TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

# Effects of BMI and Gender on Cold-induced Thermogenesis in Adult Humans

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Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktorin der Naturwissenschaften

genehmigten Dissertation.

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Die Dissertation wurde am 02.02.2022 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 09.05.2022 angenommen.

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# Abstract

Energy balance is achieved when energy intake equals energy expenditure (EE). When energy intake exceeds EE the consequences are overweight and obesity and, potentially, comorbidities such as diabetes and cardiovascular diseases. Resting energy expenditure (REE) is highly variable between individuals and although this variance is determined by many known factors, 30 % of the REE is still unexplained. In the need of finding strategies to counter obesity and modulate EE, researchers rediscovered an organ that burns energy in form of heat: brown adipose tissue (BAT). BAT gets rapidly activated during cold as part of non-shivering thermogenesis (NST) which leads to an increase in REE. However, this increase in REE and overall NST seems to be variable and the factors impacting this metabolic answer are not fully elucidated yet.

To investigate the variability in thermogenic response, the described study was developed. 173 healthy men and women were assigned to a short-term moderate cold exposure (CE) experiment to assess the body's response regarding energy metabolism and metabolism in general. CE temperature was determined individually in order to maximize NST and to minimize muscle shivering. Focus of the study was the change in REE before and after CE by indirect calorimetry. Furthermore, blood and subcutaneous adipose tissue samples were collected before and after CE to investigate changes in metabolic parameters and gene expression. Skin temperature was assessed throughout the experiment.

Mild CE led to a significant increase in REE in lean participants by 6.5 %, whereas there was no significant change in individuals with overweight and obesity. The increase in REE was comparable between males and females. The increase in REE was strongly associated with exposure temperature, which was significantly higher in women compared to men. However, adjusting for exposure temperature as well as fat free mass and age did not reveal any differences between genders. Skin temperature of the supraclavicular area decreased significantly in lean men but not in women, whereas BMI had no effect on these outcomes. Lean females displayed stronger changes in metabolites during CE such as a stronger decrease in plasma glucose and leptin levels. mRNA analysis of subcutaneous adipose tissue showed a significant upregulation in Cell death activator (CIDEA) in females with overweight and obesity, but not in males. Other browning markers, such as UCP1, PRDM16 and PGC1a remained unchanged in both genders. While there is a clear impairment in thermogenesis in overweight and obesity, the results show that males and females have a comparable thermogenic response at least regarding REE to short-term CE.

# Zusammenfassung

Man spricht von einem Energiegleichgewicht, wenn die Energieaufnahme und der Energieverbrauch sich die Waage halten. Falls jedoch die Energieaufnahme den Energieverbrauch übersteigt führt dies auf lange Sicht zu Adipositas, die mit Komorbiditäten, wie Diabetes oder Herz-Kreislauferkrankungen einhergehen kann. Der Ruheenergieverbrauch kann zwischen Individuen stark variieren, und obwohl viele Determinanten für diese Variabilität bereits erforscht sind, so bleiben 30 % davon weiterhin unerklärt. Auf der Suche nach Strategien im Kampf gegen Adipositas und um den Energieverbrauch zu modulieren, haben Wissenschaftler ein Organ wiederentdeckt, das Energie in Form von Wärme verbrennt: das braune Fettgewebe. Braunes Fettgewebe ist Teil der „zitterfreien“ Thermogenese (NST) und wird durch kalte Temperaturen stimuliert was zu einem Anstieg im Ruheenergieverbrauch führt. Dieser Anstieg und die Kapazität von NST scheinen sehr variabel zu sein und die hierfür verantwortlichen Faktoren sind noch weitestgehend unklar.

Die hier beschriebene Studie wurde konzipiert, um diese Variabilität in der kälteinduzierten Thermogenese zu untersuchen. 173 gesunde Frauen und Männer nahmen an einem kurzen und milden Kälteexperiment teil, um die Reaktion des Metabolismus und Körpers allgemein zu ergründen. Die Temperatur der Kälteexposition wurde individuell ermittelt für eine Maximierung von NST und zur Minimierung von Zittern. Der Fokus lag hier auf den kälteinduzierten Veränderungen im Ruheenergieverbrauch. Zudem wurden vor und nach Kälteexposition Blut- und subkutane Fettgewebsproben entnommen, um Veränderungen von zirkulierenden Metaboliten und der Genexpression im Gewebe zu erfassen. Oberflächentemperaturen wurden während des gesamten Experimentes erfasst.

Die Kälteexposition führte zu einem Anstieg des Ruheenergieverbrauchs bei schlanken Personen um 6,5 %, wohingegen Probanden mit Übergewicht und Adipositas keine signifikante Veränderung zeigten. Der Anstieg im Ruheenergieverbrauch war stark mit der Expositionstemperatur assoziiert, die bei Frauen signifikant höher lag als bei Männern. Es gab keinen Geschlechtsunterschied im Anstieg des Ruheenergieverbrauchs, auch nicht unter Berücksichtigung dieser Temperatur, fettfreier Masse und Alter. Die Oberflächentemperatur im supraclaviculären Bereich sank signifikant bei Männern aber nicht bei Frauen und war unabhängig vom BMI. Schlanke Frauen zeigten stärkere kälteinduzierte Veränderungen des Metabolismus, wie zum Beispiel einem stärkeren Abfall bei Blutzucker- und Leptinwerten. Die Analyse von mRNA im subkutanen Fettgewebe zeigte eine Hochregulierung von Cell death activator (CIDEA) bei

Frauen mit Übergewicht und Adipositas, aber nicht bei Männern. Andere browning Marker, wie UCP1, PRDM16 und PGC1a zeigten keine signifikante Veränderung nach Kälteexposition. Die Ergebnisse zeigen eine Verminderung der thermogenetischen Antwort bei Übergewicht und Adipositas, die nicht geschlechterspezifisch ist, zumindest was den Anstieg des Ruheenergieverbrauchs nach kurzer, milder Kälteexposition betrifft.

# Abbreviations

| Abbreviation | Full name |
|---|---|
| ANOVA | Analysis of variance |
| AR | Adrenergic receptor |
| ARID5B | AT-rich interactive domain-containing protein 5B |
| ATP | Adenosine triphosphate |
| BAT | Brown adipose tissue |
| BIA | Bio impedance analysis |
| BMI | Body mass index |
| CE | Cold exposure |
| CIDEA | Cell death-inducing DNA fragmentation factor-like effector A |
| CRP | C-reactive protein |
| DRKS | Deutsches Register Klinischer Studien |
| DWD | Deutscher Wetterdienst |
| EDTA | Ethylenediaminetetraacetic acid |
| $FeCO_2$ | Fractional content of expired $CO_2$ |
| FFM | Fat free mass |
| FREECE | Effect of FTO on Resting Energy Expenditure after a defined Cold Exposure |
| $fT_3$ | Free triiodothyronine |
| FTO | Fat mass and obesity associated |
| HOMA-IR | Homeostatic model assessment of insulin resistance |
| IRX3/IRX5 | Iroquois homeobox protein 3/ Iroquois homeobox protein 5 |
| kcal | Kilo calories |
| mRNA | Messenger ribonucleic acid |
| NEFA | Non-esterified fatty acid |
| NST | Non-shivering thermogenesis |
| PET/CT | Positron emission tomography / computed tomography |
| PGC1a | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| REE | Resting energy expenditure |
| RQ | Respiratory quotient |
| scWAT | Subcutaneous white adipose tissue |
| SERCA | Sarcoplasmic reticulum $Ca^{2+}$-ATPase |
| SNP | Single nucleotide polymorphism |

| | |
|---|---|
| SNS | Sympathetic nervous system |
| ST | Shivering thermogenesis |
| TEE | Total energy expenditure |
| TG | Triglycerides |
| TN | Thermoneutrality |
| TRP | Transient receptor potential |
| TRPV1 | TRP vanilloid receptor 1 |
| TUM | Technical university of Munich |
| UCP1 | Uncoupling protein 1 |
| $VCO_2$ | Volume of expired carbon dioxide |
| $VO_2$ | Volume of inspired oxygen |
| v/v | Volume per volume |
| VAT | Visceral adipose tissue |
| WZW | Wissenschaftszentrum Weihenstephan |

# 1 Introduction

## 1.1 Components of energy expenditure

When energy intake exceeds energy expenditure (EE) the plus of calories is stored as fatty acids in adipose tissue and will ultimately lead to overweight and obesity. Assessing EE is important as energy balance defines weight gain or loss. EE is composed of different parts that sum up to total EE (TEE). Resting EE (REE) is the energy expenditure a body spends for basal needs to maintain its function (e.g. breathing and organ functions), as well as for growth and reproduction (Müller and Bosy-Westphal, 2013; Poehlman et al., 2003). REE is measured in a fasted, resting and wake state without any movement of the muscles at thermoneutrality and is defined as obligatory energy expenditure (Lowell and Spiegelman, 2000). Additionally, voluntary movements of the body (activity energy expenditure, AEE) as well as energy that is needed for eating, digestion and metabolism, called diet-induced thermogenesis (DIT), contribute to TEE (Westerterp, 2013). At maximum, DIT can acutely increase the metabolic rate up to 40 % (Lowell and Spiegelman, 2000). In general, AEE and DIT represent 30-40 % and 10 % of TEE, respectively (Westerterp, 2017). As TEE is highly variable even intraindividually (Champagne et al., 2013; Donahoo et al., 2004), depending on the energy balance of each day, REE is preferred when investigating general EE. 60 % of TEE are determined by REE (Black et al., 1996; Levine, 2005). Assessing REE is important as energy balance defines weight gain or loss. Strikingly, a low REE is considered as a risk factor for the development of obesity (Wijers et al., 2009).

## 1.2 Factors influencing EE

Assessing REE is a more reliable methodological approach compared to TEE, as it is less subjected to daily variation unlike physical activity or dietary behavior (Levine, 2002). However, there are many factors that can influence REE short- and long-term, respectively. By far the biggest shaper of REE is the body's composition. Different components influence the REE at different expenses. Skeletal musculature alone comprises 40 % of mammalian body mass and accounts for 30 % of REE (Zurlo et al., 1990). Total fat free mass (FFM) is the main contributor of REE with 63 % (Johnstone et al., 2005; Ravussin et al., 1986). Adipose tissue or fat mass comprises only 4.5 kcal/kg fat but due to its high mass it explains 6.3 % of total REE (Javed et al., 2010; Johnstone et al., 2005). As males possess more FFM compared to females, they have a

significantly higher REE (Donahoo et al., 2004). Also, REE decreases with age caused by a shift from FFM to a higher fat/muscle ratio (Keys et al., 1973; Tzankoff and Norris, 1977).

Hormonal fluctuations are also responsible for the variance in REE. For instance, triiodothyronine is known to elevate oxygen consumption, heat production and thus metabolic rate (Al-Adsani et al., 1997; Astrup et al., 1992). In extreme cases of hyper- or hypothyroidism, energy expenditure can be doubled or cut in half, respectively (Bianco and McAninch, 2013). It is highly discussed, whether REE is influenced by the menstrual cycle with women displaying higher values during the luteal phase (Henry et al., 2003; Melanson et al., 1996; Solomon et al., 1982).

Tightly connected with physiology, lifestyle contributes to REE albeit in a more acute fashion. For instance, high intensity interval training increases REE significantly for up to 48 h after the actual workout, a phenomenon called excess post-exercise energy consumption (Burt et al., 2014; Paoli et al., 2012). Furthermore, dietary habits shape REE and also the rate of fat oxidation (Madzima et al., 2014; Mikkelsen et al., 2000).

Besides lifestyle, other determinants of REE are known that are not caused by the subject itself. Ambient temperature, for example, can influence REE as well as noises and music (Snell et al., 2014; Turner et al., 2016). Additionally, methods of measuring REE can have an impact on the outcome (Cooper et al., 2009). Haugen et al. demonstrated that even the time of day, in this case morning versus afternoon, significantly influences REE (Haugen et al., 2003).

All these factors have to be taken into account for assessing the REE of an individual in order to truly define their metabolic rate and, thus, energy demand for maintaining a stable body weight.

### 1.3 Unexplained variability of REE

Although there has already been a lot of scientific work in investigating REE and its determinants, the variability between individuals is still not fully elucidated yet. Most of the interindividual variability can be explained by differences in FFM, fat mass (FM) and age (Javed et al., 2010; Johnstone et al., 2005), but 26 % of the variance is still unexplained. Some of this unknown variance may be explained by genetics. In fact, BMI is influenced by heritability in a range from 40 to 80 % (Speliotes et al., 2010). Around 140 polygenic loci are known that are associated with obesity and BMI such as variants in the melanocortin 4 receptor (MC4R) and fat mass and obesity associated (FTO) gene (Locke et al., 2015; Pigeyre et al., 2016).

FTO is associated with an increased risk for the development of obesity (Reddon et al., 2016). It displays the strongest genome-wide association in its introns 1 and 2 with 89 common variants in the European population (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007). Due to the high variety of single nucleotide polymorphisms (SNP) within this gene locus, no causal variant for the phenotype is detected yet.

As the prevalence of obesity and its comorbidities has reached epidemic dimensions, new strategies to counteract weight gain in the general population are necessary, as prevention still seems to be in a fledgling stage around the globe (Collaborators, 2017; Roberto et al., 2015). Strategies that aim to reduce energy intake, either by lifestyle modification or by medication, have been proven useful, but with side effects (Silverstone, 1992). Furthermore, weight loss is accompanied by a decrease in REE which complicates weight maintenance (Hall and Kahan, 2018). Another possibility to reduce weight is by means of increasing energy expenditure. Clearly, there are medications that increase EE, but have considerable side effects such as on the cardiovascular system (Chen et al., 2020). Exercise programs are possible, but depend on the compliance and health status of individuals. Therefore, new strategies to curb EE are warranted.

## 1.4 Thermogenesis and BAT

A possible strategy to combat weight gain is non-shivering thermogenesis (NST). As endotherms, humans keep their body core at a constant temperature, independent of the surrounding climate. Body hair and subcutaneous fat are the first protectors of core temperature, but if this is not sufficient, adaptive thermogenesis has to produce heat. Adaptive thermogenesis consists of two components: shivering and non-shivering thermogenesis (ST and NST, respectively). ST is a powerful tool facilitated by skeletal muscles which start to contract involuntarily, when skin temperature drops below a certain threshold (Blondin et al., 2014a). At maximum, muscles can increase oxygen consumption by approximately 40 % to produce heat energy (Eyolfson et al., 2001). However, ST is energy consuming and disturbing locomotor activity which is difficult to sustain over longer periods.

If heat loss exceeds heat production the risk of hypothermia increases (Haman, 2006; Meigal, 2002; Palmer and Clegg, 2017). NST is heat energy that is produced without involvement of muscle contractions. For longer periods at cold temperatures, NST is indispensable for defending the body to maintain endothermy. In human newborns and in small mammals, such as rodents, a specialized organ contributes to this NST: brown adipose tissue (BAT). For a long time,

researchers believed that active BAT is restricted to newborn humans and disappears during development (Cannon and Nedergaard, 2004). Due to their small body volume to surface ratio, increased heat loss is a consequence that cannot be compensated by ST alone (Palmer and Clegg, 2017). Therefore, a permanent low grade BAT activation is necessary. For example, human infants are able to double their EE without shivering (Himms-Hagen, 1995). Adult humans on the other site do not need such effective mechanisms to maintain their core temperature.

A breakthrough occurred in 2009, when active BAT was observed in human adults by several independent groups. During [$^{18}$F] fluorodeoxyglucose positron emission coupled tomography-computed tomography ($^{18}$F-FDG-PET/CT) scans active BAT depots were visible in the neck and supraclavicular area, especially after cold exposure (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Saito et al., 2009; Virtanen et al., 2009). Since then, publications investigating BAT activity in humans are spreading up like mushrooms, resulting in a panoply of information, but there is still a number of open questions (Rosen and Spiegelman, 2014).

## 1.5 Physiology and characteristics of BAT

In contrast to white adipocytes (diameter of 25-200 µm), brown adipocytes are rather small (15-60 µm) and packed with mitochondria and many small lipid droplets instead of one big droplet, hence they are called multilocular (Jeanson et al., 2015). Brown adipose tissue (BAT) displays a brownish color which is explained by the high iron content of the highly abundant mitochondria (Cinti, 2001). The physiological function of BAT is quite different from white adipocytes, as it burns fat and thereby releases energy in form of heat instead of storing it. BAT gets rapidly activated by the sympathetic nervous system (SNS) via adrenergic factors that act on correspondent receptors on the brown adipocyte's surface (Tupone et al., 2014). Consequently, free fatty acids are released by lipolysis which trigger the activation of uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004). Due to its multilocular morphology, the increased surface of lipid droplets in BAT simplifies lipolysis and thus mobilization of fuel (Darcy and Tseng, 2019).

UCP1 is the central player in this process which separates respiratory chain activity from ATP synthesis by redirecting protons through the inner mitochondrial membrane creating a proton leak (Fig. 1). The resulting proton-motive force is released as heat thus making BAT a thermogenic organ (Cannon and Nedergaard, 2004; Fedorenko et al., 2012). Due to its distinct vascularization, not only substrates are easily transported to the adipocytes, but also generated heat can be distributed into the system (Cao, 2013). Free fatty acids predominantly derived from intracellular

triglyceride lipolysis serve as main fuel for BAT (Blondin et al., 2017a), but also circulating glucose, lipids and other substrates are used (Coolbaugh et al., 2019; Orava et al., 2011; Ouellet et al., 2012; Townsend and Tseng, 2014; Yoneshiro et al., 2019).

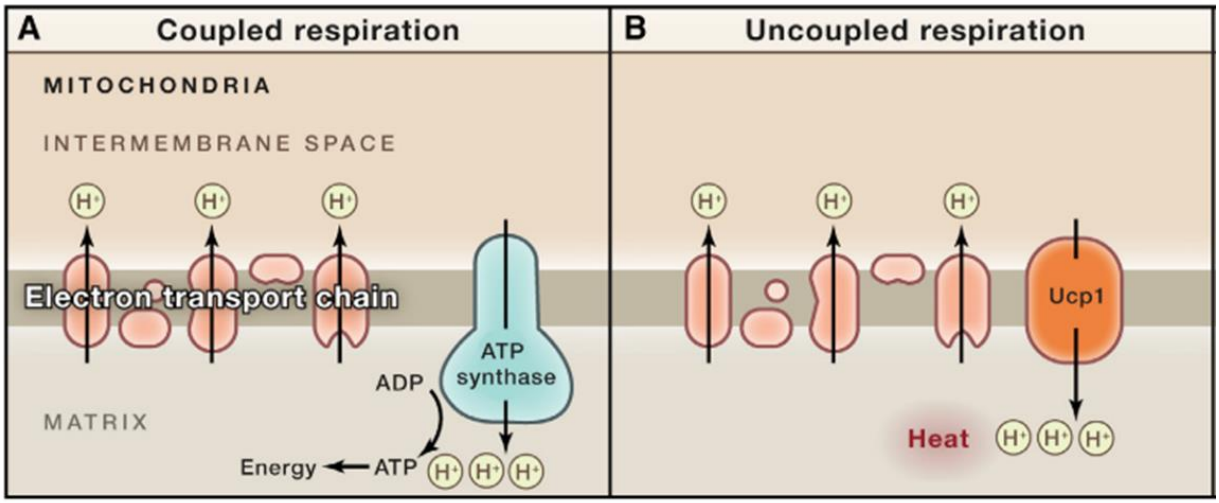


**Figure 1 Mechanism of UCP1 on respiration** A) coupled respiration in the absence of uncoupling protein 1 (UCP1) which generates energy in form of adenosine triphosphate (ATP); B) thermogenesis is uncoupled by UCP1, by transporting protons back to the mitochondrial matrix without the involvement of ATP synthase; energy is released by heat; adapted from (Chen et al., 2016)

Classically and physiologically, BAT is activated by cold which is sensed by the body in form of thermoreceptors in the skin. This process leads to the activation of transient receptor potential channels (TRP) via the brain. TRP channels are opened perpetually and react to a plethora of stimuli, for example redox reagents, ATP, and sensory stimuli leading to an activation of the SNS (Liu et al., 2018). BAT is highly innervated by efferent fibers of the sympathetic nervous system (SNS) whose release of noradrenaline or $\beta_3$−adrenoceptor agonists (AR) binds to the $\beta_3$–adrenergic receptors residing on the brown adipocyte surface. This leads to an initiation of a signaling cascade which in turn activates lipolysis of intracellular triglycerides (Collins et al., 2004; Saito, 2014). Furthermore, gap junctions between neighboring adipocytes probably amplify the signal sent by noradrenergic nerves (Cannon and Nedergaard, 2004; Himms-Hagen, 1989). The crosstalk of brain and BAT becomes even more evident considering the fact that bilateral denervation of BAT leads to an impairment of thermogenesis and whitening albeit the vasculature is still intact (Blaszkiewicz et al., 2019).

The highest proportion of human adult BAT is in the supraclavicular area, a region above the collarbone (Leitner et al., 2017). However, BAT is found in several depots of mice and humans (Hankir and Klingenspor, 2018), but their prevalence and composition can vary. Whereas murine BAT consists of true classical brown adipocytes, human BAT is rather a mixture out of brown, white and so called beige adipocytes (Jespersen et al., 2013; Sharp et al., 2012; Shinoda et al., 2015; Wu et al., 2012).

## 1.6 Beige Adipocytes

The so-called “beige” adipocytes reside within white adipose tissue (WAT), hence they are also called “brite” (brown in white) adipocytes (Petrovic et al., 2010; Schulz et al., 2011). Beige adipocytes can adapt either white or brown adipocyte properties, depending on the surrounding conditions (Fig. 2).

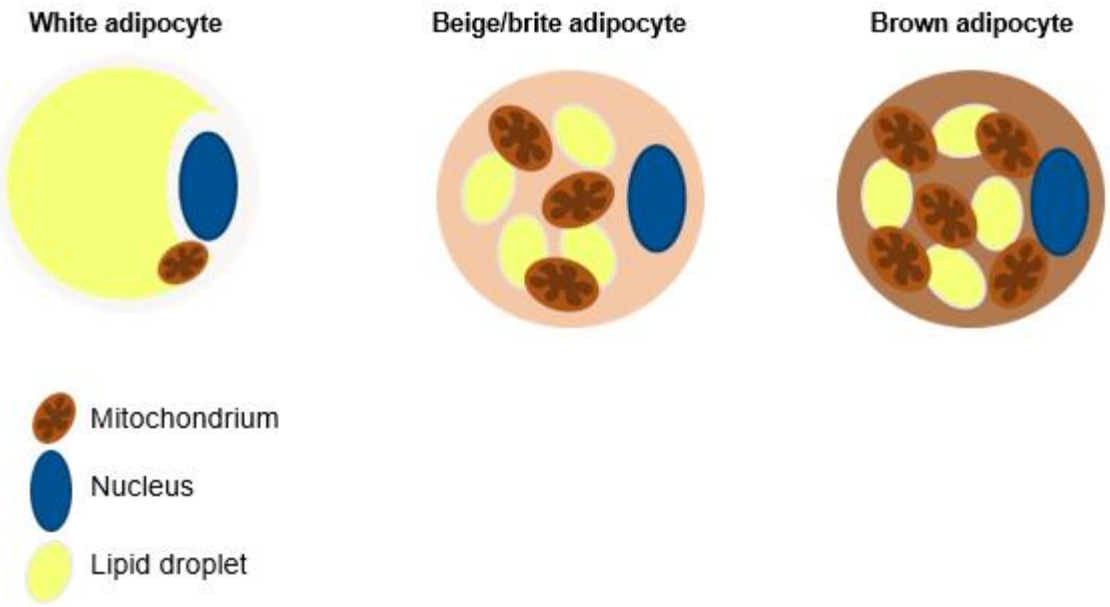


**Figure 2: Morphology of white, beige and brown adipocytes.** White adipocytes possess one big lipid droplet (unilocular) and only few mitochondria, while brown adipocytes consist of many small lipid droplets (multilocular) and have a high number of mitochondria for thermogenesis. Beige/brite adipocytes resemble white adipocytes until proper stimuli induce the browning which leads to a brown-like morphology.

Regarding their developmental lineage, beige adipocytes seem to have a different origin compared to brown adipocytes. Whereas classical brown adipocytes share $Myf5^{+}$ and $Pax7^{+}$

signatures with skeletal muscle cells, at least a subset of beige adipocytes originates from mesenchymal precursors that also give rise to smooth muscle cells (Long et al., 2014). Two hypotheses exist regarding the development of beige adipocytes: a) the de novo differentiation from precursor cells (Wang et al., 2013) or b) the transdifferentiation from mature white adipocytes (Barbatelli et al., 2010; Lee et al., 2012). Recently it was shown that at least the latter is true for mature scWAT in humans in a novel *in vitro* experiment (Harms et al., 2019). Either way, beige adipocytes display a white phenotype unless they are exposed to proper stimuli. Like classical brown adipocytes, beige adipocytes react to adrenergic stimuli but by changing its expression pattern in favor of UCP1 and other thermogenic genes (Kajimura et al., 2015). Brown adipocytes, on the other hand, express high UCP1 levels independent of stimuli (Kalinovich et al., 2017). Beige fat cells can reach UCP1 mRNA levels comparable to BAT, but only the expressed UCP1 protein can induce thermogenesis. Merely 8 % of UCP1 protein content of classical BAT can be found in fully stimulated beige adipocytes in mice (Kalinovich et al., 2017). The process of browning is reversible: it was shown that at least in mice, the beige phenotype diminishes after two weeks without adrenergic stimulation via autophagy induced mitochondrial clearance (Altshuler-Keylin et al., 2016). Interestingly, classical brown adipocytes are resistant to those temperature changes (Roh et al., 2018).

The capability of browning decreases with age, just like the occurrence of BAT in general (Berry et al., 2017; Cypess et al., 2009). Theoretically, this reduced activity leads to a decreased EE and may promote adiposity which might explain the age-related shift towards a higher fat percentage (Yoneshiro et al., 2011a).

## 1.7 Strategies to exploit thermogenesis

There is an increasing interest in augmenting BAT volume and its activity and, thus, REE by using different approaches, such as food agents. For instance, capsaicin, the pungent substance common to plants of the genus *Capsicum*, acts on the TRP vanilloid receptor 1 (TRPV1) of sensory neurons in the oral cavity and gastric mucosa, which in turn sense the pungency. At this point, the following cascade is comparable to that during cold sensation (Saito, 2014). Capsinoids are structurally very similar to capsaicin, but they lack almost completely the pungent component (Kobata et al., 1999). Nevertheless, they are capable of activating the TRPV1 leading to an activation of BAT activity and also browning of adipocytes in the murine system (Baskaran et al., 2016; Ohyama et al., 2016; Shintaku et al., 2012). In humans with considerable BAT activity, these

substances can increase EE by 36.3 kcal/h (Yoneshiro et al., 2012). It is of note that relatively high amounts of these agents, over 10 mg/d, have to be consumed to reach such results (Okla et al., 2017). Other food agents, such as grains of paradise (*Aframomum melegueta*), resveratrol or catechin-rich beverages are potential activators of BAT or browning (El Hadi et al., 2019; Nirengi et al., 2016; Sugita et al., 2014). Even the bitter components of the domestic hops (*Humulus lupulus*) promote UCP1 augmentation, although the exact underlying mechanism is not elucidated yet (Morimoto-Kobayashi et al., 2015).

The $\beta_3$-agonist mirabegron which is used to ameliorate overactive bladder syndrome also increases REE in accordance with $^{18}$FDG uptake into BAT as well as induction of thermogenesis in beige fat cells (Cypess et al., 2015; Finlin et al., 2018). Sildenafil, originally used to treat erectile dysfunction, inhibits phosphodiesterase type 5 (PDE5). PDE5 suppresses lipolysis in BAT and by its deactivation browning is promoted (Li et al., 2018a). Unfortunately, both medications can affect the cardiovascular system which limits their possible application for weight management. This may be due to $\beta_3$-ARs within the human heart (Michel et al., 2011) or also by the low specificity of agents on the different ARs. For instance, $\beta_3$-AR knockout mice do not display any impairment of REE following cold exposure, probably due to compensation by $\beta_1$- and $\beta_2$-ARs (Evans et al., 2019).

Newer approaches try to bypass the adrenergic receptors and to act further downstream of the signaling cascade on G protein-coupled receptors in order to activate UCP1 (Schnabl et al., 2019). Secretin, a hormone which is secreted prandially by the gut, activates thermogenesis without involvement of the SNS (Li et al., 2018b). Furthermore, exercise can not only increase EE by the work which is done by skeletal muscle and elevated metabolism, but also by inducing WAT browning. By training, the two myokines irisin and meteorin-like (METRNL) are upregulated and can promote beige adipocyte development (Lee et al., 2014a; Rao et al., 2014). Lactate, produced by anaerobic glycolysis during exercise, also contributes to WAT browning (Carrière et al., 2014).

However, the real effect of these treatments can hardly be assessed, if the high variability in BAT activity and prevalence is not understood. Furthermore, the understanding of the circumstances of browning in beige adipocytes is still not fully elucidated. Therefore, there is a need to investigate which factors influence the activation of brown fat and browning to develop proper treatments for certain disease conditions.

## 1.8 Aim of the thesis

This thesis represents a subanalysis of an ongoing study which aims to detect possible contributors to the high variability in REE and also thermogenic response.

In 2015, ae SNP (rs1421085) in the *FTO* gene locus was identified that is highly related to adipose differentiation in adipose tissue and, thereby, may at least partially explain the higher BMI in risk allele carriers. Claussnitzer et al. identified that the switch from thymine to cytosine leads to a disruption in the ARID5B motif. By investigating chromatin interactions, it was possible to identify eight target genes of *FTO* whose expression is influenced by this SNP (Claussnitzer et al., 2015). Amongst them were IRX3 and IRX5, two master regulators involved in adipocyte development. Mice, deficient in IRX3, were shown to have a reduction of 25 to 30 % in body weight, mainly explained by less fat mass (Smemo et al., 2014). Investigating genome-wide expression patterns showed that IRX3 and IRX5 play major roles in the repression of thermogenesis. In non−risk allele carriers, the ARID5B motif represses IRX3 and IRX5 thus facilitating the thermogenic program of preadipocytes leading to a development of beige adipocytes. Due to the nucleotide switch in risk allele carriers, IRX3 and IRX5 repress the browning process and fewer beige adipocytes can develop resulting in a shift to white adipocytes, a reduced thermogenesis and increased lipid storage. *In vitro* experiments showed that preadipocytes of risk allele carriers showed a reduced reaction to adrenergic stimuli compared to healthy controls (Claussnitzer et al., 2015).

To shed light on these questions, we elaborated a human clinical study under controlled and defined conditions. We named it FREECE as an acronym for “the Effect of **F**TO on **R**esting **E**nergy **E**xpenditure after defined **C**old **E**xposure”.

The primary aim of the study was to assess the changes in REE and circulating metabolites after a short and mild cold exposure and to elucidate whether these responses differ between genotypes.

The study is still ongoing to collect sufficient data for analyzing genotype effects on thermogenesis. With regard to this thesis, we analyzed the data of 170 participants that were recruited up to that time point. It is still not elucidated how feasible approaches aiming to activate BAT and browning are for different groups in order to control body weight. The incorporated publications (Manuscripts 1 and 2) investigated the effect of gender and BMI on the thermogenic response in healthy adults. Furthermore, changes in mRNA expression of subcutaneous WAT in subjects with overweight and obesity were examined to elucidate the capacity of browning in those individuals. The results

should help to get a better understanding on how NST works in different cohorts that should be considered in future weight management studies.

# 2 Study Design and Methods

## 2.1 Project Fundamentals

The FREECE study is a project conducted at the Chair of Nutritional Medicine and it was initially funded by the Institute for Food & Health (ZIEL) of the Technical University of Munich (TUM). It is a cross-sectional, uncontrolled, single-arm study. The study centers were located at the Technical University of Munich, one on Campus D in Munich and the other in Freising-Weihenstephan. The study protocol was reviewed and approved by the Ethics Commission of the Faculty of Medicine of TUM (project no. 236/16). All personal data of the participants was anonymized to ensure privacy. Furthermore, the subjects were carefully informed on the methods and risks of the study and gave their written informed consent to participate, before starting the examination. Participants received a small honorarium to compensate for the time they spent to take part in the study. The study was registered at the German Clinical Register DRKS with the number DRKS00010489.

Experiments were conducted from October 2016 to May 2017, from October 2017 to May 2018 and from October 2018 to April 2019. The study is still ongoing and recruitment started in November 2019.

## 2.2 Study population

A total of 173 participants was recruited on a voluntary basis starting in August 2016. The study was advertised via bulletins on several campuses of TUM, posts on the faculty-owned website and Facebook page. Furthermore, participants were recruited by sending an e-mail to the students at the Wissenschaftszentrum Weihenstephan (WZW). The participants' eligibility was assessed with a detailed screening questionnaire that was conducted during a phone call. Volunteers were only assigned to the study after checking the inclusion and exclusion criteria (Table 1), after being informed on the purpose and possible risks of the study, and after giving written informed consent.

**Table 1: Inclusion and exclusion criteria for eligibility**

| Inclusion criteria | Exclusion criteria |
|---|---|
| Age over 18 years | Severe disease |
| BMI between 18.5 and 50 kg/m² | Diabetes mellitus |
| Non-smoker | Professional athletes (more than 10 h of high intensity training per week) |
| Healthy | Breastfeeding or lactating women |
| Caucasian origin | Pregnant women |
| Written informed consent | Fluctuations in body weight of more than 3 kg within the last 3 months |

BMI: Body Mass Index;

After checking the inclusion and exclusion criteria, participants were asked to visit the study unit once before the measurements in order to pick up the materials (checklist and dietary protocol). Furthermore, this offered the opportunity for the participants to meet the research team and the environmental setting beforehand. Any questions regarding the study procedures could be asked at this point or later via e-mail or phone call. In case of a potential fat biopsy, participants were invited one or two weeks prior to the examination for an informative conversation with the study physician. Furthermore, there was a clinical examination of the subjects and blood was taken in order to rule out a dysfunction of the coagulation system.

Three days prior to the appointment, participants were asked to fill out a dietary protocol to assess their eating habits. They recorded all foods and beverages consumed, the method or means of preparation, the quantity or the estimated portion sizes. In the presence of the participant, the researchers reviewed the dietary recall for completeness and any necessary clarifications were made. They should keep an eye on their checklist to avoid any circumstances that could hamper the examinations. Participants had to refrain from physical activity 24 h prior to the study, as well as drinking beverages containing caffeine (Fig. 3).

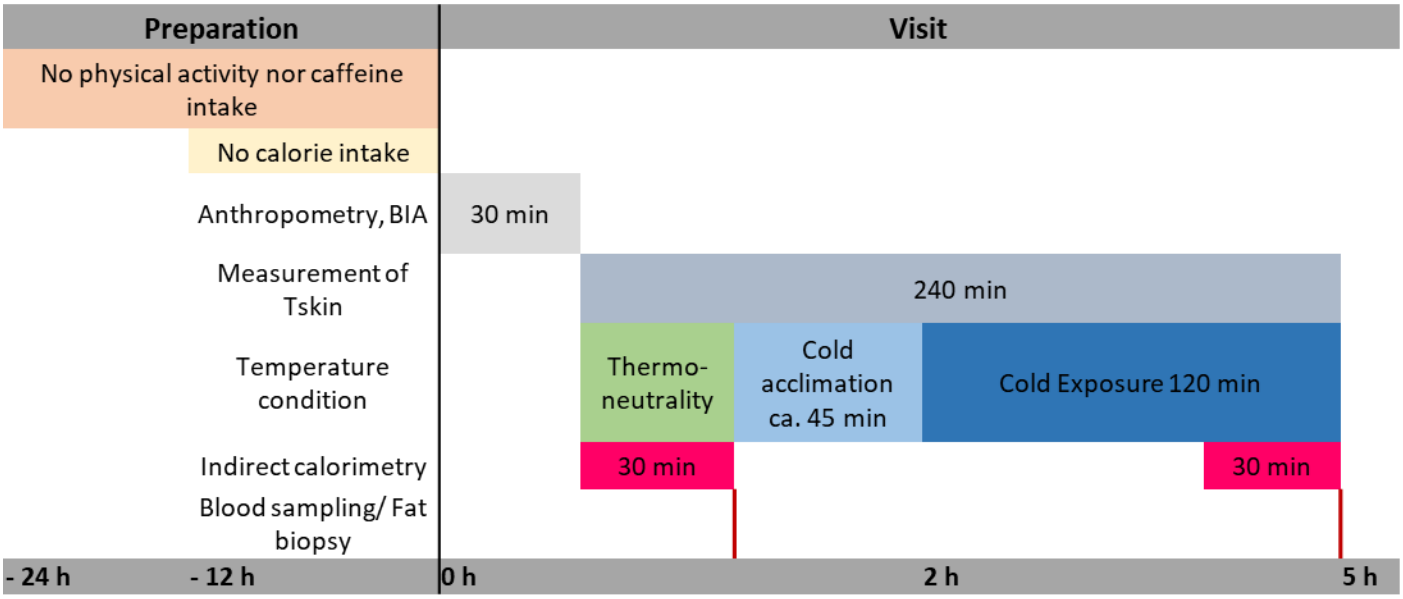


**Figure 3: Timetable of the intervention** BIA, Bioimpedance Analysis; Tskin, Skin temperature; Tcore, Body core temperature;

## 2.3 Phenotyping

Anthropometric parameters were determined according to established standard operating procedures (SOPs). Measurements were taken in the morning between 7:30 am and 8:30 am after an overnight fast. Body height (cm) was measured using a stadiometer (Seca, Hamburg, Germany) to the nearest 0.1 cm. Body weight and body composition were measured using the TANITA Body Composition Analyser Type BC-418 MA (Tanita Europe GmbH, Sindelfingen, Germany). In Freising, the SECA mBCA 515 was used additionally. Measurements were performed barefoot in underwear and after emptying the urinary bladder. BMI was calculated by dividing weight in kg by height in meter squared (kg/m²). Waist was assessed with a soft measuring tape midway between the lowest rib and the iliac crest. Hip circumference was measured at the widest part of the gluteal region. Both was measured to the nearest 0.1 cm.

Systolic and diastolic blood pressure and pulse rate were assessed in triplicates in a sitting position after five minutes resting time using Omron M8 comfort (Mannheim, Germany).

## 2.4 Temperature assessment

Skin temperature was measured by using iButtons (DS- 1921 H, Thermochron; Maxim, Dallas, United States) which are wireless thermosensors that were attached to the skin by adhesive tapes. The validity of these sensors regarding this task was already tested elsewhere (van Marken Lichtenbelt et al., 2006). Eight of these sensors were required to assess the overall skin temperature according to ISO 9886 (ISO9886, 2004).

$$T_{skin} = T_{forehead} * 0.07 + T_{shoulder} * 0.175 + T_{chest} * 0.175 + T_{upper\ arm} * 0.07 + T_{lower\ arm} * 0.07 + T_{hand} * 0.05 + T_{upper\ leg} * 0.19 + T_{lower\ leg} * 0.2$$

**Equation 1: Calculation of the mean skin temperature** according to DIN ISO 9886:2004-05

The ninth sensor was attached to the supraclavicular fossae to assess temperature changes at this specific site. These iButtons collected data once a minute with a resolution of 0.125 °C. Data was transferred to a separate EXCEL sheet for each participant.

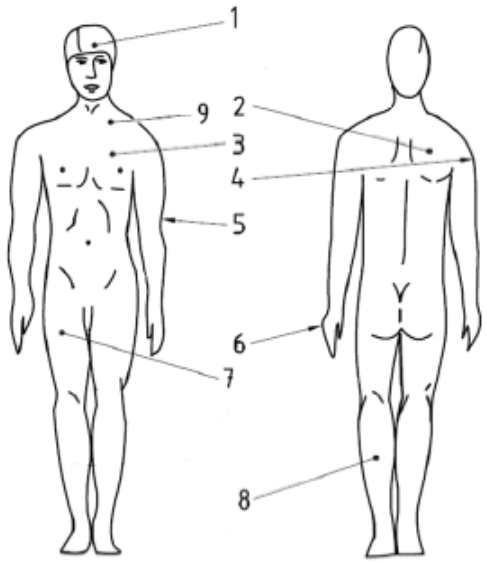


**Figure 4: Skin measurement locations** 1-8: defined by ISO 9886 to calculate mean skin temperature; 9: supraclavicular region; 1) forehead 2) right shoulder blade 3) left chest 4) left upper part of the upper arm 5) left lower part of the upper arm 6) back of left hand 7) right upper leg 8) left lower leg + 9) above the collar bone

Ambient temperature data was obtained from the website of the German Meteorological Service (Deutscher Wetterdienst, www.dwd.de) for the study site in Munich. For Freising, data was obtained from the meteorological station in Freising (https://www.wetter-weihenstephan.de/) which is run by the DWD and the LfL (Bayerische Landesanstalt für Landwirtschaft).

## 2.5 Indirect calorimetry

REE was assessed by using a canopy hood system by Cosmed (COSMED Quark RMR, Fridolfing, Germany). Each day, the cart was calibrated after a 30 min warm-up phase. The flow calibration was performed using a 3 L calibration syringe at the beginning of every test day. Gas was analyzed before each indirect calorimetry measurement following the manufacturers' instructions.

Before measurements, participants were bedded between two water perfused mattresses in a supine position and were asked to rest for 5 minutes. Afterwards, the canopy was placed above the participants' head and the coat was gently tucked in underneath the body, to avoid any leakage of air. During measurements, the participants were not allowed to talk, to move or to fall asleep. When starting the measurement, the individual breathing volume was assessed using a flow meter. This is required to achieve a steady state, where variances in the parameters are as low as possible. Regarding this, the critical parameter was $FeCO_2$ (fraction of expired carbon dioxide), which should range between 0.9 and 1.1 %. This step required around 5 to 10 min. Afterwards, data was collected for 30 min.

The conversion of the measurements of $VO_2$ (consumption of oxygen (ml/min)) and $VCO_2$ (production of $CO_2$ (ml/min)) to energy expenditure [kcal/day] was done by application of the shortened Weir equation (Weir, 1949):

$$RMR\left[\frac{kcal}{24\ h}\right] = \left[3.9 * VO_2\left(\frac{ml}{min}\right) + 1.1 * VCO_2\left(\frac{ml}{min}\right)\right] * 1.44$$

**Equation 2: Shortened equation for calculation of resting metabolic rate (RMR)** according to Weir (Weir, 1949)

The Respiratory Quotient (RQ) was calculated as shown in Equation X. The RQ describes the percental fraction of macronutrients that were oxidized for energy production:

$$RQ = \frac{VCO_2\ (\frac{ml}{min})}{VO_2\ (\frac{ml}{min})}$$

**Equation 3: Calculation of the respiratory quotient (RQ)** (Stipanuk and Caudill, 2013)

At both sites, identical devices were used (Cosmed Quark RMR 1.0, Fridolfing, Germany), but during a maintenance period, the device in Freising was exchanged by a newer version (Cosmed Quark RMR 2.0, Fridolfing, Germany), from March to May 2017.

For analyzing REE, results of each indirect calorimetry were checked for their variability in parameters during the measurements according to Fulmer and colleagues (Fullmer et al., 2015). If one of the parameters $VCO_2$ or $VO_2$ had a higher coefficient of variance of 10 % over the period of 30 minutes, a stable timeframe of 4 minutes was assessed that led to stable results. Participants who did not meet a stable four-minute window in the respective REE measurement(s) were excluded from analysis.

## 2.6 Individualized cooling protocol

To assure stable and controllable temperature conditions, subjects were laid between two water perfused mattresses that were connected to a cooling device (WiseCircu type WCR-P8, Witeg Labortechnik, Wertheim, Germany). The device was run with double distilled (bidest) water which could be determined with an accuracy of 0.1 °C. Subjects were separated from the mattresses by sheets by means of hygiene. One sheet was placed on top in order to create a cooling compartment in case of big and tall participants that were not fully covered by the mattresses themselves. As the mattresses were limited to 162 cm in length, it was important that the chest region was covered. Feet were allowed to stick out if necessary. Participants were asked to put their arms underneath the sheets and not to bend their legs, nor to roll to the site during the cold exposure.

For assessing the REE at basal conditions, the water inlet temperature was set to 32 °C. For some participants this seemed to be too warm, so temperature was decreased by one or two degrees, until subjects felt comfortable. After the first indirect calorimetry and blood drawing, cooling procedure was started. The water inlet temperature was decreased stepwise (2 °C each 5 min) following an adapted protocol by Bakker et al. (Bakker et al., 2014). The subjects were asked to tell any reaction of the body caused by cold to assess shivering threshold. As soon as shivering occurred, water inlet temperature was increased by 2 °C. Afterwards, 120 min of cold exposure started. If shivering occurred during that time, temperature was increased further until shivering stopped.

## 2.7 Blood and adipose tissue collection

Immediately after each indirect calorimetry, blood was drawn. Routine parameters (listed in Table 2) were analyzed at the certified laboratory SynLab Labordienstleistungen (Munich, Germany). Plasma fasting glucose values were determined by using fresh EDTA blood for analysis with HemoCue Glucose $201^{+}$ System. For in house lab measurements plasma was collected (EDTA KE monovettes, Nümbrecht, Sarstedt) and centrifuged at 2,500 g for 10 min at room temperature. By centrifugation, plasma was separated from red blood cells. In between these two layers the so-called buffy coat was formed, which was collected separately for isolation of DNA. Serum was collected (Serum Gel monovettes, Nümbrecht, Sarstedt), left for 30 min at room temperature to allow clotting, followed by centrifugation (2,500 g for 10 min at 20 °C). Plasma, buffy coat and serum were distributed into aliquots and stored at -80 °C for later measurement of selected biochemical parameters.

**Table 2: Parameters analyzed by SynLab Labordienstleistungen**

| Parameters | Source | P01 | P02 | P03 |
|---|---|---|---|---|
| Blood cell count/thrombocyte | EDTA-Plasma | ✓ | | ✓ |
| Clinical chemistry (ALT, AST, γ-GT) | Serum | ✓ | | |
| Electrolytes (Na+, K+, $Ca^{2}$+) | | | | |
| Lipid profile (HDL, LDL, total cholesterol, Triglycerides) | | | | |
| TSH, $fT_3$, CRP (sensitive) | | | | |
| $fT_3$, CRP, Triglycerides | Serum | | ✓ | |
| Coagulation (PTT, INR, Quick) | Citrate-Plasma | | | ✓ |

ALT=alanine transaminase, AST=aspartate transaminase, γ-GT=Gamma-glutamyltransferase, $Na^+$=sodium, $K^+$=potassium, $Ca^{2+}$=calcium, HDL=high-density lipoprotein, LDL=low-density lipoprotein, TSH=thyroid stimulating hormone, $fT_3$= free triiodothyronine, CRP=C-reactive protein, PTT= partial thromboplastin time, INR= international normalized ratio, P01= blood drawing after first IC, P02= blood drawing after second IC; P03: blood drawing prior to examinations, necessary for fat biopsy

In order to obtain subcutaneous adipose tissue, needle aspiration biopsy was conducted after each indirect calorimetry, subsequent to blood sampling. The subjects' skin was desinfected thoroughly, followed by local anesthesia of the region lateral from the umbilicus with 1 % lidocaine. In cases of hair-growth regarding the respective area, skin was shaved beforehand. The biopsy needle (Strauss-cannula; 2.0 x 43 mm; 14G) was inserted into the superficial fat layer and 4 ml of 0.9 % sodium chloride solution (NaCl, 0.9 %, Braun, Melsungen, Germany) was injected. Aspirated adipose tissue was mixed with 0.9 % NaCl-Heparin (50 IE/ml). Under sterile conditions, the tissue was filtered through a mesh and washed with 0.1 % Krebs-Ringer-buffer, before further processing. For RNA processing, fat tissue was aliquoted into tubes containing sterilized zirconia-glass-beads (Carl Roth, Karlsruhe, Germany), RLT-buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany) and 1 % (v/v) ß-mercaptoethanol (#M3148, Sigma-Aldrich, St. Louis, Missouri, USA) and was immediately frozen and stored at -80 °C. For fat histology, fat tissue was fixed in 4 % buffered formalin (pH 7.4) for 24 h and transferred into 70 % ethanol until further usage. Samples were stored in the dark at 4 °C.

## 2.8 Laboratory analyses

Plasma adiponectin, leptin (both R&D, Wiesbaden, Germany) and insulin (DRG Instruments, Marburg, Germany) were assayed by using commercially available enzyme-linked immunosorbent assays (ELISA) according to manufacturers' protocol. All parameters were pipetted manually, with duplicates for each sample. In case of high variations in the duplicates, or ODs that were outside the range of the given standards, measurements were repeated for these samples, if necessary in a diluted concentration. ODs were measured using the Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm measuring wavelength and 620 nm reference wavelength.

Plasma nonesterified fatty acid (NEFA) levels were measured using a reagent enzymatic colorimetric method (Wako Chemicals, Neuss, Germany). The method relies on the ACS-ACOD-method resulting in the oxidative condensation of 3-Methyl-N-Ethyl-N-(ß-Hydroxyethyl)-Aniline (MEHA) and 4-aminoantipyrine into a blue pigment, which can be measured by photometry. All samples were pipetted by hand in duplicates on a 96 well plate. Addition of the kit substances and mixture of samples was done by the Infinite M200. Measurements occurred at 546 and 660 nm absorbance.

For miRNA and mRNA isolation mirVana miRNA isolation kit (ThermoFisher Scientific, Dreieich, Germany) was used. The amount of tissue varied for each sample and ranged between 50 and 250 mg. Tissue samples from umbilical area were shred, either with cooled mortar and pestle or by using a Fast Prep device (MP Biomedicals). Experiments were carried out with Fast Prep and autoclaved UV-light treated microtubes filled with beads (Carl Roth GmbH). Using this isolation kit, mRNA and miRNA were isolated at the same time, however for this thesis, only mRNA was of relevance.

Afterwards, mRNA was transcribed to complementary DNA (cDNA) according to the protocol of High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Langenselbold, Germany). Quantitative PCR was carried out with SYBRgreen MasterMix (QIAGEN GmbH, Düsseldorf, Germany) to measure the expression of beiging markers UCP1, PGC1a, PRDM16, and CIDEA along with PPIA, GAPDH, and IPO8 as housekeeping genes. This was done in scWAT samples of 17 individuals before and after CE. Results were normalized accordingly (target/housekeeping).

## 2.9 Statistical methods

Data was analyzed using Graphpad Prism Version 5.2 and R programming environment. All data is presented as mean ± standard deviation. Results were considered as statistically significant when $p < 0.05$.

For comparing different groups (males/females, BMI categories) student's t-test or ANOVA were used for normally distributed data. If a Gaussian distribution was not given, Mann-Whitney U test or Kruskal-Wallis test with post-hoc testing by Bonferroni testing were used. For comparing different time points (TN vs. CE) a paired student's t-test and a Wilcoxon signed rank test were used for normally and not normally distributed respectively. Linear regression analysis and Spearman correlation coefficients were determined for the relationships.

# 3 Manuscript overview

This thesis contains two first author manuscripts. For each manuscript the publication status, a short summary and a detailed description of each authors' contribution is given.

**Manuscript 1**

**Gender differences in the response to short-term cold exposure in young adults**

Laura A. Mengel, Hatti Seidl, Beate Brandl, Thomas Skurk, Christina Holzapfel, Lynne Stecher, Melina Claussnitzer and Hans Hauner

Published 2020 in *The Journal of Clinical Endocrinology & Metabolism*, March 2020,

Summary

Humans display a sexual dimorphism that is also expressed as difference in body composition and metabolism. While women possess a higher fat ratio, men have a higher fat free mass content. In times of overweight and obesity and thus comorbidities such as diabetes, cardiovascular diseases and even some kinds of cancer, new strategies to tackle overnutrition are warranted. Brown adipose tissue (BAT) is an organ whose adipocytes possess a plethora of mitochondria and which is able to release energy as heat. Brown adipocytes burn energy with the aid of the uncoupling protein 1 (UCP1) which is bound to the inner matrix of mitochondria. UCP1, in its active state, uncouples the protons generated by substrate oxidation from adenosine triphosphate (ATP) production. This mechanism is classified into the so called non shivering thermogenesis (NST), a mode of action of the body to defend the body from cold temperatures, apart from shivering by skeletal muscles, called shivering thermogenesis. The investigation of BAT and NST as putative therapy for weight management is relatively new. Overall consensus is that there is a high variability between individuals in change of resting energy expenditure following cold exposure and thus NST and BAT activity. However, reasons for that are still understudied as most studies consist of rather small cohorts or are simply based on retrospective studies. Especially possible gender effects were not addressed directly and observations were produced as byproduct. However, current literature is conflicting which still raises the question if there are considerable gender effects in cold induced thermogenesis.

For this reason, we recruited a large set of healthy, young, lean males and females in order to assess the differences between genders in resting energy expenditure and metabolism after cold exposure. Participants were exposed to a defined short-term cold exposure protocol that was

highly standardized. Resting energy expenditure and blood parameters were assessed for basal conditions and after treatment with cold temperatures. Surface body temperature was assessed throughout the experiment.

The results show that there is no difference between men and women by means of cold induced increase of resting energy expenditure. Females displayed stronger changes for some metabolites compared to males. Thus plasma glucose levels and leptin concentration were decreased to a higher magnitude in women compared to men, while adiponectin increased in females more than in males. Also, skin temperature of the supraclavicular area was stable in women after cold exposure whereas men showed a significant decrease in that region.

Although women showed stronger reactions in metabolic profile subsequent to cold exposure than men this was not reflected by a higher increase in resting energy expenditure. This indicates that using cold induced thermogenesis as weight management tool is applicable for men and women in the same magnitude. However, these results are taken from a short-term cold exposure protocol, giving no information on the long term effects of cold exposure on metabolism.

## Author contributions

HH and MC conceived and designed the study. LAM was responsible for recruitment, management and organization of the study. LAM and HS performed research. Data preparation and initial analysis was done by LAM, supported by LS. LAM and LS analyzed data. LAM and HH wrote the manuscript. CH, BB and TS assisted with experimental design and data interpretation. All authors were involved in the final revision of the manuscript.

## Manuscript 2

## Effect of BMI on the thermogenic response to cold exposure and associated changes in metabolism and browning markers in adult humans

Laura A. Mengel, Bahareh Nemati Moud, Hatti Seidl, Alberto Mesas Fernández, Claudine Seeliger, Beate Brandl, Thomas Skurk, Christina Holzapfel, Melina Claussnitzer and Hans Hauner

Published 2022 in *Obesity Facts*, January 2022,

Summary

Obesity is a multifactorial disease that comes along with several comorbidities such as diabetes, cardiovascular diseases or even cancer. As the prevalence of overweight and obesity has reached a pandemic character we are in need of new strategies to combat excess weight gain and to promote weight loss. New approaches are developing about the thermogenic organ brown adipose tissue (BAT). Usually, BAT gets activated by cold stimulus leading to an uncoupling of mitochondrial respiration and thus elevated energy expenditure in favor for heat production. It is known that this so called cold induced thermogenesis, which is part of non-shivering thermogenesis (NST) results in an increased energy expenditure in human adults. However, there is a high interindividual variability suggesting that there are differences in the response among different populations. Retrospective studies have shown that a higher BMI is correlated with reduced BAT activity but a direct approach assessing the differences in thermogenic response in association with the BMI are still lacking.

In this study, we exposed a cohort of 173 healthy adults of different BMI groups to an individualized short term cold exposure (CE) protocol. We assessed resting energy expenditure (REE) and circulating metabolites at basal conditions and after CE. For suitable candidates with overweight or obesity, a subcutaneous white adipose tissue (scWAT) biopsy was conducted at both conditions to assess changes in browning marker genes due to CE. Participants with normal weight displayed a significant increase in REE following CE that was not observed in BMI groups with overweight and obesity. Furthermore, there were differences in the change of circulating metabolites concentrations supporting the hypothesis that lipolysis and metabolic response are impaired in persons with overweight or obesity. Although insulation was higher in participants with obesity, offset of shivering was comparable between BMI groups suggesting an impaired NST of the group with obesity. Anyhow, participants with obesity where able to reduce their insulin levels during CE and thus improving the insulin sensitivity. We found changes in the expression of the browning

marker gene CIDEA in women but not in men hinting to a gender difference in browning capacity after short term CE.

Overall, thermogenic response was less pronounced in persons with overweight and obesity compared to those with normal weight. If this impairment is the consequence of a higher BMI or if a higher BMI is a consequence of impaired thermogenesis cannot be concluded by this cross sectional study. However, CE had beneficial effects on whole body glucose homeostasis and insulin sensitivity of participants with obesity posing a possible strategy to ameliorate the comorbidities of obesity.

## Author contributions

This study was designed by HH and MC. LAM, BNM, HS, AMF, and CS performed the experiments. LAM analyzed data and wrote the manuscript. LAM was responsible for and CH, BB and TS assisted with experimental design, recruitment of volunteers and data interpretation. All authors were involved in the final revision of the manuscript.

# 4 Discussion

The research presented within this thesis provided further insight into the understanding of non-shivering thermogenesis and browning regarding different population groups.

## 4.1 Thermogenic response in females and males after cold exposure

In manuscript 1, we explored the thermogenic response of healthy, lean, and young adults to a short-term individualized CE as change in REE, metabolic blood parameters, and skin temperatures. While a higher prevalence of women having active brown adipose tissue was reported in retrospective studies (Au-Yong et al., 2009; Brendle et al., 2018; Cypess et al., 2009), cross-sectional studies could not detect any differences in the change of REE between males and females (Celi et al., 2010; van der Lans et al., 2013; van Marken Lichtenbelt et al., 2009). However, the aforementioned studies were rather small and did not focus on the gender effect on cold-induced thermogenesis. With our study, we sought to elucidate the discrepancy in the literature by exposing a larger and coherent cohort to a defined and strict cold exposure protocol. Regarding the relevant increase in energy expenditure we could not find significant differences in the relative increase of REE. The magnitude of increase was according to what was found in other studies (Gashi et al., 2019; Hanssen et al., 2014; van der Lans et al., 2016; van Ooijen et al., 2001).

The individualized cooling protocol revealed that while women have an earlier onset of shivering compared to males and thus are exposed to a higher temperature throughout the experiment their increase in REE after CE was comparable to that of men. After adjustment for exposure temperature, women showed a modestly higher increase in REE compared to men, but this difference was not significant. The overall increase in REE was significantly associated with exposure temperature, but this was not dependent on BMI, fat mass or fat ratio. When it comes to REE women reacted to the same magnitude as men when exposed to cold, independent of the exposure temperature.

This stays in contrast to our findings on changes of skin temperature. The superficial skin temperature of the supraclavicular area, the region where the biggest brown adipose tissue depot is usually located, decreased significantly in males, but not in females. There was even a tendency of increase at that location in women which didn't reach statistical significance. A decrease in supraclavicular skin temperature (that was also negatively correlated with BAT activity) in men was reported by several research groups (van der Lans et al., 2016; Yoneshiro et al., 2011b),

while one other small study conducted in females also reported no significant differences in skin temperature over this area (Martinez-Tellez et al., 2019). Another study carried out in both genders with considerable BAT activity, the decrease in supraclavicular skin temperature was as well associated with BAT activity (Gashi et al., 2019). Considering that BAT burns energy to produce heat, it seems logical that the supraclavicular fossae might experience a lower temperature loss compared to other areas. Even skin areas that were not exposed to the cold (e.g. the forehead) experienced a marked drop in temperature. This could be a hint that BAT activity is higher in women than in men.

Furthermore, there were substantial differences in the response of circulating metabolites upon CE. Women displayed a stronger decrease in plasma glucose and leptin levels when exposed to cold, while there was a significantly higher increase in adiponectin compared to men. Changes in NEFA and TG levels were comparable between genders. BAT uses predominantly intracellular fatty acids as fuel, but is also a sink for circulating substrates such as TGs and glucose (U Din et al., 2018; Weir et al., 2018). Orava and coworkers did observe a significantly higher cold-induced uptake of glucose into BAT in women than men which is supporting our findings (Orava et al., 2013), whereas one other study could not find differences in glucose uptake (Saito et al., 2009). An increase in NEFAs that is comparable between genders was also reported (Celi et al., 2010). Women displayed higher circulating leptin levels compared to men, this was also seen in our results for both conditions. However, the relative decrease of leptin levels upon CE was significantly higher in females than in males. Assessment of 24 h profiles of serum leptin in humans has shown a diurnal pattern of leptin with a peak in the night and early morning and the nadir around noon and midafternoon (Sinha et al., 1996). This could lead to the assumption that our observation could be a mere time effect, as also stated by Iwen and colleagues (Iwen et al., 2017). However, Zeyl et al. did also observe a reduction of plasma leptin levels after short-term CE (25 to 60 min) by around 22 % (Zeyl et al., 2004), and also other groups were able to show a significant drop in leptin levels after CE (Blondin et al., 2017a; Ricci et al., 2000). A recent study also showed a gender-based effect on leptin levels upon CE (Sun et al., 2020). Leptin is a central player in energy expenditure and appetite control. Secreted predominantly by adipocytes, it mediates information to the hypothalamus, whether food intake is required or not (Stephens et al., 1995). High levels of leptin reduce appetite, at least in healthy individuals. Decreasing levels of leptin enhance the thrive for energy intake, a logical consequence after energy consumption after cold exposure. It may be argued that the decrease in leptin levels is caused by a reduced blood flow (Zeyl et al., 2004), however, adiponectin, which is also secreted by WAT, increases its levels

during CE. Therefore, the drop in leptin concentrations might either be explained by reduced secretion or an increased uptake of leptin by receptors.

We observed a significant increase in plasma adiponectin concentrations in both men and women. Other research groups described a decrease in adiponectin after CE in healthy lean men (Blondin et al., 2017a; Iwen et al., 2011). Otherwise, Imbeault and colleagues reported that CE enhances adiponectin levels in men (Imbeault et al., 2009). It is of note that these studies were only conducted in men and not in women. Adiponectin contributes to WAT browning in scWAT by directly binding to M2 macrophages and inducing proliferation of these cells after CE (Hui et al., 2015). As widely known, women possess higher levels of adiponectin for unknown reasons (Laughlin et al., 2007) which might contribute to the fact that females display a higher capacity of CIT.

## 4.2 Thermogenic response and obesity

To elucidate the effect of BMI and body composition on cold-induced thermogenesis, participants with overweight and obesity were exposed to the same short-term CE as the participants with normal weight in manuscript 1. Manuscript 2 deals with the thermogenic response by means of change in REE, metabolites and skin temperatures in different BMI groups. Furthermore, biopsies of subcutaneous adipose tissue were taken at thermoneutrality and after CE from participants that were overweight or obese.

Fat free mass and fat mass are major contributors to REE in humans (Javed et al., 2010; Johnstone et al., 2005). Clearly, individuals with higher bodyweight also have a higher absolute fat (free) mass leading to a higher REE as observed in manuscript 2. However, this effect disappeared after short-term CE, as persons with lower BMI increased their REE, whereas REE of overweight and obese remained unchanged. As a consequence, participants with overweight or obesity show an impaired response in the increase of REE after cold stimulation compared to normal weight individuals which is according to published literature (Brychta et al., 2019). However, this short-term CE had several beneficial effects for individuals with overweight and obesity such as a reduction in circulating glucose and insulin levels. Even if an acute activation of NST and BAT might not be efficient against overweight or obesity regarding weight loss, it improves whole body glucose homeostasis and lipid metabolism. It is of note, that this cross-sectional study represents only a snapshot of the possibilities NST could offer. Cold acclimation studies in lean individuals showed that repeated mild CE can augment BAT volume and activity

assessed by PET-CT and indirect calorimetry (Blondin et al., 2014b; van der Lans et al., 2013; Yoneshiro et al., 2013). One study conducted in men with overweight and obesity also found significant increases in BAT volume and improvements of glucose and insulin levels after cold acclimation, although no increase in EE was observed (Hanssen et al., 2016). A long-term inpatient study conducted on a small group of young, lean men has shown that one month of mild CE (16 °C) leads to an augmentation of BAT volume and an increase of fat metabolic activity (Lee et al., 2014b). One study conducted in men and women with a CE duration of 6 weeks (1 h each day) showed an increase of REE compared to the control group, but without any changes in the metabolic profile (Romu et al., 2016). If human BAT is able to significantly improve metabolic health and to reduce excessive weight in overweight and obese patients is questionable and will be discussed in detail in the following chapter.

## 4.3 Difficulties in the assessment of BAT activity

Due to different techniques in assessing BAT volume and activity, the impact of BAT on human metabolism is highly and controversially debated. Depending on the source of information, BAT can account for 1-7 % of EE in humans (Loh et al., 2017), or can increase REE by 200-400 kcal/day, when fully activated (Kajimura and Saito, 2014). It is also estimated that 50 g of BAT can utilize up to 300 kcal/day if maximally stimulated (Rothwell and Stock, 1983). Newer estimations propose a rather small contribution of around 25 kcal/day that are burned by BAT when fully activated. This means that in one year about 1.9 kg of fat can be lost, given a continuous stimulation of BAT (Muzik et al., 2013). Others, however, state that 70 g of fully activated BAT can lead to a body fat reduction of at least 4 and up to 18 kg per year (Gerngroß et al., 2017; Virtanen et al., 2009) or a body weight loss of 3 to 8 % (Peng et al., 2015). For comparison, conventional medication therapy for weight loss accounts for 2.9 – 8.6 kg/year (Apovian et al., 2015) and a combination of dieting, exercising and behavioral counseling leads to a weight reduction of 5 – 10 % at best (Hall and Kahan, 2018). If there is a possibility to continuously activate BAT in humans, this might represent a powerful tool for weight loss therapy.

Unfortunately, an evaluation of the benefit of activated BAT is difficult to define due to the following reasons: On the one hand, BAT appearance is variable from one individual to another ranging between 34 to 913 g in mass across studies (Marlatt et al., 2018). This makes it difficult to use BAT activity over a wide range of individuals for weight maintenance and loss, especially if one considers that elderly and individuals with a higher BMI show a lower volume and activity of BAT

(Brychta et al., 2019; Hanssen et al., 2016; Saito et al., 2009). The real contribution of BAT to EE is still not clearly assessable as its influence may be easily over- or underestimated, depending on the methods to measure size and activity. Assessing BAT activity by indirect calorimetry might be overestimating its capacity, when shivering thermogenesis is not fully excluded but regarded as BAT contribution, or when NST is facilitated by other organs (Haman and Blondin, 2017; u Din et al., 2016).

On the other hand, BAT activity might also be underestimated. Estimations are mostly calculated by glucose or fatty acid uptake into BAT. But as mentioned earlier, BAT uses different kinds of substrates, especially intracellular ones, which makes a true estimation of the contribution challenging. Furthermore, some labelled substrates exhibit short half-life impeding an accurate assessment (Marlatt et al., 2018). In addition, BAT has to be stimulated to the maximum to investigate the true capacity of energy consumption. Although $^{18}$FDG-PET-CT is the gold standard for assessing BAT activity (Gatidis et al., 2016; Kiefer, 2017), it only traces one substrate and may not display the full capacity of activity if the stimulus is not sufficient. PET/CT scans can only track one kind of substrate. Considering that BAT relies on intracellular substrates and can utilize both fat and glucose as fuel, it is in all probability that BAT contribution is underestimated (Carpentier et al., 2018; Gashi et al., 2019).

### 4.4 Browning as potential therapy for weight loss

In contrast to BAT, which is a relative small organ in humans, WAT and thus beige adipocytes residing within it, can make up 3 to 70 % of the human body (Parlee et al., 2014). As beige adipocytes can differentiate from precursor cells, but can also develop by transdifferentiation of mature white adipocytes, their potential to contribute to NST is very promising (Inagaki et al., 2016; Kajimura et al., 2015). In an attempt to map BAT and “brownable” depots, Leitner et al. proposed that fully activated brownable depots could increase EE by more than 520 kcal/d in healthy young men (Leitner et al., 2017). So far, in humans considerable browning of subcutaneous WAT has only been observed in patients with severe burning (Patsouris et al., 2015; Sidossis et al., 2015) and cancer cachexia (Kir et al., 2014; Petruzzelli et al., 2014), states of high adrenergic stress and disproportional release of catecholamines.

There is a rising number of studies aiming to elucidate the browning capability of scWAT induced by different interventions. As UCP1 is the central player of brown adipocyte activity and thus thermogenesis, it’s mRNA expression is widely used as measure for browning capacity. Recently,

several interventional studies were conducted in order to assess the effects of cold acclimation or short-term CE on body weight, composition and metabolism, while others used medication, weight loss or exercise interventions. Selected examples will be discussed in the following chapters.

### 4.4.1 Cold exposure

So far, only one study was able to show increases in UCP1 mRNA expression of scWAT following short-term CE (Kern et al., 2014), while others reported no changes (Celi et al., 2010; Chondronikola et al., 2014). Kern et al. saw a significant increase of UCP1 and PGC1a mRNA expression, but only in participants that attended the experiment during the summer. In samples taken during the winter months, basal expression of UCP1 was higher compared to those taken during summer, but there was no further increase subsequent to CE. However, there was no increase of UCP1 mRNA in participants with a BMI higher than 30 kg/m². One study conducted in both genders could observe upregulations of browning genes after a cold acclimation protocol (10 days, each day 30 min CE), but only in summer (Finlin et al., 2018). On the contrary, van der Lans and coworkers used a similar approach and exposed lean and young participants for 10 days for a maximum of 6 h each day to cold. They could not observe any change of mRNA expression in scWAT but an increase in glucose uptake into BAT (van der Lans et al., 2013). Others also reported changes in BAT volume and activity following cold acclimation (Blondin et al., 2014b). Regarding changes in metabolites, one study conducted in lean males undergoing 4 weeks of cold acclimation (5 cold exposure sessions per week) did not lead to a significant change in metabolites (Blondin et al., 2017b) as well as a 10-day cold acclimation study in overweight and obese men (Hanssen et al., 2016). While cold is the strongest physiological driver of BAT activity and NST, it might not be suitable as weight management approach in humans as a hyperphagic response could lead to an increase in fat mass and body weight (Loh et al., 2017).

### 4.4.2 Exercise

The beneficial effects of exercise on the human body are well known and physical exercise may also have positive effects on BAT activity and browning. However, in middle-aged men, a 12-week exercise intervention led only to small increases in UCP1 mRNA expression in scWAT (Norheim et al., 2014). Another study, conducted in lean young men consisting of 6-week exercise intervention had no significant effect on BAT volume or activity (Motiani et al., 2019) and a similar

study carried out in individuals with obesity also did not reveal changes in mRNA expression of scWAT (Tsiloulis et al., 2018). A combination of exercise and caloric restriction in women for six weeks did not result in an upregulation of browning markers in scWAT (Nakhuda et al., 2016) leading to the assumption that it requires a longer stimulation in order to achieve measurable effects. To date, there is only one study that carried out an exercise intervention in both genders. Here, physical activity for 3 days per week over a period of 12 weeks was applied for different BMI groups (Otero-Díaz et al., 2018). While this intervention had no significant effect on body weight and BMI, hip circumference was reduced as well as lipid metabolites such as triglycerides and total cholesterol. Furthermore, there was an increase in expression of browning markers. Regarding UCP1 mRNA, after intervention the expression levels of individuals with obesity were comparable with those of individuals with normal weight. Overall, exercise might elicit browning or even activation of BAT that contribute to the overall beneficial effects achieved by this intervention. However, physical exercise is not always applicable especially if the health status of patients prohibits a proper work out.

### 4.4.3 Medication

As exercise is not applicable for all patients as a treatment option for weight loss, other ways of increasing EE are needed. In the past, drugs that act on EE and have been used, such as dinitrophenol, but were retracted from the market as soon as the severe side effects were evident (Colman, 2007). As mentioned earlier, BAT is stimulated by the SNS via adrenergic activation of $\beta_3$-adrenergic receptors. These receptors can be found on the surface of white and brown adipocytes and also the bladder (Cannon and Nedergaard, 2004; Yamaguchi and Chapple, 2007). However, selectivity of drugs acting of $\beta_3$-adrenergic receptor agonists is not exclusive resulting in an activation of the two other β-adrenergic receptors (Arch, 2011; Michel et al., 2011). This is why medications that aim to treat for instance overactive bladder syndrome have side effects that affect the cardiovascular system (Khullar et al., 2013). However, mirabegron that is currently used to treat overactive bladder syndrome, is now used to activate BAT in human intervention studies. A 10-day intervention study with mirabegron in healthy males led to an upregulation of browning markers (Finlin et al., 2018). Another study that was carried out in males, who displayed sufficient BAT activity beforehand, were treated with 200 mg mirabegron for a period of 12 weeks. There was an increase of 13 % in REE compared to placebo and glucose uptake in BAT was increased reaching values that are normally seen during CE (Cypess et al., 2015). However, circulating glucose and NEFA concentrations increased during treatment as well as HOMA-IR. It is important

to note, that 200 mg of mirabegron are 4 times more than the currently approved dose of 50 mg per day for treatment of the overactive bladder syndrome. In a very recent study, improvement of glucose homeostasis and browning of scWAT were observed after a twelve-week treatment with mirabegron in participants with obesity (Finlin et al., 2020). Further human studies are certainly needed to examine if mirabegron or derivatives have a true potential and are safe enough to become a medication for the treatment of obesity.

### 4.4.4 Weight loss

Weight loss per se may also have beneficial effects on BAT activity. Dadson and colleagues assessed BAT activity via PET-CT in women with obesity before and six months after bariatric surgery (Dadson et al., 2018). At baseline, BAT activity in the supraclavicular area was significantly lower in females with obesity compared to the control group with normal weight. After bariatric surgery, women lost around 23 % of the initial body weight and improved whole body glucose homeostasis and lipid metabolism. Furthermore, BAT activity increased six months post-surgery. The proportion of supraclavicular brown fat was comparable with the control group. Even if BAT activity is reduced in individuals with obesity and might have limited contribution to weight loss, it might help at later stages for weight maintenance.

Regardless of the kind of therapy, one should keep in mind that the effect of beiging is temporary and a reversible process (Altshuler-Keylin et al., 2016). Therefore, long-term treatment is necessary for sustainable results in browning and putative weight loss. Although experiments in the murine system have shown that scWAT is more susceptible to browning than visceral fat depots, in humans it is quite the opposite (Bettini et al., 2019; van den Beukel et al., 2015; Zuriaga et al., 2017). Browning might occur more rapidly in visceral adipose depots which are not accessible by small-scale tissue biopsy leading to an underestimation of browning capacity in adult humans.

## 4.5 Other contributors to NST

One should keep in mind that BAT is not the only organ that is contributing to NST. In BAT, UCP1 uncouples respiration and shuttles protons across the inner membrane of mitochondria in brown and beige adipocytes (Cannon and Nedergaard, 2004). In skeletal muscle, a similar process takes place which also leads to futile cycling. Under normal conditions, the sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump transports $Ca^{2+}$ ions into the sarcoplasmic reticulum upon ATP hydrolysis for maintenance of the proton gradient (Smith et al., 2013). The membrane-bound protein sarcolipin modulates the activity of SERCA by uncoupling the ATP hydrolysis from the calcium pump, whereby work is released as heat (Kozak and Young, 2012). Mice that were ablated of BAT and shivering thermogenesis were still able to maintain core body temperature due to sarcolipin activity (Rowland et al., 2015b). In humans, Wijers and colleagues demonstrated that a single day of mild cold exposure (16 °C) is sufficient to significantly increase mitochondrial uncoupling in skeletal muscle of lean men (Wijers et al., 2018). Not only skeletal muscle is able to use SERCA mediated calcium cycling for heat generation. This mode of action was also observed recently in beige adipocytes of mice and humans (Ikeda et al., 2017). Therefore, cold-induced NST in adult humans might be increased and used for weight management. However, further investigations are warranted to shed light on the physiological relevance of these processes.

# 5 Summary

Energy expenditure is highly variable between individuals and a plethora of studies has addressed this topic to understand what causes this variability and to explain why some people gain more weight than others. While most of this variance is explained by physiological and external determinants, around 30 % are still unknown.

The contribution of brown and beige adipocytes to human energy expenditure and metabolism is still far from being elucidated. Albeit adult humans only possess small depots of brown adipose tissue, – it is highly variable between individuals and impacted by several determinants such as BMI, age, and lifestyle - there are effects visible on metabolism and energy expenditure. Especially the high plasticity of beige adipocytes complicates the correct assessment of brown/beige adipocyte contribution to thermogenesis. This work aimed to elucidate certain effects on the variability in thermogenic response by means of changes in resting energy expenditure and circulating metabolites. There were gender differences detectable, but they were restricted to rather small observations implicating that men and women have comparable responses to cold exposure. In addition, a higher BMI and fat mass are associated with an impaired thermogenesis expressed as unchanged resting energy expenditure following short-term cold exposure. This appears daunting if cold treatment may be considered as anti-obesity therapy. However, the FREECE study used a short-term cold exposure intervention and even this short exposure time was sufficient to elicit improvements in whole body glucose homeostasis and first hints of browning, at least in women. A longer period of cold exposure or repeated exposure, potentially in combination with medications such as mirabegron or supported by exercise interventions might increase the effect of BAT and browning on weight loss and metabolic health.

# 6 Outlook

The presented results of the FREECE study are only covering parts of the possible research questions that can be investigated in this cohort. The recruitment for this study is still ongoing and the following topics should be investigated in future analyses:

As mentioned earlier, variants in the *FTO* gene locus are associated with BMI and other weight-related traits, but the causative variant(s) and its/their function is/are still not fully identified. The *FTO* SNP rs1421085 is potentially involved in cold-induced thermogenesis in humans and needs confirmation from studies *in vivo*. Genotyping of the FREECE cohort will help to elucidate whether the findings of Claussnitzer and colleagues are also applicable to the *in vivo* situation. Any correlation of this and other specific SNPs with parameters of energy expenditure will be analyzed in future studies, but is not topic of this thesis.

So far, no specific circulating browning marker has been detected in humans yet, but the expression of some micro RNAs (miRNA) is associated with BAT activity and browning (Goody and Pfeifer, 2018). Especially miRNA-92a, which was detected in human serum, was inversely correlated with BAT-activity and might thus represent a relevant circulating browning marker (Chen et al., 2016). As there are difficulties in assessing BAT activity, circulating biomarkers could help to uncover the true potential of BAT. Serum samples of the FREECE cohort are currently investigated for any changes in circulating miRNA levels during CE.

Magnetic Resonance Imaging (MRI) could represent a non-invasive biomarker of tissue fat content by measuring the proton density fat fraction (PDFF). PDFF in BAT is lower compared to PDFF in scWAT. A high PDFF difference between the two compartments may reflect a different adipose tissue composition between both depots. In collaboration with the University Hospital “Klinikum rechts der Isar” participants that took part in the FREECE study were invited for an additional MRI measurement as established recently (Franz et al., 2018). The obtained data from the MRI measurements will be correlated with the REE data of the FREECE study to elucidate whether there are associations between PDFF and the increase in REE upon CE.

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# Acknowledgements

This work would not have been possible without the help and support of many people. First of all, I want to thank Univ.-Prof. Dr. med. Hans Hauner for giving me the opportunity to work on this interesting project. Having a totally different scientific background, this task was challenging but also very exciting and gave me the possibility to gain more insights to human nutrition and metabolism. Thank you for your trust in my abilities and work within these years.

Furthermore, I want to thank Prof. Dr. med. Thomas Skurk, for being my mentor during this time and giving me useful advice whenever I needed it. Special thanks go out to Beate Brandl, PhD, who always lend me an ear especially in the beginning when I was a greenhorn in this field. I want to express many thanks to Christina Holzapfel, PhD, for helpful suggestions regarding administrative or scientific issues and helping me coordinating the study at the two study centers in Munich and Freising.

Many thanks go to the ladies of the Study Unit, supporting me during long days of examinations. Also, I am thankful for our study physicians for their expertise and excellent patient care.

Further, I want to thank all my colleagues for the joyful time in the lab, long walks after lunch, visits of Volksfest and Wiesn, collection of lab memes for our office door, and for the great support and friendship throughout the years.

Finally, I want to thank my family and friends and my everything, Fil, for always being at my site, for your patience and love. Without you this work wouldn’t have been possible.