinal fossa

*From (Sacks and Symonds 2013); UCP-1: Uncoupling protein 1.

1.4.2 Morphological composition and differences between brown and white adipocytes

Brown adipocytes have an abundance of mitochondria, in contrast with WAT. In the inner mitochondrial membrane is located the main marker of BAT, UCP-1, which produces heat. These types of adipocytes have a smaller size and also include multilocular fat droplets. The high requirement of oxygen used by BAT, leads to its greater degree of vascularisation compared to WAT (Cinti 2006, Frühbeck, Becerril et al. 2009). The main differential characteristics between brown and white adipocytes are summarised in Table 1.3.

Table 1.3 Summary of the differences between brown and white adipocytes.

Characteristics*	WAT	BAT
Primary Functions	Energy storage Secretory activity Lipogenesis and lipolysis	Thermogenesis
Main location of the depots	Visceral: Retroperitoneal Intra-abdominal Perigonadal Subcutaneous: Abdominal, inguinal, perirenal and gluteal	Thyroid/tracheal Mediastinal Paracervical Supraclavicular Parathoracical Supra and perirenal
Colour	White (ranging from light ivory to strong yellowish)	Brown (ranging from light pinkish to dark reddish)
Vascularisation	Adequate amount of vascularisation	Abundant blood flow and number of vessels
Nervous system controlling innervation	Mainly sympathetic and also parasympathetic	Sympathetic
Shape	Ranging from polyhedral to spherical	Polygonal
Size	Varying from 25 to 200μm	Smaller from 16 to 60μm
Lipid droplets	Unilocular in one large droplet, occupying the 90% of the cell	Multilocular with abundant small droplets
Mitochondria	Few, small, elongated	Abundant, large, round
UCP-1 expression	No	Yes

*From (Frühbeck, Becerril et al. 2009); WAT: White adipose tissue, BAT: Brown adipose tissue, UCP-1: Uncoupling protein 1.

Even though white and brown adipocytes differ, they can be found in the same tissue depot. Through histologic analysis, Figure 1.5, brown and white adipocytes have been detected in the same location, such as the cervical and supraclavicular tissue (Cypess, Lehman et al. 2009).

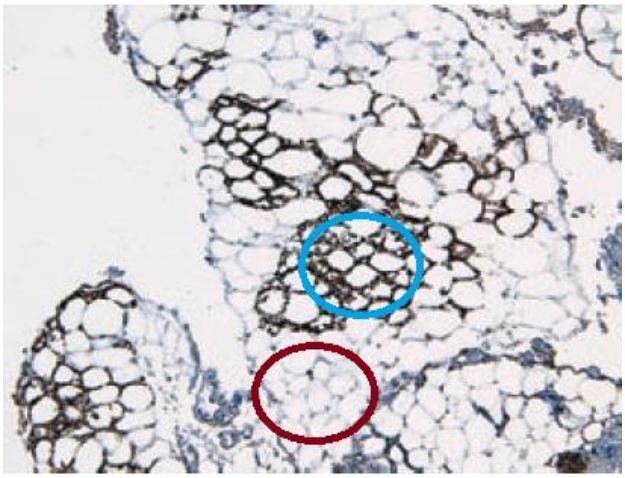


Figure 1.5 Histological image of brown and white adipocytes.
Red circle: white adipocytes; Blue circle: brown adipocytes. This is a biopsy from a 48 years old woman with parathyroidectomy. BAT was stained with antibody to UCP-1. BAT: Brown adipose tissue, UCP-1: Uncoupling protein one. From (Cypess, Lehman et al. 2009).

1.4.3 Thermogenic process

The sympathetic nervous system (SNS) influences the activity of BAT (Admiraal, Holleman et al. 2013). Exposure to cold and certain types of food ingredients can stimulate the SNS. In turn, the sympathetic nerves signal the adrenal medulla (the inner part of the adrenal gland) to secrete the stress hormone norepinephrine (NE), also known as noradrenaline, starting a signal induction pathway. NE, in turn, acts on β-adrenergic receptors, primary the β3-adrenoreceptor in BAT, which are found on the plasma membrane. The β3-adrenergic receptor couples with guanine nucleotide-binding (G) proteins, resulting in the activation of the enzyme adenyl cyclase (AC), and

thus, increasing the concentration of intracellular cyclic adenosine monophosphate (cAMP), catalysing the production of ATP (Soeder, Snedden et al. 1999).

As levels of intracellular cAMP rise, this messenger binds with the regulatory subunit of cAMP-dependent protein kinase (protein kinase A (PKA)) converting it to an active form. The role of PKA is to add phosphate groups to proteins, so it phosphorylates the hormone-sensitive lipase (HSL). This intracellular neutral lipase hydrolyses the stored TG in brown adipocytes, which results in lipolysis.

The primary function of the lipid droplets is to store any potentially toxic free fatty acids (FFAs), such as TGs (Listenberger, Han et al. 2003). At the end of lipolysis, TGs are broken down by HSL, resulting in the production of one molecule of glycerol and three molecules of FFAs. The next step is to activate the FFAs and this is completed with the enzyme acetyl coenzyme A (acyl-CoA) synthetase. After acylation, the fatty acyl-CoA enters the mitochondria with the help of two carrier proteins (carnitine acyltransferase I and II) and is used as substrate for β-oxidation in brown adipocytes (Saponaro, Gaggini et al. 2015). During β-oxidation, the proton released is "stolen" from UCP-1, instead of used in the ATP synthase, converting the energy from the oxidation into heat (Cannon and Nedergaard 2004), Figure 1.6.

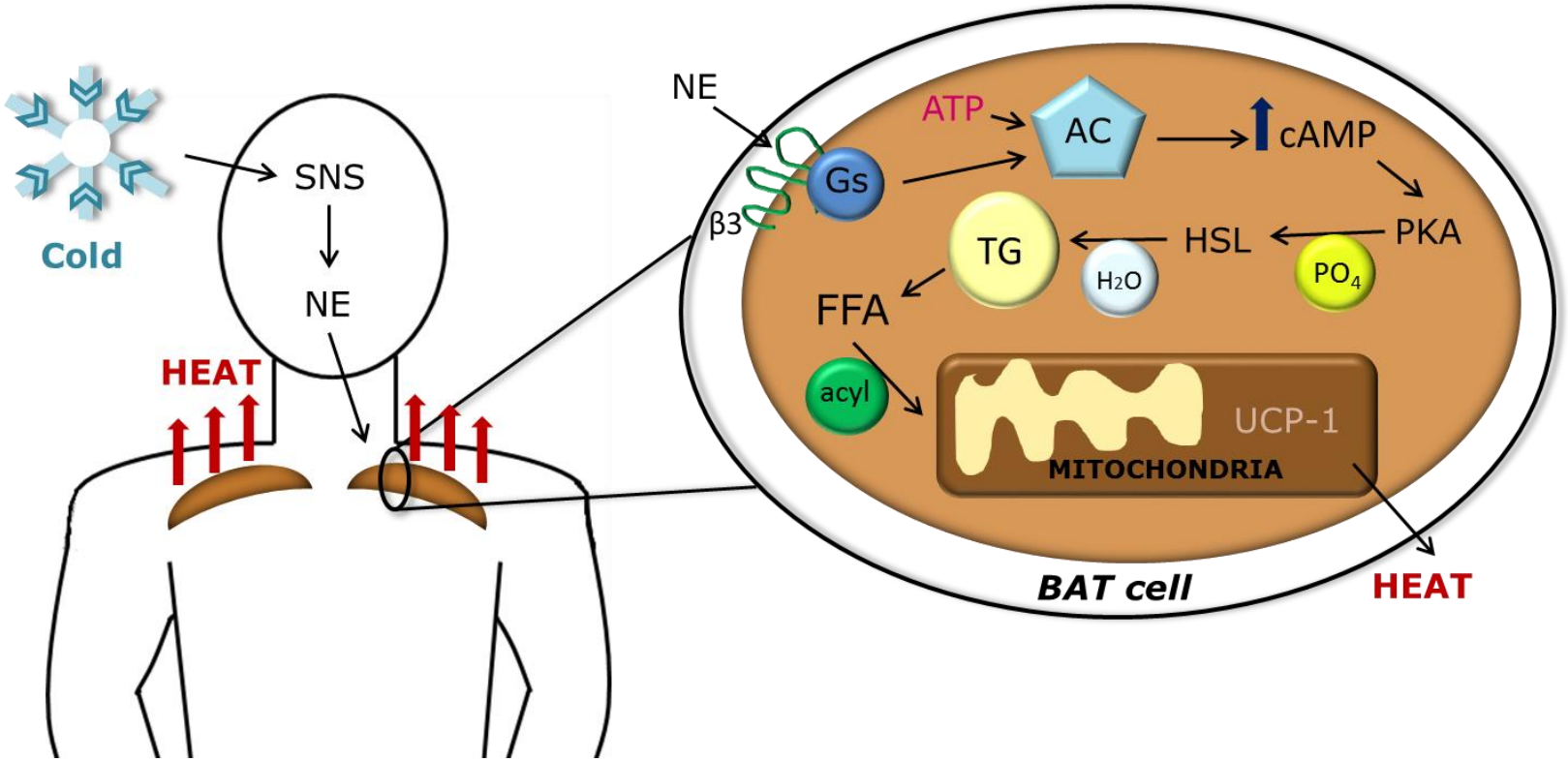


Figure 1.6 How heat is produced in brown adipose cells after cold exposure.

The abbreviations denote - SNS: Sympathetic nervous system, NE: Norepinephrine, β3: β3-adrenoreceptor, Gs: Guanine nucleotide-binding protein, ATP: Adenosine triphosphate, AC: Adenyl cyclase, cAMP: Cyclic adenosine monophosphate, PKA: Protein kinase A, PO_4: Phosphate, HSL: Hormone-sensitive lipase, H_2O: Water, TG: Triglyceride, FFA: Free fatty acid, acyl: Acyl group, UCP-1: Uncoupling protein one, BAT: Brown adipose tissue.

1.4.4 Factors affecting the activity of brown adipose tissue

1.4.4.1 Age and gender

It has long been established that the age of a person is inversely related to BAT; BAT activity declines with age (Schosserer, Grillari et al. 2018). Since 1972, it has been known that interscapular BAT slowly disappears up to the age of 30 with a sudden drop afterwards (Heaton 1972). Yoneshiro and colleagues (Yoneshiro, Aita et al. 2011a) scanned using PET/CT, 213 healthy adults aged 20-73 years old in a room at 24°C after cool exposure at 19°C for 1 hour. Active BAT was found in more than 50% of participants in their twenties but, for those who were aged above 50 years, the proportion with active BAT dropped dramatically to 10%. Amongst study participants aged 5-21 years, those children aged between 13-15 years old had the highest percentage of active BAT and the rate of active BAT was almost four times higher in children (44.2%) than in adults (10%) (Drubach, Palmer et al. 2011). Similar results were reported in the absence of thermal stimulation, i.e. thermoneutral conditions (Cypess, Lehman et al. 2009, Pfannenberg, Werner et al. 2010). Conversely, the same results were demonstrated using the technique of infrared thermography (IRT) in the SCR region in both the thermoneutral and stimulation periods (Symonds, Henderson et al. 2012).

Apart from research in humans, the same results are seen using immunohistochemistry. Heaton, took samples from 18 different areas from 52 cadavers aged up to 80 years. He revealed that during the first decade of life, BAT was present and there was a wide distribution through the body (Heaton 1972). The same results were observed

after analysing 22 samples of human perinephric adipose tissue (15 form alive humans and the rest from cadavers), from subjects aged between 3-74 years (Cunningham, Leslie et al. 1985). These findings suggest that the decline in BAT activity with ageing, is due to a decreased amount of BAT depots, Figure 1.7 (Schosserer, Grillari et al. 2018).

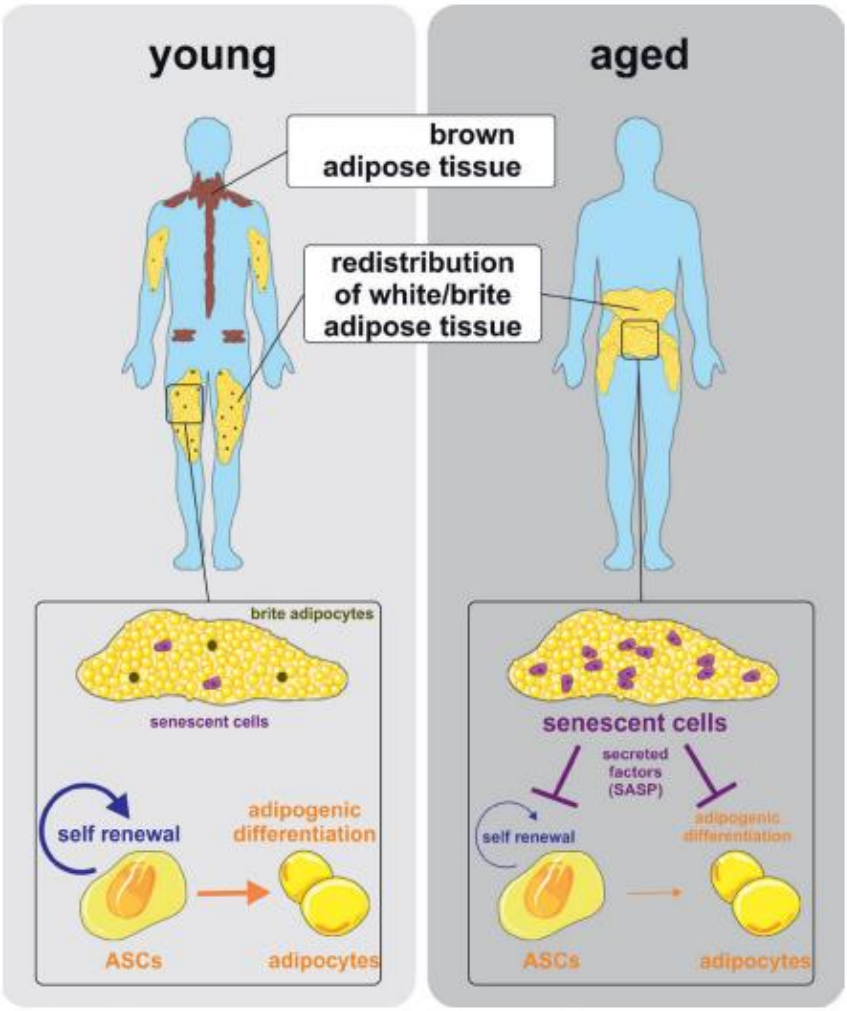


Figure 1.7 Anatomical distribution of white, beige and brown adipose tissues in young and aged humans.

Increasing in age leads to loss of BAT and redistribution of the adipose tissue contributing to the loss of the subcutaneous white fat and increase of the adiposity in the trunk and visceral areas. Also, through ageing, due to the decline of the brite adipocytes, the phenotype of the white adipocyte is more common, resulting in the reduction of the browning effect. The self-renewal of the adipose stromal cells is declining in older ages, minimising the overall functionality of the

adipose tissue. ASCs: Adipose stromal cells, BAT: Brown adipose tissue. From (Schosserer, Grillari et al. 2018).

It is considered that BAT is more commonly visible in females than males (Cypess, Lehman et al. 2009, Pfannenberg, Werner et al. 2010, Wang, Zhang et al. 2015, Becker, Nagel et al. 2016). To feel comfortable in a room with 50% humidity and a standard clothing, women will require an environmental temperature of 26^{o}C, but men need 25^{o}C (Rohles and Frederick 1971). Taking this in account, performing a study using the same environmental temperature and clothing in both sexes, means more women than men will feel cold, leading to an increased likelihood of BAT activation (Nedergaard, Bengtsson et al. 2010). There is also another explanation regarding the higher BAT prevalence in women. Many studies in rodents, have revealed that BAT metabolism is sex dependant, showing more BAT in female rodents (Rodŕiguez-Cuenca, Pujol et al. 2002, Rodriguez, Monjo et al. 2002, Monjo, Rodriguez et al. 2003). Compared to males, female Wistar rats have higher UCP-1 abundance with more multilocular cells, demonstrating greater BAT activity and sensitivity to β_3-adrenoceptor stimulation with norepinephrine (Rodŕiguez-Cuenca, Pujol et al. 2002). Also, adding testosterone to brown fat precursor cells from mice, inhibits UCP-1 mRNA expression during adrenergic stimulation by norepinephrine. However, the opposite was seen in the cells treated with progesterone (Rodriguez, Monjo et al. 2002).

1.4.4.2 Body composition

In addition to age and sex, the presence of BAT is inversely related to BMI and body fat. Van Marken Lichtenbelt and colleagues compared 10 lean healthy participants with 13 obese healthy participants having active BAT, observing that the activity of BAT was reduced with obesity (van Marken Lichtenbelt, Vanhommerig et al. 2009). In another study where 4842 people were scanned, with PET/CT, without the use of any stimulation, such as cold exposure, at a steady room temperature of 24°C, BMI was inversely associated with activation of BAT (Ouellet, Routhier-Labadie et al. 2011). Using the technique of IRT in children, BAT activity decreased with BMI percentile (Robinson, Ojha et al. 2014). In another study using IRT, in 28 males and 30 females NW adults, both resting and stimulated BAT was inversely related to body weight, BMI and fat mass (Muros-Molina, Vazquez Rocha et al. 2019).

1.4.4.3 Ambient temperature and season

BAT is environmentally stimulated by cold temperatures (van Marken Lichtenbelt, Vanhommerig et al. 2009, Robinson, Law et al. 2016). In 1963, Davis was the first to show that prolonged cold daily exposure for 8 hours in cold temperatures (12-13°C approximately) for almost one month, measuring participants' shivering activity with surface electrodes, revealed NST in males aged 25-28 years old (Davis 1961). Fifty years later, 17 healthy people were exposed to cold for 10 consecutive days to 15-16°C for 6 hours per day resulting in a rise of NST by 7% (van der Lans, Hoeks et al. 2013). This can be explained by cold induced activation of the SNS (Orava, Nuutila et al. 2011).

Seasonal variation is another important factor in BAT activity. Comparing BAT activity in 45 healthy lean young male adults between winter and summer after 2 hours of cool exposure at 19°C, revealed an increased EE as well as in the cold induced thermogenesis during winter, but not in the summer (Yoneshiro, Matsushita et al. 2016). Hence, BAT activity in adults during the winter is more likely than in the summer (Au-Yong, Thorn et al. 2009, Saito, Okamatsu-Ogura et al. 2009).

1.4.4.3.1 Cool and cold temperatures

In 2009, one of the first studies in adult humans revealed that BAT was metabolically active during acute mild cold exposure (16°C) but not at thermoneutrality (22°C) (van Marken Lichtenbelt, Vanhommerig et al. 2009). Further studies, using PET/CT scanning and a range of cooling stimuli, have confirmed that cool and cold exposure can increase the uptake of ^{18}F-fludeoxyglucose (^{18}F-FDG) in BAT, Table 1.4.

Other investigations using the IRT technique in children or adults have studied cool and cold water stimulation by placing the participants' hand or feet in cold water, after swimming or drinking ice-water (Lee, Ho et al. 2011, Symonds, Henderson et al. 2012, Robinson, Ojha et al. 2014, Robinson, Law et al. 2016, Law, Chalmers et al. 2019, Muros-Molina, Vazquez Rocha et al. 2019, Muros, Green et al. 2019).

Table 1.4 BAT stimulation using cool and cold exposure with the method of PET/CT.

Study	Method of cooling
(Yoneshiro, Aita et al. 2011b)	19°C cool air temperature for 2 hours and intermittently placing legs on an ice block (c. 4-5 minutes)
(Orava, Nuutila et al. 2011)	17°C cool air temperature for 2 hours and placing one foot in water (8±1°C; 5 minutes in / 5 minutes out)
(Muzik, Mangner et al. 2013)	15.5°C cold room temperature for 120 minutes
(Ouellet, Labbé et al. 2012)	18°C liquid-conditioned suit of cold water for 180 minutes
(Cypess, Chen et al. 2012)	14°C cooling vest for 120 minutes
(Vrieze, Schopman et al. 2012)	16-18°C cool air for 2 hours
(van Marken Lichtenbelt, Vanhommerig et al. 2009)	15-16°C cool air; 2, 4 and 6 hours for 10 days
(Vijgen, Bouvy et al. 2011)	Personalised cooling protocol until shivering with air and water cooling using a Blanketrol (Weiss Technik North America, Inc, Ohio, USA)

PET/CT: Positron emission tomography/computed tomography.

1.4.5 Potential factors for brown adipose tissue stimulation

1.4.5.1 Cannabidiol

Cannabidiol (CBD) is a phytocannabinoid that does not have any psychotropic effects (Mechoulam and Shvo 1963). Few studies have been undertaken to demonstrate its effect on BAT. CBD administration to rodents resulted in body weight reduction (Ignatowska-Jankowska, Jankowski et al. 2011). A later study revealed that CBD can suppress lipogenesis and, at the same time, promote mitochondrial activity both in vitro and in vivo (Silvestri, Paris et al. 2015). Three years ago, it was found that adding CBD to

cell lines can promote the expression of brown genes and up-regulate UCP-1, suggesting that CBD can contribute in BAT activity (Parray and Yun 2016). The latest study revealed that stimulating cells with 5 μM of CBD, promoted the expression of UCP-1 is observed. This result suggests that this phytocannabinoid could increase the thermogenesis (Fellous, De Maio et al. 2020). However, at this point, no research has been published in humans, and a model of brown adipose stimulation has not been proposed.

There is also another link between cannabinoids and BAT, the orphan receptor G protein-coupled receptor 3 (GPR3). This receptor is highly expressed in the central nervous system (Saeki, Ueno et al. 1993, Heiber, Docherty et al. 1995) and is involved in neurodegenerative diseases (Ruiz-Medina, Ledent et al. 2011, Oeckl, Hengerer et al. 2014). GPR3 has been identified as a target for the CBD meaning they could be potentially used to treat Parkinson or Alzheimer's disease, by targeting the GPR3 (Morales, Isawi et al. 2018). Mice lacking GRP3 have reduced UCP-1 and an impaired metabolic response of iBAT that can lead to increased fat deposition (Godlewski, Jourdan et al. 2015).

1.4.5.2 Prolactin

In 1990, an attempt to discover the role of prolactin in BAT during lactation led to the conclusion that this peptide acts as a mediator of BAT suppression (Chan and Swaminathan 1990). Though, in 1995, in fetal and newborn rodents, high levels of prolactin receptors were expressed in brown adipocytes (Royster, Driscoll et al. 1995). However, after a decade, research in cell-line, revealed that BAT can express functional prolactin receptors, although the expression of

UCP-1 could not be modulated by prolactin (Viengchareun, Bouzinba-Segard et al. 2004). In addition, enhancement of nutrition by 50% above the recommended allowances during late maternity in ewes, promoted the fetal BAT maturation, which was positively related to the high abundance of the 15-kD isoform of the long form of the prolactin receptor, compared to the controls with the normal metabolised energy requirements (Budge, Bispham et al. 2000). Also, an investigation of the prolactin administration in rats through gestation in BAT was performed to further establish the effect of prolactin in BAT (Budge, Mostyn et al. 2002). In the latter study, prolactin administrated during pregnancy (24μg/kg per day) resulted in a significant increase in the UCP-1 abundance in late gestation foetus and new-born rats.

It has been demonstrated that BAT differentiation requires signalling from prolactin receptors, and a proposed model of brown adipose regulation by prolactin pathway has been formulated (Viengchareun, Bouzinba-Segard et al. 2004). This model includes activation of a series of signalling pathways, such as mitogen-activated protein kinase and phosphatidyl-inositol 3-kinase, which are induced in the prolactin receptors of the brown adipocyte after the binding of prolactin. This signalling leads to the transcriptional activation of insulin-like growth factor 2, promoting the BAT thermogenesis through UCP-1.

1.5 Imaging techniques and tracers used to localise the brown adipose tissue

There are different techniques to localise BAT activity. These include a variety of radiopharmaceuticals used in nuclear medicine. PET/CT using ^{18}F-FDG scanning has been characterised as a valid method to measure BAT in humans. For example, during FDG-PET/CT, glucose uptake is estimated using the appropriate glucose analog FDG and is expressed as a standardised uptake value (SUV). It is the measurement of the intensity of the signal taken from the PET-CT (Chen, Cypess et al. 2016). However, other techniques such as magnetic resonance imaging (MRI) (Hu, Wu et al. 2014), dual energy computed tomography (Borga, Virtanen et al. 2014), enhanced contrast ultrasonography (Flynn, Li et al. 2015) and near-infrared time resolved spectroscopy (Nirengi, Yoneshiro et al. 2015, Nirengi, Inoue et al. 2016) have been recently used as an alternative in a small amount of studies, though further validation should be performed.

1.5.1 Single-photon emission computerised tomography using ^{123}I-Metaiodobenzylguanidine

Metaiodobenzylguanidine (MIBG) is a molecule that has similar action as NE (Wieland, Wu et al. 1980), whereas ^{123}I, a radioisotope of iodine, can function in the SNS (Kline, Swanson et al. 1981). The imaging process is performed using single-photon emission computerised tomography (SPECT) scanning, creating three dimensional images (Gelfand, Elgazzar et al. 1994). This method is mostly used to

diagnose tumours (Leung, Shapiro et al. 1997, Okuyama, Ushijima et al. 2002). After revealing that BAT can concentrate MIBG in female rats (Okuyama, Sakane et al. 2002), this technique was used in a study performed during winter in 266 children to evaluate the results after been treated for neuroendocrine or other types of tumours. ^{123}I-Metaiodobenzylguanidine (^{123}I-MIBG) uptake in the region of the neck was found in 12% of children, showing that it can identify active BAT (Okuyama, Ushijima et al. 2003). ^{123}I-MIBG is used as a marker of the sympathetic activity, whereas ^{18}F-FDG is a marker for metabolic activity (Admiraal, Holleman et al. 2013).

1.5.2 Positron emission tomography using 6-[$_{18}$F]-fluorodopamine

The compound 6-[$_{18}$F]-fluorodopamine ([$_{18}$F]-FDA) targets sympathetic neuron transporters. Hence, the mechanism of the uptake of [$_{18}$F]-FDA in the human body, is similar to MIBG through PET scanning (Goldstein, Chang et al. 1990). A study using [$_{18}$F]-FDA tracer to assess the activity of BAT in 67 patients, found that almost 18% of the participants had active BAT, suggesting that [$_{18}$F]-FDA may also be a radiopharmaceutical that can be used to localise BAT activity (Hadi, Chen et al. 2007).

1.5.3 Positron emission tomography/ computed tomography using ^{18}F-fludeoxyglucose

The technique of PET/CT using ^{18}F-FDG, is a diagnostic tool to identify tumours (Bar-Shalom, Yefremov et al. 2003, Kwee and Kwee 2009). The uptake of ^{18}F-FDG shows the glucose uptake in metabolically

active tissues, including BAT, and not in the SNS. The action of this radiopharmaceutical, after an introduction of a stimulus, is believed to be through the stimulation of β3-adrenoreceptor resulting in substantial glucose uptake (Hadi, Chen et al. 2007). This tracer is used most commonly in BAT measurement and it is used in most of the studies (Cohade, Osman et al. 2003, Hadi, Chen et al. 2007).

1.5.3.1 Identifying the best tracer for imaging brown adipocyte tissue

After recognising the above 3 tracers, to identify BAT, there was the need to determine which one of these would be best for BAT detection. Therefore in a retrospective study (Hadi, Chen et al. 2007) among 96 patients, it was reported that higher BAT activity was demonstrated with the use of ^{18}F-FDG by 19.2%, than in ^{123}I-MIBG and $_{18}$F-FDA, 18% approximately for both, concluding that ^{18}F-FDG could give better results for BAT visualisation. On the other hand, after performing ^{123}I-MIBG SPECT and ^{18}F-FDG PET/CT in 10 healthy lean Caucasian males, there was no significant difference when trying to localise the anatomic locations with these two methods (Admiraal, Holleman et al. 2013).

1.5.4 Limitations of positron emission tomography/ computed tomography and single-photon emission computerised tomography

PET/CT and SPECT has many limitations apart from the high cost of the scanner, which include:

1. The ionising radiation (c. 8mSv) produced from a single PET/CT scan exceeding 5 to 10 times more than the usual annual environmental radiation dose for a person.

2. It is an invasive method given that to put the tracers in the human body, injection of radioactive tracer is needed. The radiopharmaceutical injection performed needs to equilibrate before scanning begins. For example in ^{18}F-FDG will need 1 hour and for ^{123}I-MIBG SPECT will take at least 24 hours and sometimes 48 hours (Hadi, Chen et al. 2007).

3. Setting a SUV threshold that is too low to measure BAT can lead to overestimation in activity (Steinberg, Vogel et al. 2017).

4. In addition, underestimation of BAT could result, especially after consuming a meal, where insulin is released, and glucose uptake is higher in the muscles than in BAT (Vosselman, Van der Lans et al. 2012), without any change in the oxidative metabolism (Orava, Nuutila et al. 2011), as it measures glucose uptake and not the thermogenic capacity (Olsen, Csikasz et al. 2017). A good example is a study that took biopsies in human participants to measure UCP-1. It was found that both participants defined as BAT positive and negative, had high UCP-1 (Lee, Swarbrick et al. 2011). Another issue is the underestimation of glucose metabolism in BAT, as the location of BAT depots vary between patients and can be found even in WAT (brite cells). Therefore the metabolic volume measured will not necessarily equal the true cell volume (Steinberg, Vogel et al. 2017). Unfortunately, BAT uptake is not always linked with its metabolic activity and

can lead to misleading results. The intracellular TG can influence BAT uptake but not the metabolic rate. This was observed in a study, where the participants with higher BAT TG took up less FDG leading to a lower SUV (Blondin, Labbé et al. 2015).

5. Patients could suffer from claustrophobia or anxiety during the procedure (Griffeth 2005).
6. Low repeatability (Crandall, Gajwani et al. 2019).
7. Cannot be used in a field study.
8. The time of injection is a crucial factor, as lower BAT activity is observed in the morning than in the afternoon, which could compromise repeatability when a patient is late for a session (Steinberg, Vogel et al. 2017). Also, a lower injected dose can increase the image "noise" and result in a higher SUV. Also the time after the injection when PET/CT is performed can lead to lower or higher SUV (Chen, Cypess et al. 2016).
9. It is not able to determine, BAT cell volume or number (Chen, Cypress et al. 2016).
10. Obese participants might have higher SUV in non-WAT tissues that could cofound the interpretation, so a cut off used in lean subjects, could be very low for obese participants (Tahari, Chien et al. 2014).
11. It measures just activity at a specific time and not over a prolonged period as with thermal imaging. Therefore, real time measurements, in order to see any changes pre and post stimulation are not readily feasible with PET/CT.

12. Better results with SPECT are seen after 24 rather than 4 hours (Admiraal, Holleman et al. 2013) but in some participants it might take 48 hours (Hadi, Chen et al. 2007). This makes the study sessions, long and not feasible to follow, especially if the participants have to come back for 2 or more study sessions.

1.5.5 Infrared thermography or thermal imaging: an non-invasive alternative to measure metabolic brown adipose tissue

1.5.5.1 Definition and principle of infrared thermography

The terms "therm" is the Greek word for heat and "graph" the representation of an object. Thus, IRT is the method of presenting, as an image, an object's temperature by converting its emitted infrared energy (not visible to the human eye) to temperature (e.g. heat). Every object which has a surface temperature above absolute zero (0 Kelvin or -273.15^{o}C) can emit electromagnetic radiation (or infrared radiation) according to its surface temperature (Jones 1998, Modest 2013). The wavelength range of infrared is 0.75-1000μm. However, in medical use, a wavelength between 8 to 12μm is used (Qi and Diakides 2007). During IRT, there are several variables which should be taken into consideration, such as the emissivity of human skin among others (Law, Chalmers et al. 2018).

1.5.5.2 History of infrared thermography in medical use

Sir William Herschel, a British astronomer, introduced the existence of infrared radiation in 1800. While using a prism to disperse sunlight, he accidentally saw an invisible light on the outside of red light when he increased the temperature of an object; an electromagnetic wave (Herschel 1800). In 1871, the thermal measurement and, consequently, the establishment of the temperature as an indicator of illness was demonstrated by Wunderlich (Wunderlich 1871). After this discovery, almost one century passed until the capture of the first infrared image of a human by Czerny in Frankfurt (Czerny 1929). In 1925, the medical use of a single detector infrared bolometer was used for diagnostic purposes (Schwamm and Reeh 1953). Thermography was first applied in medicine in 1956 for research in breast cancer (Lawson 1956). Since then, IRT has been widely used in the medicinal field (Diakides, Bronzino et al. 2012, Lahiri, Bagavathiappan et al. 2012).

1.5.5.3 Use of infrared thermography to measure metabolic brown adipose tissue

After the discovery of active BAT in adults in 2009 using PET/CT technique (Virtanen, Lidell et al. 2009), IRT has been used in studies to measure BAT since 2011 (Lee, Ho et al. 2011). Since then, various study protocols in humans have been used to explore factors that can stimulate BAT (Lee, Ho et al. 2011, Symonds, Henderson et al. 2012, Robinson, Ojha et al. 2014, Robinson, Law et al. 2016, Law, Chalmers et al. 2019, Muros-Molina, Vazquez Rocha et al. 2019). In these studies, the main BAT depot measured was the SCR, which has been

previously characterised as the most active and (Leitner, Huang et al. 2017) also the most accessible location.

In 2018 (Law, Morris et al. 2018), IRT was evaluated alongside the established technique of PET/CT. In the latter study, SCR BAT from 8 healthy male adults was measured using PET/CT (measuring the metabolic rate of glucose) and IRT. In the results, a correlation between IRT and the metabolic rate of glucose of PET/CT was found. Therefore, IRT has been established as an alternative to the PET/CT technique to explore the SCR BAT activity.

1.5.5.4 Advantages and disadvantages of infrared thermography in assessing the brown adipose tissue

This non-invasive technique, without any ionising radiation such as the technique of PET/CT, is suitable in all ages, even in children (Symonds, Henderson et al. 2012, Robinson, Ojha et al. 2014). Therefore, it is vital to study humans ethically and according to the Good Clinical Practice, using the IRT technique instead of exposing the participants in radiation through PET/CT scanning. Also measuring in real time and handling a portable equipment, can lead to successful investigation of BAT in environments outside the laboratory such as in schools (Robinson, Ojha et al. 2014).

However, there are some limitations to be considered during IRT. Firstly, carotid arteries could act as a confounder during BAT assessment; though this has been reported as not significant in the current methodology (Law, Chalmers et al. 2018). Also, there is not

any international standardised protocol, as at least recommendations have been established for ^{18}F-FDG PET/CT use in humans (Chen, Cypess et al. 2016), and therefore, the precision needed in image analysis could lead to over or underestimation of BAT.

1.6 Hypothesis and aims

An overview of the advances in the field of BAT activation and its imaging in humans was presented in this Chapter. BAT can metabolise stored FFAs and contribute to EE when it is active, releasing energy in the form of heat, suggesting BAT as a potential target in the prevention of obesity (Frühbeck, Becerril et al. 2009). Taking these into consideration, in the following studies, I have explored the current methodology and established functional, as well as acceptable, approaches to stimulate BAT in adults.

I aimed to further explore the current methodology of IRT in adults in Chapter 2. In a first study (Chapter 2, Section 2.9.3.5), my main hypothesis was to establish that the 15^{o}C of cool water stimulation in adults could be used even in participants living in warmer countries and just arrived in UK. Furthermore, as acclimatisation is very crucial prior to any intervention, in a second study (Chapter 2, Section 2.12), I hypothesised that longer acclimatisation would be preferable to a shorter one, to avoid any masking of the effects of the intervention due to BAT pre-activation.

In a cross-over study (Chapter 3), I hypothesised that after a seven days period of daily cool showers, BAT activity would be greater than prior to the cool showers in healthy adult males.

In a blinded randomised control study (Chapter 4), my primary hypothesis was that, after acute (single day) or chronic (post seven days) ingestion of 582mg CBD, BAT stimulation would be enhanced in both lean and OW healthy adult males.

In a first feasibility study inside a hospital clinic environment (Chapter 5), I hypothesised that patients with hyperprolactinaemia would have higher BAT stimulation prior treatment with dopamine agonists (DA).

In a second feasibility study in a hospital clinic setting (Chapter 6), my primary hypothesis was that prior to weight loss in morbidly obese patients BAT would not be active.

2 Materials and methods

2.1 Sample size and justification

All calculations for the studies in Chapters 3 and 4 were performed using the PS Power and Sample Size Version 3.1.2 (Dupont and Plummer Jr 1990). For the pilot studies in Chapters 5 and 6, an achievable sample size was set of at least 10 participants for each study.

2.2 Recruitment of the participants

In the studies of Chapters 2, 3 and 4, healthy adult volunteers were invited to take part. They were recruited either by word of mouth or through posters/fliers (Chapter 3: CoR-BAT Flier v1.0 23/2/2016) within the campuses of the University of Nottingham. Eligibility of the participant was assessed with screening questionnaire (Chapter 3: CoR-BAT Participant Screening Questionnaire v1.0 23_2_16, Chapter 4: Eligibility assessment), the participants information sheet (PIS) (Chapter 2, Section 2.9.3.5: 1. Temperature of the cooling stimulus: BITS Information Leaflet V1.2 01.10.15, 2. Section 2.12: BITS Information Leaflet V1.2 01.10.15; Chapter 3: CoR-BAT Participant Information Leaflet v1.0 23_2_16; Chapter 4: Healthy Volunteers Information Sheet (Final Version 3.0: 21.6.2017)) was given at least 24 hours before taking written informed consent form (Chapter 2, Sections 2.9.3.5 and 2.12: Healthy Volunteer's Consent Form Version 2.1 1: 1st October 2015; Chapter 3: Healthy Volunteer's Consent Form Final version 1.0 23/2/2016; Chapter 4: Healthy Volunteers Consent Form (Final Version 3.0:23.06.2017) in which PhD student Salahaden

Sultan took the written informed consent and kept all of the study's paperwork). One copy of the consent form was kept by the participant and one was held securely in the investigator site file (ISF).

For the studies in Chapters 5 and 6, potential participants were recruited either from the endocrine clinic (Chapter 5) or from the bariatric clinic (Chapter 6). The patient was identified by the hospital doctor, who was also the principal investigator and a member of the participant's usual care team, during standard service appointments. In the clinic, PIS (Chapter 5: Participant information sheet (Hyperprolactinaemia arm), Version 2, 16/03/2017, IRAS number 212218; Chapter 6: Participant information sheet (Bariatric surgery), 16/03/2017, Version 2, IRAS number 212218) was made available for the potential eligible participant containing contact details of the research team in case more information was required. The principal investigator answered any questions, and each potential participant was free until their next routine out-patient visit (typically 3-6 months for the endocrine clinic and 3-4 months for the bariatric clinic) to decide whether they wished to participate. In case of successful recruitment, a meeting with me was arranged (usually after patient's clinic appointment) in which I explained the study again and informed written consent (Consent Form version 2, 16/03/2017, IRAS number 212218) was received by myself or a research team member who knew the principles of Good Clinical Practice and had undergone proper training. In the consent form, the participant had the option to inform their general practitioner (GP) about their participation in the study and if chosen a note was sent to their GP (GP information

letter TIBATH Version 2, 16/03/2017, IRAS number 212218). In a case where the clinician, in Chapter 5 only, had the opinion of giving DA as treatment immediately, the potential participant was given less than 24 hours to decide whether to take part or not in the study according to the protocol. The recruitment for both of the Chapters 5 and 6, started on September 2017 and finished on December 2018.

One copy of the consent form was kept by the participant, one was held securely in the ISF and one was filed in the participant's clinical notes, in accordance with Derby Teaching Hospitals National Health Service (NHS) Foundation Trust's standard operating procedure (only for Chapters 5 and 6).

A unique study code was assigned to each participant, used to anonymise on any further study paperwork, e.g. questionnaires or databases. A screening log and a master file were kept locked separately to link personal identifiable information to the study code. It was explained to each volunteer, in all of the studies, that they were free to withdraw at any time, without giving any explanation, but it was noted that all of the data collected so far will be used for future research purposes according to the informed written consent. The rights of the participant to privacy and written informed consent adhered to the Data Protection Act 1998.

2.3 Anthropometric assessment

In Chapters 2, 3 and 4, I carried out the anthropometric measurements, whereas, in Chapters 5 and 6, the measurements were taken from the nurses at the Royal Derby Hospital during patient's standard appointment.

2.3.1 Age

The age of the participant was found using the following formula in Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA).

$$\text{Age} = \text{INT}\left(\text{YEARFRAC}(\text{Date of birth}, \text{Session Date})\right)$$

Where "INT" rounds a number down to the nearest integer, "YEARFRAC" returns the year fraction representing the number of whole days between the start date and end date. For the start date I have set up the "Date of birth" and end date the "Session Date".

2.3.2 Height

Height was measured using the Leicester height measure (Seca GmbH & Co. KG, Hamburg, Germany). Before the measurement, the participant was asked to remove any headgear. Then it was asked to stand on the 'footprints' without wearing shoes, with the heels together and touching the backstop. The legs of the participant were straight and the position of the buttocks and the shoulder blades touching the uprights. The shoulders were relaxed and arms were placed to the side as the head was facing straight. It was then verified that the participant's head was in the "Frankfort plane", a midline

position of an imaginary line from the centre of the ear hole to the lower border of the eye socket. After that, the head plate was brought down onto the head, ensuring it rests on the crown of the head, which is the top back half. Two readings of height were made to verify the outcome.

2.3.3 Weight

The participant was asked to remove everything from pockets, to obtain correct results, using the Seca 899 medical scale (Seca GmbH & Co. KG, Hamburg, Germany). Then, they were asked to step onto the scale and stand still on the centre of the scale with the body weight evenly distributed between both feet. The arms were hanging freely by the sides of the body with the palms facing the thigh and the head looking straight forward. Weight was recorded with a digital readout. Two measurements of weight were performed to verify the result.

In physics, mass (m) is a measure of the amount of an object, and is related to the number and type of atoms present inside an object (Hecht 2011). Weight (w) has a similar mean to mass, however, in this term, the gravitational force (g) acts upon it (Hecht 1994). To calculate weight, the following formula is used in physics:

$W = m \times g$

Therefore, for example, if the same study is performed on another planet rather than Earth such as the Moon, the mass of the participants will be the same, however as the Moon has a different gravity than Earth (Minetti 2001), the weight of the participants will

change. In this thesis, the meaning of weight was used, as the same term is used in similar studies (Muros, Green et al. 2019).

2.3.4 Blood pressure and heart rate

In Chapter 3, blood pressure and heart rate were measured on the left hand, with a Boots pharmaceuticals advanced clinically validated blood pressure arm monitor model 5690404 (Boots UK Ltd., Nottingham, UK). After 5 minutes rest from the beginning of the session, the participant sat down next to a table with the arm resting on the table and the feet being flat on the floor. It was checked that the arm was at the same level of the heart and was relaxed. The correct cuff size was used, after measuring the upper arm at the midpoint between the shoulder and elbow. Then the cuff was placed 2cm above the elbow. The tubing was placed at the centre of the arm, facing the front. After, the end of the cuff was pulled so it was wrapped evenly and firmly around the arm. Before starting, the participant was asked to relax for 2 minutes, and then the "start" button was pressed. While the measurement was taken, the participant was requested to keep still, breathe normally and be silent. Two readings were taken, each two minutes apart, and the average was computed. In case the two readings differed more than 5mmHg, one additional reading was obtained before measuring the average.

2.4 Cortisol assessment

2.4.1 Saliva sampling

The samples were collected only after obtaining informed written consent from the participant. All of the procedures regarding the

samples were performed under the Human Tissue Act (HTA) 2004 codes of practice. On 13th of June 2017, a HTA audit took place where the salivary samples of this study were successfully audited. All of the documentation of the tissue tracking was in order, including the laboratory documentation and the procedure of the analysis of the samples.

At the beginning of the session, in preparation of taking salivary samples, the participant was asked to drink 90 ml of water. After the anthropometric measurements, two samples were collected from the participant, each a minute apart. The participant washed his hands and, after drying them, sat down in a chair and opened the cap and removed the swab from the Salivette© cortisol, code blue (SARSTEDT AG & Co. KG, Nümbrecht, Germany). Then, the swab was placed in his mouth and gently chewed for 60 seconds. After collection, the samples were kept in a secured box with wet ice for relocation. Then the samples were centrifuged for 2 minutes in 1.000 x g (Model: Centrifuge 5810/5810 R; Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany). After disposal of the swab as clinical waste, the samples were stored and locked inside a freezer at -80°C in Academic Child Health, School of Medicine, University of Nottingham, in a HTA approved facility, for later analysis.

2.4.2 Laboratory procedure

Laboratory procedures were carried out at the Academic Child Health Department, School of Medicine, University of Nottingham. All chemicals, reagents and laboratory procedures were assessed and implemented in compliance with the UK health and safety executive's

control of substances hazardous to health (SI No. 1657, 1988) and risk assessment guidelines. The procedures used in the study were in line with the ones recommended by the manufacturer. Samples were analysed using the "Cortisol: expanded range, enzyme immune assay, kit research" (Catalogue Number: 1-3002-SAL; Stratech Scientific Ltd, Ely, UK), designed for use on human saliva. Dr Ian Bloor helped me when necessary, during the laboratory analysis of the cortisol samples and Mr Mark Pope with the laboratory regulations and safety issues. A total of 38 samples were analysed in duplicates and inter and intra assay precisions were calculated.

On the day of analysis, the samples were removed from the freezer and placed on wet ice to be thawed. The reagents were brought to room temperature, as well as the microtiter plate with the foil pouch and the plate strips closed. When the samples were thawed, they were centrifuged (Model: Centrifuge 5810/5810 R, Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) at 1.500g x 15 minutes at 4^{o}C and the supernatant was subsequently aspirated for cortisol analysis. Preparation of wash buffer was made, using 900ml of deionised water and 100ml of Wash Buffer Concentrate. Into a disposable tube, 24mL of Assay Diluent were inserted and replacements in G1 and H1 wells with blue ones were made. Into standards (0.012 – 3.0µg/dL), controls, high, lows (all these four in duplicates), in all of the saliva samples wells, 2 wells serving zero and into 2 wells of non-specific binding wells, 25µL of Assay Diluent were pipetted.

Upon the constitution of enzyme conjugate (horseradish peroxidase), 15μL of enzyme conjugate were diluted into the 24mL Assay Diluent and added to each well. The plate was mixed on a plate rotator for 5 minutes at 500rpm and then it was incubated at room temperature for 1 hour with aluminium foil on top. After the incubation, the plate was washed 4 times with the wash buffer; as a result, no liquid was left due to the binding of the cortisol. With a multichannel pipette, 200μL of tetramethylbezidine substrate solution were added to all of the wells and then the plate was covered with an aluminium foil and mixed on a plate rotator for 5 minutes at 500rpm. It was incubated at room temperature for a further 25 minutes and the reaction stopped by adding 50μL of the Stop solution. The plate was then spun for 3 minutes at 500rpm, until the colour was yellow, and then read using the software KCjunior™ (BioTek Instruments Inc., Vermont, USA).

2.4.3 Cortisol standard curve

The standard curve of the saliva cortisol was inside the standards, Figure 2.1.

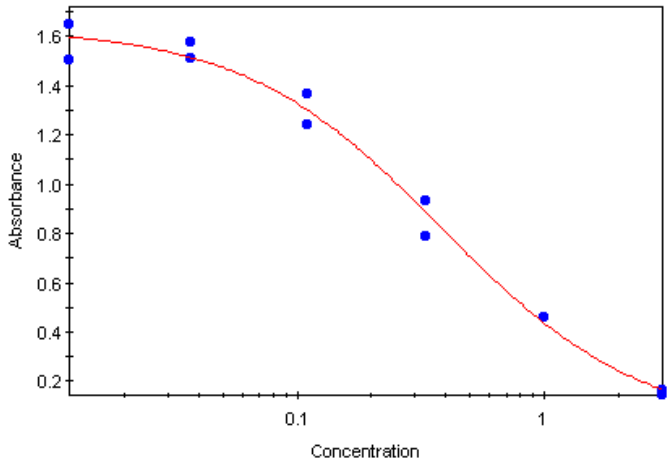


Figure 2.1 The cortisol standard curve of the saliva samples.

The blue circles show the standards whereas the red line is the standard curve. Parameter (y= (A-D)/ (1+(x/C)^B)+D), A=0.0035, B=-1.0750, C=0.3859, D=1.6360, R^2=0.9900.

2.4.4 Saliva cortisol verification

The first step to obtain robust results was to verify which sample would be used for the analysis. Prior and post the intervention, no difference in the salivary samples was observed. The only significant change found was between the samples collected post the intervention, Table 2.1.

Table 2.1 Comparison of the two salivary cortisol samples prior and post the intervention (n=6, mean±SD).

Samples	**Salivary cortisol**	
	Prior	**Post**
Sample 1 (μg dl^{-1})	0.39±0.17	0.52±0.13*
Sample 2 (μg dl^{-1})	0.37±0.17	0.42±0.09*

*p=0.036.

From the above table, as the first salivary cortisol sample was measured higher, post the intervention, I used the second sample for the analysis.

2.5 Skin temperature

2.5.1 Measurement of the skin temperature

Careful cleaning of the area of interest, to place the skin sensors, with an alcohol based wipe (Clinell wipe alcohol based 70% alcohol and 2% chlorhexidine, GAMA Healthcare, Hertfordshire, United Kingdom) was performed. In Chapter 3, two iButtons® (Model DS1921H-F50, Maxim Integrated, California, USA) had been already programmed before participant's arrival with the One Wire Viewer hardware (Maxim Integrated, California, USA). These were positioned with 3M Micropore™ medical tape 1530-1 (3M, Minneapolis, USA) on the participant to measure the skin temperature every minute. The participant was asked to place one iButton® on the area below the clavicle bone at the left upper chest and the other one over the third metacarpal of the left hand (for Chapter 3 only).

In Chapter 4 the participant was asked to place iButtons® on the left side of the head, left paravertebral, dorsal 3rd metacarpal of the left hand, left anterior thigh, left shin, left arm at the antecubital fossa and between the 3rd and 4th metatarsal of the left dorsal foot.

2.5.2 Analysis of the skin temperature

In Chapter 3, the data were put into a Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) file and medians of the last 5 minutes of the acclimatisation period and of the last 5 minutes of stimulation period were calculated for each site. Paired t-test was used to assess any difference prior and post intervention and with

one-way analysis of variance (ANOVA) the skin temperature was assessed between the three periods of the session.

In Chapter 4, the following equation of Hardy/DuBois (Hardy, Du Bois et al. 1938), which has been previously evaluated (Mitchell and Wyndham 1969), of a total measurement of 7 skin sites, was performed:

Mean skin temperature (7 points) = (0.07 × head) + (0.14 × antecubital fossa) + (0.05 × hand) + (0.07 × foot) + (0.13 × shin) + (0.19 × thigh) + (0.35 × paravertebral)

In Chapter 5 and 6, as standardised clothes, e.g. shorts, were not provided, measurements of the thigh and paravertebral could not be taken. For this reason, the above equation could not be used. Therefore, a paired t-test was used to compare the values for each site individually as described above for Chapter 3.

2.6 Indirect calorimetry assessment

2.6.1 Pre-assessment equilibration

Using the Fitmate pro© (Cosmed, Rome, Italy) the assessment of the RMR was conducted in Chapter 3. The measurement of the inspired and expired flow and volume was measured with an optoelectronic reader, to calculate the RMR of the participant. The Fitmate pro©, conforms to the standards of the international organization for standardisation and is CE marked.

The participant was asked to sit relaxed on a chair and place the RMR mask that is attached to a flowmeter over their mouth and nose. The

elastic cords were pulled tightly to eliminate any leaks. The height, weight, gender and date of birth of the participant were input in the equipment to be used in the subsequent calculations.

2.6.2 Resting metabolic rate protocol

After ensuring that the participant was relaxed and ready to start, the "Resting Metabolic Rate" from the test menu was selected. The equipment was automatically calibrated prior to each test, which lasted 15 minutes (the first 5 minutes of data were discarded and 10 minutes of the acquisition phase), according to the manufacturer's instructions, during which, the participant was asked to breathe normally. Any problems (e.g. air leak through the mask, participant not relaxed) during the test, were automatically detected by internal sensors of the equipment and a warning message would appeared on the screen, though such thing did not happen.

The values measured, were compared with the predicted ones calculated automatically with the Harris-Benedict formula (Harris and Benedict 1918).

2.6.3 Resting metabolic rate analysis

RMR correction was performed to eradicate misleading results. Thus, in case the measured percentages of the RMR were different in each participant prior and post the 7 days period of the cool showers, I used the highest prediction percentage of the RMR and corrected the lowest one. For example, if the RMR prior the cool showers had a prediction of 96% and post the cool showers was 83%, the RMR after the 7 days period of the cool showers was corrected with the rule of

three to 96%. This was performed for each participant individually. Paired t-test was used in the analysis to identify any differences prior and post the intervention.

2.7 Questionnaires and diaries

In Chapter 3, to check the eligibility of the participant a questionnaire was sent via e-mail (CoR-BAT Participant Screening Questionnaire v1.0 23_2_16). After receiving the informed written consent form, a demographic questionnaire (CoR-BAT Study: CoR-BAT Participant demographics v1.0 23_2_16) was completed. At the end of the first visit, two food and exercise diaries ("CoR-BAT Study Food and Exercise Diary 1 V1.0 23/2/16" and "CoR-BAT Study Food and Exercise Diary 2 V1.0 23/2/16") were provided to the participant in order to record the food consumption and any exercise for 3 specific days prior and during the intervention. A cool shower diary (CoR-BAT Study: Shower Study v1.0 23_2_2016) was provided to be completed, including information about the date, time and temperature of the cool showers that the participant had. If a warm shower was taken immediately after, the duration and temperature were also recorded. In Chapter 4, a follow-up sheet was provided to each participant, including the date and time of the capsules' ingestion.

2.8 Clinical research file

For the studies in Chapters 5 and 6, a clinical research file was conducted. This document included all of the data extracted from the participant's routine out-patient clinical records during the clinical appointment and before the IRT session. It included demographics,

anthropometric measurements, pulse, systolic and diastolic blood pressure and laboratory tests.

2.9 Infrared thermography

2.9.1 Location of the infrared thermography

2.9.1.1 Investigation room

In all of the studies, the rooms used during IRT met specific criteria. One of them was the adequate size of space where the procedure of IRT was performed. The distance between the participant and thermal camera was at least 1m, to capture the region of interest (ROI) to study BAT activity, meaning that a minimum size of 2m x 3m room was required. Participant changed clothes in a different room when necessary. Reflective surfaces, such as mirrors behind the participant, were removed, to avoid any reflections that might disturb the image analysis. For the studies in Chapters 2 and 3, there was a panic button, in case an emergency occurred, connected to the Division of Child Health, but was never needed. For Chapters 4, 5 and 6, sometimes there was another investigator, e.g. principal investigator or trainee students (after receiving consent from the patient), with me while undertaking the studies and the studies, which were performed inside the hospital were the staff was aware of the sessions. All the rooms were equipped with fresh water supply for BAT stimulation.

2.9.1.2 Room and ambient temperature

For the studies in Chapters 3 and 4 the room temperature was maintained in 22^{o}C (Law, Morris et al. 2018), for at least 1 hour prior to participant's arrival for a study and was kept at the same

temperature throughout. In order to control the temperature in the room, either an air conditioner unit or a radiator was used, or the room was temperature controlled. Between the air condition unit and the participant, there was a curtain, to avoid direct draughts which can be detected in the thermal images and therefore the airflow was kept in low levels. For the studies in Chapters 5 and 6 that were undertaken in rooms inside the hospital area where the room temperature could not be regulated, the temperature was regularly measured with an Extech MO297 moisture psychometre (Extech Instruments, New Hampshire, USA) and any changes were noted. The ambient temperature data for all of the studies was obtained from the Meteorological office site (https://www.metoffice.gov.uk/).

2.9.1.3 Computer and other equipment

All of the equipment producing heat, such as laptop, were located away from the participant to avoid heat disturbance.

2.9.2 The imaging system

2.9.2.1 Thermal camera characteristics

The forward-looking infrared (FLIR) E60 (FLIR Systems Inc., Oregon, USA) was used, with a resolution of 320x240 pixels, thermal sensitivity of <0.05^{o}C and temperature range from -20^{o}C to 650^{o}C.

2.9.2.2 Calibration and service of the camera

Frequent service was obtained from an external private company. During service, the company also checked the camera calibration. However, self-check of the camera was performed after measuring

some water with a water-thermometer and then configuring the result with the thermal camera before each session.

2.9.2.3 Mounting the thermal camera

For all of the studies, a tripod model Slik Able 300DX Tripod with 3-Way Pan Tilt Head 9 (SLIK Corporation, Saitama, Japan) was used, to provide vertical height adjustment, depending on the height of each participant and to avoid any tilting of the camera.

2.9.3 Procedure of the infrared thermography

2.9.3.1 Standard operating procedures and risk assessments

During my studies, in collaboration with Dr James Law, we created the standard operating procedure with the title "Infrared thermography (or Thermal Imaging) of SCR brown adipose tissue in humans using the 'Blanketrol II' (Weiss Technik North America, Inc, Ohio, USA) cooling unit". That document, also included the risk assessment form, containing all the possible hazards while performing IRT. This was used as a guideline when performing IRT using the 'Blanketrol II' (Weiss Technik North America, Inc, Ohio, USA) cooling equipment.

2.9.3.2 Intra-observer variability

To determine the reproducibility of the IRT, the coefficient of variation (CV), using Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) was calculated in three different trial studies, performed in three different occasions under BITS ethics (Section 2.2). The first one, included the comparison of two sessions conducted at

the same day by myself where the CV was found at 1.93%. In the next one, after 5 days of taking thermal images from the same participant and at the same time, using hand cooling as a stimulus, when I compared the same image during stimulation the CV was found to be 0.537%. On the third one, I used one image taken on the second trial and when I analysed the same thermal image in 4 different days, with a 5 days gap during each analysis, CV found in the right and left SCR side was similar, 0.01% and 0.02% respectively.

In another intra-observer study, 7 participants took part, including myself, to identify any differences when analysing the thermal images using FLIR and MatLab (MathWorks, Massachusetts, USA) softwares. In FLIR, an old technique of analysis, we drew the ROI, whereas in MatLab, we placed points on the ROI, which was drawn automatically by the software. Eight different thermal images were analysed for each method with at least a week between the two techniques, Figure 2.2. All of these studies demonstrate that I am able to apply the method of IRT and analyse the data within an acceptable amount of intra-observer variability, lower than 2%.

2.9.3.3 Pre-imaging equilibration

On arrival at the investigation room, the participant was informed of the context of the study. After receiving written informed consent, the participant was asked to change into standardised clothing, consisting of a light vest-style cotton top (Chapters 2, 3, 5 and 6), so as the ROI will not be covered by clothes; shorts were also provided (Chapter 4). In all of the studies, the participant removed the shoes and socks

from both feet. Any jewellery was removed and hair was placed in such a way so as not to obscure the ROI.

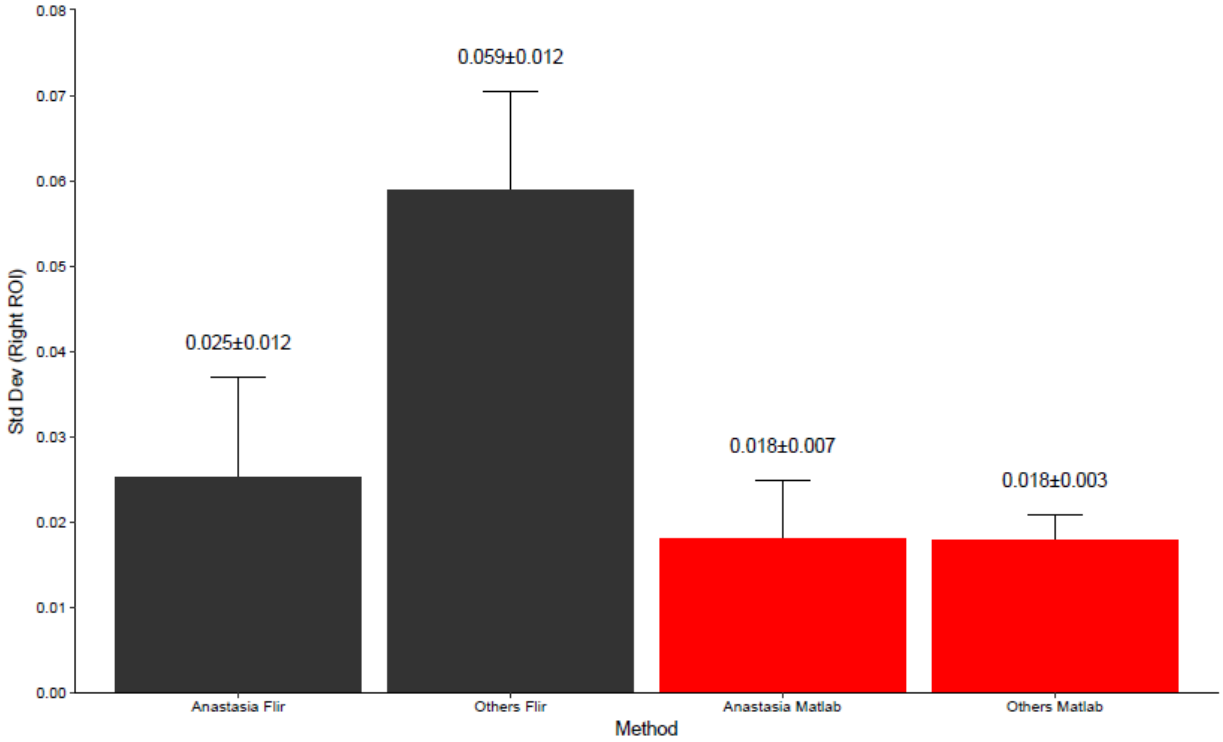


Figure 2.2 Bar charts assessing the results of thermal image analysis between my results and the rest of the participants' using two different procedures.

The mean standard deviation±standard error are presented in each bar. The two black bars, show my results (Anastasia FLIR) comparing to the ones of the rest of the participants (others FLIR), using the old technique of drawing the area of interest by ourselves (n=7, mean±SD). The red bars, depict my outcomes (Anastasia Matlab) and the rest of the participants' outcomes (Others Matlab), using the most recent technique of analysing the thermal images. Std Dev: Standard deviation, ROI: Region of interest.

2.9.3.4 Procedures prior to the commencing of infrared thermography

Three measurements of the tympanic temperature were taken using a Braun ThermoScan 4520 Ear Thermometre (Braun GmbH, Kronberg im Taunus, Germany). The temperature of the room and humidity were measured with an Extech MO297 moisture psychometre (Extech Instruments, New Hampshire, USA).

Once the participant was positioned comfortably, Figure 2.3, the camera was manually focussed and adjusted to capture the ROI. The reflected temperature was measured, holding a reflector (made from cardboard and a creased aluminium foil; foil was selected as it reflects heat). This parameter, along with the ambient (room) temperature, humidity and distance were inserted in the camera settings. The emissivity was always set to 0.98 (used to measure the skin temperature).

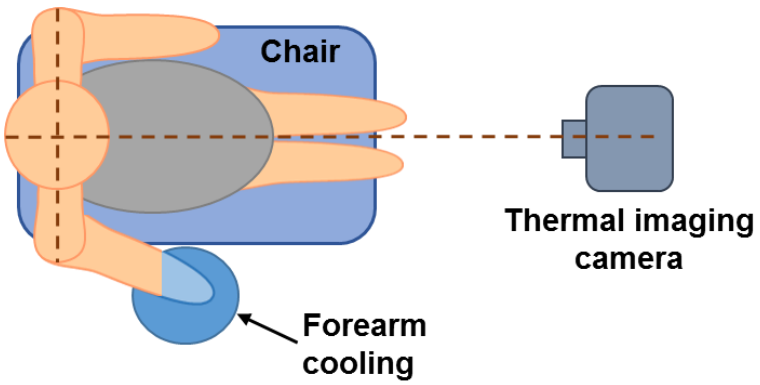


Figure 2.3 Thermal imaging session set-up.

The participant was sat comfortable in a chair throughout the IRT. The camera and cooling stimulus settings were in specific places. IRT: Infrared thermography.

2.9.3.5 Assessing the optimal cooling stimulus to activate the brown adipose tissue in adults

In previous studies using the technique of IRT, the mean temperature was set at 15°C (Robinson, Ojha et al. 2014, Robinson, Law et al. 2016). At lower temperatures, skeletal muscles can shiver, i.e. shivering thermogenesis (Tansey and Johnson 2015). A temperature that could be used repeatedly without shivering, especially in participants that just came in the UK from warmer countries, has not been studied. Therefore my hypothesis was that the 15°C cool stimulus used in previous studies would induce NST.

The conceptualised and designed of the study was made by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds. The study protocol was already written and approved for use by Dr. James Law, Professor Michael E. Symonds and Professor Helen Budge. Apart from the study sessions, I also performed the recruitment and obtained consent from the participants. Having obtained approval from the University's ethics committee (REC number: D19062014 SoM Child, Appendix I), I recruited 6 healthy female adults (mean age: 23.7±2.6 years and BMI: 22.08±2.65kg/m^2) that had moved to UK from Spain recently, during August of 2016. The recruitment was performed as described in Section 2.2 of this Chapter and the study took place in the facilities of the University of Nottingham located in Queens Medical Centre campus, Nottingham, UK. The inclusive criteria for this study were:

- adult participant

- participant without any scar tissue affecting the ROI
- participant without any medical conditions and medications that might affect BAT.

This trial, consisted of one study visit, in which after receiving written informed consent, I asked from the participant to change into standardised clothes including V-neck style cotton top and shorts and measures of height and weight were taken (Sections 2.3.2 and 2.3.3). Then IRT was performed, as described in Section 2.9 of this Chapter. During the baseline, the participant was sitting comfortable for 20 minutes in a 22°C room temperature and then, the cooling blanket Blanketrol II (Weiss Technik North America, Inc, Ohio, USA) was placed around the left arm at 17°C for 10 minutes. Every 10 minutes, the blanket's temperature was lowered for 1°C, until 10°C was reached and then it was set to 1°C higher. All of the sessions started in the morning and the participants were fasted for at least 4 hours and avoid any drinks except water as well as smoking for at least 2 hours.

In the results of this study, one female felt discomfort deriving from shivering at 14°C, whereas one participant felt shivering on 13°C and one on 12°C; the rest of the three participants shivered at 11°C. Therefore, I concluded that I will use the already established method of 15°C, which will not cause shivering.

2.9.3.6 Brown adipose tissue stimulation using cold water

During the acclimatisation, the participant was asked to place the right hand in an empty bucket before introducing the stimulus (cool

water) and remain as still as possible throughout the session. During the stimulation period, the bucket was filled up with water at 15°C and the right hand was immersed to the level of the middle ulnar. The water temperature was continuously measured with a tank thermometer and in case of rise detection in temperature, cool water was added (Chapters 4, 5 and 6). Another cooling stimulus used was the cooling blanket Blanketrol II (Weiss Technik North America, Inc, Ohio, USA) set at 15°C (Chapter 2, Sections 2.9.3.5 and 2.12 and Chapter 3) which was located around the arm.

2.9.3.7 Baseline and stimulation periods during infrared thermography

The IRT was performed, in two different periods. Thermal pictures were taken manually every 20 seconds and the picture number, or else reference number, including time were written in the data collection sheet. The time periods for both of the sessions were as follows:

1. Pre-stimulation period (acclimatisation or baseline period)

For 15 minutes there was no stimulus induced. At the last 5 minutes, the participant was informed about the introduction of the cool stimulus.

2. Stimulation period

Immediately after the pre-stimulation period, cool water was added to the empty bucket where the participant's right hand was resting. The temperature of the stimulus was measured by the digital thermometer "Splash-Proof Super-Fast" (Electronic Temperature

Instruments Ltd, West Sussex, UK) after the cool stimulus was introduced and lasted 15 minutes. In case of the use of the cooling blanket, the cooling blanket Blanketrol II (Weiss Technik North America, Inc, Ohio, USA) was securely located around the left arm.

In Chapter 3, the above two periods lasted for 20 minutes each and a third period was added in the IRT; the post-stimulation period. At this period, after the cooling blanket was removed from the participant's hand, a series of images for another 5 minutes were taken.

2.10 Thermal image analysis

2.10.1 Converting thermal images to numbers for analysis

At the end of each session, the thermal images were retrieved. A cross, Figure 2.4, was used to identify the ROI and also for the measurement of the reflected temperature.

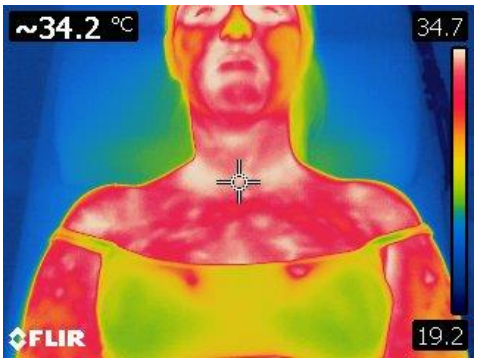


Figure 2.4 Example of a thermal image.
The cross indicates the ROI and it is adopted to measure the reflected temperature to be used as input in the camera settings. The various colours seen in this picture are due to the differences in the temperature of the body and the environment. As the participant is getting hotter, the colour is turning from deep blue to green, yellow, red and finally to white. ROI: Region of interest.

An automated method for the analysis of thermal images developed at the University of Nottingham (Law, Morris et al. 2018), was used

via MatLab R2013b (MathWorks, Massachusetts, USA). The script to process the images "thermal_export" was selected. This script was used to define the ROI in the SCR area.

After the execution of the script, a dialog box appeared, Figure 2.5. From this box, "File" and then "Open Folder" was selected.

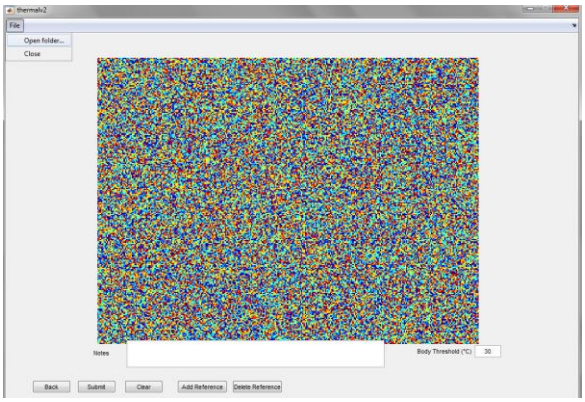


Figure 2.5 Dialog box used to select the region of interest.
The definer dialog box appeared after selecting the BAT-ROI definer and it was used to select the ROI. BAT: Brown adipose tissue, ROI: Region of interest.

After the selection of the proper folder containing the thermal images to be analysed, the images appeared in the dialog box, Figure 2.6.

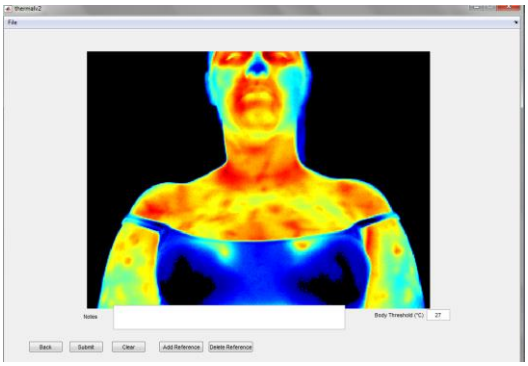


Figure 2.6 Dialog box after selecting the appropriate folder for analysis.
The chosen folder was set into order for the ROI definition. ROI: Region of interest.

Then, adjustment of the body threshold in the proper temperature was made, to minimise any emerging of the heat from the background

in the results, such as hair temperature, Figures 2.7a and 2.7b. If, for example, a body threshold at 27°C was chosen for the analysis of the Figure 2.6, the hair of the participant is now visible due to the low threshold and thus there would be blue colour next to the neck, Figure 2.7a. If this image was analysed without using the proper temperature of body threshold, the outcome would be biased results due to the heat leak induced from the hair, Figure 2.7b.

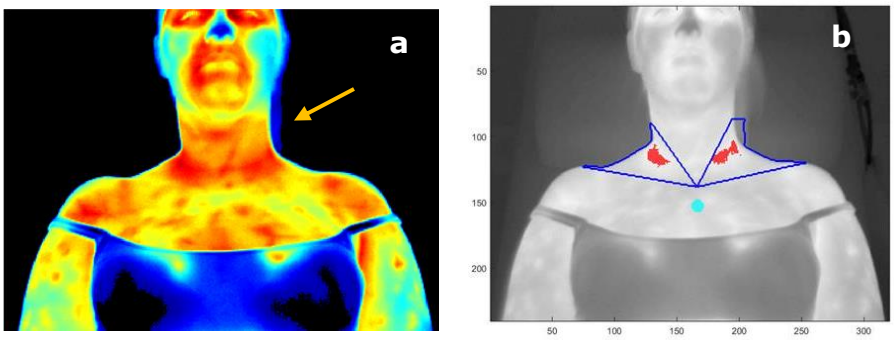


Figure 2.7a and b Images with a heat leak.
In the left image (a), there is a heat leak next to the neck, pointed with the yellow arrow. Therefore, in the results (b), we can see that the right triangle, uses values from the background (hair temperature) to calculate BAT activity. BAT: Brown adipose tissue.

After adjusting the body threshold at the appropriate temperature, the next step of analysis was followed, which is the selection of the ROI.

In this image 6 dots needed to be placed, to format the two triangles, Figures 2.8a and 2.8b:

- at the reference point
- at the acromion of the left shoulder
- at the acromion of the right shoulder
- at the right side of the middle of the neck
- at the left side of the middle of the neck

- approximately 2cm below the sternal notch.

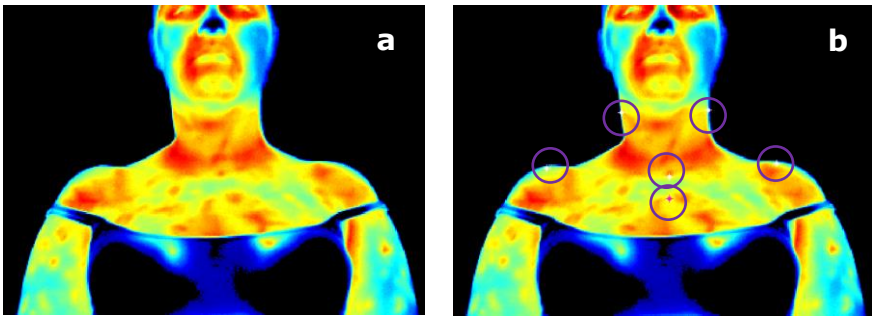


Figure 2.8a and b Thermal images prior and post the input of the 6 dots to select the region of interest.

The thermal image appears in the dialog box (a) and 6 dots are manually inserted (b). The dots spotted in purple circles, depict the points that were placed to obtain correct results.

Once all the 6 dots were positioned in the right place, "Submit" was selected and then the program moved on to the second picture automatically. When a point was missed, the message "Not enough points" would appear. This process was repeated until all images had proper dots on them and the message "All done!" appeared on the screen to notify that the process had finished.

When unsuitable images for the analysis were detected, the programme's notes section would be used. Figures 2.9a and 2.9b show some examples of unsuitable images because a part of the ROI is hidden.

In the first thermal image, Figure 2.9a, the hand is covering a big area of the ROI, whereas the same happens for the second image, Figure 2.9b, with the participant's hair. For these reasons, it would be constructive to mention that these images were unsuitable for analysis and the results were not taken into consideration.

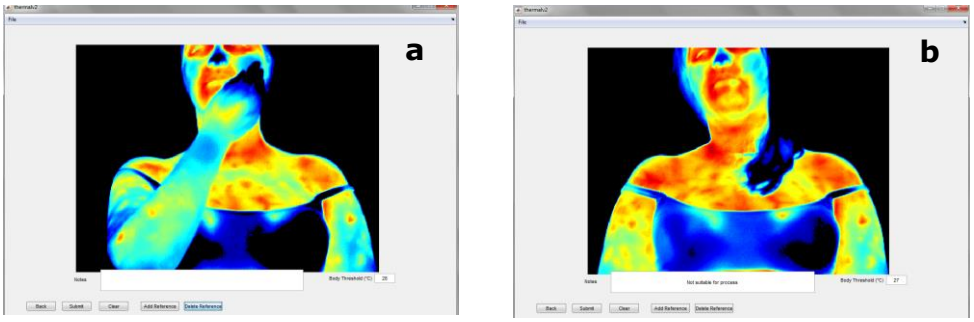


Figure 2.9a and b Unsuitable images for analysis.

In both of the images there is something obscuring the ROI, either the hand (a) or hair (b). In the later analysis these images will not be used, as they will not give us a robust outcome. ROI: Region of interest.

After completing the first step of the analysis, the second step commenced. By opening the next script "process", an options menu was popped up, showing the processing options that I could choose for my analysis, Figure 2.10.

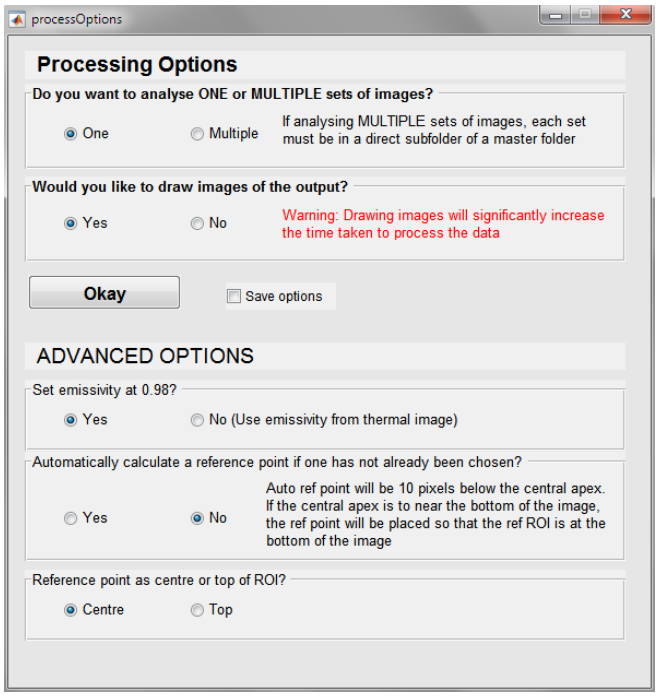


Figure 2.10 Processing options menu.

In the menu, there are options to analyse images from one or multiple sessions, draw lines around the ROI, correct the emissivity for human skin and calculate the reference point if it hasn't been added during the defining process. ROI: Region of interest.

The script would go through each pixel and calculate the "hotspot" within the ROI; the median upper decile of point within the ROI of BAT activity. After running the script, a Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) document was created in the same folder where the new MatLab images were saved depicting BAT activity, Figure 2.11. From the Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) document, the median temperatures of the 10% upper percentile of the hottest area of the ROI, from the right and left area and the median temperatures of the reference point were used for analysis.

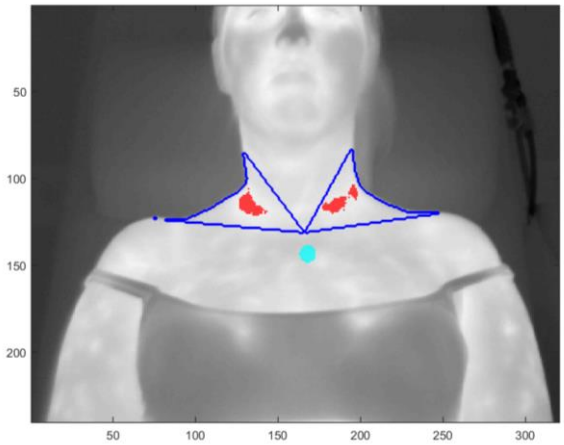


Figure 2.11 Right and left triangle of the region of interest drawn automatically after analysis.

In blue lines the 2 triangles depict the right and left ROI and the red areas illustrate the right and left temperature 10% upper percentile hotspot. The light blue dot represents the reference point. ROI: Region of interest.

From the Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) document, the average of the last 5 minutes of the acclimatisation was calculated and that depicted the baseline temperature ($T_{baseSCR}$). To find the maximum temperature, the function "=max" was used in Microsoft® Office Excel 2013 (Microsoft Corporation, Washington, USA) in all of the data during the

stimulation period. Then, in the number deriving from the above function, an average of ±20 seconds was calculated to depict the maximum SCR temperature (T_{maxSCR}). I used this way, as the maximum temperature could be a false result, deriving from a small movement of the participant. Thus finding the average of one image before (-20 seconds) and one image after (+20 seconds) the maximum temperature resulted to a more robust outcome.

To calculate the change of temperature (ΔT) of either SCR or reference temperatures, the equation used was:

$$\Delta T = T_{base} - T_{max}$$

where the T_{base} was the average baseline temperature and the T_{max} the maximum temperature ±20 seconds.

Also the relative temperature (ΔT_{REL}) for both the baseline and stimulation periods was calculated using the equation:

$$\Delta T_{rel} = \Delta T_{SCR} - \Delta T_{REF}$$

where ΔT_{SCR} was the ΔT of the SCR temperature and ΔT_{REF} the ΔT of the reference temperature.

According to a recent published paper regarding the use of IRT in human studies (James et al. 2018), T_{base} and T_{max} are the main outcome measures of the IRT. It is also noted that the ΔT is used when the participants have a high BMI in order to minimise any effects of obesity in the results.

2.11 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 24.0 statistical software for Windows (IBM Corporation, New York, USA). Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating that the data are normally distributed. Comparisons between two groups were made using a paired t-test unless otherwise is stated.

The software GraphPad Prism version 7.0 (GraphPad Software, California, USA) was used to produce the statistical graphs and data are presented as mean±standard deviation. Further details of statistical analysis will be included in the individual sections, to provide a better understanding.

2.12 Development of the current methodology – Duration of the acclimatisation period

2.12.1 Introduction and hypothesis

Environmental temperature (Au-Yong, Thorn et al. 2009) and participant's stress (Robinson, Law et al. 2016) can be crucial factors affecting BAT activation. Therefore, I wanted to investigate whether a longer acclimatisation period was needed, in which participant's BAT would reach a plateau in case of already active BAT, instead of a short one. The acclimatisation period of the participant is very crucial to avoid any BAT pre-activation, masking the effects of interventions targeted at activating BAT. In this study, my hypothesis was whether a longer (30 minutes) period is preferable to a shorter one (5 minutes) during acclimatisation.

2.12.2 Study design

The conceptualised and designed of the study was made by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds. The study protocol was already written and approved for use by Dr. James Law, Professor Michael E. Symonds and Professor Helen Budge. Apart from the study sessions, I also performed the recruitment and obtained consent from the participants.

Having obtained approval from the University's ethics committee (REC number: D19062014 SoM Child, Appendix I), during the recruitment, Section 2.2, the eligibility to participate was checked.

The inclusive criteria included:

- male gender
- age between 18-30years
- participant without any scar affecting the ROI
- participant without any medical conditions (hyperthyroidism, hypothyroidism and history of CVDs, diabetes mellitus) or medications that could affect BAT activity (beta-blockers).

Two study sessions were held for each participant. The first session, included 30 minutes of acclimatisation and 15 minutes of stimulation period (i.e. the Long session) while the other had 5 minutes of acclimatisation and 15 minutes of stimulation (i.e. the Short session), Figure 2.12. Random allocation was defined through the function "=RAND" in Microsoft® Office Excel 2013 (Microsoft Corporation,

Washington, USA). During the stimulation, the cooling blanket Blanketrol II (Weiss Technik North America, Inc, Ohio, USA) set in 15°C (Section 2.9.3.7) was used. Height and weight were measured at the beginning of each session (Sections 2.3.2 and 2.3.3). All of the sessions took place during morning hours and the participants were fasted for at least 4 hours and avoid any drinks except water as well as smoking for at least 2 hours and abstain from any physical activity 12 hours prior the session.

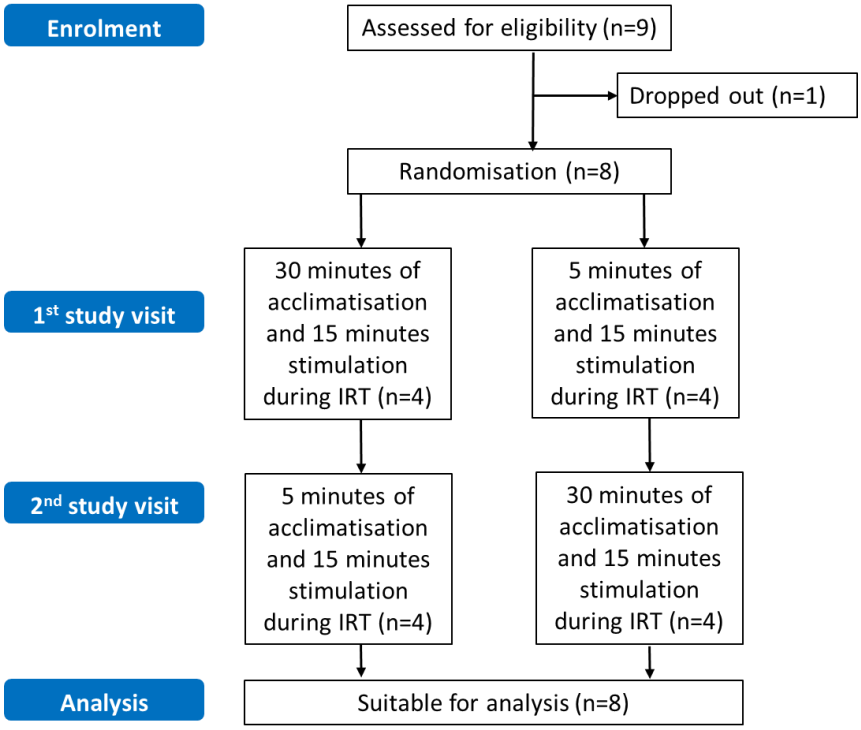


Figure 2.12 Timeline of the acclimatisation study.

The participants were randomised to have either the 30 minutes of acclimatisation (participants: 1, 4, 5 and 7) or the 5 minutes of acclimatisation (participants: 2, 3, 6 and 8) on their first study visit. IRT: Infrared thermography.

2.12.3 Analysis

Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating that the data are normally distributed. First, determination of the effect of the outside

temperature in BAT was performed. Pearson's correlations were used to assess the correlation between the SCR and reference temperatures with the outside temperature. Paired t-test was used to identify any differences between the Long and Short sessions and also to explore the effect of BAT activation during Long and Short acclimatisation.

2.12.4 Results

The mean age of the participants was 27.1±4.1 years and the mean BMI was 25.54±2.68kg/m^2.

2.12.5 Room and ambient temperature

The mean room temperature on the first visit was at 21.5±0.7^{o}C and on the second day at 21.3±0.6^{o}C. The mean environmental temperature was 9.2±1.6^{o}C on the first visit, and 9.8±2.8^{o}C on the second visit, with individual values presented in Table 2.2.

Table 2.2 Date and outdoor environmental temperatures for each study visit (n=8).

Participant	Date of study on the 1st study visit	Date of study on the 2nd study visit	Environmental temperature (°C)	
			1st	2nd
1	21/01/2016	22/01/2016	7.3	11.7
2	26/01/2016	12/02/2016	12	7
3	18/02/2016	19/02/2016	8	9
4	18/02/2016	19/02/2016	9	9
5	23/02/2016	25/02/2016	9	6
6	15/03/2016	13/04/2016	8	14
7	14/10/2016	17/10/2016	11	13
8	01/11/2016	03/11/2016	10	9

2.12.6 Correlations of supraclavicular and reference temperatures

The outside temperature did not have any effect on either the baseline SCR (Long: R^2=0.006, r=0.080, p=0.849; Short: R^2=0.343, r=-0.585, p=0.127) or the maximum SCR (Long: $R^2=6.092^{e-005}$, r=0.007, p=0.985; Short: R^2=0.283, r=-0.532, p=0.174) temperatures. The same results were found for the reference baseline (Long: R^2=0.073, r=0.270, p=0.516; Short: R^2=0.154, r=-0.392, p=0.335) and reference maximum (Long: R^2=0.114, r=0.337, p=0.413; Short: R^2=0.418, r=-0.647, p=0.082) temperatures.

2.12.7 Supraclavicular and reference temperatures

Median SCR baseline BAT temperatures in the Long session differed from those in the Short one (Long: 34.3±0.4ºC, Short: 34.5±0.4ºC), Figure 2.13. Although, there was no difference in the maximum temperatures (Long: 34.5±0.4ºC, Short: 34.6±0.5ºC; p=0.475). Also, there was not any significant difference in baseline (Long: 32.2±0.9ºC, Short: 32.6±0.7ºC; p=0.267) or maximum (Long: 32.6±0.7ºC, Short: 32.8±0.8ºC; p=0.291) reference temperatures between the Short and Long acclimatisation.

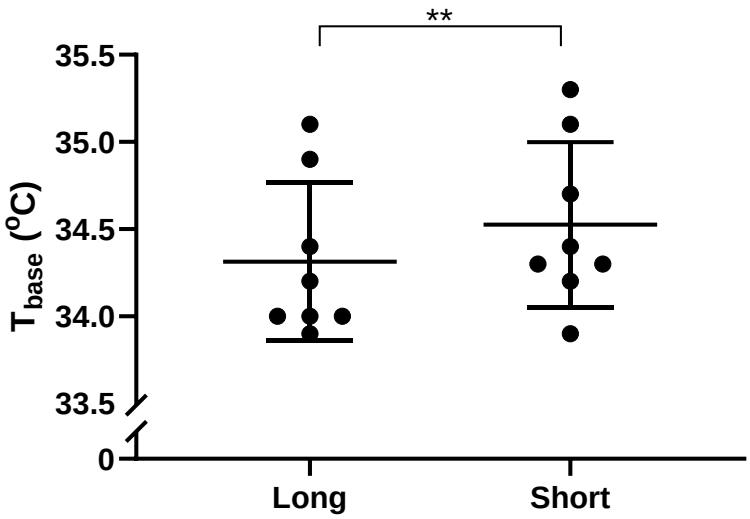


Figure 2.13 Baseline supraclavicular temperatures between Long and Short acclimatisation.

Longer acclimatisation shows a significant lower SCR temperature than the Short one; **p=0.008 (n=8). T_{base}: Baseline supraclavicular temperature, SCR: Supraclavicular.

2.12.8 Cooling stimulus effect on brown adipose tissue

During the Long session, there is a significant effect between baseline and stimulation (Figure 2.14a and 2.15a and b) whereas the effect of the cooling stimulus does not seem to affect BAT after the Short acclimatisation (Figure 2.14b and 2.15c and d).

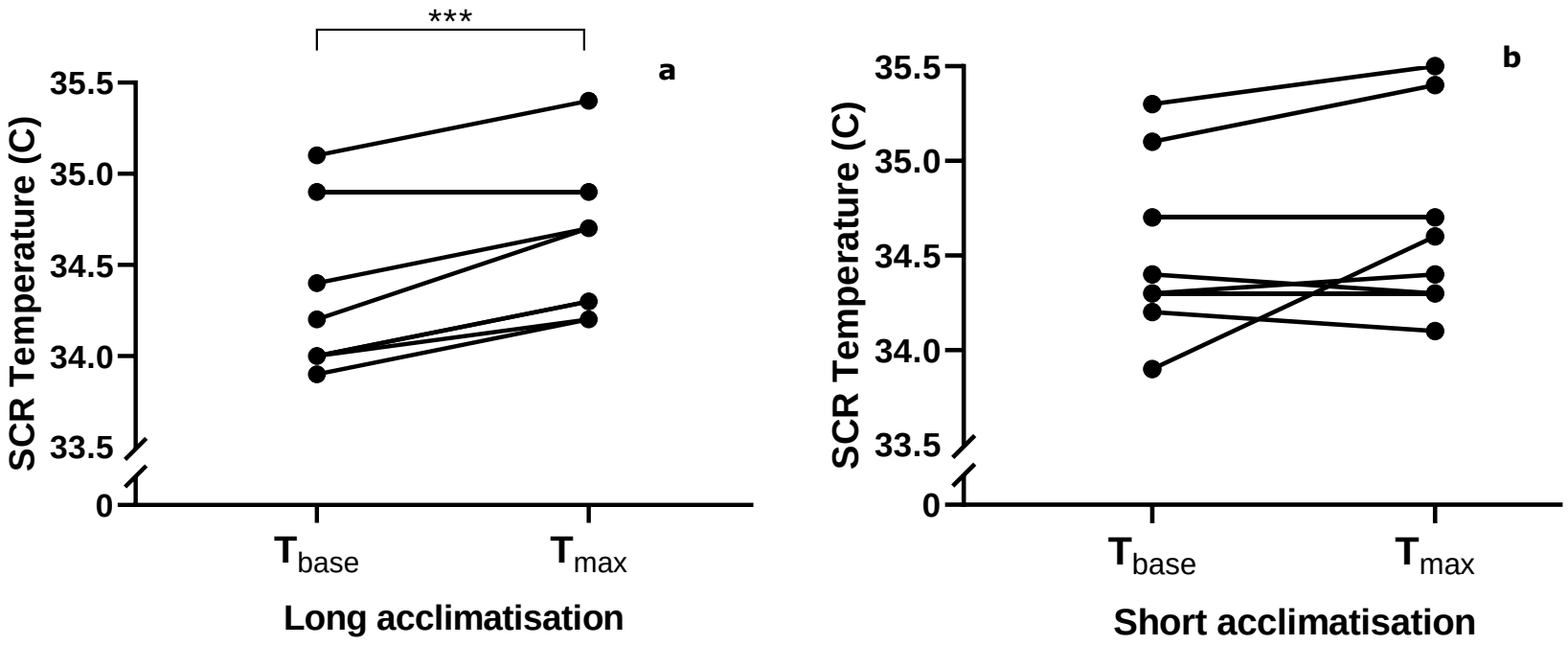


Figure 2.14a and b Cool stimulus effect on brown adipose tissue between Long and Short acclimatisation.

In Long acclimatisation (a), there is a significant effect on BAT temperature after the introduction of the cool stimulus (T_{base}: 34.3±0.4ºC, T_{max}: 34.5±0.4ºC; ***: p=0.0008, n=8, mean±SD). In Short acclimatisation (b) there is not any change in BAT temperature (T_{base}: 34.5±0.4ºC, T_{max}: 34.6±0.5ºC; p=0.188, n=8, mean±SD). SCR: Supraclavicular, BAT: Brown adipose tissue, T_{base}: Baseline supraclavicular temperature, T_{max}: Maximum supraclavicular temperature.

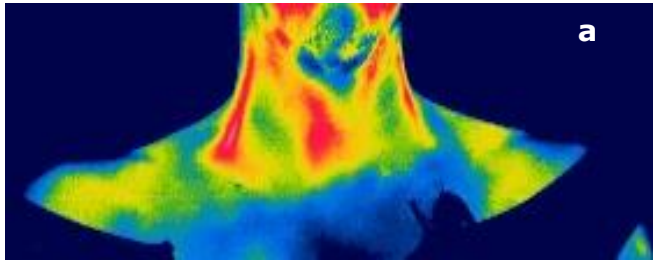
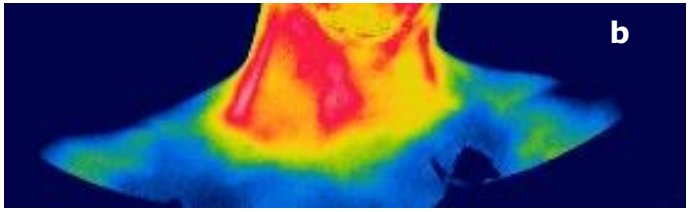
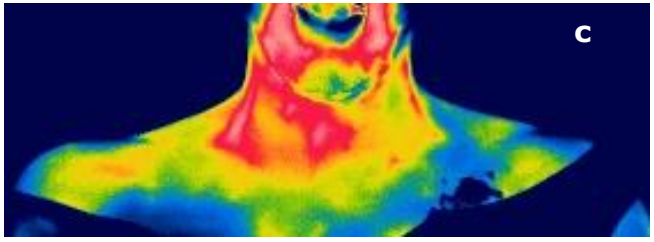
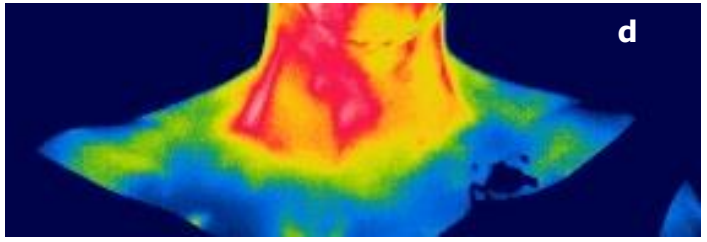
Type of acclimatisation	End of Acclimatisation	Maximum cool stimulus
Long		
Short		

Figure 2.15a, b, c and d Thermal images of the same participant during acclimatisation and stimulation between Long and Short sessions.

During the Long acclimatisation (a), participant's BAT was 'settled down' and after applying the cooling stimulus (b) there was a significant change in BAT activity. However, during the Short acclimatisation, there was not any visible difference between the acclimatisation (c) and the effect of the applied stimulus on BAT (d). BAT: Brown adipose tissue.

2.12.9 Conclusions

As it was hypothesised, external factors might affect BAT activation before participant's study session and thus, a Longer acclimatisation is needed to be applied. For this reason, in all of the studies of this thesis, a reasonable time of approximately 15 minutes of acclimatisation was chosen, by comparing the time series of the thermal data from all participants. It was seen that the participants had reached a plateau temperature over the first 15 minutes of acclimatisation. In this way, any BAT activation prior to study session masking the effects of the stimulus was prevented.

3 The effects of cool showers on brown adipose tissue function in healthy adult males

3.1 Introduction

BAT can be activated by environmental factors, such as exposure to cold (Muros, Green et al. 2019). This stimulation occurs by increased heat production following the induction of NST and activation of the UCP-1 (Cannon and Nedergaard 2004). In 2009, BAT was reported to be metabolically active during acute mild cold exposure (16°C) but not at thermoneutrality (22°C) in adults (van Marken Lichtenbelt, Vanhommerig et al. 2009). Further studies using PET/CT scanning, with a range of cooling stimuli, have established that cold exposure can increase the uptake of fluorodeoxyglucose (FDG) in BAT (Vijgen, Bouvy et al. 2011, Cypess, Chen et al. 2012, Vrieze, Schopman et al. 2012, van der Lans, Hoeks et al. 2013). However, in a prospective study, daily 1 hour exposure in cold temperatures for six weeks, revealed higher RMR with no detectable difference in BAT activity, using the technique of MRI (Romu, Vavruch et al. 2016).

Application of IRT in both adults and children, can quantify changes in heat produced from BAT after stimulation, including placing the participants' hand or feet in cool water, swimming or drinking ice-water (Lee, Ho et al. 2011, Symonds, Henderson et al. 2012, Robinson, Ojha et al. 2014, Robinson, Law et al. 2016,

Law, Chalmers et al. 2019, Muros-Molina, Vazquez Rocha et al. 2019, Muros, Green et al. 2019). Mild anticipatory stress is another trigger which not only results in BAT activation, measured by IRT, but also to high salivary cortisol levels (Robinson, Law et al. 2016). As summarised above and in Chapter 1, Section 1.4.4.3.1, although many studies have confirmed cold exposure as a trigger for BAT activation, a practical and routine application to promote this function in everyday life is still needed.

3.1.1 Hypothesis

Given that cold stimulation activates BAT, I hypothesised that BAT activity will be enhanced in healthy male adults after they were exposed to daily cool showers for a week. I also hypothesised that post the 7 days of cool exposure, a higher RMR will be seen. As the intervention used in this work was not expected to be stressful to the human body, I hypothesised that the salivary cortisol concentration would not be altered post the intervention.

3.2 Materials and methods

Approval was taken from the research ethics committee from the University of Nottingham (REC reference No: H15032016 CoR-BAT; Appendix II). This study took place in the facilities of the University of Nottingham, located in the Queens Medical Centre campus, Nottingham, UK. The conceptualisation and design of the study was developed by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds. The study protocol was written by me and Professor Helen Budge who contributed to any

corrections, and sent all of the documentation to obtain ethical and regulatory approvals. I performed the recruitment and obtained consent from the participants.

3.2.1 Participants

The process of recruitment can be found in Chapter 2, Section 2.2. The inclusion criteria were:

- male gender
- BMI between 18.5-24.9 kg/m^2
- age between 18-30 years.

Exclusion of the participant was made on case of any of the following characteristics:

- diabetes mellitus
- hypothyroidism
- hyperthyroidism
- history of CVDs, asthma or breathing problems
- excessive PA
- use of β-blockers.

3.2.2 Sample size

Based on previously published data using IRT, the response within each subject group should exhibit a normal distribution with standard deviation 0.212 (Symonds, Henderson et al. 2012). In such case, with a true difference in means of 0.3°C, at least 7 participants should be studied, in order to reject the null hypothesis with a probability of correctly rejecting the null hypothesis (power of the study) of 0.85 and significance level of 0.05 (Chapter 2, Section 2.1). I aimed to

recruit 13 participants calculating an attrition rate of 46%. The attrition rate was set that high, as the study was undertaken during autumn and winter and one of the adverse events (which did not happen) was that the participants due to the cold showers, might get ill and not be able to participate.

3.2.3 Study design

During the 2-week study period, each participant undertook two assessments and completed two food and exercise diaries on three consecutive days prior and during the cool showers (Chapter 2, Section 2.7). The intervention included daily showers at cool water temperature (18-20^{o}C), each lasting for about 3 minutes on 7 consecutive days, Figure 3.1. After the cool showers, the participant could take a warm shower measuring the water temperature and duration.

Before the first study visit, the eligibility of the participant was checked through a screening questionnaire (Chapter 2, Section 2.7) and after ensuring eligibility, an electronic copy of the PIS was sent at least 24 hours before he provided written informed consent (Chapter 2, Section 2.2). The participant was asked to avoid eating for at least 4 hours, avoid any drinks except water as well as smoking for at least 2 hours and abstain from any excessive exercise for 12 hours before the session.

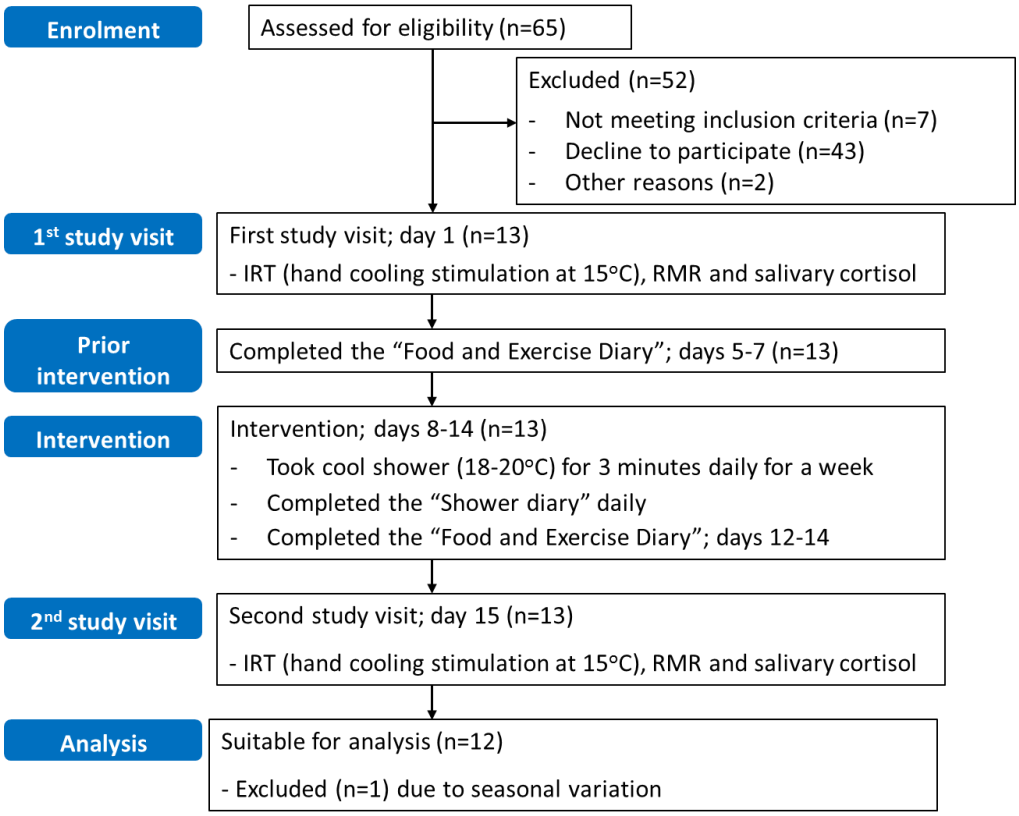


Figure 3.1 Study design of the cool shower intervention.

On days 1 and 15 thermal images were taken for each participant prior and post to the cool showers. During the 7 days of the intervention, the time and duration of the showers and a "Food and Exercise Diary" were recorded. IRT: Infrared Thermography, RMR: Resting metabolic rate.

On the 1st visit the participant changed to the study's clothes (Chapter 2, Section 2.9.3.3) and it was asked to drink 90 ml of room temperature water, as a preparatory step, before the collection of the salivary samples. Then, anthropometric and physiologic measurements were assessed, including height, weight, blood pressure and heart rate (Chapter 2, Section 2.3). A demographic questionnaire was also completed by the participant, including parameters such as ethnicity (Chapter 2, Section 2.7).

Two salivary samples were provided by the participant (Chapter 2, Section 2.4.1). Then, using indirect calorimetry (Chapter 2, Sections 2.6.1 and 2.6.2), the RMR of the participant was measured.

IRT was performed to depict the BAT activity of the SCR (Chapter 2, Section 2.9.3). For the purposes of the study, cool stimulation was induced by placing the participant's left arm in a cooling blanket Blanketrol II (Weiss Technik North America, Inc, Ohio, USA), set at 15^{o}C (Chapter 2, Section 2.9.3.6). Then, thermal images were obtained during baseline, stimulation and post-stimulation periods (Chapter 2, Section 2.9.3.7). During IRT, the skin temperature of the participant was measured using skin sensors (Chapter 2, Section 2.5.1) on the left side of the body.

In addition, the participant was asked to refrain from extreme PA during the 2 weeks of the study. At the end of the first session, each participant was provided with a thermometer to measure the water temperature of the cool showers (and of any warm shower taken right after the cool one) and detailed explanation, as well as the investigator's contact details were provided. Reminders in the form of texts or e-mails were sent to each participant throughout the whole study period in order to remind them to take the showers (CoR-BAT Participant Shower reminder e-mail and text v1.0 23/2/2016), complete the given diaries (CoR-BAT Participant food diary reminder e-mail and text v1.0 23/2/2016) and other information regarding the follow-up visit (CoR-BAT Participant Study Visit Reminder e-mail and text v1.0 23/2/2016). At the end of the session, a thank you card with a thermal image of the participant and a £20 Amazon voucher

were provided for the participation in all of the study visits. All of the sessions took place in the morning.

3.3 Laboratory procedures

Salivary samples were collected at both study visits and the samples were properly stored according to HTA regulations. All of the samples were analysed using the "Cortisol: expanded range, enzyme immune assay, kit research" (Catalogue Number: 1-3002-SAL, Salimetrics LLC, UK), designed to detect the human cortisol. A complete description of the methods used in this study are presented in Chapter 2, Section 2.4.

3.4 Data analysis

Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating that the data were normally distributed. Prior to the main analysis of the results, determination of the effect of the outside temperature in BAT was performed. One participant studied during summer season (first visit: 15/07/2016, environmental temperature 20^{o}C, second study visit: 02/08/2016, environmental temperature 23^{o}C), was excluded from all of the analysis of this Chapter, as seasonal variation can affect BAT (Au-Yong, Thorn et al. 2009). Therefore data of 12 participants were analysed for the needs of this study. Pearson's correlations were used to assess the correlation between the SCR and reference temperatures with the outside temperature. In case of relationship was found, multivariate analysis of variance (MANOVA) was

performed to identify whether the outside temperature had any effect on the SCR and reference temperatures.

Repeated measures analysis of variance ANOVA was used to identify differences in prior and post temperatures. Paired t-tests were performed to compare any differences of the same period, e.g. stimulation, prior and post the intervention (Chapter 2, Sections 2.5.2, 2.6.3, 2.10, and 2.11). Paired sample t-test was used for the analysis to identify any differences prior and post the intervention in the levels of the salivary cortisol.

3.4.1 Food and exercise diaries analysis

The food and exercise diaries were processed in the online food platform "Chronometer" (https://cronometer.com/), a database including all the UK food products. Macronutrients and estimated kilocalories (kcals) expended during the exercise, were used for the data analysis. The mean carbohydrate, protein, fat, total energy and total kcals deriving from any PA were calculated for each of the two food and exercise diaries of each participant and comparisons were made using paired t-test.

3.5 Results

3.5.1 Sample characteristics

Out of the 86 participants who expressed an interest in taking part in the study, 13 consented to take part and 12 were analysed. No participant withdrew during the sessions and there was no missing data. The majority of the participants' ethnicity was British (n=4), followed by Chinese (n=3), any other white ethnic background (n=3),

any other Asian background (n=1) and other mixed ethnicities (n=1). Participants' median age at the time of the assessment was 23.1±3.6 years; range 18.9-29.8 years.

3.5.2 Anthropometric data

Anthropometric measurements taken prior and post to the cool showers remained stable, Table 3.1.

Table 3.1 Anthropometric measurements of the participants prior, and post, the 7 day period of cool showers (n=12, mean±SD).

Measurements	Prior	Post
Weight (kg)	67.6±8.2	67.6±8.5
Height (m)	1.77±0.06	
BMI (kg/m^2)	21.52±2.04	21.51±2.51
Systolic (mmHg)	117.1±2.9	117.5±3.9
Diastolic (mmHg)	70.3±6.3	69.8±6.3
Heart Rate (p/min)	69.0±10.6	72.5±9.6
Tympanic Temperature (°C)	35.7±0.2	35.8±0.2

BMI: Body mass index

3.5.3 Room and ambient temperature

The room temperature was set at 22°C and did not change during the experimental procedures. The mean environmental temperature was 7.1±3.8°C on the first visit, and 6.5±3.8°C on the second visit (p=0.673), with individual values given in Table 3.2.

Table 3.2 Outside environmental temperatures in relation to the time of year throughout which each session was conducted in each participant of the study (n=12).

Participant	Date of study prior the intervention	Date of study post the intervention	Environmental temperature (°C)	
	Prior	Post	Prior	Post
1	19/10/2016	02/11/2016	15	11
2	08/11/2016	22/11/2016	6	8
3	20/10/2016	03/11/2016	10	9
4	09/11/2016	23/11/2016	4	7
5	14/11/2016	28/11/2016	10	3
6	15/11/2016	29/11/2016	11	6
7	21/11/2016	05/12/2016	8	-0.6
8	24/11/2016	08/12/2016	7	12
9	29/11/2016	13/12/2016	4	7
10	30/11/2016	14/12/2016	5	10
11	14/02/2017	28/02/2017	1.3	2
12	14/02/2017	28/02/2017	4	4

3.5.4 Correlations of supraclavicular and reference temperatures with the environmental temperature

The baseline SCR prior to the intervention (Figures 3.2a, b, c and d,) and the maximum reference temperature post intervention were inversely related to the outside temperature (Figures 3.3a, b, c and d). Though, outside temperature did not had any effect on either SCR or reference temperatures prior ($F=0.608$, $p= 0.842$) or post ($F=1.863$, $p= 0.519$) intervention.

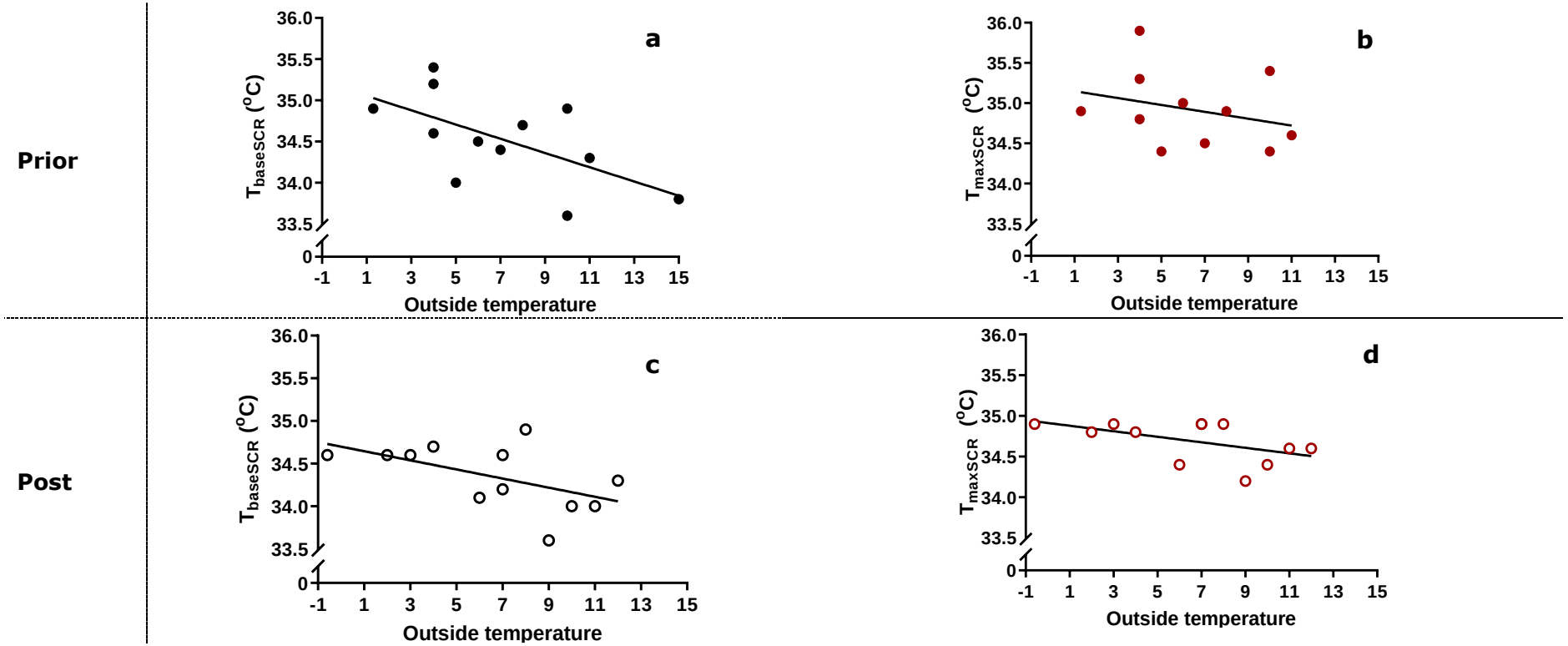


Figure 3.2a, b, c and d Correlations between the outside temperature with the baseline and maximum supraclavicular temperatures prior and post the intervention.

SCR temperatures (n=12) prior (closed circles) and post the intervention (open circles), including baseline (black) and maximum (red) temperatures. Only Figure a showed an inverse correlation between SCR and outside temperature (a: R^2=0.338, r=-0.581, p=0.037, b: R^2=0.256, r=-0.526, p=0.073, c: R^2=0.184, r=-0.541, p=0.070, d: R^2=0.438, r=-0.526, p=0.079). SCR: Supraclavicular, $T_{baseSCR}$: Baseline supraclavicular temperature, T_{maxSCR}: Maximum supraclavicular temperature.

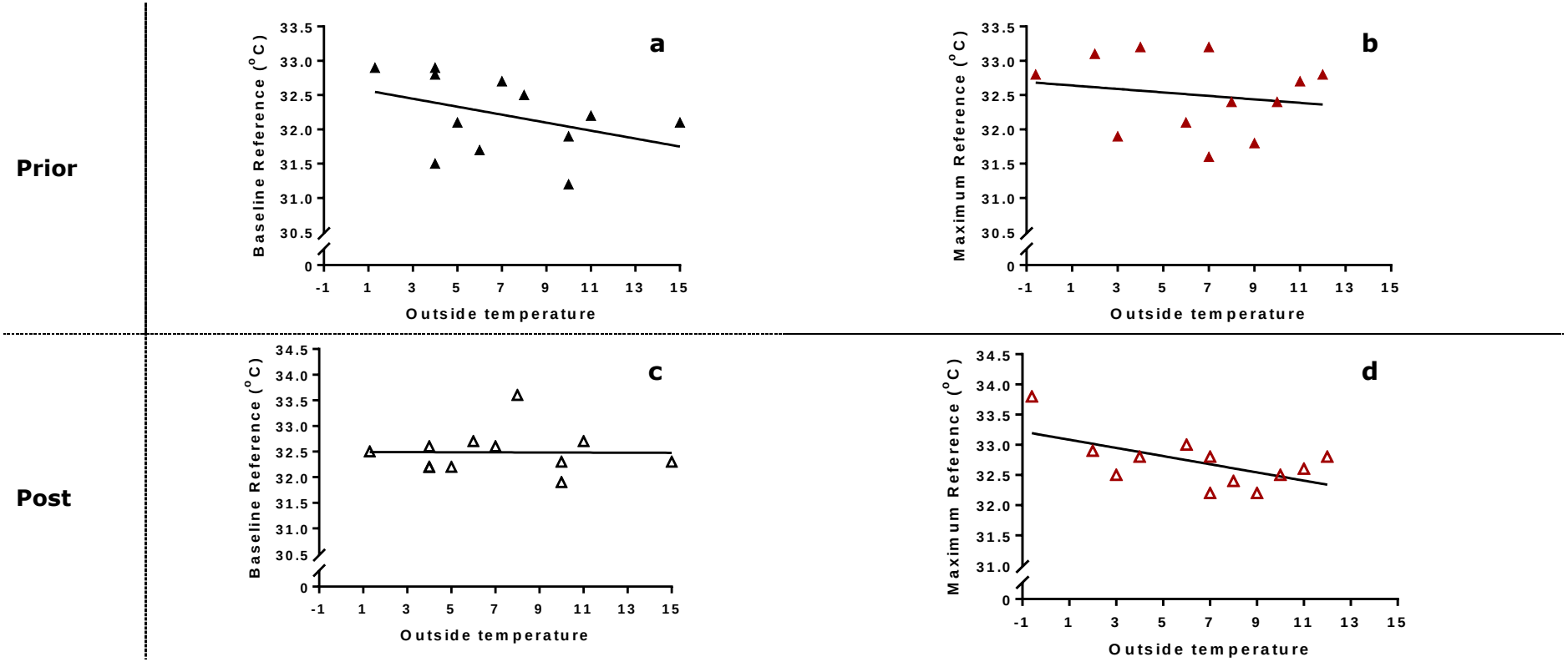


Figure 3.3a, b, c and d Correlations between the outside temperature with the baseline and maximum reference temperatures prior and post the intervention.

Reference temperatures (n=12) prior the intervention (closed triangles) and post the intervention (open triangles), including baseline (black colour) and maximum (red colour) temperatures. Only Figure d showed an inverse correlation between reference and outside temperature (a: R^2=0.228, r=-0.478, p=0.098; b: R^2=0.184, r=-0.429, p=0.142; c: R^2=0.0497, r=-0.223, p=0.464; d: R^2=0.360, r=-0.600, p=0.038).

3.5.5 Supraclavicular and reference temperatures

Differences between prior and post the 7 days of the cool showers in both SCR and reference temperatures were identified, Figure 3.4.

Specifically, the maximum SCR temperature, Figure 3.5, declined between the study visits.

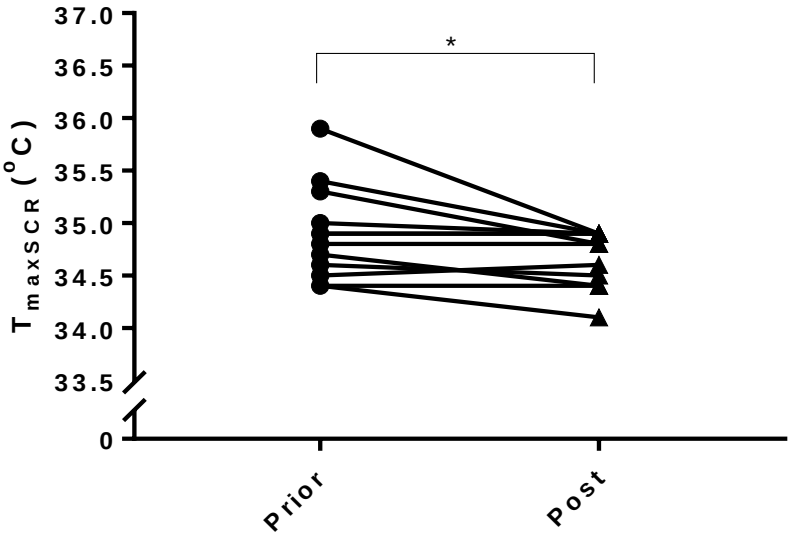


Figure 3.4 Effect of the cool showers at the maximum supraclavicular temperature prior and post 7 days the period of cool showers.

Closed circles: maximum SCR temperature prior to the intervention and closed triangles: maximum SCR temperature post the 7 day period of cool showers. SCR: Supraclavicular, T_{maxSCR}: Maximum supraclavicular temperature; *p=0.031, n=12.

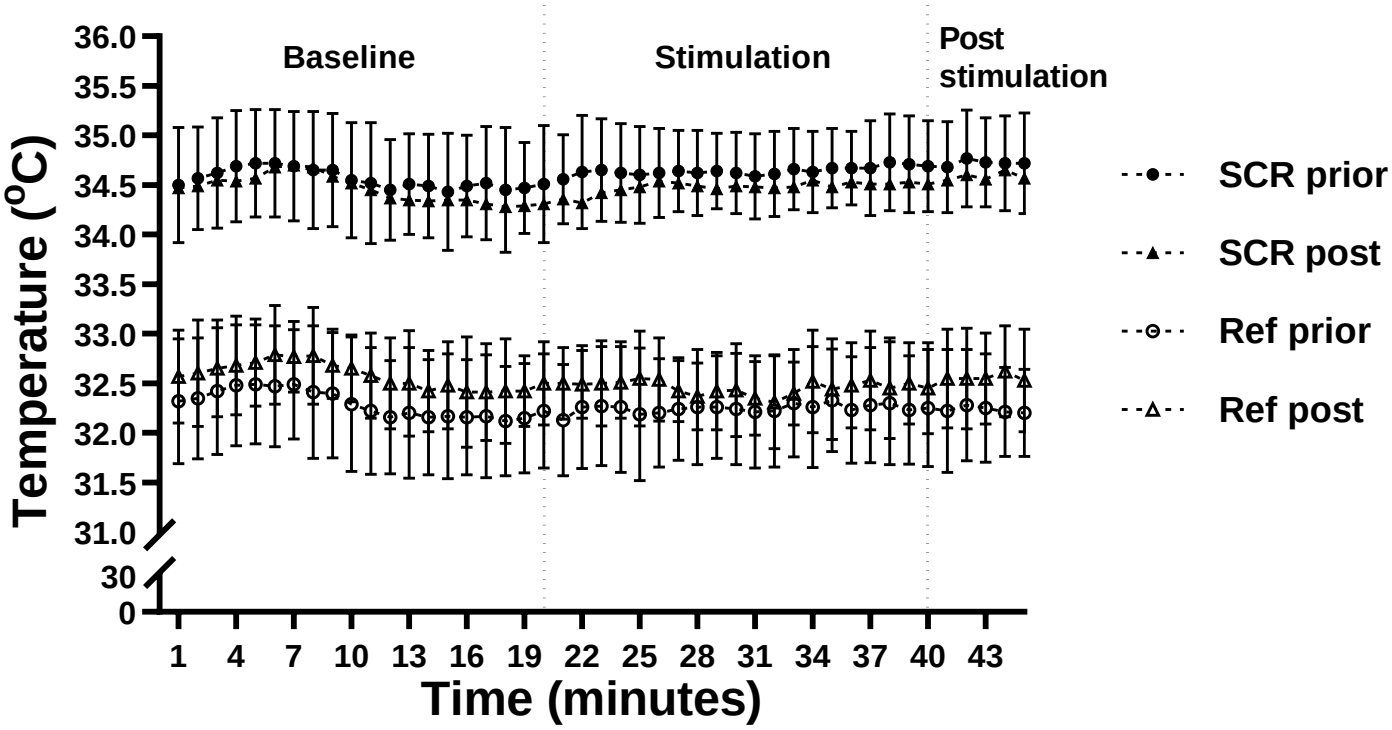


Figure 3.5 Supraclavicular and reference temperatures prior and post the 7 days of cool showers.

In this figure, the 3 periods of each visit are separated by a dotted line (n=12, mean±SD). Baseline and stimulation periods lasted for 20 minutes whereas post-stimulated period, without involving any stimulus, lasted for 5 minutes. SCR ($F=10.621$, $p=0.002$; prior: closed circles, post: closed triangles) and reference ($F=11.311$, $p= 0.002$; prior: open circles, post: open triangles) depict the temperatures in real time during the IRT. SCR: Supraclavicular, Ref: Reference, IRT: Infrared thermography.

The reference temperature, however, significantly increased post the 7 day period of cool showers, Figure 3.6.

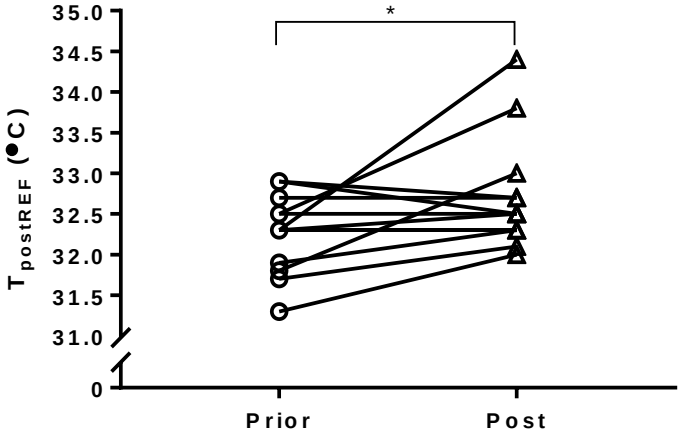


Figure 3.6 Effect of the cool showers on the reference temperature at the post-stimulation period.

Open circles: post-reference temperature prior the intervention and open triangles: post-reference temperature post the 7 day period of the cool showers. $T_{postREF}$: Post-stimulation reference temperature; *p=0.045, n=12.

3.5.6 Relative temperature

ΔT_{REL} was decreased by 0.5°C in both baseline and maximum phases, Table 3.3.

Table 3.3 Differences between the supraclavicular and reference temperatures of the baseline and maximum periods prior and post the 7 days of cool showers (n=12).

Measurement	Prior	Post
Baseline ($\Delta T_{baseREL}$)	2.3±0.4*	1.8±0.6*
Maximum (ΔT_{maxREL})	2.4±0.5†	1.9±0.4†

Similar superscripts denote statistical significance; *p=0.049, †p=0.027. $\Delta T_{baseREL}$: Baseline relative temperature, ΔT_{maxREL}: Maximum relative temperature.

3.5.7 Hand and clavicle mean skin temperatures

Clavicular skin temperature did not change between the study visits. However, in response to the cool stimulation, the contralateral hand skin temperature significantly changed during the session, without identifying any changes prior and post the intervention, Table 3.4.

Table 3.4 Clavicular and hand skin temperatures prior and post to the intervention, and among periods (n=12, mean±SD).

Period	Prior		Post	
	Clavicle	Hand	Clavicle	Hand
Baseline (°C)	31.2±0.7	31.4±2.0[a,b]	31.6±0.7	31.7±1.4[c,d]
Stimulation (°C)	31.2±0.7	31.0±1.7[a]	31.5±0.4	30.8±1.4[c,e]
Post-stimulation (°C)	31.2±0.6	30.1±2.0[b]	31.5±0.6	29.9±1.5[d,e]

Similar superscripts denote statistical significance; a: p=0.036, b: p=0.0002, c: p=0.004, d: p<0.0001, e: p=0.001.

3.5.8 Salivary cortisol response, resting metabolic rate, macronutrients, exercise and shower diaries

No alterations were seen in salivary cortisol concentrations between the two study visits (prior: 0.38±0.16mgdl^{-1} and post: 0.41±0.16mgdl^{-1}; p=0.584). There were also no differences in the RMR measurements (prior: 1550±153 kcal/day, post: 1549±147 kcal/day; p=0.866), macronutrients intake (Table 3.5) and exercise (prior: 542±454kcal, post: 520±421kcal; p=0.883).

There was no consistency in the time of day that the participants took their showers. The mean cool shower temperature the participants took was 18.9±1.7°C with duration of 3.05±0.13 minutes. Immediately after the cool shower, they took a shower at a normal temperature, 28.2±9.6°C, and duration of 6.72±5.40 minutes.

Table 3.5 Comparison of energy, carbohydrate, protein and fat consumption prior, and post, the 7 day period of cool showers (n=12, mean±SD).

Macronutrient consumption	Prior	Post
Energy (kcal)	2318±520	2374±516
Carbohydrate (gr)	266.2±64.3	248.4±83.1
Protein (gr)	95.3±35.1	107.3±41.6
Fat (gr)	87.2±25.7	97.3±22.2

Kcal: Kilocalories.

3.6 Discussion

This prospective study is the first to my knowledge to report a drop of maximum BAT temperature after cool stimulation post having daily cool showers for a week and controlling for the environmental temperature. Previous studies using PET/CT have shown higher BAT activation following the application of cold air (Vrieze, Schopman et al. 2012, Muzik, Mangner et al. 2013) or exposure to cold (Orava, Nuutila et al. 2011, Yoneshiro, Aita et al. 2011b) for either a short period, acute exposure e.g. 1–2 hours, or a longer period, chronic exposure, e.g. 10 days (van der Lans, Hoeks et al. 2013). However, none of the results seen in these studies are in line with the ones presented in this Chapter.

As earlier studies used acute cold exposure that could be one of the main reasons of the difference seen in the outcomes with my study. In some of the above studies, in order to achieve increased BAT activity, an ice block (Yoneshiro, Aita et al. 2011b) or cold water

(Orava, Nuutila et al. 2011) was used on top of the cooling exposure. Another reason that the results of the present study are not similar to the above studies could be that most of the interventions lasted about 2 hours, but the intervention used in this study was 3 minutes/day. Taking into account that, in a single session, BAT can be activated as long as some parameters, such as the environmental temperature, are controlled (Chen, Cypess et al. 2016). In the present study, all of the participants were at home when taking a shower so the environmental temperature could be set that high at the time of the shower, preventing BAT stimulation. For PET/CT studies, a panel has made some recommendations for human studies investigating BAT (Chen, Cypess et al. 2016). It suggests that the duration of any study should be a minimum of 60 minutes prior to injection of the radio-isotope and another 60 minutes after the injection. Therefore, as the cool exposure was for hours rather than minutes, there is a higher probability to show active BAT.

On the other hand, only one study using cold exposure without reporting shivering, daily for an hour for 6 weeks, did not reveal any variation in BAT activity (Romu, Vavruch et al. 2016). These results are in line with my results, however, the temperature of the cold exposure in the above study is not mentioned, thus the participants might have been exposed to higher temperatures e.g. 22^{o}C. Using IRT to assess maximum SCR in acute exposure after swimming in cold water (Law, Chalmers et al. 2019) and hand immersion in cool water (Muros-Molina, Vazquez Rocha et al. 2019) – using the same water temperature in my study - showed a rise in BAT deriving from the

stimulation. As the study of this Chapter was a chronic one where cool showers were taken on 7 consecutive days, the drop of maximum SCR could be attributed to the prolonged and repeated exposure to the cold and, therefore, adaptation of BAT was developed, as the duration and temperature of the cool showers remained stable though the intervention. This theory is further supported by a study that investigated the changes in body temperature, in which short exposure to 16^{o}C in nine males was accompanied by a significant decrease in core temperature (van Marken Lichtenbelt, Schrauwen et al. 2002). Notably, cool shower stimulation has not been previously reported.

IRT studies, performing acute exposure of cold in BAT (Law, Morris et al. 2018, Law, Chalmers et al. 2019), have showed that the relative temperature was significantly increased after BAT activation, whereas, in the present study, a decrease of 22% in the relative temperature between the two sessions was found, deriving from the lower activation of BAT after the showers. Using swimming for 250m in 7^{o}C, as BAT trigger (Law, Chalmers et al. 2019), revealed rise in the relative temperature, accompanied by a lower reference temperature. Thus, my findings differ from those previously observed. This may be attributed to the participants' body adaptation to the chronic effect of mild cold exposure due to the repeated activation of BAT over the course of a week. Thus, a greater cold trigger may be needed to further activate BAT. This is further supported by the increase in post-stimulation reference temperature and by the decrease in the maximum SCR temperature.

There are two likely explanations for the observed decrease mean hand temperature during, and after, having the hand inside the cooling blanket. Firstly, during the IRT technique used in this study, participants sat still to facilitate the robust collection of data. Despite wearing standardised clothing, and being in a temperature-controlled room, the participants may have started cooling down due to their lack of any PA. This theory is further supported by the drop in the mean skin temperature reported by another IRT study (Robinson, Law et al. 2016). Secondly, peripheral vasoconstriction, a thermoregulatory defence mechanism (Carlson and Marriott 1996), may have contributed to the observed decrease in the mean hand skin temperature. Vasoconstriction maintains the core temperature of the human body when cold is applied, resulting in a decrease of the peripheral skin temperature (Charkoudian 2010, Vosselman, Van der Lans et al. 2012, van der Lans, Hoeks et al. 2013, Maeda 2017). This also explains the reason why the mean clavicle skin temperature did not change during the cold stimulation in this work, as it is a central area.

Cortisol is a well-known stress biomarker (Lupien, Ouellet-Morin et al. 2006) linked with many physiological changes such as metabolism and fat distribution (Dallman, la Fleur et al. 2004). Increases in cortisol have been previously found to be associated with differences in BAT activity of adult females, without using any other stimulus such as cold stimulation (Robinson, Law et al. 2016). Thus, the measurement of the participants' cortisol was undertaken to assess whether the intervention caused chronic stress. Notably, no change

in salivary cortisol concentration was detected between the two study visits. Therefore, it is likely that the daily cool showers did not act as a stressor and that the BAT activation measurements were not affected by altered cortisol.

Previous studies in cold activation of BAT reported a small increase in RMR of approximately 13% (Cypess, Weiner et al. 2015), and in the EE (Yoneshiro, Aita et al. 2011b, Yoneshiro, Aita et al. 2013). However, others have reported an increase in total EE with significantly elevated BAT activation in the absence of a rise in the RMR (van der Lans, Hoeks et al. 2013). Studies using cool stimulation for longer periods presented a variety of results. In a 6-week study with a 1 hour daily cooling protocol, RMR was increased by 4% without any rise in BAT volume (Romu, Vavruch et al. 2016). In contrast, when a cooling protocol over 10 days (2 hours on the first day, 4 hours on the second day, and 6 hours per day for the remaining days) was implemented, no changes in the RMR were observed, despite an increase in BAT activation (van der Lans, Hoeks et al. 2013). The latter study is in line with outcomes of the RMR in the present work.

There was a non-significant trend for higher protein and lipid intake from the participants between prior, and post intervention in my study. Current evidence shows that a high-fat diet can minimise BAT glucose uptake when using the technique of PET/CT image (Williams and Kolodny 2008). Male rats, following a cafeteria diet (high in fat), show the same levels of thermogenesis as those exposed to cold temperatures (Rothwell and Stock 1980) showing also interscapular

hypertrophy (Young, Saville et al. 1982). The opposite results are seen in rats following a high protein diet, where the interscapular BAT weight was less, and after stimulation, with NE, there was no increase the interscapular temperature compared to the control group (Brito, Brito et al. 1992). The relationship between DIT and cold-induced thermogenesis (CIT) has been previously supported (Wijers, Saris et al. 2007, van Marken Lichtenbelt, Vanhommerig et al. 2009, Lee, Smith et al. 2014), but at the same time other studies have not observed such a relationship (Vosselman, Van der Lans et al. 2012, Schlögl, Piaggi et al. 2013, Peterson, Lecoultre et al. 2016). In line with the findings from the present study, participants in previous work who were exposed to mild cold for 2 hours consumed the same amounts of food during thermoneutrality and after exposure to a cold trigger (Langeveld, Tan et al. 2016). Thus, my results support that DIT does not effect in CIT.

Despite earlier work in cancer patients revealing an association between PA and BAT activation (Dinas, Nikaki et al. 2015), a more recent study on a larger young healthy population suggested that this association is not present in healthy subjects (Acosta, Martinez-Tellez et al. 2018). Although PA of participants did not change during the intervention to affect BAT activation in the present work, the PA behaviour of participants has not been previously assessed in a prospective study.

The main strength of this study is that the intervention was not performed in a laboratory setting, and its results are applicable to real-life situations. Even though the environmental temperature was

inversely related to the baseline SCR prior the intervention and maximum reference temperature post the 7 days of cool showers, the effects of the cool showers weren't affected by the outside temperature; this is the first study to assess the outside temperature in combination with the intervention's results. Additionally, because the technique of IRT was used in this work, a time series of BAT activity was explored throughout the session, rather than in a single moment as with PET/CT. Also, the sample size is of a standard size for this type of work and is sufficiently powered to detect any differences.

However, my study has some limitations. For example, the cool showers were performed for only 3 minutes per day, and it is possible that a shower water temperature set to 18–20°C was insufficient to further trigger BAT activation, as most of the studies have been performed temperatures ranging from 7°C-17°C (van Marken Lichtenbelt, Vanhommerig et al. 2009, Cypess, Chen et al. 2012, van der Lans, Hoeks et al. 2013, Law, Chalmers et al. 2019, Muros-Molina, Vazquez Rocha et al. 2019). Taking a warm shower, after the cool one might have stopped any further trigger of BAT activity and could have minimised the overall heat produced by each participant. Furthermore, this study was performed during the autumn/winter and, as BAT is affected by the outside temperature (Au-Yong, Thorn et al. 2009), different results might be seen in a similar study conducted during the summer. Another limitation is that only healthy, lean, young males were used in the present work. Therefore, the

findings might not be applied to patients with various health conditions or individuals who are obese, older, and/or female.

3.7 Conclusions

I investigated BAT activation via daily cool showers for 7 consecutive days in young healthy males. Both the maximum SCR and the relative temperatures were decreased after the cool shower intervention, and a higher temperature was observed in the non-BAT region, which acted as a reference point, during the post-stimulation period, Figure 3.7. Together, these findings suggest that a stronger stimulus (such as lower temperature and/or more minutes of cool shower) is needed to activate BAT. Therefore, a maximum BAT stimulation might have been achieved through the cool showers intervention, leading to a further body adaptation.

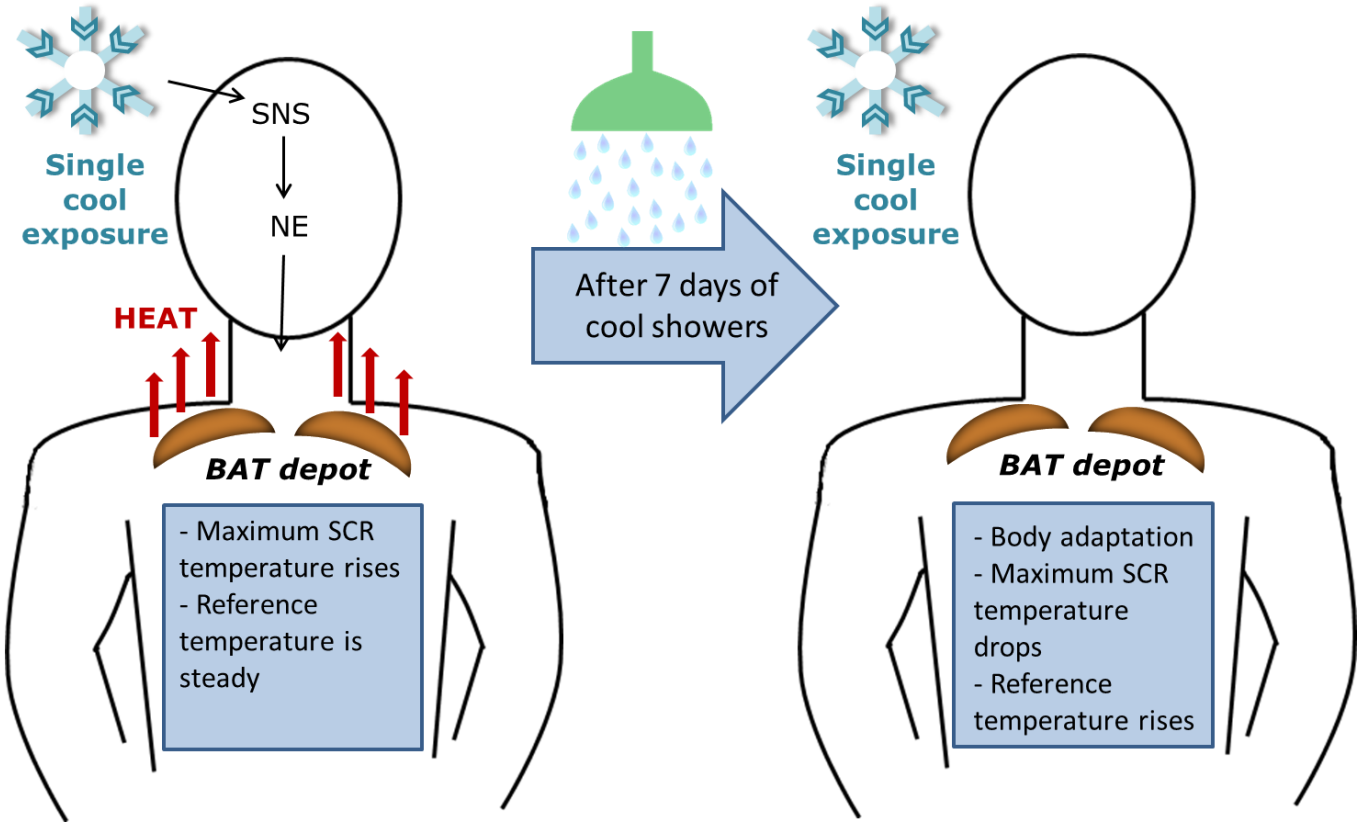


Figure 3.7 Summary diagram of the study.

On the left, SCR temperature rises after acute exposure to cold. On the right, chronic exposure to cool showers for a week, does not activate BAT, revealing further adaptation to mild cold. SNS: Sympathetic nervous system, NE: Norepinephrine, BAT: Brown adipose tissue, SCR: Supraclavicular.

4 Does cannabidiol affect the brown adipose tissue function as assessed by infrared thermography in healthy males?

4.1 Introduction

CBD was firstly isolated in 1940 from the plant cannabis sativa (cannabis) (Adams, Hunt et al. 1940). In 1963, CBD was characterised as a non-psychotropic phytocannabinoid (Mechoulam and Shvo 1963). The first botanical drug containing CBD, Sativex® by GW Pharmaceuticals Ltd, was approved and marketed in Spain in 2010 (GW-Pharmaceuticals 2011) and has now obtained regulatory authority approval in many European countries (GW-Pharmaceuticals 2013b). This oromucosal spray is indicated as a treatment for pain in adults with moderate to severe spasticity due to multiple sclerosis (Turri, Teatini et al. 2018, Markovà, Essner et al. 2019) and is currently used as a treatment in the NHS (NHS 2018). Nowadays, CBD is widely indicated for several medical applications, including breast cancer (McAllister, Murase et al. 2011), rapid eye movement, sleep behaviour disorder (Linares, Zuardi et al. 2019), schizophrenia (Leweke, Piomelli et al. 2012, McGuire, Robson et al. 2018) and epilepsy (French, Thiele et al. 2017, Devinsky, Patel et al. 2018).

In rodents, CBD administration can decrease body weight through cannabinoid type 2 (CB_2) receptors, suggesting that these receptors could contribute to the weight regulation (Ignatowska-Jankowska, Jankowski et al. 2011). Also in vitro and in vivo, CBD can reduce the

intracellular lipid by inhibiting lipogenesis and subsequently could enhance mitochondrial activity (Silvestri, Paris et al. 2015). It is currently not known whether CBD can impact on BAT , although this has been suggested from in vitro studies (Parray and Yun 2016). In latter study, it was demonstrated that 10μM of CBD in 3T3 fat cell line (3-day transfer inoculum 3×10^5 cells) stimulated the expression of brown genes and UCP-1. It is possible that the therapeutic use of CBD could prevent obesity, promoting thermogenesis in BAT. No previous studies have been conducted to date in humans, with respect to CBD and BAT activation, and this is the first study to determine whether CBD can affect BAT function in healthy adults as assessed using IRT.

4.1.1 Hypothesis

As CBD has been linked with BAT activation pathways, my primary hypothesis was that BAT activity will be enhanced by both acute and chronic exposure after 7 days of ingestion of CBD tablets. Given that BMI has a negative relation with BAT activity (Saito, Okamatsu-Ogura et al. 2009, van Marken Lichtenbelt, Vanhommerig et al. 2009, Vijgen, Bouvy et al. 2011, Muros-Molina, Vazquez Rocha et al. 2019), I also hypothesised that OW participants, who have less BAT activity, would be less responsive.

4.2 Materials and methods

The study took place in one of the clinical assessment rooms at the Division of Medical Sciences and Graduate Entry Medicine and Health, University of Nottingham, Royal Derby Hospital, Derby, UK. For this study, approval was received from the research ethics committee

from the University of Nottingham (Ethics Reference No: E14112016; Appendix III). After written informed consent, the procedures were performed according to the approved protocol. The data protection of the participants' information adhered to as required by the Data Protection Act 1998.

It was part of study designed to assess the effects of CBD on the human cardiovascular system. The conceptualisation and design of this sub-study was developed by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds. The study protocol was written by Dr Timothy England, Dr Saoirse O'Sullivan and Mr Salahaden Sultan and the sub-study details by me and Professor Helen Budge who contributed to any corrections. The collaborators obtained ethical approval. In collaboration with Mr Salahaden Sultan, we performed the recruitment and he obtained consent from the participants. I performed all of the study data collection.

4.2.1 Participants

The recruitment was as described in Chapter 2, Section 2.2. The inclusion criteria were:

- healthy male (e.g. no acute or chronic illness)
- aged 18-40 years
- no underlying cardiovascular or other medical conditions
- not exposed to cannabis within the last month.

Volunteers were not eligible to take part in the study if they had one more of the following characteristics:

- female; women were excluded due to the potential gender differences in response to CBD, as BAT is more commonly visible in females than males and it can be potential confounding effect of the menstrual cycle (Cypess, Lehman et al. 2009, Wang, Zhang et al. 2015, Muros-Molina, Vazquez Rocha et al. 2019)
- patient with cardiovascular disease or any other disease
- receiving any type of medication
- older than 40 years
- recent exposure to cannabis (within the last month), however addiction was not assessed.

4.2.2 Sample size

Based on previously published data using this thermal imaging, the response within each subject group should exhibit a normal distribution with standard deviation 0.212 (Symonds, Henderson et al. 2012) and with a difference in population means of 0.3°C, 10 experimental participants and 10 control participants each, would give 85% power to detect the difference with an significance level of 5% for a two-tailed t-test (Chapter 2, Section 2.1). As this was a sub-study, the main study aimed to recruit 13 participants in each group; a sample size that is acceptable for the present work. Thus, 13 participants were recruited in each group; CBD and placebo.

4.2.3 Study design

This is a randomised control trial (RCT) regarding the effect of CBD on BAT. Each participant underwent a baseline IRT measurement while having his right hand in cold water during his first visit. At the

second visit IRT was performed before and after administration of a single oral dose of 3 tablets of either placebo (composition of each capsule: 95% microcrystalline cellulose and 5% silica), control group, or 582mg of CBD (composition of each capsule: 38% CBD, 57% microcrystalline cellulose and 6% silica), study medication group. Each participant took the tablets, either placebo or CBD, three per day, for the next 5 days. Then, he came back for a third visit and ingested the final 3 tablets during the study session.

The dose chosen was based on a study published from the investigators (Jadoon, Tan et al. 2017). The duration of the study was designed for the needs of the cardiovascular outcomes but also covered any potential effect of CBD in BAT during chronic exposure. The placebo and CBD tablets were provided for free by Phivida Neutrafuels (Lot number 0717PV 0101. There was not reported any difference in the taste or appearance from the participants. The randomisation sequence was performed using computerised random number generation by Dr Timothy England.

4.2.4 Study assessment

The PIS was sent to the eligible volunteers at least 24 hours before providing written informed consent on the first visit (Chapter 2, Section 2.2). Each eligible participant was asked to fast for at least 6 hours, not to receive any vitamin supplementation for 72 hours, avoid any physical exercise, medication, caffeine, alcohol and cigarette and e-cigarette smoking for at least 24 hours before each visit. During the eight days of the study period, 3 visits were conducted, as

summarised in Figure 4.1. All of the study sessions started between 8:00 – 10:00 a.m.

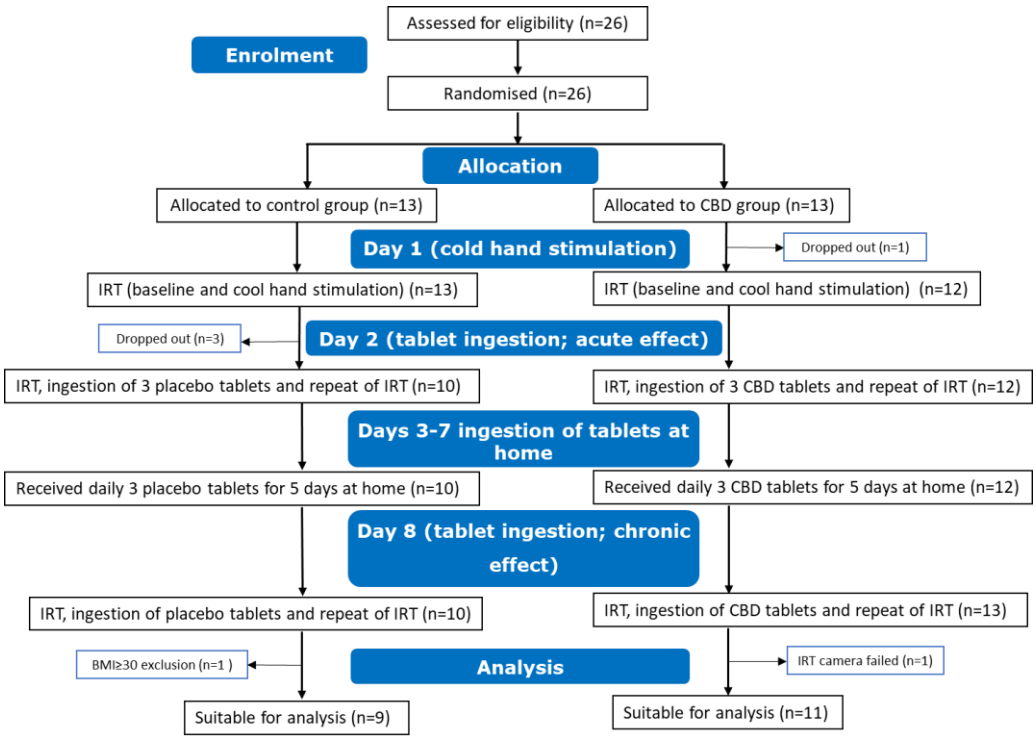


Figure 4.1 CONSORT 2010 flow diagram of the study.

The participants attend three study visits after ensuring their eligibility. Three CBD or placebo tablets were ingested for a 7 day period. CBD: Cannabidiol, IRT: Infrared thermography, BMI: Body mass index.

After ensuring that the participant was eligible to take part in the study, at the first visit, on Day 1, informed written consent was taken. Anthropometric measurements were assessed, including height and weight (Chapter 2, Sections 2.3.2 and 2.3.3). Supraclavicular BAT was measured using IRT (Chapter 2, Section 2.9). For the purposes of this study, cool water was used as a stimulant while the participants had already the hand in a bucket (water temperature 15°C, Chapter 2, Section 2.9.3.6). During the IRT, the mean skin temperature of the participant was measured using skin sensors for 7 different body

locations; on the head, antecubital fossa, hand, foot, shin, thigh and paravertebral (Chapter 2, Section 2.5.1).

At the second session, Day 2, ongoing consent was verbally confirmed and anthropometric measurements were taken. Then, for 15 minutes, the participant was asked to sit straight in a reclining bed with a pillow under his head while IRT was performed precisely with the same way as in the previous session, without any cooling stimulation, while skin temperature was measured with skin sensors. After that, the participant was asked to orally take 3 capsules of either placebo or CBD in a blinded fashion (neither the participant nor the assessor knew which treatment was provided) and then remained in the bed for another 80 minutes. During this time, the participant was allowed to use his phone, read or take a rest, though PA was not allowed. Afterwards, a further series of thermal images were taken for another 15 minutes. At the end of the session, a meal was offered and a follow-up sheet was given to be completed (Chapter 2, Section 2.7). The participant was asked to orally take 3 tablets daily at the same time of the day for the following 5 days and complete the follow-up sheet while being at home.

On the third study visit, Day 8, the follow-up sheet was checked to make sure that the correct doses of the tablets were received from the participant. Then, the same protocol used in the second study visit was followed, starting with verbal confirmation of the ongoing consent and the anthropometric measurements. The tolerance of CBD and placebo was assisted with a diary provided including any side effects the participants may have experienced. At the end of the

session, a meal and a £30 Amazon voucher were provided for participation in all of the three visits.

4.3 Data analysis

Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating normal distribution. Out of the 26 eligible participants interested in participating in the study, 25 consented, of which 3 subsequently dropped out. In total, data collection was made for 22 participants, with missing data from one of the participants due to failure of the IRT camera. From the remaining 21 participants, of the analysis, 1 participant was excluded due to being obese (BMI≥30), Figure 4.1. There was a higher number of OW participants taking part than expected, so they were separated into different groups for subsequent analysis. However, only one was actually obese and as BAT is reduced with obesity (van Marken Lichtenbelt, Vanhommerig et al. 2009) he was excluded from the analysis.

Assessment of the outside temperature with the SCR and reference temperatures was made with Pearson's correlations. Associations between the BMI and SCR temperature were analysed using Pearson's correlations. Repeated measures ANOVA was used to identify any difference between the NW and OW SCR and reference temperatures during the mild cold stimulation.

For the needs of the second part of analysis, the participants were divided into 4 groups; 1. Control NW (n=4), 2. Control OW (n=4), 3. CBD NW (n=7) and 4. CBD OW (n=5). Repeated measures ANOVA

was performed to measure the difference between the CBD and placebo in acute and chronic periods in NW and OW participants. The skin temperature was analysed as described in Chapter 2, Section 2.5.2.

4.4 Results

4.4.1 Anthropometric data and side effects

Anthropometric measurements for NW and OW participants are seen in Table 4.1. The mean weight of the participants in both groups, did not alter between the visits.

Table 4.1 Anthropometric measurements of the normal weight and overweight participants on the first day of assessment (mean±SD).

Measurement	NW CBD (n=4)	NW Control (n=7)	OW CBD (n=4)	OW Control (n=5)
Age (years)	24.5±3.6	29.1±5.8	24.1±5.4	23.1±2.6
BMI (kg/m^2)	22.3±1.3	23.4±0.8	26.9±1.2	27.1±1.1

NW: Normal weight, OW: Overweight, CBD: Cannabidiol, BMI: Body mass index.

Post-CBD administration, side effects reported included lack of appetite on day 4 (n=1), headache on day 3 (n=1), insomnia on days 2 and 3 (n=1), hyperactivity on days 2 and 3 (n=1), and dysuria on days 5 and 6 (n=1). Following placebo administration, participants reported migraine on day 4 (n=1) and light-headedness on day 6 (n=1)

4.4.2 Room and ambient temperature

The room temperature was maintained at 22°C and the mean environmental temperature did not significantly change (Day 1: 5.7±4.6°C, Day 2: 5.1±5.1°C, Day 3: 5.9±4.3°C; p=0.516), Table 4.2.

Table 4.2 Outside environmental temperatures in relation to the time of year throughout which each session was conducted in each participant of the CBD study (n=20).

	Day 1	Day 2	Day 8	Environmental temperature (°C)		
				Day 1	Day 2	Day 8
1	19/09/2017	22/09/2017	28/09/2017	7	4	15
2	04/10/2017	06/10/2017	12/10/2017	10	10	11
3	27/09/2017	11/10/2017	17/10/2017	13	13	11
4	18/10/2017	10/11/2017	16/11/2017	11	9	10
5	09/10/2017	22/11/2017	28/11/2017	11	13	5
6	13/10/2017	03/11/2017	09/11/2017	14	11	9
7	19/10/2017	01/11/2017	07/11/2017	11	9	10
8	17/11/2017	12/12/2017	18/12/2017	1	-1	2.4
9	05/12/2017	06/12/2017	12/12/2017	0	9	0
10	05/12/2017	07/12/2017	13/12/2017	3	2	3
11	05/12/2017	08/12/2017	14/12/2017	3	2	2.3
12	26/02/2018	27/02/2018	05/03/2018	-0.7	-2	1
13	19/01/2018	25/01/2018	31/1/2018	3	5	6
14	31/01/2018	20/03/2018	26/03/2018	6	2.9	4
15	05/02/2018	07/02/2018	13/02/2018	1	-2	4
16	14/02/2018	16/02/2018	22/02/2018	0	1	1
17	21/02/2018	09/03/2018	15/03/2018	5	1	7
18	08/02/2018	23/02/2018	01/03/2018	2	-2	0
19	16/03/2018	18/04/2018	24/04/2018	7	10	9
20	16/03/2018	25/04/2018	01/05/2018	7	8	9

CBD: Cannabidiol.

4.4.2.1 Correlations of supraclavicular and reference temperatures with the environmental temperature

There were not any correlations between the environmental and SCR or reference temperatures, Table 4.3.

Table 4.3 Correlations of the environmental temperature with the supraclavicular or reference temperatures in normal weight and overweight participants.

Group	Intervention	Baseline SCR	Maximum SCR	Baseline reference	Maximum reference
NW	**Hand cooling stimulus (n=11)**	R^2=0.190, r=-0.436, p=0.179	R^2=0.290, r=-0.539, p=0.087	R^2=0.003, r=0.060, p=0.859	R^2=0.001, r=-0.031, p=0.925
OW	**Hand cooling stimulus (n=9)**	R^2=0.107, r=-0.328, p=0.388	R^2=0.398, r=-0.631, p=0.068	R^2=0.136, r=-0.369, p=0.327	R^2=0.196, r=-0.443, p=0.232
NW Control	**Acute (n=7)**	R^2=0.309, r=0.556, p=0.444	R^2=0.571, r=0.755, p=0.244	R^2=0.353, r=0.594, p=0.405	R^2=0.094, r=0.307, p=0.692
NW CBD	**Acute (n=4)**	R^2=0.308, r=-0.555, p=0.195	R^2=0.383, r=-0.619, p=0.132	R^2=0.0004, r=-0.020, p=0.964	R^2=0.0005, r=0.023, p=0.953
OW Control	**Acute (n=5)**	R^2=0.060, r=-0.245, p=0.754	R^2=0.552, r=-0.743, p=0.256	R^2=0.169, r=0.412, p=0.587	R^2=0.390, r=-0.625, p=0.375
OW CBD	**Acute (n=4)**	R^2=0.317, r=0.563, p=0.322	R^2=0.680, r=0.824, p=0.085	R^2=0.449, r=0.670, p=0.215	R^2=0.615, r=0.784, p=0.116
NW Control	**Chronic (n=7)**	R^2=0.008, r=0.094, p=0.905	R^2=0.078, r=0.280, p=0.719	R^2=0.322, r=0.567, p=0.432	R^2=0.449, r=0.670, p=0.329
NW CBD	**Chronic (n=4)**	R^2=0.068, r=-0.261, p=0.571	R^2=0.158, r=-0.397, p=0.377	R^2=0.012, r=0.110, p=0.813	R^2=0.026, r=0.161, p=0.729
OW Control	**Chronic (n=5)**	R^2=0.073, r=-0.270, p=0.729	R^2=0.007, r=0.084, p=0.915	R^2=0.033, r=0.183, p=0.816	R^2=0.337, r=-0.580, p=0.304
OW CBD	**Chronic (n=4)**	R^2=0.020, r=0.144, p=0.816	R^2=0.552, r=-0.743, p=0.149	R^2=0.009, r=-0.097, p=0.875	R^2=0.372, r=-0.610, p=0.274

NW: Normal weight, OW: Overweight, SCR: Supraclavicular; CBD: Cannabidiol.

4.4.3 Hand cooling effect and body mass index during hand cooling stimulation

No correlations were found between BMI and the baseline (R^2=4.593^{e-005}, r=0.006, p=0.977) or stimulated SCR temperatures (R^2=0.002, r=0.049, p=0.835). The same results were observed with the reference temperature (Baseline: R^2=0.0433, r=0.1208, p=0.378; Maximum: R^2=0.047, r=0.217, p=0.356).

No difference was found between the NW and OW groups in either SCR or reference temperatures, Figure 4.2.

4.4.3.1 Skin temperature

There was not any change in the mean skin temperature between NW and OW participants as measured at baseline (NW: 30.8±0.6°C, OW: 30.6±1.1°C; p=0.531) or stimulation (NW: 30.7±0.6°C, OW: 30.4±1.0°C; p=0.431) periods.

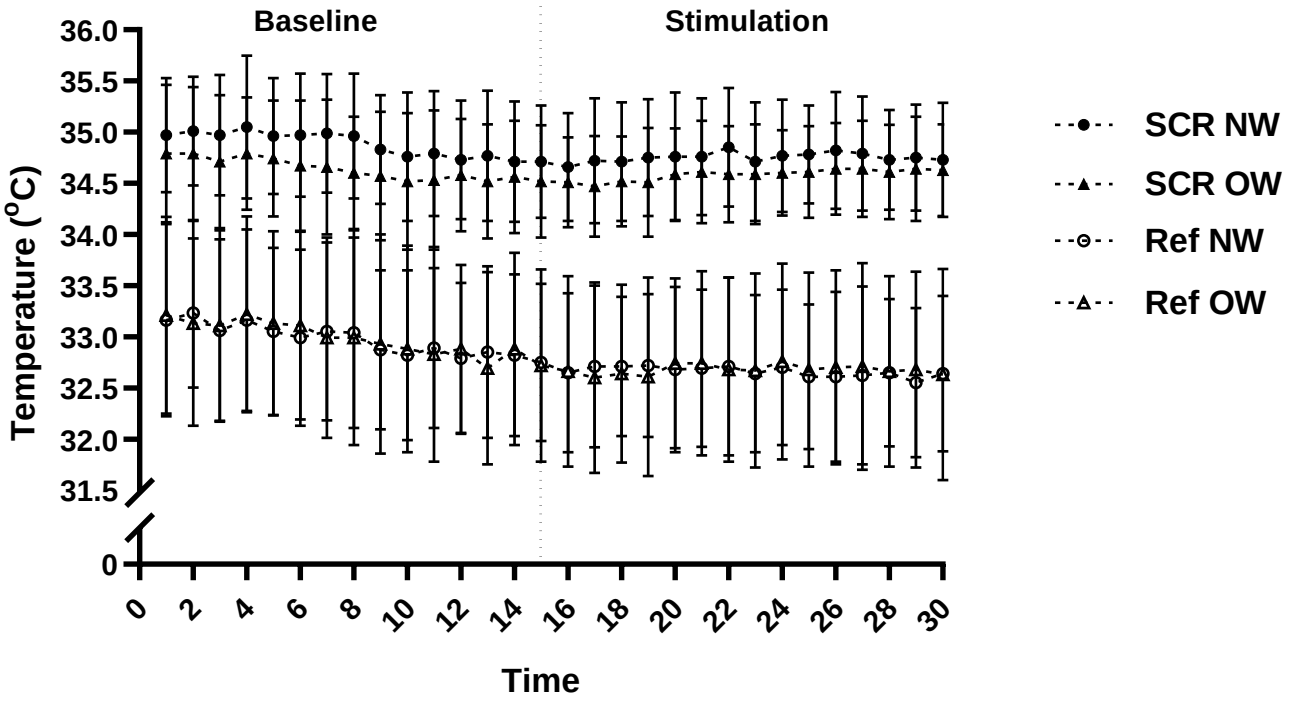


Figure 4.2 Supraclavicular and reference temperatures between normal weight and overweight participants.

The baseline and stimulation periods are separated from a dotted line lasting 15 minutes each (mean±SD). There is not any difference between the SCR temperatures (F=0.609, p= 0.445) in NW (closed circles; n=11) and OW (closed triangles; n=9) males or the reference temperatures (F=0.688, p=0.418; NW: open circles, OW: open triangles).
SCR: Supraclavicular, NW: normal weight, OW: Overweight, Ref: Reference.

4.4.4 Acute effect of the cannabidiol in the supraclavicular, reference and relative temperatures

There was not any difference found between NW placebo and CBD participants in SCR and reference temperatures, Figure 4.3. The same results were found for the OW participants after acute ingestion of placebo or CBD, Figure 4.4. Relative temperature did not alter between the groups, Table 4.4.

Table 4.4 Relative temperature in baseline and maximum periods in acute placebo or cannabidiol exposure in normal weight and overweight participants (mean±SD).

Measurement	**NW Control** (n=4)	**NW CBD** (n=7)	**OW Control** (n=4)	**OW CBD** (n=5)
Baseline ($\Delta T_{baseREL}$)	1.8±0.6	1.3±0.8	1.9±0.7	2.0±0.7
Maximum (ΔT_{maxREL})	1.4±1.0	1.6±0.6	2.3±0.2	2.1±0.6

NW: Normal weight, OW: Overweight, CBD: Cannabidiol.

4.4.4.1 Skin temperature

No change was observed in the mean baseline and stimulated skin temperature between the CBD and control participants for both BMI groups, Table 4.5.

Table 4.5 Mean skin temperatures during baseline and after ingestion of placebo or CBD in normal weight and overweight participants (mean±SD).

Period	**NW Control** (n=4)	**NW CBD** (n=7)	**OW Control** (n=4)	**OW CBD** (n=5)
Baseline (°C)	31.0±0.7	31.8±0.7	30.5±1.3	30.7±1.5
Stimulation (°C)	31.2±1.0	31.5±0.9	29.8±0.9	30.5±1.5

NW: Normal weight, OW: Overweight, CBD: Cannabidiol.

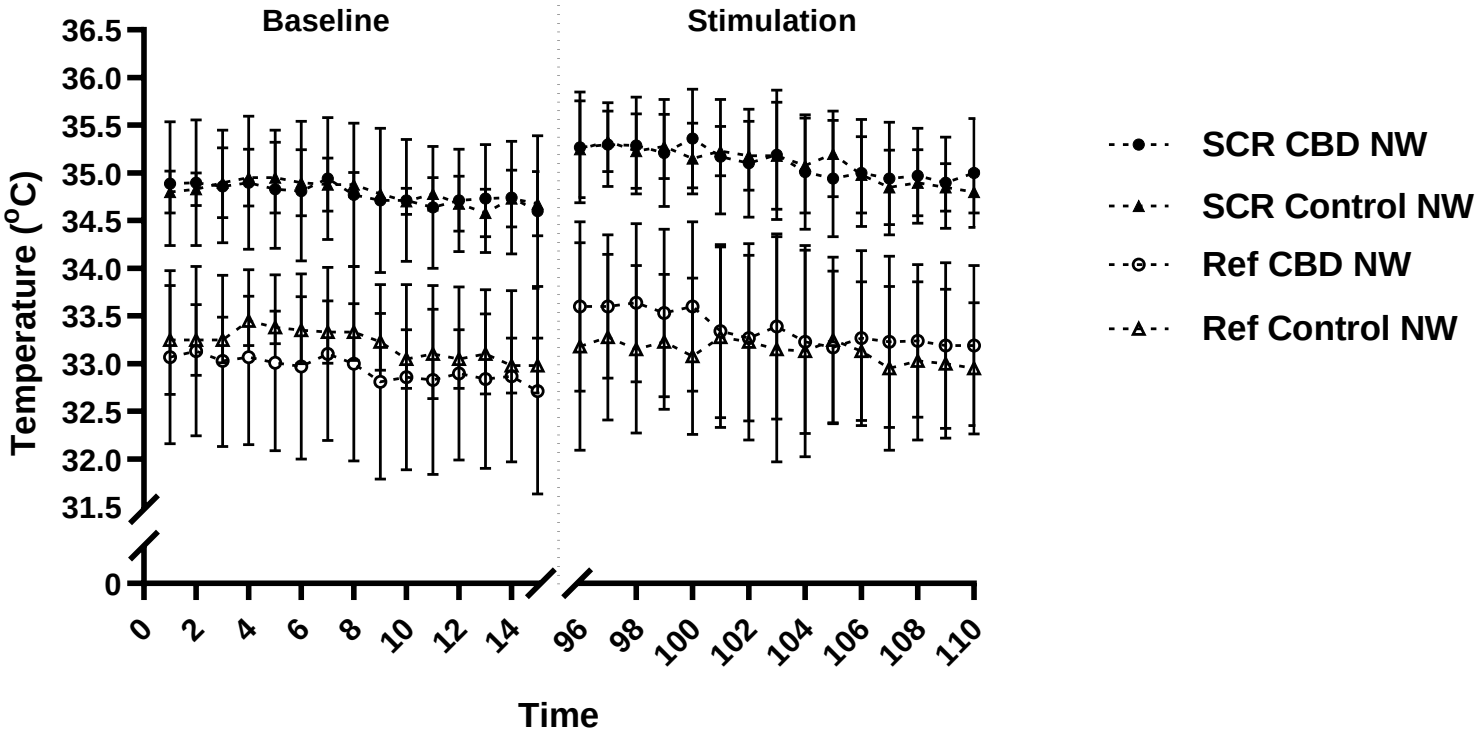


Figure 4.3 Acute effect of cannabidiol in the supraclavicular and reference temperatures in normal weight participants.

The two periods are separated with a dotted line. At the end of the baseline, the three tablets were ingested from the participant and after 80 minutes, IRT measurements were performed again for another 15 minutes (mean±SD). There were not any differences in SCR ($F=0.147$, $p=0.711$) or reference temperatures ($F=1.537$, $p=0.246$) between CBD (circles; n=7) and control (triangles; n=4) participants. SCR: Supraclavicular, CBD: Cannabidiol, Ref: Reference, IRT: Infrared thermography.

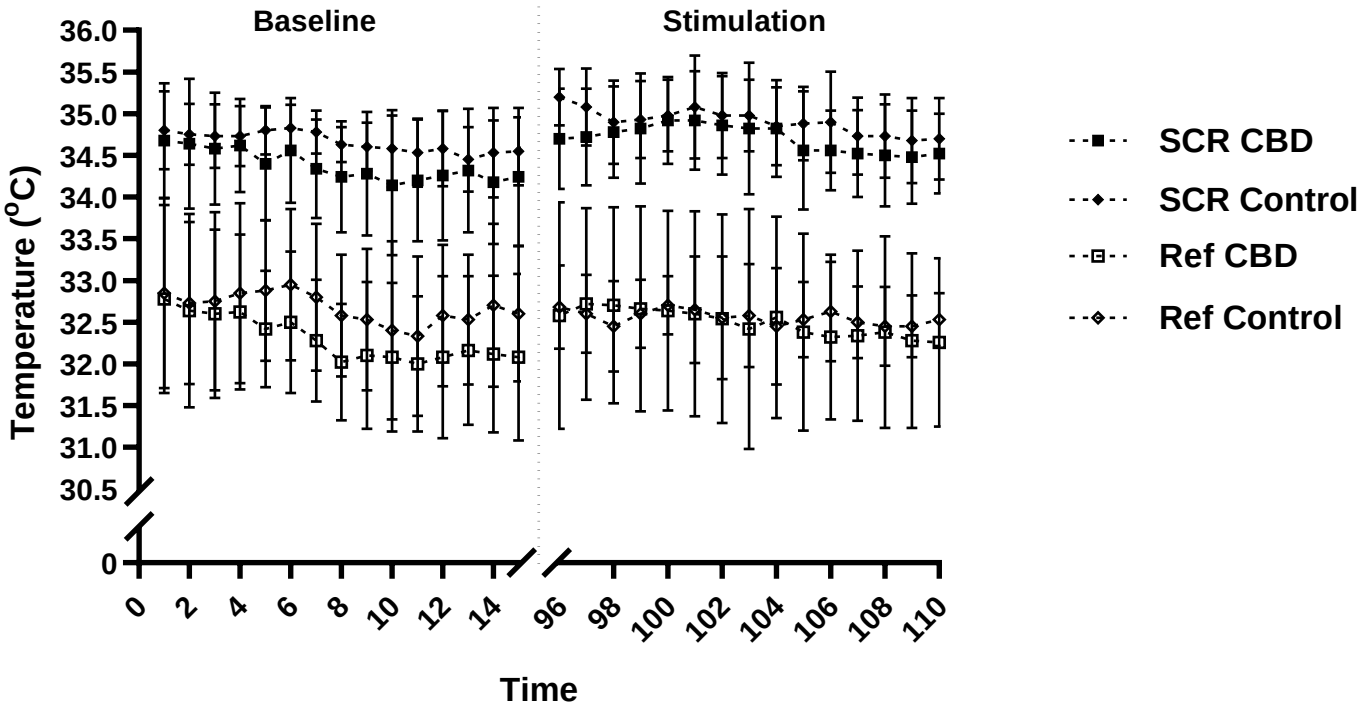


Figure 4.4 Acute effect of cannabidiol in the supraclavicular and reference temperatures in overweight participants.

The dotted line separates the baseline with the stimulation period. At the end of the baseline, the three tablets were ingested and after 80 minutes, IRT measurements were performed for another 15 minutes (mean±SD). There were not any differences in SCR ($F=1.775$, $p=0.225$) or reference ($F=0.934$, $p=0.366$) temperatures between overweight CBD (square; n=5) and control (rhombus; n=4) participants. SCR: Supraclavicular, CBD: Cannabidiol, Ref: Reference, IRT: Infrared thermography.

4.4.5 Chronic effect of the cannabidiol in supraclavicular, reference and relative temperatures

After daily ingestion of the tablets for 7 days, there was not any difference found between the two groups for both SCR and reference temperatures in NW participants, Figure 4.5. The same outcomes were seen in OW participants, Figure 4.6. Relative temperature did not alter between the study groups, Table 4.6.

Table 4.6 Relative temperature in baseline and maximum periods in chronic placebo or cannabidiol exposure in normal weight and overweight participants (mean±SD).

Measurement	NW Control (n=4)	NW CBD (n=7)	OW Control (n=4)	OW CBD (n=5)
Baseline ($\Delta T_{baseREL}$)	1.6±0.4	1.9±0.4	1.6±0.7	1.6±0.5
Maximum (ΔT_{maxREL})	1.7±0.4	1.9±0.4	1.7±0.4	1.9±0.6

NW: Normal weight, OW: Overweight, CBD: Cannabidiol.

4.4.5.1 Skin temperature

There was not any difference in the mean baseline or stimulated skin temperature between the CBD and control groups for both of the BMI groups, Table 4.7.

Table 4.7 Mean skin temperatures during baseline and after ingestion of placebo or CBD in normal weight and overweight participants (mean±SD).

Period	NW Control	NW CBD	OW Control	OW CBD
Baseline (°C)	31.6±1.0	31.7±0.8	30.8±1.2	31.6±1.3
Stimulation (°C)	31.3±1.1	31.2±0.7	30.6±0.8	30.1±0.9

NW: Normal weight, OW: Overweight, CBD: Cannabidiol.

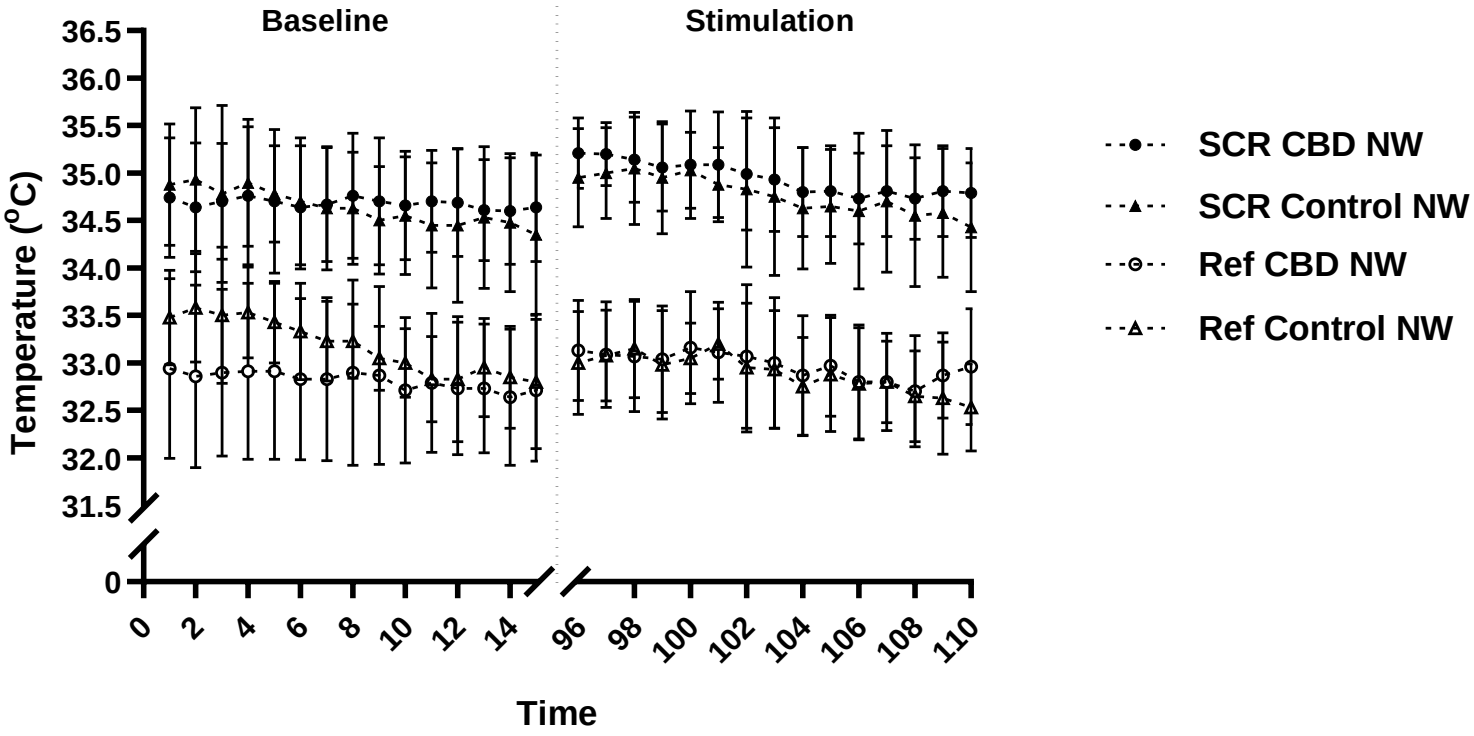


Figure 4.5 Chronic effect of cannabidiol in the supraclavicular and reference temperatures in normal weight participants.

The dotted line separates the baseline with the stimulation period. At the end of the baseline, the three tablets were ingested from the participant and after 80 minutes, IRT measurements were performed again for another 15 minutes (mean±SD). There were not any differences in SCR ($F=0.108$, $p=0.750$) or reference ($F=2.116$, $p=0.180$) temperatures between CBD (circles; n=7) and control (triangles; n=4) participants. SCR: Supraclavicular, CBD: Cannabidiol, Ref: Reference, IRT: Infrared thermography.

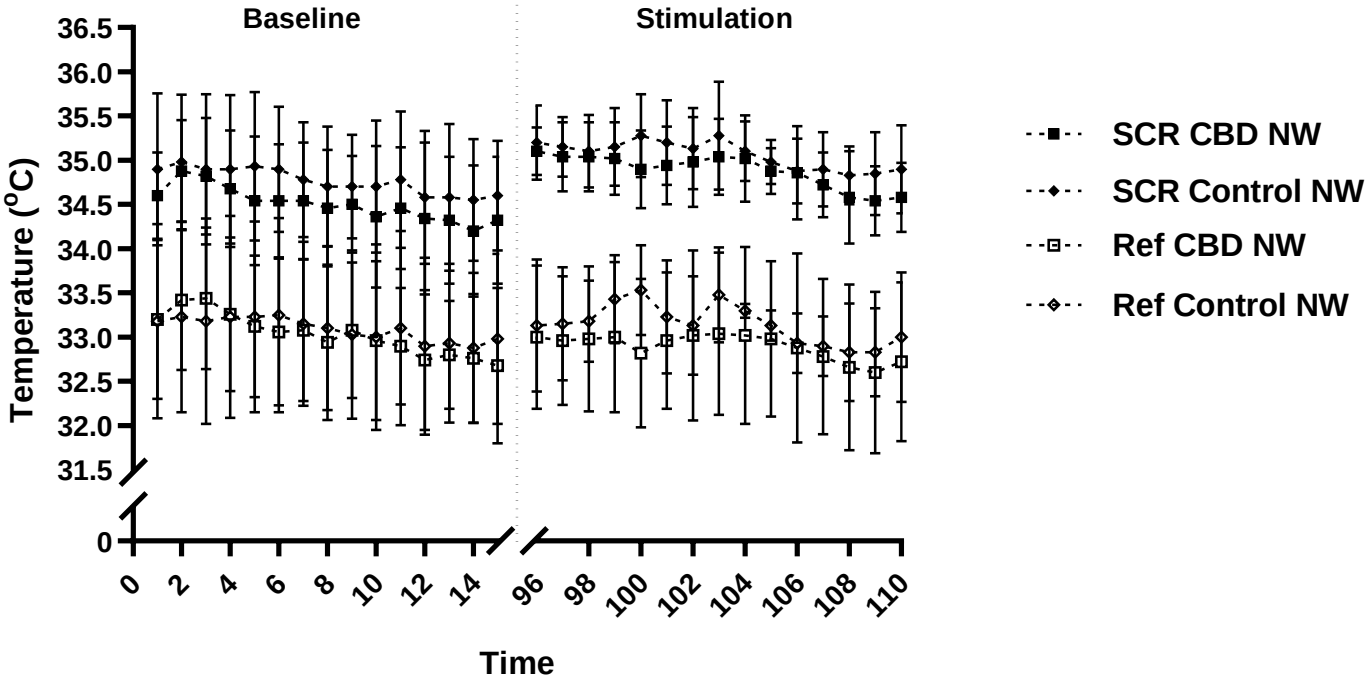


Figure 4.6 Chronic effect of cannabidiol in the supraclavicular and reference temperatures in overweight participants.

The dotted line separates the baseline with the stimulation period. At the end of the baseline, the three tablets were ingested from the participant and after 80 minutes, IRT measurements were performed again for another 15 minutes (mean±SD). There were not any differences in SCR (F=0.017, p=0.901) or reference (F=0.114, p=0.746) temperatures between CBD (square; n=5) and control (rhombus; n=4) participants. SCR: Supraclavicular, CBD: Cannabidiol, Ref: Reference, IRT: Infrared thermography.

4.5 Discussion

Acute or chronic use of CBD did not reveal any difference in SCR or reference temperatures compared to the control group in NW and OW healthy adult males. To my knowledge, this is the first study in humans using CBD to activate BAT. In vitro (Parray and Yun 2016), 10μM of CBD incubated in 3T3-L1 cell line, elevated the brown fat specific genes and proteins, including UPC-1, as well as mitochondrial biogenesis in the cultured white adipocytes, concluding that CBD promotes the brown adipogenesis. One of the features of CB receptor antagonists is the activation of thermogenesis (Hsiao, Shia et al. 2015). In vivo, BPR0912, a cannabinoid receptor 1 antagonist, was proposed to have thermogenic effects on BAT after its administration of 10 mg/kg for 20 days in diet-induced obese male mice (Hsiao, Shia et al. 2015). In this study, UCP-1 protein was elevated in BAT and lipolysis was enhanced, suggesting that its activation was mediated from the β2-adrenoreceptors.

After stimulation of the CB_2 receptor, which can also be stimulated through CBD (Ignatowska-Jankowska, Jankowski et al. 2011), the UCP-1 levels were significantly elevated in the obese group. Though, the above results are not in line with the ones found in this RCT study, in which, no difference in BAT activity was revealed during acute or chronic exposure to CBD. The above study, administrated a specific dose to the rodents according to their weight. Therefore in humans with higher body weight a higher dose should be given compared to the dose taken from the normal weighted ones. Also, the chronic duration of the rat study was between 20 days but mine only lasted

7 days. Therefore, a longer period of exposure to CBD might be needed to reveal a significant effect in human.

A well-known trigger used widely to activate BAT in humans is cold exposure (Lee, Ho et al. 2011, Symonds, Bosman et al. 2011, Symonds, Henderson et al. 2012, Law, Chalmers et al. 2013, Robinson, Ojha et al. 2014, Muros-Molina, Vazquez Rocha et al. 2019, Muros, Green et al. 2019). However, a factor that can decrease BAT activity is high BMI. Studies comparing lean with obese children, in thermoneutral environment (Gatidis, Schmidt et al. 2016), or lean adults during cold induced stimulation (Muros-Molina, Vazquez Rocha et al. 2019), showed that BMI is inversely related to BAT activity. Though, the results of the present study, using hand cooling stimulation, did not show any relationship between BMI and BAT activity, probably due to the small sample size or the low difference in the BMI between NW and OW participants.

The outcomes of this study could be different in a same study design with a large number of participants involved. For this reason, having obtained the results from the Chapter, I used the Power and Sample Size Calculations Version 3.1.6 (21) to calculate the sample size needed to perform any similar study in the future. If the true difference in the experimental and control means is 0.207, with standard deviation 0.339, we will need to study 43 participants and to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with 80% power. For OW participants, if the true difference in the experimental and control means is 0.220, standard deviation 0.267, we will need to study 24

participants to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with 80% power.

One of the main limitations of this study was the small number of participants in each of the four groups, used to better depict BAT activity in different BMI classifications. Also, the mean environmental temperatures, Table 4.2, during the study was at 5^{o}C approximately for all of the sessions, as it was performed during autumn/winter. As season can affect BAT (Au-Yong, Thorn et al. 2009), the results of this study could be different when performed during spring/summer. On the other hand, this study has many strengths, as is the first one in humans attempting to trigger BAT with CBD oral intake, in a laboratory setting and also in real life, through the daily ingestion of tablets at home for 5 consequent days. Also, the study design was blinded for both the volunteers and investigator to minimise the risk of bias and there was concealment of allocation. Others include the non-invasive use of the IRT technique and the advantage of this technique to take a series of real-time images.

4.6 Conclusions

This is the first blinded RCT to investigate the effects of the CBD in BAT compared to placebo, in NW and OW young male adults. The comparison showed that neither the NW group nor the OW group revealed any metabolic BAT activation or any changes in the relative and mean skin temperatures. Also, there was not found any relationship between the BMI and the baseline or maximum SCR

temperatures. However, the sample size calculation can be valuable for any future studies aiming to activate BAT in humans using CBD administration.

5 A feasibility study to assess the brown adipose tissue activity in patients with hyperprolactinaemia

5.1 Introduction

Prolactin is a peptide and its genetic, structure and binding properties are similar to those of the growth hormone and human placental lactogen (Melmed and Jameson 2014). It is synthesised in the anterior pituitary gland, in the lactotroph cells (Ra 2016). Prolactin release is controlled by dopamine, which acts as an inhibitory factor on the pituitary dopamine type 2 receptors located in the lactotroph cells (Melmed and Jameson 2014). In contrast, serotonin promotes the stimulation of prolactin (Balsa, Sánchez-Franco et al. 1998). In adults, high prolactin concentrations in the serum (normal reference range in ages >18 years; males: 45-375mU/L and females (not pregnant or breast feeding): 59-619mU/L) (NHS 2019), can cause hyperprolactinaemia (Serri, Chik et al. 2003). The causes of this condition can vary from physiological, e.g. stress and pregnancy, pharmacological, e.g. antipsychotics, antidepressants, oestrogens and opioids and pathological, e.g. tuberculosis, primary hypothyroidism, cirrhosis, chronic renal failure and untreated Parkinson's disease (Majumdar and Mangal 2015). DA are usually used to restore prolactin to normal (Wang, Mullan et al. 2012).

Prolactin is well known for its effect on the mammary glands, including the stimulation of mammogenesis (growth and development

of the mammary glands), lactogenesis (synthesis of milk) and galactopoiesis (maintenance of milk secretion). Also, it has a role in regulating the immune system, osmotic balance and angiogenesis (Freeman, Kanyicska et al. 2000). In BAT, increasing maternal food intake through gestation promotes fetal BAT maturation and raises the abundance of the prolactin receptor (Budge, Bispham et al. 2000). Further investigation of the effect of prolactin administration in rats through gestation (Budge, Mostyn et al. 2002), showed five times more UCP-1 abundance in BAT of both late gestation foetus and the new-born, establishing the effect of the prolactin on BAT activity. Another study (Viengchareun, Servel et al. 2008), found that BAT differentiation requires signalling of the prolactin receptors. In the latter study, a proposed model of brown adipose regulation by prolactin pathway was proposed, through the activation of signalling pathways, such as mitogen-activated protein kinase, after the binding of prolactin leading to NST. Having all this in mind, in this Chapter, I tried to test the feasibility to recruit patients with hyperprolactinaemia to assess their BAT activity.

5.1.1 Hypothesis

The primary hypothesis of this feasibility study is that BAT in patients with hyperprolactinaemia will have a higher BAT activity before the treatment with DA.

5.2 Materials and methods

Approval was taken from the Health Research Authority from the NHS (IRAS project ID: 212218, Appendix IV; REC reference: 17/NW/0167,

Appendix V). The conceptualisation and design of the study was made by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds and Professor John Alcolado. The study protocol was already written and approved for use by Professor John Alcolado, Professor Michael E. Symonds and Professor Helen Budge. Apart from performing all of the study sessions and data collection, I also took part in the recruitment process (which was primarily performed by Professor John Alcolado) and written informed consent was obtained by myself or Professor John Alcolado.

This study took place at the Royal Derby Hospital, endocrinology clinic, Derby, UK. All data listed in the clinical research file (Chapter 2, Section 2.8), have been collected from the nurses working at the Royal Derby Hospital and the anthropometric measurements were made as part of the routine clinical care for the needs of the participants inside the Royal Derby Hospital from the NHS staff. For the thermal imaging purposes, data were collected after taking written informed consent from the participants.

5.2.1 Participants

The recruitment can be found in Chapter 2, Section 2.2. The inclusion criteria were:

- patient over 18 years old
- patient with hyperprolactinaemia from any cause, where the supervising clinician has made the decision that treatment is required with DA and where the patient has agreed to this form of therapy

- patient willing and able to give informed consent.

Patient was not eligible to take part in the study if there was any of the following criteria:

- patient will not be receiving DA therapy for the duration of their involvement in the study
- patient is pregnant, breastfeeding or planning pregnancy during her involvement in the study
- patient's supervising clinician is of the opinion that DA therapy must commence within 48 hours
- patient is terminally ill
- mental incapacity to consent
- already participating in the present research.

5.2.2 Sample size

As this was a trial study, I aimed to recruit at least 10 patients with hyperprolactinaemia that will be treated with DA.

5.2.3 Study design

This feasibility study was designed to include 3 study sessions; one prior to treatment with DA and the rest two were performed post to treatment with DA, at least 6 months apart, Figure 5.1. The DA used was Cabergoline at the dose of 0.5 mg twice a week, i.e. one 0.5 mg tablet was taken in the morning with breakfast on Mondays and Thursdays, for all of the participants. IRT was performed at the end of the patient's clinically indicated out-patient visits.

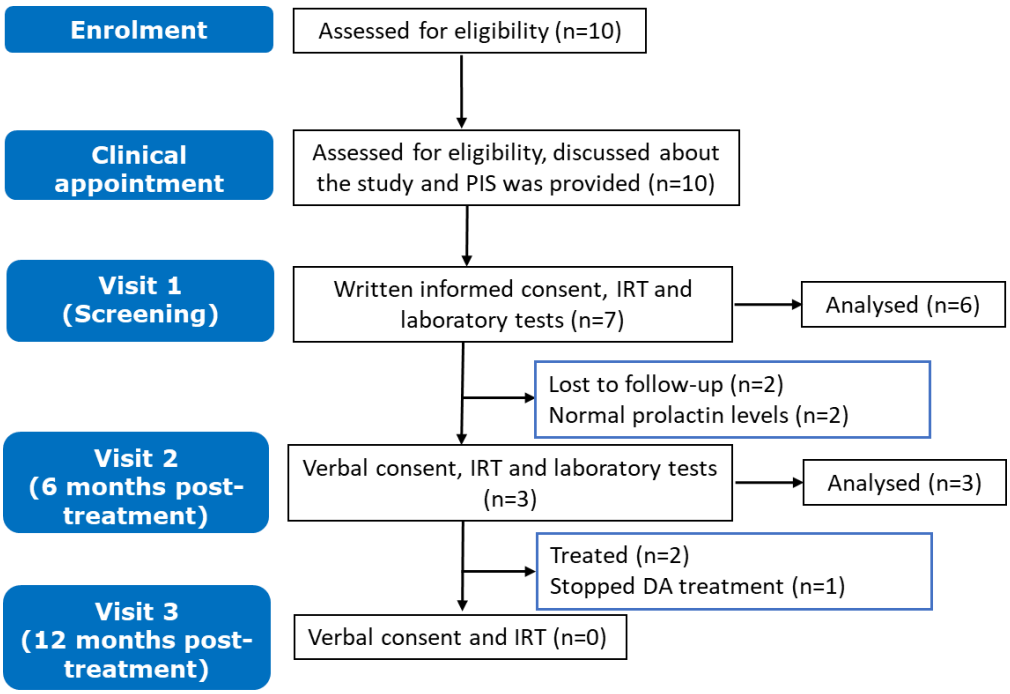


Figure 5.1 Study design of the prolactin patient's follow-up.

This trial study was designed to include 3 study visits, one prior and two post to DA treatment. PIS: Participant information sheet, IRT: Infrared thermography, DA: Dopamine agonist.

The patient was approached on the clinical appointment by the principal investigator, who made available the PIS and explained the study design to the eligible patient (Chapter 2, Section 2.2). Then, the first visit was arranged after the patient had read the PIS and had the opportunity to ask for further questions. The patient was not required to fast or refrain from drinking or smoking. This decision was made because from the time the patient arrived at the clinic until the time that the IRT was performed was between 1:30-3:00 hours. The visits were undertaken between 12:00-15:00. During the visits, the patient was asked to sit in a chair and then skin sensors were placed in 7 different places on the left side of the body. As two of the places were the knee and thigh, and the patient could not lift the trousers, the sensors were placed only in 5 locations (Chapter 2.5, Section

2.5.1). Afterwards, IRT was performed to depict the BAT activity of the ROI (Chapter 2, Section 2.9). In this study, the cooling method used was immersing of the right hand in cool water (Chapter 2, Section 2.9.3.6), kept at 15^{o}C. Date of birth, weight and height were also recorded by the nurses of the patient's appointment on the day of the IRT session. As part of the routine clinical care, laboratory tests to measure the serum prolactin concentrations and pregnancy test were performed and recorded in the clinical research file from the NHS staff (Chapter 2, Section 2.8).

5.3 Data analysis

Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating that the data were normally distributed. Assessment of the effect of the outside temperature in BAT was performed using Pearson's correlations to assess any correlation between the SCR and reference temperatures. Also, Pearson's correlations were used to identify any correlation between the prolactin levels of the patients and SCR temperature among the six patients.

Although the study was designed for 3 study visits, due to insufficient compliance, only the data of the first two visits of three patients were used for the analysis. Repeated measures ANOVA was used to identify any change between prior and post to DA treatment in SCR and reference temperatures. The following results do not include one male patient, assessed only in the first visit, as he was an outlier (Age: 63 years - Prolactin concentration= 2168mlU/L with $T_{baseSCR}$=34.2^{o}C and

T_{maxSCR}=34.4°C). Skin temperature was analysed as described in Chapter 2, Section 2.5.2.

5.4 Results

In this study, 6 patients, of which 1 was male, were analysed (mean age: 34.5±8.8 years; range: 32-44 years).

5.4.1 Anthropometric Data

Two of the participants were classified as NW, one as OW, two as obese class I and one as obese class III. All anthropometric measurements performed prior to DA treatment of the six participants are presented in Table 5.1.

Table 5.1 Anthropometric measurements of the prolactin patients prior the dopamine agonist treatment (n=6; mean±SD).

Measurements	Prior
Weight (kg)	87±19.9
Height (m)	1.65±0.05
BMI (kg/m²)	32.16±9.5

BMI: Body mass index.

5.4.2 Room and environmental temperature

Air-condition was not available and the room temperature prior to treatment was at 22.6±0.8°C and the mean environmental temperature prior to DA intake was at 7.1±3.7°C, Table 5.2.

Table 5.2 Environmental temperatures in relation to the time of year throughout which each session was conducted (n=6).

Participant	Date of study before the intervention	Date of study after the intervention	Environmental temperature (°C)	
	Prior	Post	Prior	Post
1	08/10/2017	16/04/2018	12	11
2*	30/10/2017	-	8	-
3*	20/11/2017	-	11	-
4	05/02/2018	22/10/2018	3	10
5	05/02/2018	19/10/2018	4	6
6*	12/02/2018	-	5	-

*Participants did not attend their second clinic appointment.

5.4.2.1 Correlations of supraclavicular and reference temperatures with the environmental temperature

The outside temperature was not related to either SCR (Prior - baseline: R^2=0.989, r=0.994, p=0.064; maximum: R^2=0.946, r=0.972, p=0.149; Post – baseline: R^2=0.361, r=0.600, p=0.589; maximum: R^2=0.310, r=0.556, p=0.624) or the reference temperatures (Prior - baseline: R^2=0.031, r=-0.178, p=0.885; maximum: R^2=0.069, r=-0.262, p=0.830; Post - baseline: R^2=0.0.57, r=-0.240, p=0.845; maximum: R^2=0.031, r=-0.176, p=0.887).

5.4.3 Correlations between prolactin concentrations and supraclavicular temperature

Among the participants, who came on the screening session prior to DA treatment, it was seen that as the prolactin concentration got high, the maximum SCR temperature was lower, Figure 5.2. Though no difference was found in the baseline SCR (R^2=0.726, r=-0.852,

p=0.066). There was also data from one outliner that was not plotted in Figure 5.2, due to very high prolactin levels (Female participant – Prolactin= 1226mIU/L, $T_{baseSCR}$= 35.3°C and T_{maxSCR}= 35.6°C). As the prolactin concentration of this female participant were substantially higher than all other measurements (compared to the maximum prolactin level that the participants had of 702mIU/L) , after visual identification and consideration it was decided to be an outlier (Aggarwal and Ranganathan 2016).

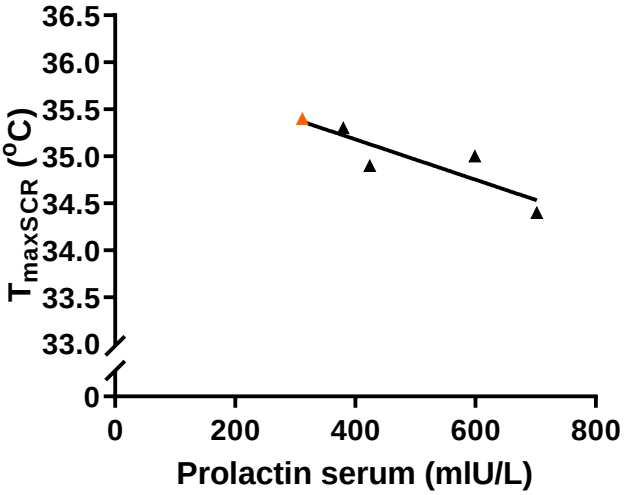


Figure 5.2 Correlation of the prolactin levels with the maximum supraclavicular temperature in patients with hyperprolactinaemia (n=5).

Five maximum SCR temperatures are presented (triangles; 1 male in orange) for each participant prior to DA treatment (R^2=0.778, r=-0.882, p=0.047). SCR: Supraclavicular, T_{maxSCR}: Maximum supraclavicular temperature, DA: Dopamine agonists.

5.4.4 Supraclavicular and Reference temperatures

For the three participants (Table 5.2: Participants 2, 3, 6; mean age: 39.3±6.4 years - range: 32-44 years, weight: 71.3±8.7kg, height: 1.67±0.005m, BMI: 25.38±3.28kg/m^2; room temperature – Prior: 22.3±0.5°C and Post: 23.1±0.1°C; environmental temperature – Prior: 6.3±4.9°C and Post: 9.0±2.6°C), there was not any difference

prior and post to DA treatment in SCR, though, there was a change in the reference temperature, Figures 5.3 and 5.4a, b, c and d.

5.4.5 Relative temperature

The relative temperature did not alter between prior and post the intervention (Baseline: Prior - $\Delta T_{baseREL}$: 2.3±0.3, Post - $\Delta T_{baseREL}$: 2.4±0.5, p=0.579; Maximum: Prior - ΔT_{maxREL}: 2.3±0.6, Post - ΔT_{maxREL}: 2.4±0.6; p=0.528).

5.4.6 Skin temperature

There were not any detectable changes between the baseline and maximum temperatures of the head, arm, clavicle, hand and foot sites, Table 5.3.

Table 5.3 Baseline and maximum skin temperatures in patients prior and post to dopamine agonist treatment (n=3; mean±SD).

Body sites	Baseline (°C)	Maximum (°C)
Head	Prior: 33.9±0.7 Post: 34.1±0.1	Prior: 34.2±0.6 Post: 34.4±0.1
Arm	Prior: 33.1±1.5 Post: 32.4±0.7	Prior: 33.5±2.0 Post: 32.4±0.7
Clavicle	Prior: 32.7±0.7 Post: 32.8±0.9	Prior: 32.7±0.9 Post: 33.0±0.6
Hand	Prior: 31.8±1.8 Post: 31.1±2.8	Prior: 31.9±1.2 Post: 31.5±3.0
Foot	Prior: 28.4±2.7 Post: 32.0±1.8	Prior: 28.4±2.6 Post: 31.9±1.9

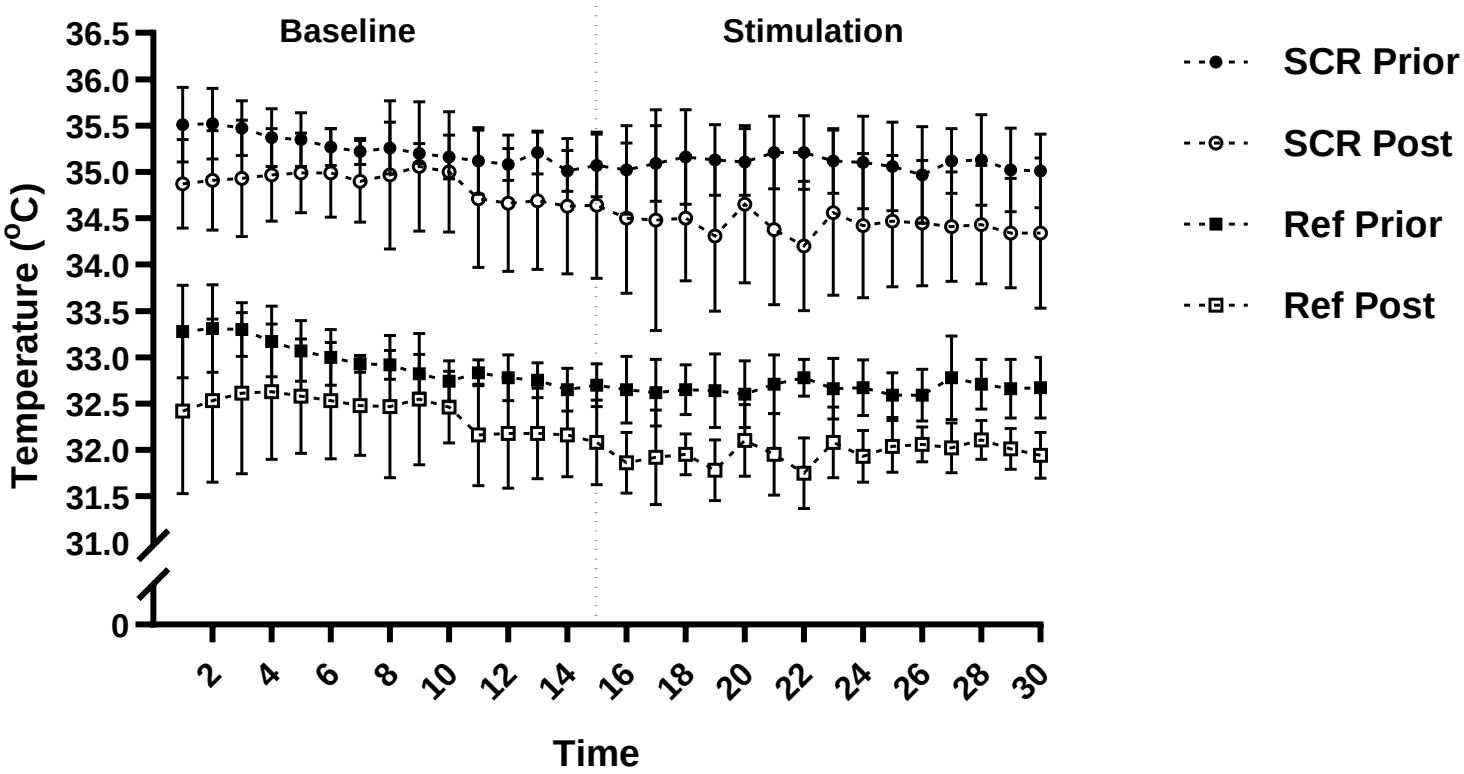
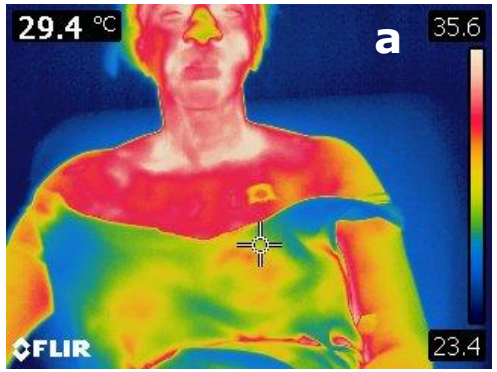
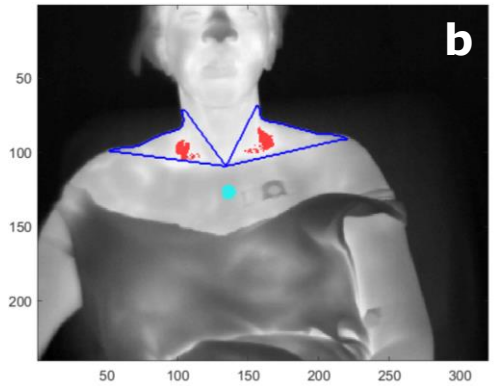


Figure 5.3 Temperature of the supraclavicular and reference areas prior and post the dopamine agonists treatment.

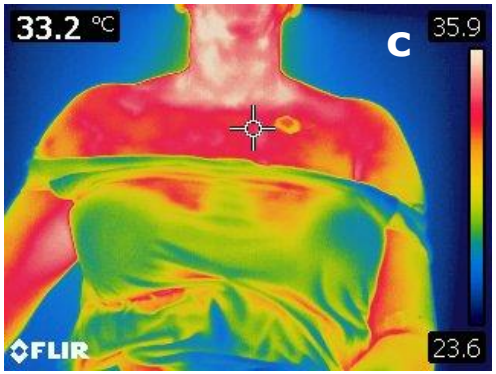
In this figure (n=3), the two periods of each visit are separated by a dotted line. Baseline and stimulation periods lasted for 15 minutes each. SCR (Prior: closed circles, Post: open circles; F=4.971, p=0.090) and reference (Prior: closed squares, Post: open squares; F=11.832, p=0.026) depict the temperatures in real time during the IRT. SCR: Supraclavicular, Ref: Reference, IRT: Infrared thermography.

Prior DA treatment





Post DA treatment



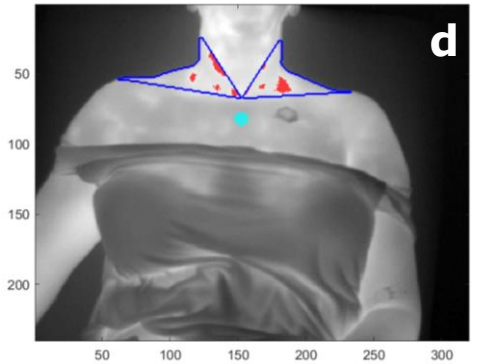


Figure 5.4a, b, c and d Examples of the thermal images prior and post analysis and treatment of the same participant.

Example of a female participant aged 44 years with BMI 24.44 kg/m^2. The Figures a and c are in JPEG format at the maximum BAT stimulation. The Figures b and d are PNG images deriving from analysis; the blue triangles are separating the right with the left ROI, the red colour shows the overall SCR temperature and the light blue circle is the reference point. PNG: Portable network graphics, JPEG: Joint photographic experts group, DA: dopamine agonist, ROI: Region of interest, SCR: Supraclavicular.

5.5 Discussion

This is the first study in humans, to my knowledge, showing that the circulating prolactin was inversely related to maximum SCR stimulation when a cold stimulus was applied. Previous research in vitro and in vivo (Viengchareun, Bouzinba-Segard et al. 2004), using a T37i brown adipose cell line and new-born mice, have investigated the effects of prolactin on BAT. These studies revealed that brown adipocytes express prolactin receptors and prolactin can trigger BAT activation, however with no rise in UCP-1. Furthermore, nutritional enrichment of pregnant sheep during late gestation, significantly increased BAT abundance and UCP-1 activity of the foetus, which was associated with increased abundance of the prolactin 1 receptor (Budge, Bispham et al. 2000). Another study (Budge, Mostyn et al. 2002) where prolactin was administrated throughout the pregnancy of rats, resulted in an increase in UCP-1 concentration of both foetus and new-borns suggesting that prolactin might act through its receptor. Further studies in rodents, have confirmed that prolactin receptors are expressed in brown adipocytes (Bole-Feysot, Goffin et al. 1998, Viengchareun, Bouzinba-Segard et al. 2004, Viengchareun, Servel et al. 2008). All these are in line with my results, as prolactin is enhancing BAT stimulation, meaning that higher prolactin could have an impact on the stimulation of BAT in adults. In this case, external factors, such as cold water, cannot further stimulate BAT.

Although the maximum SCR temperature prior and post the DA treatment does not have any difference, it seems to be a lower trend

after the DA treatment. If the outlier was included in the analysis (Female participant – Prolactin=1226ng/ml with T_{SCR}=35.3°C and T_{maxSCR}=35.6°C) the results would not differ as the correlation between the prolactin concentrations and maximum SCR temperature was lost, Figure 5.5. The same outcomes were observed in the baseline SCR (R^2=0.007, r=0.087, p=0.868).

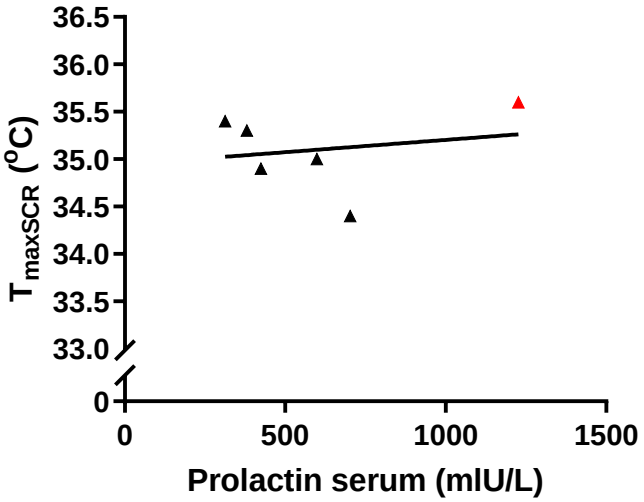


Figure 5.5 Correlation of the prolactin levels with the maximum SCR temperature in patients with hyperprolactinaemia, including the outlier (n=6).

Six maximum SCR temperatures are presented (triangles; the outlier in red) for each participant prior the dopamine agonist treatment (R^2=0.041, r=0.203, p=0.699). SCR: Supraclavicular, T_{maxSCR}: Maximum supraclavicular temperature.

Even though there is not a significant change in the maximum SCR, thermal images, Figures 5.4a, b, c and d, from a 44 years old lean female, expose that maximum BAT stimulation could be lower post to DA treatment. Only the reference temperature was significantly lower post to DA treatment among the three female patients. Prior to DA treatment, as BAT is metabolically active in a prolonged period during high levels of prolactin, the overall reference temperature is higher than post treatment. Thus, by minimising the prolactin levels in the

human body, I believe that the reference temperature drops back to its normal levels.

In this study, I tried to establish whether an intervention using prolactin would be appropriate for further exploration, as there are no relevant published papers so far, to my knowledge. Before I discuss about the main question of my study, prior to the study design, a detailed feasibility assessment was conducted with respect to the clinical relevance, sample size and the logistics at site (e.g. Good Clinical Practice training, proper facilities to carry out the study, deviation of the study from normal practise). The main question in this feasibility study, was whether a sufficient number of patients could be recruited. Out of the 10 patients identified, I successfully recruited 7, with the remaining 3 rescheduling or cancelled their next clinical appointment. The fact that the participants, did not came back for their follow-up session does not necessarily mean low compliance in the study. Although, in order to actual assess the acceptability of the patients, a questionnaire or a short interview investigating whether the participants (1) are satisfied from the study session, (2) intent to continue coming to the sessions, (3) considered that it was appropriately performed, (4) fitted with their culture and (5) receive any positive or negative feedback, would be necessary to understand in depth the acceptability of this study. This is one of the eight areas of focus that feasibility studies should address (Bowen, Kreuter et al. 2009). Of course other aspects such as demand, implementation, practicality, adaptation intergradation, expansion and limited-efficacy

testing should be set beforehand in a future clinical setting and monitored carefully after setting specific goals.

This is the first attempt to assess the achievability of using the IRT technique to capture BAT activity in adult patients with hyperprolactinaemia. I established that an IRT study could take place in a clinical setting, where each session can take in less than an hour, without involving any invasive procedures. The main limitation of this study was the small number of the patients recruited. Also, the compliance of the patients to continue with their clinical appointments was poor as the 4 out of 7 participants were lost to follow up. Furthermore, using a very small sample size (5-6 observations) to identify any correlation between the prolactin levels and SCR, might reveal a relationship though none will actually exist (Type I error)184 (Aggarwal and Ranganathan 2016).

5.6 Conclusions

The main finding of this study was the inverse correlation of the prolactin levels and maximum SCR stimulation, meaning that prolactin could affect BAT stimulation, Figure 5.5. This trial study, using the innovative and non-invasive technique of IRT, showed that it is acceptable from the patients with hyperprolactinaemia. Also, the engagement of the patients was very good, as none of them left during the sessions.

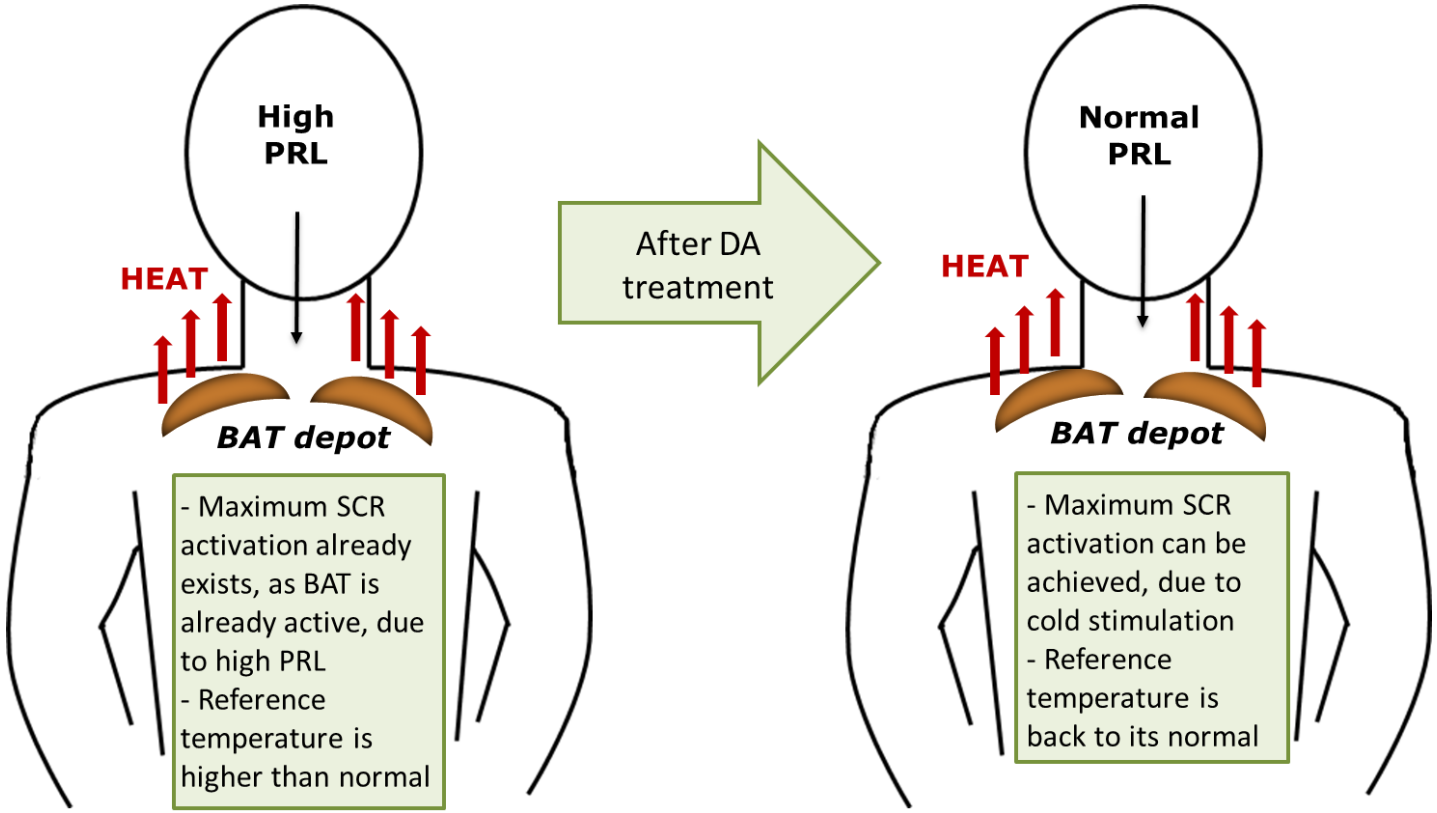


Figure 5.6 Summary diagram of the study trial in patients with hyperprolactinaemia.

On the left, SCR temperature is already risen due to high prolactin concentrations during cold exposure. On the right, after DA treatment, the concentrations of prolactin came back to normal, revealing a lower reference temperature during cold stimulation. PRL: Prolactin, BAT: Brown adipose tissue, SCR: Supraclavicular, DA: Dopamine Agonist.

6 A feasibility study to assessment of the usefulness of thermal imaging in quantifying brown adipose tissue activation in bariatric clinic

6.1 Introduction

Obesity induced inflammation can lead to various adverse health conditions including CVDs (Hubert, Feinleib et al. 1983, Van Gaal, Mertens et al. 2006) and diabetes (Dandona, Aljada et al. 2004, Wellen and Hotamisligil 2005) which are in the 10 main global causes of death, according to the WHO (WHO 2018b). Currently, there are various treatments to tackle obesity in adults such as exercise combined with diet (Shaw, Gennat et al. 2006), as well as, bariatric surgery (Colquitt, Pickett et al. 2014). In the last decade, another treatment has been proposed, the activation of BAT (Frühbeck, Becerril et al. 2009). Stimulated BAT generates heat through NST (Cannon and Nedergaard 2004, Saito, Okamatsu-Ogura et al. 2009). Maximal BAT activation could lead to a 4.1kg of weight loss (Virtanen, Lidell et al. 2009) in a year. Previous studies have revealed several triggers such as mild cold exposure (van Marken Lichtenbelt, Vanhommerig et al. 2009), cold water (Orava, Nuutila et al. 2011) and food ingredients (Yoneshiro, Aita et al. 2012, Yoneshiro, Aita et al. 2013) that can elevate BAT activity in adults.

However, after application of a stimulus, BAT might not be active, as its activity, as determined by PET/CT, is inversely related to BMI and

fat percentage (van Marken Lichtenbelt, Vanhommerig et al. 2009, Pfannenberg, Werner et al. 2010, Vijgen, Bouvy et al. 2011, Orava, Nuutila et al. 2013, Muros-Molina, Vazquez Rocha et al. 2019). Therefore, in a study assessing BAT activity through air and water perfused mattress stimulation, only 3 out of 15 morbidly obese participants were BAT-positive (Vijgen, Bouvy et al. 2011). After identifying BAT-positive individuals, another study explored the possibility of further activating BAT after weight loss was induced with a laparoscopic adjustable gastric banding (Vijgen, Bouvy et al. 2012). Only 2 female participants were BAT-positive before surgery, whereas half of the participants, showed active BAT after the surgery. Also, in the latter study, the mean body weight of the participants was reduced by approximately 28kg in a year's time after the surgery. All of the above studies suggest that activation of BAT is a promising target to reduce obesity. In this Chapter, I tested the feasibility to recruit morbidly obese patients from NHS bariatric clinic in Derby and also assess their BAT activity after weight loss surgery.

6.1.1 Hypothesis

My primary hypothesis in this feasibility study is that BAT will not be active before weight loss in morbidly obese patients.

6.2 Materials and methods

This study took place at the Royal Derby Hospital, medical bariatric clinic, Derby, UK. Approval was taken from the Health Research Authority from the NHS and the research ethics committee (IRAS project ID: 212218; Appendix IV, REC reference: 17/NW/0167,

Appendix V). All data listed in the clinical research file (Chapter 2, Section 2.8), have been collected from the nurses working at the Royal Derby Hospital and also the anthropometric measurements were collected as part of the routine clinical care for the needs of the patients inside the Royal Derby Hospital from the NHS staff. For the thermal imaging purposes, data were collected after taking written informed consent from the patients.

The conceptualisation and design of the study was made by myself with the help of my supervisors, Professor Helen Budge and Professor Michael E. Symonds and Professor John Alcolado. The study protocol was already written and approved for use by Professor John Alcolado, Professor Michael E. Symonds and Professor Helen Budge. Apart from performing all of the study sessions and data collection, I also took part in the recruitment process (which was primarily performed by Professor John Alcolado) and written informed consent was obtained by myself or Professor John Alcolado.

6.2.1 Participants

The recruitment of the participants can be found in Chapter 2, Section 2.2. The inclusion criteria were:

- age over 18 years old
- meet the National Institute for Health and Care Excellence criteria for bariatric surgery, is on the clinical pathway for bariatric surgery and is being contemplated for bariatric surgery within next 12 to 15 months
- willing and able to give informed consent.

Patients not eligible were those met any of the following criteria:

- not due to have bariatric surgery in the next 12 to 15 months
- pregnant, breastfeeding or planning pregnancy during their involvement in the study
- terminally ill
- mental incapacity to consent
- already participating in the current research.

6.2.2 Sample size

For this trial study, I aimed to recruit at least 10 patients who would be in the bariatric surgery group (Chapter 2, Section 2.1).

6.2.3 Study design

This feasibility study was designed to include four study sessions, of which the two were performed before the bariatric surgery, at least 2 months apart, 6 months before undergoing bariatric surgery. Then the patient was followed up for 12 months after the surgery with two further sessions at 6 and 12 months post-surgery, Figure 6.1. IRT was performed at the end of the patient's clinical out-patient visits.

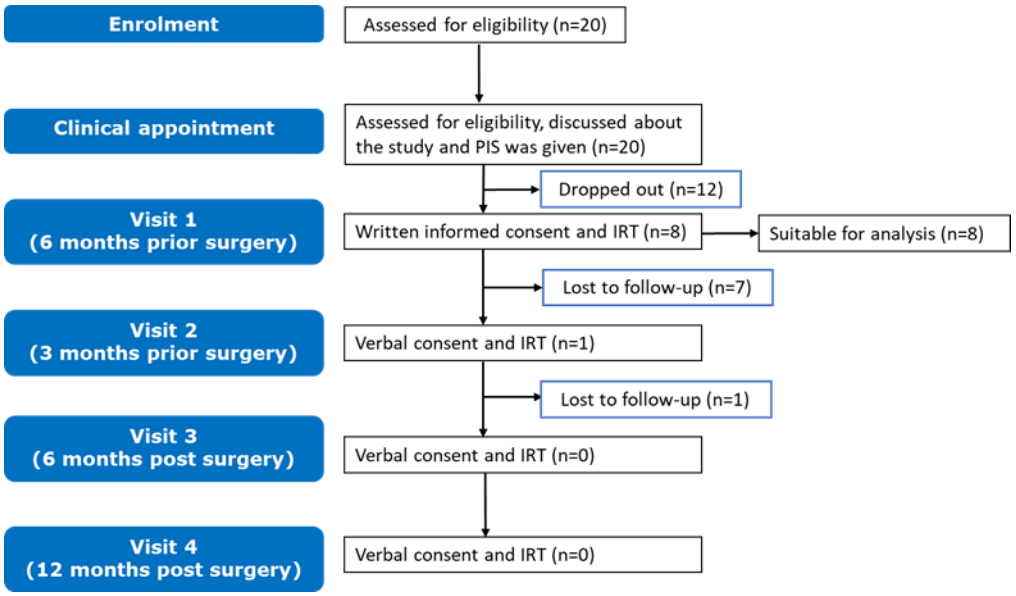


Figure 6.1 Study design of the bariatric patients' follow-up.

This trial study was designed to include four study visits, two prior and two post the bariatric surgery. PIS: Participants information sheet, IRT: Infrared thermography.

The patient was approached on the clinical appointment by the principal investigator, who made available the PIS and explained the study design (Chapter 2, Section 2.2). Then, the first visit was arranged after the patient had read the PIS and had the opportunity to ask for further questions. The patient was not required to fast or to refrain from drinking or smoking. All of the visits were undertaken in the morning between 8:00-12:00. During the visits, the patient was asked to sit in a chair and then skin sensors were asked to be placed in 7 different places on the left side of the body. As two of the places were the knee and thigh, and the patient could not lift the trousers, the sensors were placed only in 5 locations (Chapter 2.6, Section 2.5.1). Afterwards, IRT was performed to depict BAT activity of the ROI (Chapter 2, Section 2.9). In this study, the cooling method used was immersing of the right hand in cool water (Chapter 2, Section 2.9.3.6), kept at 15°C. Date of birth, weight and height were

also recorded by the nurses/dieticians of the patient's appointment on the day of the IRT session.

6.3 Data analysis

Kolmogorov-Smirnov normality test determined the data distribution with a p value of >0.05, indicating that the data were normally distributed. Assessment of the effect of the environmental temperature in BAT was performed using Pearson's correlations to assess any correlation between the outside temperature and SCR or reference temperatures.

Although the study was designed for four study visits, due to low compliance, only the data of the first visit were used for the analysis. Thus, paired t-test was performed to identify any difference, due to the cold effect on BAT, between the acclimatisation and stimulation periods (Chapter 2, Sections 2.11). Regarding the anthropometric measurements, from 1 participant, diastolic and systolic blood pressure was not measured during the clinic appointment.

6.4 Results

In this study, 8 participants were recruited and attended the first visit, of which only one came back for the second visit. The remaining seven participants did not attend another clinic appointment from September 2017 to September 2018. Participants' mean age at the time of the first visit was 51.7±7.7 years; range 42.7 to 68.8 years.

6.4.1 Anthropometric data

The anthropometric measurements performed on the first visit of the patients are presented in Table 6.1.

Table 6.1 Anthropometric measurements of the bariatric patients on their first visit (n=8; mean±SD).

Measurements	Data
Weight (kg)	133.7±11.8
Height (m)	1.68±0.06
BMI (kg/m^2)	47.1±5.0
Systolic (mmHg)*	161.3±14.7
Diastolic (mmHg)*	84.5±9.3
Heart Rate (p/min)	91.1±12.2

*n=7, BMI: Body mass index.

6.4.2 Room and ambient temperature

The mean room temperature was at 22.4±0.2^{o}C, and the mean environmental temperature was at 7.9±4.1^{o}C; individual values are presented in Table 6.2.

Table 6.2 Environmental temperatures in relation to the time of the year throughout which each session was conducted in bariatric patients (n=8).

a/a	Date of study session	Room temperature (°C)	Environmental temperature (°C)
1	14/09/2017	22.2	13
2	28/09/2017	22.9	15
3	12/10/2017	22.5	6
4	02/11/2017	22.6	8.8
5	25/01/2018	22.1	7.9
6	01/02/2018	22.5	4
7	15/02/2018	22.3	5
8	08/03/2018	22.3	4

6.4.2.1 Correlations of supraclavicular and reference temperatures with the outside temperature

The outside temperature was not related to either SCR (baseline: R^2=0.210, r=-0.459, p=0.252; maximum: R^2=0.310, r=-0.561, p=0.147) or reference temperatures (baseline: R^2=0.321, r=-0.567, p=0.142; maximum: R^2=0.224, r=-0.474, p=0.235).

6.4.3 Supraclavicular and reference temperatures

There were no differences between the baseline and maximum SCR temperatures (Figure 6.2a and Figures 6.3a, b, c and d) left or right SCR sides, Figure 6.2b. The reference temperature did not change after application of the cool stimulus, Figure 6.2c.

6.4.4 Relative temperature

The relative temperature did not alter ($\Delta T_{baseREL}$: 1.1±0.4, ΔT_{maxREL}: 1.2±0.6; p=0.756).

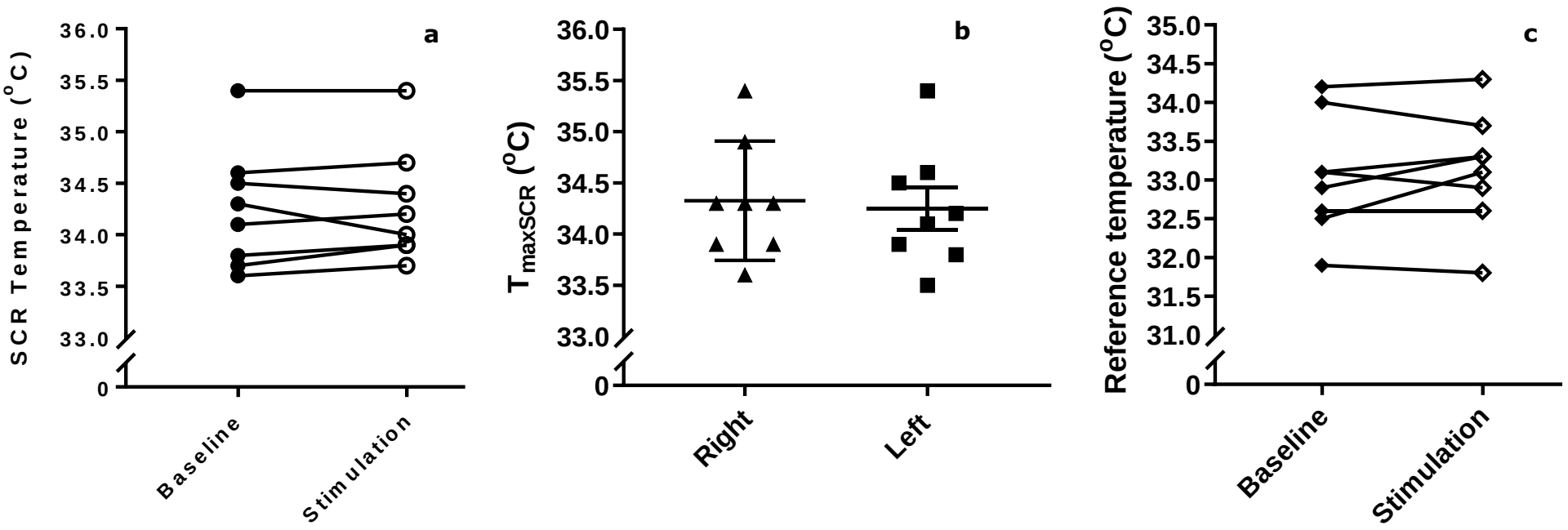
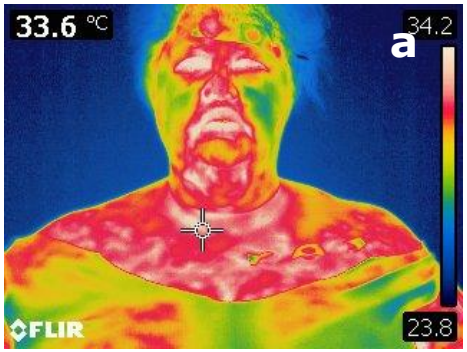
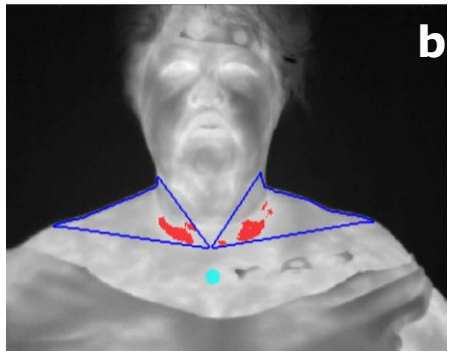


Figure 6.2a, b and c Supraclavicular and reference temperatures before and after hand cooling in morbidly obese patients (n=8).

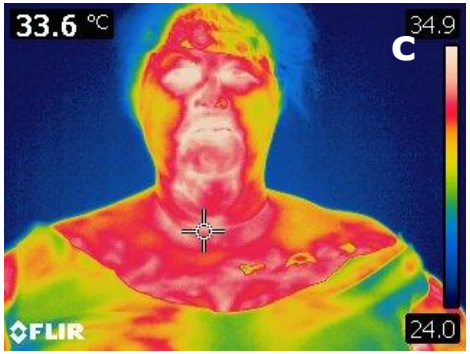
In Figure a, each participant's SCR temperature is represented by circles during baseline (closed circles) and stimulation (open circles). In Figure b, the stimulated SCR temperature is presented for both right (closed triangles) and left (closed squares) sites of the neck. In Figure c, the reference temperature is presented for both before (closed diamond) and after (open diamond) stimulation. SCR: Supraclavicular, T_{maxSCR}: Maximum supraclavicular temperature.

Baseline





Stimulation



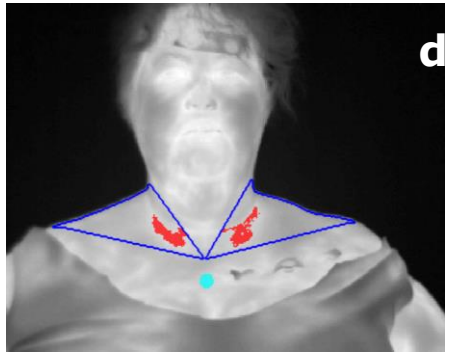


Figure 6.3a, b, c and d Examples of the thermal images before and after analysis at baseline and stimulation periods.

Example of a female participant aged 53.8 years with BMI 44.41kg/m^2. Figures a and c are the thermal images in JPEG format at the baseline and stimulation periods accordingly. Figures b and d are PNG images deriving from analysis; the blue triangles are separating the right from the left ROI, the red colour shows the overall SCR temperature and the light blue circle is the reference point. PNG: Portable network graphics, JPEG: Joint photographic experts group, ROI: Region of interest, SCR: Supraclavicular.

6.4.5 Skin temperature

There were not any detectable changes between the baseline and maximum temperatures of the head, arm, clavicle, hand and foot sites, Table 6.3.

Table 6.3 Baseline and maximum skin temperatures in morbidly obese patients (n=8; mean±SD).

Body sites	Baseline (°C)	Maximum (°C)
Head	33.5±1.0	33.7±0.7
Arm	32.8±0.9	33.3±1.4
Clavicle	33.2±0.9	33.2±0.8
Hand	32.5±2.0	32.7±1.7
Foot	30.8±2.5	30.7±2.2

6.5 Discussion

In this study, 8 adult female participants, classified as obese class III, did not show any BAT response after the application of the cool stimulus. Comparing lean and obese adults that have undergone PET/CT scanning after cold stimulation, revealed that 13 males out of 14 obese class I, exhibited lower BAT activation than lean participants (van Marken Lichtenbelt, Vanhommerig et al. 2009). Another study assessing BAT activity in obese Class I adults found that only 11 out of 30 participants had active BAT, as determined by PET/CT, and that age was inversely related to BAT (Orava, Nuutila et al. 2013). In class III obese participants, it was revealed that only 3 females out 15 adults demonstrated metabolic BAT (Vijgen, Bouvy et al. 2011). Thus,

it is less likely to detect BAT in higher obesity classes, as also seen in the results of this Chapter.

Studies using IRT in healthy lean adults (Muros-Molina, Vazquez Rocha et al. 2019), adult patients (Gatidis, Schmidt et al. 2016) and lean compared with obese children (Robinson, Ojha et al. 2014) have also shown that a higher BMI is negatively related to BAT. To support this theory, the data from this Chapter were plotted with those previously published for healthy lean adults (Muros-Molina, Vazquez Rocha et al. 2019). Pearson's correlations were not applied, as that would be a false correlation, as I am creating a dataset with my data and the previously published ones. Unpaired t-test, between the data, revealed that there were significant differences in baseline and maximum SCR temperatures between the two different groups, Figure 6.4a and b. As previously has been suggested, BMI seems to be inversely related to the baseline SCR temperature as well as the maximum SCR temperature, Figure 6.4a and b.

Failure to activate BAT with cold could be explained in part by the fact that obese people have better insulation due to more subcutaneous fat, which is reduced after bariatric surgery (Vijgen, Bouvy et al. 2012). Greater insulation in obese people results in reduced vasoconstriction, in response to cold, compared to lean subjects (Wijers, Saris et al. 2010), though is improved after bariatric surgery (Vijgen, Bouvy et al. 2012). A recent study (Brychta, Huang et al. 2019) revealed that lean and obese men have the same tolerable temperature in cold before shivering. However, it was seen that the physiology of the obese men, did not allow to initiate the

thermogenesis produced from BAT until their bodies had reached a much lower temperature.

In this study, I tried to establish whether this intervention could be feasible for further exploration, as to my knowledge there are no relevant papers published papers were sessions took place after patients' clinic appointments. Before I discuss about the main question of this study, prior to the study design, a detailed feasibility assessment was conducted with respect to the clinical relevance, sample size and the logistics at site (e.g. Good Clinical Practice training, proper facilities to carry out the study, deviation of the study from normal practise). The main question in this feasibility study, was whether a sufficient number of patients could be recruited. Out of the 20 patients identified, I successfully recruited 8, with the remaining 7 cancelling their next clinical appointment or being discharged. The fact that the participants, did not came back for their follow-up session does not necessarily mean low compliance in the study. Although, in order to actual assess the acceptability of the study to patients, a questionnaire or a short interview investigating whether the participants (1) were satisfied with from the study session, (2) intend to continue coming to the sessions, (3) considered that it was appropriately performed, (4) fitted with their culture and (5) receive any positive or negative feedback, would be necessary to understand in depth the acceptability of this study. This is one of the eight areas of focus that feasibility studies should address (Bowen, Kreuter et al. 2009). Of course other aspects such as demand, implementation, practicality, adaptation intergradation, expansion and limited-efficacy

testing should be set beforehand in a future clinical setting and monitored carefully after setting specific goals.

To my knowledge, this is the first attempt to assess the possibility of using the IRT technique in morbidly obese adults. The main strength of this study was the establishment of the IRT methodology in a clinical setting, taking less than an hour without involving any invasive procedures and be acceptable from all of the patients. However, the subsequent compliance of the patients to continue with the clinical appointments and undergo a bariatric surgery, were the main limitations of the study.

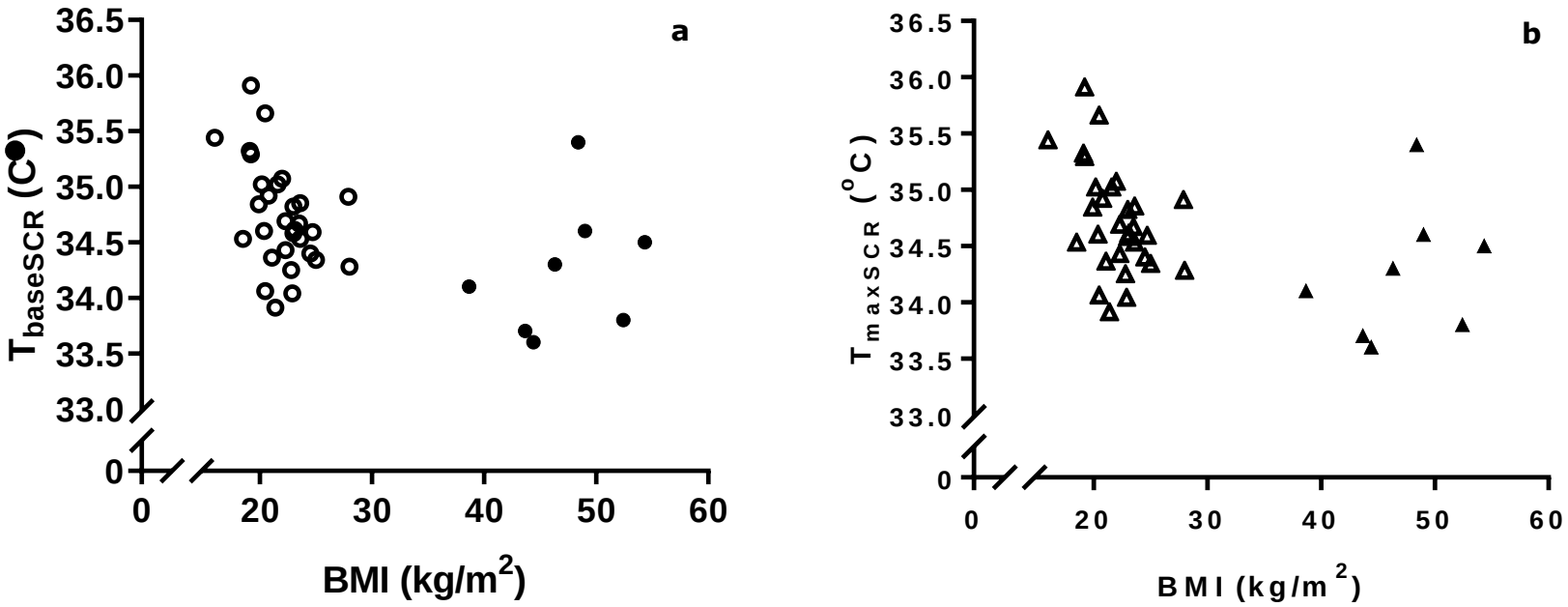


Figure 6.4a and b Correlations between baseline and maximum supraclavicular temperatures in normal weight and class III obese participants.

Figure a, baseline SCR temperatures are presented for each participant (Muros-Mulina data: open circles; n=30, Chapter's data: closed circles; n=8). Figure b, stimulated SCR temperatures are presented for each individual (Muros-Mulina data: open triangles, Chapter's data: closed triangles). Baseline SCR - Muros-Mulinos data: 34.7±0.4°C, Chapter's data: 34.2±0.5°C; p=0.020. Maximum SCR - Muros-Mulinos data: 34.9±0.4°C, Chapter's data: 34.2±0.5°C; p=0.0008. SCR: Supraclavicular, $T_{baseSCR}$: Baseline supraclavicular temperature, T_{maxSCR}: Maximum supraclavicular temperature, BMI: Body mass index.

6.6 Conclusions

This pilot study revealed that using IRT is an acceptable methodology from the patients of a bariatric clinic. All of the sessions lasted less than 40 minutes, so the interest of participating and engaging in this study was good. On the other hand, the patients were unable to comply with their fixed clinical appointments and undergo a bariatric surgery. As also seen in previous studies due to the higher BMI classification, there are fewer possibilities to detect metabolic BAT.

7 Conclusion

7.1 General aims

Each of the studies presented in this thesis aimed to investigate the effects of specific stimuli that could activate BAT and the effects of the diseases that could enhance or reduce BAT activity. This was achieved by assessing BAT status prior to, and after, a stimulus or an intervention, in healthy volunteers and patients. This final chapter summarises the key findings from all of the studies in this thesis.

7.2 Summary of the findings

7.2.1 Development of the current methodology

Many factors contribute to regulate BAT activity throughout our daily lives. These include stimuli we can modulate, such as drinking a cup of coffee (Velickovic, Wayne et al. 2019) or being stressed (Robinson, Law et al. 2016), whilst others are external and not easily modified, such as seasonal changes (Au-Yong, Thorn et al. 2009). My original hypothesis that a longer acclimatisation period is required in thermal imaging protocols was accepted and an extended acclimatisation period is now advised during any IRT experiment. By careful examination of BAT, following 5 or 30 minutes of acclimatisation, the baseline temperature during the longer, 30 minutes, acclimatisation was lower compared to the baseline temperature of the short one, 5 minutes, Chapter 2. In addition, after the short acclimatisation, the stimulation effect of BAT was masked, due to previous activation deriving from various factors such as cold weather. Therefore, no

effect was demonstrated during mild cold exposure in a short acclimatisation whereas, after the longer acclimatisation period, BAT activation was significantly higher in response to the stimulus.

7.2.2 Cool showers

After 2009, when BAT was officially reported as metabolically active in adults after mild cold exposure but not at thermoneutrality (van Marken Lichtenbelt, Vanhommerig et al. 2009), a series of investigations in adults took place using the techniques of IRT, PET/CT and MRI. These studies, included placing participants' limbs in cold water, swimming, drinking ice-water and exposure to cold air (Lee, Ho et al. 2011, Symonds, Henderson et al. 2012, Vrieze, Schopman et al. 2012, Robinson, Ojha et al. 2014, Romu, Vavruch et al. 2016, Muros-Molina, Vazquez Rocha et al. 2019, Muros, Green et al. 2019), though none investigated a prolonged effect of routine daily cool showers.

An investigation of taking daily 3 minutes cool shower (approximately 18^{o}C) for seven days was undertaken in healthy male adults with the hypothesis that BAT activity, measured using IRT, would be enhanced after the intervention, Chapter 3. Surprisingly, after the period of the cool showers, the maximum SCR temperature declined, whereas the post-stimulation reference temperature was raised. Taken together, these results suggest that the human body is adapting to a daily period of lower water temperatures. Consequently, my main hypothesis was rejected as the cool stimulus used prior to the intervention did not have anymore any effect on BAT post the seven days of the cool showers.

7.2.3 Cannabidiol

The non-psychotropic phytocannabinoid, CBD, has been suggested to contribute to the weight regulation in animals (Ignatowska-Jankowska, Jankowski et al. 2011). Around 3 years ago it was shown to impact on BAT regulation in cell lines (Parray and Yun 2016). My study is the first research investigating the effect of CBD in healthy male adults, Chapter 4. The main hypothesis of this study was that CBD could promote BAT activity in both acute and chronic exposure after 7 days of daily CBD 582mg ingestion. However, that hypothesis was rejected as no differences were identified in BAT activity in either acute or chronic CBD ingestion, in NW and OW adults.

7.2.4 Hyperprolactinaemia

Surveying the scientific literature, I identified an association between BAT activity and high prolactin during rodent development (Budge, Bispham et al. 2000, Budge, Mostyn et al. 2002). Currently, however, there have not been other studies examining this relationship in humans. The main hypothesis of my third study was that BAT temperature demonstrated with the IRT method would be higher prior to treatment with DA amongst NHS patients with hyperprolactinaemia, Chapter 5. In the results, circulating prolactin concentrations were inversely related to the maximum BAT temperature during cool application, suggesting that prolactin can have an impact on BAT activity. Also, I found that the reference temperature was higher prior to treatment compared to post treatment, suggesting that the body temperature due to BAT activity was higher in untreated patients.

7.2.5 Obesity

Obesity is a major factor that is responsible for a wide range of NCDs, including CVDs and diabetes, amongst others (Lavie, Milani et al. 2009). In this study, I looked at the feasibility of recruiting participants from the NHS, Chapter 6, and concluded that most of the patients did not attend their clinic appointments, even when they had serious health issues. However, patients that were recruited on the day of their clinic appointment had a good engagement as they all completed the 40 minutes of IRT session. After assessing morbidly obese females, it was confirmed that BMI was inversely related to BAT activity, measured using the IRT technique. Finally, it was seen that IRT sessions were quick and acceptable from the patients, making the IRT technique achievable inside a hospital setting.

7.3 Future research

Further investigations would be necessary to elucidate the findings in this thesis. In the following sub-sections, various suggestions can be found.

7.3.1 Cool showers

Single cool stimulation has already been established to activate BAT (Vrieze, Schopman et al. 2012, Romu, Vavruch et al. 2016, Muros-Molina, Vazquez Rocha et al. 2019, Muros, Green et al. 2019). However, prolonged exposure to a cool stimulus, Chapter 3, and previous work (Romu, Vavruch et al. 2016), revealed no association between the stimulus and BAT activity. Thus, future work should focus on either personalised cooling protocols (Martinez-Tellez, Sanchez-

Delgado et al. 2017) or assessing BAT activity during a single cold exposure. Other work could focus in exploring differences among ethnicities, as it has been shown that the total BAT volume and the non-shivering thermogenesis were significantly higher in Caucasians compared with South Asians (Bakker, Boon et al. 2014). Even though BAT activity has been explored in humans over the last decade, no study has been published yet in winter swimmers or open sea swimmers compared to a control group. However, a clinical trial was registered in May 2018 with the title "Winter-Swimming and Brown adipose tissue activity in middle-aged obese subjects – WinterBAT" (Clinical-Trials 2018) but there are no results from this study yet. In a similar future study, the effect in BAT immediately after acute exposure to a cool shower more than 3 minutes and/or a lower temperature is suggested.

7.3.2 Cannabidiol

As there has not been any other study in humans before to this thesis which has explored the effects of CBD on BAT, there are many things that need to be researched further. The main limitation of the study undertaken in Chapter 4, was the small sample size, due to the separation of the participants according to their BMI. A similar study with more participants could be able to possibly identify an effect of the CBD in BAT. Additionally, the exact time of the anticipated stimulation due to CBD should be assessed, as it might range in different age groups or gender.

7.3.3 Prolactin

Prolactin can be a stimulus causing BAT activation in animals (Budge, Bispham et al. 2000, Budge, Mostyn et al. 2002) and also as seen from this thesis in humans. However, as no work prior to this thesis has been undertaken in humans, it would be helpful to study the effects of hyperprolactinaemia in younger ages, where BAT is known to be active (Yoneshiro, Aita et al. 2011a). As it would not be ethical to inject high concentrations of prolactin in adults, BAT could be examined in gestating women with the technique of the IRT during, as they have high levels of prolactin due to pregnancy, and post-pregnancy (without breastfeeding, due to lower prolactin levels). The technique of IRT is passive, i.e. receives transmitted heat and uses no radiation or any other factor that could hurt the offspring or the mother and, as it has no serious adverse events, it would be easy to identify whether pregnant women have active BAT during gestation. Another interesting study could focus on the stimulation of BAT in women who exclusively breastfeed and the ones who do not, as mothers who breastfeed exclusively have significant higher prolactin concentrations than those who do not (Uvnäs-Moberg, Widström et al. 1990). Thus, BAT and prolactin could be assessed, around a month of the newborns' birth compared with mothers who do breastfeed and those who do not.

7.3.4 Obesity

During cold exposure, BAT activity is inversely related to fat mass (Robinson, Ojha et al. 2014, Gatidis, Schmidt et al. 2016, Muros-Molina, Vazquez Rocha et al. 2019). However, this should not be a barrier to explore which stimuli could affect BAT in obese adults. Due to the greater insulation and lower vasoconstriction the obese people have (Wijers, Saris et al. 2010), a stronger stimulus could be explored, such as a lower water temperature of that used in lean participants. In addition, individual cooling protocols in OW and obese participants could be assessed prior any intervention as has been suggested and performed in studies with lean adults (Chen, Cypess et al. 2016, Martinez-Tellez, Sanchez-Delgado et al. 2017).

7.4 Final remarks

The studies presented in this thesis have demonstrated how BAT can be affected by various stimuli leading to NST. Additionally, it has been seen, for the first time, that the technique of the IRT to assess BAT activity can be undertaken in a hospital setting. All these findings could help to further explore BAT during treatments or interventions in adults.

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Appendices

Appendix I

A study of brown adipose tissue activation in healthy volunteers using thermal imaging (BITS Study)

Appendix I

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+44 (0) 115 8232561
Louise.Sabir@nottingham.ac.uk

23rd December 2015

Dr James Law
Clinical Lecturer in Child Health
Division of Child Health, Obstetrics & Gynaecology
School of Medicine
QMC Campus
Nottingham University Hospitals
NG7 2UH

Dear Dr Law

Ethics Reference No: D19062014 SoM Child- please always quote
Study Title: A study of brown adipose tissue activation in healthy volunteers using thermal imaging.
Chief Investigator: Professor Helen Budge, Professor of Neonatal Medicine, Division of Child Health, School of Medicine
Co Investigators/Supervisors: Professor Michael Symonds, Professor of Developmental Physiology, Dr James Law, Clinical Lecturer, Division of Child Health, School of Medicine. NIHR Academic Clinical Fellows or Foundation Programme doctors: to be confirmed.
Student: BMedSci Project student to be assigned.
Duration of Study: 30/06/2014-30/06/2017 3yrs **No of Subjects:** 60

Thank you for your letter dated 25th November 2015 notifying the committee of amendment no 2 1 October 2015 as follows:

The following changes to the protocol were requested:

- In order to standardise the effect of stimulus volunteers will be asked to refrain from eating or drinking from midnight (except for water and sugar-free squash) on the study day session.
- Change question 9 of application form "will a disturbance allowance be offered from yes to no, be consistent with previous change to the Consent form and Information leaflets.
- Addition of those trained to take consent to include other appropriately trained members of the research team.

and the following revised documents were received:

Brown fat thermal imaging in healthy volunteers:

- Notice of Amendment form dated 1st October 2015
- FMHS Research Ethics Application Form fv1.2 01/10/15
- Protocol version 1.1: 01.10.15
- Participant Information Leaflet FV1.2 : 01.10.15
- Consent Form FV2.1: 1st October 2015

These have been reviewed and are satisfactory and the study amendment no 2: 1st October 2015 is noted and approved.

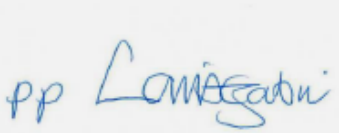
Appendix I

The University of Nottingham

Approval is given on the understanding that the Conditions of Approval set out below are followed.

1. You must 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. This study is approved for the period of active recruitment requested. The Committee also provides a further 5 year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.
4. 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, UoN Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix II

The effects of cool showers on brown adipose tissue in healthy adults: The CoR-BAT Study.

Appendix II

The University of Nottingham

Faculty of Medicine and Health Sciences

Research Ethics Committee
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham
NG7 2UH

Direct line/e-mail
+44 (0) 115 8232561
Louise.Sabir@nottingham.ac.uk

4th April 2016

Professor Helen Budge
Professor of Neonatal Medicine
Child Health, Obstetrics & Gynaecology
E Floor, East Block
School of Medicine
QMC Campus
Nottingham University Hospitals
NG7 2UH

Dear Helen

Ethics Reference No: H15032016 CoR-BAT **– please always quote**
Study Title: The effects of Cool Showers on Brown Adipose Tissue in healthy adults: The CoR-BAT study
Short Title: Cool Showers and brown adipocyte tissue (The CoR-BAT Study)
Chief Investigator/Supervisor: Professor Helen Budge, Professor of Neonatal Medicine, Child Health, Obstetrics and Gynaecology, School of Medicine
Lead Investigators/student: Miss Anastasia-Viktoria Lazaridi , PhD Student, Child Health, Obstetrics and Gynaecology, School of Medicine.
Other Key Investigators: Professor Michael Symonds, Professor of Developmental Physiology, Child Health, Obstetrics and Gynaecology, School of Medicine.
Type of Study: PhD Project, Thermal imaging, non-invasive
Proposed Start Date: 03/2016 **Proposed End Date:** 31/10/2017 18mths
No of Subjects: 18 **Age:** 18-30 years

Thank you for submitting the above application which was considered by the Committee at its meeting on 15th March 2016 and the following documents were received:

CoR-BAT Study:

- FMHS REC application form Version 1.0 23/2/2016.
- Protocol Version 1.0 23/02/2016
- Appendix 1: Participant's invitation e-mail v1.0 23_2_16
- Appendix 2: Participant Screening Questionnaire v1.0 23_2_16
- Appendix 3: Participant demographics v1.0 23_2_16
- Appendix 4: Participant reminder e-mail and text v1.0 23/2/2016
- Appendix 5: Participant food diary reminder e-mail and text v1.0 23/2/2016
- Appendix 6 Participant study visit reminder e-mail and text v1.0 23/2/2016
- Appendix 7: The Shower Diary v1.0 23/2/2016
- Appendix 8: The Food and Exercise Diaries 1&2 v1.0 23/02/2016
- Flier v1.0 23/2/2016
- Participant Information Leaflet v1.0 23_2_16
- Healthy Volunteer's Consent Form v1.0 23/2/2016
- Thank You Card v1 23_2_16
- Plan B version 1.0 23/2/2016

These have been reviewed and are satisfactory and the study is approved.

Appendix II

The University of Nottingham

Approval is given on the understanding the conditions set out below are followed:

1. You must 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. This study is approved for the period of active recruitment requested. The Committee also provides a further 5 year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.

4. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

pp Lamiegdon

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

Appendix III

Characterising the effects of cannabidiol, a phytocannabinoid, on the human cardiovascular system using ultrasound and other non-invasive techniques

Appendix III

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
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham, NG7 2UH

2 August 2017

Mr Salahaden Sultan
PhD Student
c/o Dr T England
Clinical Associate
Vascular Medicine
Division of Medical Sciences
GEM School of Medicine
Derby Royal Hospitals
Uttoexter Road
Derby DE22 3DT

Dear Mr Sultan

Ethics Reference No: E14112016 – please always quote	
Study Title: Characterising the effects of cannabidiol, a phytocannabinoid, on the Human Cardiovascular System using Ultrasound and other non-invasive techniques.	
Chief Investigator/Supervisor: Dr Timothy England Clinical Associate Professor, Dr Saoirse O'Sullivan, Associate Professor, Vascular Medicine, Division of Medical Sciences and GEM, School of Medicine, Derby Royal.	
Lead Investigators/student: Mr. Salahaden Sultan, PhD Student, Department of Vascular Medicine, Division of Medical Sciences and GEM, School of Medicine	
Other Key Investigators: Anastasia Lazaridi ,PhD student supervised by Professor Helen Budge, Professor of Neonatal Medicine and Professor Michael Symonds, Deputy Head of School of Medicine Academic Child Health & Obstetrics & Gynaecology.	
Type of Study: PhD project	
Proposed Start Date: 01/03/2017	**Proposed End Date:** 31/08/2018 17mths
No of Subjects: 26	**Age:** 18-50 years
School: Medicine	

Thank you for notifying the Committee of amendment no 1: 19.07.2017 as follows:

- Addition of thermal imaging to optimise the research potential and enable analysis of the effect of CBD on the activation of brown adipose as detailed. This is in collaboration with colleagues in the Division of Academic Child Health: Anastasia Lazaridi ,PhD student supervised by Professor Helen Budge, Professor of Neonatal Medicine and Professor Michael Symonds, Deputy Head of School of Medicine.
- Extension of study period to ensure completion from March 2018 to August 2018.

and the following documents were received:

- FMHS REC Notice of Amendment form no 1.0: 19.07.2017
- 7 day CBD study ethics-application form dated 21.06.17
- Participant Information Sheet v3.0 21.06.2017
- 7 day CBD Research Protocol V3.0 21.06.2017
- HV consennt form v3.0 21.06.2017

These have been reviewed and are satisfactory and the study 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).

Appendix III

University of Nottingham
UK | CHINA | MALAYSIA

2. You must notify the Chair of any serious or unexpected event.

3. This study is approved for the period of active recruitment requested. The Committee also provides a further 5-year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.

4. An End of Project Progress Report is completed and returned when the study has finished (please request a form).

Yours sincerely

pp Lawrenson

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

Appendix IV

An assessment of the usefulness of thermal imaging to quantify brown adipose tissue activation in clinical settings (Health Research Authority approval)

NHS
Health Research Authority

Professor John Alcolado
Head of Division. Consultant Physician
University of Nottingham
Medical School Building, Royal Derby Hospital
Uttoxeter New Road
Derby
DE22 3NE

Email: hra.approval@nhs.net

30 March 2017

Dear Professor Alcolado

Letter of HRA Approval

Study title:	**An Assessment of the usefulness of Thermal Imaging to Quantify Brown Adipose Tissue Activation in Clinical Settings**
IRAS project ID:	**212218**
REC reference:	**17/NW/0167**
Sponsor	**University of Nottingham**

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

IRAS project ID	212218

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

IRAS project ID	212218

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **212218**. Please quote this on all correspondence.

Yours sincerely

Kevin Ahmed
Assessor

Telephone: 0207 104 8171
Email: hra.approval@nhs.net

Copy to: *Ms Angela Shone, Sponsor Contact, University of Nottingham*
Dr Teresa Grieve, R&D Contact, Derby Teaching Hospitals NHS Foundation Trust

IRAS project ID	212218

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance confirmation]	V1	20 February 2017
GP/consultant information sheets or letters [GP info letter]	V2	16 March 2017
IRAS Application Form [IRAS_Form_27022017]		27 February 2017
IRAS Application Form XML file [IRAS_Form_27022017]		27 February 2017
IRAS Checklist XML [Checklist_27022017]		27 February 2017
Letter from sponsor [Sponsor Letter]	V1	20 February 2017
Other [Statement of Activities]	1	09 March 2017
Other [Schedule of Events]	1	09 March 2017
Other [Response to provisional opinion (email)]		21 March 2017
Participant consent form [Participant consent form]	V2	16 March 2017
Participant consent form [Consent form]	V1	14 February 2017
Participant information sheet (PIS) [Participant info sheet bariatric]	V2	16 March 2017
Participant information sheet (PIS) [Participant info sheet, hyperprolactinaemia]	V2	16 March 2017
Participant information sheet (PIS) [Participant info sheet, pyrexia]	V2	16 March 2017
Participant information sheet (PIS) [Participant info sheet, NAFLD]	V2	16 March 2017
Research protocol or project proposal [Appendix 7, protocol]	V2	16 March 2017
Summary CV for Chief Investigator (CI) [CV]	V1	14 February 2017
212218 17.NW.0167 FIFO 30.03.2017		30 March 2017

Appendix IV

IRAS project ID	212218

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the, *participating NHS organisations, capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Ms Angela Shone
Tel: 01158467105
Email: BB-Sponsor@exmail.nottingham.ac.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	The sponsor has submitted the HRA Statement of Activities and intends for this to form the agreement between the sponsor and study sites. The sponsor is not requesting, and does not require any additional contracts with study sites.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional

IRAS project ID	212218

Section	HRA Assessment Criteria	Compliant with Standards	Comments
			indemnity provided by their medical defence organisation covers the activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	No application for external funding has been made. No study funding will be provided to sites, as detailed at Schedule 1 of the Statement of Activities.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
All participating NHS organisations will undertake the same study activities. There is therefore only one study site 'type' involved in the research.

IRAS project ID	212218

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.

NHS organisations in England that are participating in the study **will be expected to formally confirm their capacity and capability** to host this research.

- Following issue of this letter, participating NHS organisations in England may now confirm to the sponsor their capacity and capability to host this research, when ready to do so. How capacity and capacity will be confirmed is detailed in the Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) section of this appendix.
- The Assessing, Arranging, and Confirming document on the HRA website provides further information for the sponsor and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).

The Sponsor has correctly assessed that a Principal Investigator should be appointed at study sites

GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken

Where arrangements are not already in place, network staff (or similar) undertaking any of the research activities listed in A18 or A19 of the IRAS form would be expected to obtain an honorary research contract from one NHS organisation (if university employed), followed by Letters of Access for subsequent organisations.

IRAS project ID	212218

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix V

An assessment of the usefulness of thermal imaging to quantify brown adipose tissue activation in clinical settings (research ethics committee approval)

Appendix V

NHS
Health Research Authority

North West - Haydock Research Ethics Committee
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8012

30 March 2017

Professor John Alcolado
Head of Division. Consultant Physician
University of Nottingham
Medical School Building, Royal Derby Hospital
Uttoxeter New Road
Derby
DE22 3NE

Dear Professor Alcolado

Study title:	**An Assessment of the usefulness of Thermal Imaging to Quantify Brown Adipose Tissue Activation in Clinical Settings**
REC reference:	**17/NW/0167**
IRAS project ID:	**212218**

Thank you for your submission of 21 March 2017, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

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.

Conditions of the favourable opinion

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

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

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk 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 management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

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).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see

"Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance confirmation]	V1	20 February 2017
GP/consultant information sheets or letters [GP info letter]	V2	16 March 2017
IRAS Application Form [IRAS_Form_27022017]		27 February 2017
Letter from sponsor [Sponsor Letter]	V1	20 February 2017
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Participant information sheet (PIS) [Participant info sheet, hyperprolactinaemia]	V2	16 March 2017
Participant information sheet (PIS) [Participant info sheet, pyrexia]	V2	16 March 2017
Participant information sheet (PIS) [Participant info sheet, NAFLD]	V2	16 March 2017
Research protocol or project proposal [Appendix 7, protocol]	V2	16 March 2017
Summary CV for Chief Investigator (CI) [CV]	V1	14 February 2017

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 HRA 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 Research Ethics

Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

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

17/NW/0167	**Please quote this number on all correspondence**

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

Yours sincerely

Dr Tim S Sprosen
Chair

Email: nrescommittee.northwest-haydock@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Ms Angela Shone
Dr Teresa Grieve, Derby Teaching Hospitals NHS Foundation Trust

Appendix VI

List of Suppliers

3M, St. Paul, Minneapolis, 55144-1000, USA; https://www.3m.com/

BioTek Instruments Inc., P.O. Box 998, Highland Park, 100 Tigan Street, Winooski, Vermont, 05404, USA; https://www.biotek.com/

Boots UK Ltd., D90, Thane Rd, Nottingham, NG90 1BS, UK; https://www.boots.com

Braun GmbH, Frankfurter Str. 145, Kronberg im Taunus, 61476, Germany; https://us.braun.com/en-us

COSMED, Via dei Piani di Mt. Savello 37, Albano Laziale, Rome, 00041, Italy; https://www.cosmed.com/en/

Eppendorf Vertrieb Deutschland GmbH, Peter-Henlein-Straße 2, Wesseling-Berzdorf, 50389, Germany; https://corporate.eppendorf.com/en/

Extech Instruments, 9 Townsend West, Nashua, New Hampshire, 03063, USA; http://www.extech.com/

FLIR Systems, Inc., 27700 SW Parkway Avenue, Wilsonville, Oregon, 97070, USA; https://www.flir.co.uk/

GAMA Healthcare, 2 Regal Way, Watford, Hertfordshire, WD24 4YJ, UK; https://gamahealthcare.com/

GraphPad Software Inc., 2236 Avenida de la Playa, La Jolla, California, 92037, USA; https://www.graphpad.com

IBM Corporation, 1 New Orchard Rd, Armonk, New York, 10504, USA; https://www.ibm.com/us-en/?lnk=fcc

MathWorks, 1 Apple Hill Drive, Natick, Massachusetts, 01760-2098, USA; https://uk.mathworks.com/

Maxim Integrated, 160 Rio Robles, San Jose, California, 95134 USA; https://www.maximintegrated.com/en.html

Microsoft Corporation, One Microsoft Way, Redmond, Washington, 98052, USA; https://www.microsoft.com

PS: Power and Sample Size Calculation, by William D. Dupont and Walton D. Plummer, Jr., Department of Biostatistics, Vanderbilt University School of Medicine, Tennessee, USA; http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize#Suggested_citation

SARSTEDT AG & Co. KG, Sarstedtstraße 1, Nümbrecht, 51588, Germany; https://www.sarstedt.com/en/home/

Seca GmbH & Co, Hammer Steindamm 3-25, Hamburg, 22089, Germany; https://www.seca.com/en_hu.html

SLIK Corporation, 853 Kayama, Hidaka City, Saitama, 350-1231 Japan; http://slik.com/

Stratech Scientific Ltd, Cambridge House, St Thomas' Pl, Ely, CB7 4EX, UK; https://www.stratech.co.uk/

Electronic Temperature Instruments Ltd, Easting Close, Worthing, West Sussex, BN14 8HQ, UK; https://thermapen.co.uk/

Weiss Technik North America, Inc, Cincinnati Facility, 12011 Mosteller Road, Cincinnati, Ohio, 45241, USA; https://www.cszproducts.com/