Keeping warm in the cold : human brown adipose tissue upon cold acclimation

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Keeping warm in the cold

Human brown adipose tissue upon cold acclimation

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Keeping warm in the cold

Human brown adipose tissue upon cold acclimation

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,

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CHAPTER 1

General Introduction

OBESITY

In 2008, more than 1.4 billion adults worldwide were overweight and more than half a billion obese, and these numbers are still increasing. In 2012 it was estimated that at least 3.4 million adults die each year as a result of being overweight or obese. Additionally, a high BMI is a risk factor for the development of cardiovascular diseases, diabetes and endometrial, breast and colon cancer. Overweight and obesity are the leading health risks for global deaths (WHO 2008&2012). Overweight only develops when energy intake exceeds energy expenditure (EE), or in other words, when someone is in a long-lasting state of a positive energy balance (1). Research in recent years studied how energy balance can be maintained, mainly focusing on reducing energy intake by means of food restriction, and/or by increasing EE by increasing physical activity. However, the majority of these treatment strategies have failed to be effective on the long term. As a consequence long-lasting positive health outcomes are rarely seen with these kind of interventions (2).

COLD EXPOSURE

Cold exposure can result in unpleasant sensations and thermal discomfort. Prolonged exposure to severe cold can result in decreased physical and mental performance. Cold could also aggravate symptoms of chronic diseases and it is well known that the coldest season is associated with increased morbidity and mortality (3, 4). The more efficient heating systems combined with better insulation in houses and offices has resulted in less cold exposure in the last decades. Johnson et al. (5) even suggest the existence of a causal link between the decreased time spent in colder environments and increased adiposity in the population. Additionally, the use of proper clothing caused a decreased exposure to severe cold, especially of the extremities. In that perspective severe cold is less encountered nowadays, however mild cold exposure still occurs during daily life activities. Therefore in this thesis the effects of mild cold (i.e. without shivering) exposure on energy expenditure are studied.

Upon cold exposure core body temperatures can be maintained via two mechanisms, by reducing heat transfer from core to skin via vasoconstriction (6), and/or by increasing heat production. Extra heat can be produced by means of involuntary muscle contractions called shivering thermogenesis (ST) or by non-shivering thermogenesis (NST). Shivering is a very efficient way to produce heat, and peak-shivering intensity can increase EE five times (7). However, ST cannot be

maintained for longer periods of time, it impairs coordination and is uncomfortable. When the environment is mildly cold shivering does not start immediately (8) and in this condition NST precedes shivering (Figure 1).

Figure 1. Heat production and EMG signal preceding and during the onset of shivering. Top panel: Heat production is lowest during baseline condition (thermoneutral), increases preceding the onset of shivering, and EE is highest during shivering. Lower panel: EMG signal is low during baseline and preceding the onset of shivering. The first (1) arrow indicates start of cold exposure (removal of duvet) and the second arrow (2) indicates onset of shivering. NST, non-shivering thermogenesis; ST, shivering thermogenesis; EMG, electromyography; W, watt (Joule/s). Image obtained from (8).

Van Ooijen et al. showed a significant inter-individual variation in NST upon mild cold exposure (9). The changes in energy expenditure upon mild cold exposure in young adults ranged from -0.15 to 1.45 MJ/day. Moreover, it was shown that in winter a significant higher NST was found (mean cold response: 11.5% of basal metabolic rate; BMR) compared to summer (7% of BMR) in the Netherlands (9). Again, considerable individual differences were observed in this metabolic response, which remained throughout the season. It has been shown that NST can be as high as 30% of BMR. In the long run increases in EE of 5% or more (> 1 MJ/d) may have considerable impact on body weight and body fat stores (10).

CHRONIC COLD EXPOSURE

As stated before, cold exposure can increase EE. The regulated rise in EE upon cold is called *facultative thermogenesis* (FT). FT protects the organism against cold and thereby heat balance is restored (1). Besides acute cold, chronic cold exposure can increase EE as well.

Acclimatization are the physiological or behavioral changes occurring within an organism that reduce the strain caused by stressful changes in the natural climate, while *acclimation* refers to the physiological or behavioral changes occurring within an organism that reduce the strain caused by experimentally induced stressful changes in particular climatic factors (11). When placed in a cold environment, rodents will acutely defend their body temperature by shivering and thereby they can increase their heat production five times. When this cold exposure continues, shivering gradually decreases and NST increases and this keeps total EE elevated (12). The acquired rise in energy expenditure upon chronic cold exposure is called *adaptive thermogenesis* (AT). Thus, FT is the regulated increase in EE to acutely defend internal body temperatures, while AT develops over time. The classical study on AT in humans is from Davis and was already published in 1961 (13). Daily cold (12 °C) exposure revealed a gradually decrease of shivering in time, while heat production remained elevated (Figure 2). This decreased shivering with elevated metabolism strongly resembles the results from cold acclimated rodents. At that time it was unknown which tissue was responsible for the increased EE in human adults.

BROWN ADIPOSE TISSUE

The above-described AT in chronically cold-exposed rodents is fully attributable to brown adipose tissue (BAT) (14). The extra heat produced during ST comes from involuntary muscle contractions, while BAT is the main site for NST. Mammals have two distinct types of fat tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). The main function of WAT is storing energy and it can grow from 6 to 50% of body weight during the development of obesity (15). In contrast, the main function of BAT is the production of heat. Heat is produced by the BAT-specific uncoupling protein-1 (UCP1). Uncoupling proteins transport either protons into the mitochondrial matrix and/or non-esterified fatty acid anions out of the matrix, and thereby lower the proton gradient. Extra heat is now produced, and less ATP (16). This heat facilitates body temperature maintenance during cold exposure.

Figure 2. Mean heat production (Metabolic rate; left axis / solid line) and shivering (right axis / dashed line) during a cold acclimation protocol (daily cold (8 °C) exposure for 8 hours a day). Shivering decreases during the first 10 days of cold exposure while EE remains elevated. Image adapted from (13).

Huttunen et al. (17) showed in 1981 that necropsies from the neck region contained more multilocular adipose tissue in outdoor workers compared to controls. In 2007 Nedergaard and Cannon (18) were the first who described the possible metabolic function of active BAT depots in human adults based on studies in the field of nuclear medicine. The development of new imaging techniques made it possible to show BAT depots in human adults. By means of positron-emission-tomography and computed-tomography (PET/CT), with the injection of the radioactive glucose tracer 2-deoxy- ^{18}F -fluoro-D-glucose (^{18}F FDG) glucose uptake can be visualized. Thus, with ^{18}F FDG-PET/CT-imaging metabolic active tissues can be studied. Using this technique three research groups showed in 2009 that human adults possess cold-activated BAT depots (19-21). The presence of BAT was confirmed with UCP1-positive stainings in adipose tissue from the supraclavicular region. From that moment on several studies have investigated the role of BAT in adult humans.

Figure 3. BAT in humans. Left panel: A negative relation between BAT activity and BMI was found. Healthy men with high BMI have low BAT activity. Right panel: [^{18}F]FDG-PET image of the upper body after cold exposure. BAT is shown supraclavicular, cranial, in the neck and along the spinal cord. Image obtained from (19).

In two studies a negative correlation was found between BMI and body fat percentage with BAT activity (19, 21). Subjects with a high BMI and body fat percentage have low BAT activity levels (Figure 3). This is in line with earlier observations of a blunted cold-induced thermogenesis (CIT) in obese subjects upon exposure to 16 °C (22). Orava et al. (23) showed low cold-activated BAT activity in obese compared to lean subjects. Weight loss caused a non-significant increase in BAT activity ($P=0.058$). Vijgen et al. (24) showed that weight loss (induced by gastric surgery) increases BAT activity, illustrating the plasticity of BAT. If BAT can be recruited by other mechanisms in obese subjects remains further elucidation.

OUTLINE OF THE THESIS

This thesis focuses on the effect of acute and chronic cold exposure on BAT activity and recruitment in lean and obese subjects, and how mild cold exposure can be used as a new approach to increase energy expenditure. In **Chapter 2** methodological considerations with regard to the measurement of BAT in humans are reviewed. So far, the gold standard to determine BAT in humans is [^{18}F]FDG-PET/CT-imaging. However, this technique requires the use of a radioactive tracer. Therefore we explored the feasibility to study human BAT with the less invasive and non-radioactive methodology MRI (**Chapter 3**), and with supraclavicular skin temperature measurements (**Chapter 4**). **Chapter 5** describes a proof-of-principle study on the effects of cold acclimation on BAT in lean, healthy subjects. Since the

effects of cold acclimation are expected to be beneficial for obese subjects in particular, we repeated the cold acclimation study in healthy obese men (**Chapter 6**). In **Chapter 7** we evaluated the effects of cold acclimation on immune markers in skeletal muscle. Finally, in **Chapter 8**, the results obtained in this thesis are discussed and the implications for further research are presented.

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CHAPTER 2

Cold-activated brown adipose tissue in human adults: methodological issues

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ABSTRACT

The relevance of functional brown adipose tissue (BAT) depots in human adults was undisputedly proven approximately seven years ago. Here we give an overview of all dedicated studies that were published on cold-induced BAT activity in adult humans that appeared since then. Different cooling protocols and imaging techniques to determine BAT activity are reviewed. BAT activation can be achieved by means of air- or water-cooling protocols. The most promising approach is individualized cooling, during which subjects are studied at the lowest temperature for non-shivering condition, probably revealing maximal non-shivering thermogenesis.

The highest BAT prevalence (i.e., close to 100%) is observed using the individualized cooling protocol. Currently, the most widely used technique to study the metabolic activity of BAT is deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG)-positron emission tomography/computed tomography (PET/CT) imaging. Dynamic imaging provides quantitative information about glucose uptake rates, whereas static imaging reflects overall BAT glucose uptake, localization, and distribution. In general, standardized uptake values (SUV) are used to quantify BAT activity. An accurate determination of total BAT volume is hampered by the limited spatial resolution of the PET image, leading to spillover. Different research groups use different SUV threshold values, which make it difficult to directly compare BAT activity levels between studies. Another issue is the comparison of ^{18}F FDG uptake in BAT with respect to other tissues or upon with baseline values. This comparison can be performed by using the “fixed volume” methodology.

Finally, the potential use of other relatively noninvasive methods to quantify BAT, like magnetic resonance imaging or thermography, is discussed.

INTRODUCTION

Based on histological criteria Heaton et al. (1) showed already in 1972 that there is a wide distribution of brown adipose tissue (BAT) in humans. In the field of nuclear medicine the presence of active BAT was shown by among others, Cohade et al. (2); an evaluation of 359 positron emission tomography/computed tomography (PET/CT) examinations, after the administration of 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) showed in 4% of all patients ^{18}F FDG uptake in fat tissue. This technique enables the study of metabolic active tissues on a glucose substrate. This fat tissue was called “USA-fat” of unknown etiology, which might cause confusion with tumor and/or metastases. These findings indicated that BAT is present in at least a significant fraction of human adults; however, its physiological importance required further investigation (3).

The relevance of BAT in human adults was eventually demonstrated in 2009. Dedicated studies using cold exposure revealed active BAT depots in 46–100% of young healthy subjects (4-6). This showed that BAT in human adults, like in rodents and human infants, is linked to the cold-induced metabolic response. Besides the visual data (PET/CT imaging), the three groups confirmed the presence of BAT with positive staining for uncoupling protein-1 (UCP1) in biopsies from the supraclavicular region. UCP-1 is a hallmark of BAT as it uncouples the electron transport chain and thereby heat is produced (7). Since the discovery of the relevance of BAT depots in human adults, at least 20 dedicated studies have confirmed that human adults possess active BAT depots (Fig. 1 summarizes all dedicated studies on cold-activated BAT quantified by PET/CT imaging in humans). Cold-induced human BAT is usually found in the neck, in acromial-clavicular, supraclavicular, para-aortic, axillary, paravertebral, and in perirenal depots (Fig. 2). Based on cell-specific markers UCP1-positive cells from human adipose tissue depots can be classified as “classical” (8) or “inducible” (beige/brite) (9). Seale et al. (8) showed in 2008 that “classical” BAT cells derive from a myf5+, a muscle-like lineage, whereas “inducible” cells derive from a myf5-cell lineage, a fat-like lineage (8). “Classical” BAT cells express high levels of UCP1 under basal conditions, whereas clusters of “inducible” cells can only be recognized in white adipose tissue (WAT) after stimulation (10).

Summary diagram on human BAT studies

Cooling techniques

- Air cooling; BAT prevalence 60-96%*
- Air cooling with additional cooling; BAT prevalence 36-100% in lean and 31% in obese**
- Individualized cooling; 94-100% in lean and 50% in obese***
- Water cooling including shivering****

Influences

BAT activity

Influences

Detected by

Subject characteristics

- Body composition
- Gender
- Age
- Feeding status
- Cold adaptation
- Others

Tracers

- [^{18}F]FDG to study glucose metabolism
- [^{15}O]H₂O to study tissue perfusion
- [^{15}O]O₂ and [^{15}O]CO to study total BAT oxidation
- [^{18}F]FTHA to study fat oxidation
- [^{15}C]acetate to study tissue oxidative activity

* (3 studies; upper grey area); ** (12 studies; upper white area);

*** (5 studies; lowest grey area); **** (1 study; lowest white area)

Method of cooling	Method of scanning	Subjects	BAT activity	BAT prevalence	Study
16 °C cool air for 2 hours	Static [^{18}F]FDG Threshold (unpublished; 0.9 kBq)	24 healthy M only 10 lean BMI 23.2 ± 1.2 15 overweight BMI 30.3 ± 4.2	Lean 428 ± 394 kBq Overweight 102 ± 93 Bq	23 of 24 BAT+ (96%)	Van Marken Lichtenbelt et al. NEJM, 2009 (5)
16-18 °C cool air for 2 hours	Static [^{18}F]FDG Threshold 2.0 SUV	10 healthy M only BMI 20-24	13.1 SUV max (range 6.1 – 27.6)	6 of 10 BAT+ (60%)	Vrieze et al. J Nucl Med, 2012 (46)
16 °C cool air for 2 hours	Static [^{18}F]FDG Threshold 2.0 SUV	6 healthy Gender unknown BMI 25.4 ± 4.9	2.9 ± 0.5 SUV mean	12 of 16 BAT+ (75%)	Schlogl et al J Clin Endocrin Metab, 2013 (48)
17-19 °C cool air for 2 hours Placing feet in ice water 5-9 °C, 5 minutes	Dynamic [^{18}F]FDG Threshold not indicated	5 healthy Gender unknown BMI unknown	Not indicated, example; 12.2 µmol/min /100g	5 of 5 BAT+ (100%)	Virtanen et al. NEJM, 2009 (6)
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [^{18}F]FDG Greater than background	56 healthy 31 M and 25 F BMI range 16.2 – 31.0	Not indicated	19 of 56 BAT+ (46%)	Saito et al. Diabetes, 2009 (4)
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [^{18}F]FDG Greater than background	13 healthy M only BMI 20.0 ± 2.0	3.0-18.3 SUV max in BAT+ group	6 of 13 BAT+ (46%)	Yoneshiro et al. Obesity, 2011 (30)

Continued from previous page					
Method of cooling	Method of scanning	Subjects	BAT activity	BAT prevalence	Study
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [¹⁸ F]FDG Threshold 2.0 SUV	162 healthy 103 M and 59 F BMI 22.1 ± 3.0	9 SUV max in cohort 20s 6 SUV max in cohort 30s	67 of 162 BAT+ (41%)	Yoneshiro et al. Obesity, 2011 (41)
17 °C cool air for 2 hours Placing feet in ice water 8 °C, 5 minutes	Dynamic [¹⁸ F]FDG and [¹⁵ O]H ₂ O Threshold 3.0 μmol/min/100g	27 healthy 7 M and 20 F BMI unknown	9.1 ± 5.1 μmol/min /100g	19 of 27 BAT+ (70%)	Orava et al. Cell Metab, 2011 (15)
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [¹⁸ F]FDG Threshold 2.0 SUV	18 healthy M only BMI 18.7 – 24.3	Not indicated	10 of 18 BAT+ (56%)	Yoneshiro et al. Am J Clin Nutr, 2012 (29)
20 °C cool air and cooling vest (14 °C) for 2 hours	Static [¹⁸ F]FDG Threshold 2.0 SUV	10 healthy 4 M and 6 F BMI 23.7 ± 1.4	6.2 SUV max (interpreted from figure)	10 of 10 BAT+ (100%)	Cypess et al. PNAS USA, 2012 (26)
16 °C cool air with fans	Dynamic and static [¹⁸ F]FDG [¹⁵ O]H ₂ O [¹⁵ O]CO [¹⁵ O]O ₂ Threshold 2.0 SUV	14 healthy 5 M and 9 F BMI 19.1 – 27.4	7.4 ± 3.7 SUV max in BAT+ group	5 of 14 BAT+ (36%)	Muzik et al. Front Endocrinol, 2012 (27)
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [¹⁸ F]FDG Greater than background	199 healthy 125 M and 74 F BMI 21.9 ± 0.2	Not indicated	88 of 199 BAT+ (44%)	Yoneshiro et al. Int J of Obes, 2012 (51)
19 °C cool air for 2 hours Placing legs on an ice block, 4 minutes	Static [¹⁸ F]FDG Threshold 2.0 SUV	51 health Males only BMI 22.0 ± 0.4	5.5 ± 0.6 SUV mean in BAT+ group	27 of 51 BAT+ (53%)	Yoneshiro et al. JCI, 2013 (50)
15.5 °C cool air with fans	Dynamic and static [¹⁸ F]FDG [¹⁵ O]H ₂ O [¹⁵ O]CO [¹⁵ O]O ₂ Threshold 2.0 SUV	25 healthy 10 M and 15 F BMI 23.4 ± 3.5	10.7 ± 3.9 SUV max in BAT+ group	9 of 25 BAT+ (36%)	Muzik et al. J Nucl Med, 2013 (40)
17 °C cool air for 2 hours Placing feet in ice water 8 °C, 5 minutes	Dynamic [¹⁸ F]FDG and [¹⁵ O]H ₂ O Threshold not indicated	36 obese 11 M and 25 F BMI 34.0 ± 4.1	3.9 μmol/min /100g (interpreted from figure)	11 of 36 BAT+ (31%)	Orava et al. Diabetes 2013 (24)
Individualized cooling protocol; air cooling with water cooling (mattress) for 2 h	Static [¹⁸ F]FDG (unpublished; 0.9 kBq)	15 morbid obese 2 M and 13 F BMI > 35	5.5 ± 14.8 kBq (range 0.0-54.5)	3 of 15 BAT+ (20%)	Vijgen et al. PLoS ONE, 2011 (34)
Individualized cooling protocol; Air cooling with water cooling (mattress) for 2 h	Static [¹⁸ F]FDG Threshold 1.5 SUV	10 healthy 2 M and 8 F BMI 29.8 ± 4.2	2.8 ± 3.9 before and 6.3 ± 3.2 SUV max after in BAT+ group	5 of 10 BAT+ (50%)	Vijgen et al. J Clin Endocrin Metab, 2012 (25)
Individualized cooling protocol; Air cooling for 2 h	Static [¹⁸ F]FDG Threshold 1.5 SUV	10 healthy M only BMI 21.6 ± 1.6	891 ± 1027 SUV total	10 of 10 BAT+ (100%)	Vosselman et al. Diabetes, 2012 (28)

Continued from previous page					
Method of cooling	Method of scanning	Subjects	BAT activity	BAT prevalence	Study
Individualized cooling protocol; Air cooling for 2 h	Static [^{18}F]FDG Threshold 1.5 SUV	6 healthy M only BMI 22.4 ± 2.1	7.2 ± 2.1 SUV mean	6 of 6 BAT+ (100%)	Vosselman et al. Am J Clin Nutr, 2013 (47)
Individualized cooling protocol; Suit cooling for 2.5 h	Dynamic and static [^{18}F]FDG Threshold 1.5 SUV	17 healthy 8 M and 9 F BMI 21.6 ± 2.2	14.1 ± 7.0 SUV max 6.9 ± 3.0 $\mu\text{mol}/\text{min}/100\text{g}$ before cold acc	16 of 17 BAT+ (94%)	Van der Lans et al. JCI, 2013 (39)
Tube suit with 18 °C water flow for 2,5h (subjects shivered)	Dynamic and static [^{18}F]FDG [^{18}F]THA [^{11}C]acetate Threshold 1.0 SUV	6 healthy M only BMI $23.7 - 31.0$	10.8 ± 4.5 $\mu\text{mol}/\text{min}$ (range 1.3 – 28.7)	6 of 6 BAT+ (100%)	Ouellet et al. JCI, 2012 (14)

Figure 1. All studies published since 2009 that studied cold-activated BAT in humans with PET/CT imaging are shown and the diagram summarizes the studies. The use of an individualized cooling protocol to activate BAT is preferred due to the highest probability to detect BAT. The effect of body composition, gender, age, feeding status, and cold adaptation are discussed in the paragraph individual variation. BMI is expressed in kg/m^2 , M males; F females.

Their coexpression of UCP1 makes it likely they both reveal the same functional role in adaptive thermogenesis. For an extensive review on the development and function of both “classical” and “inducible” fat, we refer to Harms and Seale (10). It is known that human depots show a mixed composition of both “classical” and “inducible” cells (11-13). Current imaging techniques cannot distinguish between the two BAT cell types. Therefore, classification is currently only possible based on biopsy material. Irrespective of their classification, only depots with increased glucose uptake compared with surrounding tissue are visualized with [^{18}F]FDG-PET/CT imaging. [^{18}F]FDG is the most commonly used radiotracer to quantify human BAT activity. But other tracers, like labeled fatty acids (14) to study fatty acid metabolism or labeled water (15) to study BAT perfusion, are used as well. To quantify human BAT activity without the use of radioactive tracers, scanning techniques like magnetic resonance imaging (MRI) (16, 17) and thermography (18, 19) are also explored.

This article evaluates BAT activation protocols and scanning techniques. First, different ways to activate BAT are discussed, with the focus on cold exposure; air- and water-cooling techniques are explained followed by the differences between “fixed” and “individualized” cooling protocols. Second, static and dynamic PET-imaging techniques are discussed. To compare BAT activity with other tissues additional analyses methods on “detectable” BAT volume and the “fixed” volume are discussed. Finally, the restrictions of [^{18}F]FDG-PET/CT imaging and potential alternative techniques are discussed.

Figure 2. Uptake of deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) in brown adipose tissue (BAT). Left: ^{18}F FDG-positron electromagnetic tomography (PET) image of a subject showing BAT activity. Arrows indicate depots where human BAT is usually found. The dashed line indicates the site of the transversal slice. Right: transversal computed tomography (CT) (top), PET (middle), and PET/CT fusion (bottom) slices of the supraclavicular region showing BAT activity.

BAT ACTIVATION

Cold exposure activates the sympathetic nervous system to secrete norepinephrine (NE), which activates brown fat cells (Fig. 3) (7). NE binds to the β_3 -receptor on the cell surface, after which intracellular signaling cascades lead to the degradation of triglycerides into free fatty acids (FFA) (20). FFA interact with UCP1 and this leads to respiration uncoupled from ATP synthesis (3). The energy from the combustion of substrates is therefore directly released as heat. Mitochondria in BAT oxidize apart from internal stored FFA, glucose and FFA from the circulation. Besides cold, insulin might also stimulate glucose metabolism in BAT. BAT is a highly insulin-sensitive tissue, possibly due to a high expression of GLUT4 (7). Insulin infusion resulted in an increased glucose uptake in BAT, comparable with uptake in skeletal muscle (15). However, no increased perfusion was found. This indicates that insulin under these circumstances does not increase thermogenesis. Therefore, the authors state that insulin is not a significant player in the NE-driven cold activation of BAT. On the contrary, cold exposure decreases plasma insulin concentrations (15). The activation of BAT by cold and the uptake of glucose and ^{18}F FDG are schematically described in Fig. 3.

Figure 3. BAT activation and thermogenesis. Upon cold exposure brown fat cells are stimulated via norepinephrine (NE) released from the sympathetic nervous system. NE binds to the β_3 -receptor and intracellular signaling processes lead to degradation of triglycerides (TG) into free fatty acids (FFA). FFA interacts with uncoupling protein-1 (UCP1), which leads to respiration uncoupled from ATP synthesis. All energy from the combustion of substrate is therefore directly released as heat. Besides intracellular FFA released from TG, mitochondria in the brown fat cell also oxidize glucose and FFAs from the circulation. Insulin also results in glucose and [18 F]FDG uptake via GLUT4 activation, but no heat is produced. Both [18 F]FDG and glucose are taken up by members of the glucose transporter family (GLUT). After phosphorylation by hexokinase (HK), FDG-6-phosphate cannot be further metabolized and is trapped in the cell and is visualized by PET imaging. Adapted from (3, 15, 20, 21).

Tissues that take up glucose are visualized with [18 F]FDGPET imaging. [18 F]FDG is a glucose analog, therefore, [18 F]FDG-PET imaging provides information about glucose uptake and phosphorylation. After phosphorylation by hexokinase, FDG 6-phosphate is trapped in the cell (Fig. 3). Radioactivity values represent tracer uptake values. As illustrated in Fig. 3, glucose uptake is increased in activated BAT and therefore active BAT depots are visible as hotspots on the PET image. Thus BAT activity values reported in several articles represent glucose uptake values. The question remains if a glucose tracer is an adequate tool to study BAT oxidation. Recent work from Shabalina et al. (22) showed that “brite” cells utilize glucose from the circulation for oxidation, confirming increased glucose metabolism in activated cells. However, the proportion of glucose oxidation to total BAT oxidation is currently unknown in humans.

Mild cold exposure may induce non-shivering thermogenesis (NST). Rodent studies revealed that BAT is the main contributor to non-shivering thermogenesis (7). Therefore, most studies investigate BAT in humans by cold activation. So far, cold is the strongest and most widely tested activator of BAT in humans, for other activators we refer to the review of Harms and Seale (10). Since BAT is involved in NST, the studies using cold exposure are most representative to obtain insight on BAT prevalence and activation. Retrospective studies showed that active BAT depots could be detected in ~6% of adult population (23, 24). These studies included images of patients that are scanned for diagnostic reasons in which no active cooling protocol is applied. Dedicated studies that investigated human BAT using cooling protocols reveal a BAT prevalence of 20–31% in morbidly obese subjects (25, 26) and 40–100% (4-6, 27-31) in lean healthy subjects. Although the variation is still high, these results indicate that the prevalence of BAT in humans is much higher than previously appreciated. Besides individual variation, group (e.g., age, gender), and differences in ethnicity, the cooling protocols themselves may affect the probability to detect BAT and thus its presence and activity, as described below.

Shivering and non-shivering thermogenesis. Upon cold exposure internal body temperature can be maintained by means of increased heat production [shivering thermogenesis (ST) and/or NST] by reducing heat loss via vasoconstriction in the extremities or a combination of both (32). When assessing the contribution of BAT to energy expenditure (EE; heat production), it is necessary to distinguish between ST and NST. Rodent studies revealed that ST is due to involuntary muscle contractions, whereas for NST the extra heat is mainly produced in BAT. Whether this applies to humans is currently unknown. NST is defined as the “thermogenesis that replaces shivering” (7), which is totally UCP dependent (33). When rodents are adapted to cold environmental temperatures, shivering gradually diminishes while EE remains elevated, illustrating the increased NST.

Human BAT metabolism should be studied in the absence of shivering. Otherwise, active skeletal muscles (shivering is due to muscle contractions) would take up the [^{18}F]FDG, leaving less tracer available for other tissues. Moreover, no distinction can be made between ST and NST. To this end, most studies check the absence of shivering by visual inspection and/or a subjective measure (by asking the subject), sometimes combined with electromyography (EMG). However, not all research groups use absence of shivering, as illustrated by Ouellet et al. (14), who reported that $1.6 \pm 0.5\%$ (range 1.1 to 2.4% of maximal voluntary

contraction) shivering occurred, which makes it difficult to distinguish NST from ST. However, it should be noted that other studies have relied on subjective measurements, and it is our personal experience that subjects not always indicate shivering correctly. The use of EMG to measure the onset of shivering is complicated by individual variation in the muscles used for shivering. Therefore, we advise the combination of asking subjects, visual inspection, and EMG to check for the absence shivering during cooling protocols where maximal NST is studied.

Individual variation. NST and BAT activity show a high individual variability; this can partly be explained by the following factors.

Body composition. A thicker subcutaneous fat layer results in a higher insulation. As a consequence, obese subjects have a later onset of shivering (34). Indeed, after a “fixed” cooling protocol (16°C for 48 h) obese subjects show a blunted NST (35). It is possible that the cold stimulus given in this protocol was not enough to induce maximal NST in the obese subjects. It is likely that the use of an individualized cooling protocol is required to induce NST in obese subjects. Upon weight loss environmental temperatures for maximal NST condition increases, moreover an increased BAT activity was found (26), and in this study an individualized cooling protocol was used.

Gender. On average, lower temperatures are needed to induce shivering in males compared with females. The higher BAT prevalence in females reported in retrospective studies (23, 36, 37) can therefore be biased by indoor temperatures in hospitals, which may be low enough to induce NST in females but not in males. Indeed the thermoneutral zone, a range of environmental temperatures that does not result in regulatory changes in evaporative heat loss or changes in metabolic heat production, is in general higher for females compared with males (38). Only a few dedicated studies investigated cold-activated BAT activity in both females and males. Interestingly, two studies showed similar levels of cold-activated BAT for both sexes (27, 39). On the other hand, Orava et al. (25) and Muzik et al. (28, 40) reported, after fixed cooling protocols (see Fig. 1 for description of cooling protocols used), higher BAT prevalence in females (Orava et al.: 87% females in BAT+ group, 58% females in BAT-group, Muzik et al.: BAT+ group 8F/1M, BAT-group 7F/8M). However, both groups used a “fixed” cooling protocol, therefore, it is possible that the cold stimulus in both studies was too mild for the male subjects.

Age. Based on [¹⁸F]FDG-PET/CT imaging there is only one dedicated study that showed that elderly show a gradual decrease in BAT activity levels with increasing

age (41). Pfannenberger et al. (37) showed in a retrospective study that BAT activity declines with increasing age. Both studies are in line with a decline in cell-specific markers for BAT with increasing age found in biopsy material (1, 42). A decrease in BAT with increasing age is in line with the blunted or absence of NST in elderly found in other studies that investigated EE and not BAT activity (43, 44). With respect to the decreased BAT activity in elderly, Nedergaard et al. (45) speculate that ageing results in lower sex hormones levels, which might lower BAT activity and favors expansion of WAT.

Feeding status. Because of the lower BAT activity found in subjects with higher body mass index, it is suggested that BAT is involved in body weight regulation. Normally, human BAT is investigated in fasted subjects. Cold-exposed healthy male subjects showed lower BAT activity in the fed state compared with the fasted state (46). Intake of a high-caloric, high-carbohydrate meal showed that BAT is active in the postprandial phase; however, the contribution of BAT to diet-induced thermogenesis requires further elucidation (47). No BAT activation was found upon a 24-h 200% overfeeding protocol (48). However, it is possible that BAT activity was underestimated due a diet-induced change in availability of the tracer. The contribution of BAT on diet-induced thermogenesis and body weight regulation remains further elucidation.

Cold adaptation. Huttunen et al. (49) already showed in 1981 that necropsies taken from neck arteries and pericardium have a higher occurrence of BAT in outdoor workers compared with control subjects. Indeed, recent [^{18}F]FDG-PET studies confirmed that cold acclimation increases BAT activity in parallel with an increased NST (39, 50).

Besides the above-mentioned inter-individual variations, BAT presence and activity and NST vary within groups. This is illustrated by the wide range in BAT activity and NST, both in morbidly obese subjects and lean healthy men (14, 34) (BAT activity: 0–54.5 kBq, EE: 35.5–51.1 J/s and 1.3–28.7 $\mu\text{mol}\cdot\text{min}^{-1}\cdot 100^{-1}$, EE 2.3–4.6 kcal/min, respectively). The reason for this variation is unclear yet possible explanations could be the degree of cold adaptation or genetic variation in for example UCP-1 (51). Besides the above-mentioned individual differences, the cooling protocols themselves have an influence on NST and BAT activity levels found, as described below.

Fixed cooling protocols. The first studies on cold-activated BAT in human adults used “fixed” cooling protocols to activate BAT. Cold exposure consisted of placing subjects in an environmental temperature of 16 °C for 2 h (5) or a slightly higher

environmental temperature (19°C) with leg cooling in ice water (4, 6, 31). BAT prevalence in these studies ranged from 46% to 100%. Other groups used in addition air fans (40) or a cooling vest (27). The use of “fixed” cooling protocols with an ambient temperature below 17°C results in highest probability to detect BAT in lean subjects. The probability to detect active BAT depots is decreased when subjects are placed in an ambient temperature of 19–20 °C. In those cases additional air fans did not increase the probability (40), whereas the use of a cooling vest resulted in 100% BAT detection (27).

Individualized cooling protocols. When using a fixed low temperature protocol an obese subject will experience less intensive cold than a lean subject. This is due to extra subcutaneous adipose tissue and lower surface-to-volume ratio, which results in a higher insulation (52). This was confirmed in pilot experiments where obese subjects had a lower onset temperature of shivering (34). From that moment on our research group uses individualized cooling protocols. Subjects are cooled until shivering and then temperature is set slightly (1–2 °C) above the temperature that causes the onset of shivering. With this protocol BAT activity is measured during maximal non-shivering thermogenesis, most likely representing maximal BAT activity. We found that by means of an individualized cooling protocol the probability to detect BAT is nearly 100% (94–100%) in lean young subjects (29, 39, 47).

Air- versus water-cooling. BAT activation can be achieved by means of air cooling (cold room or tent) and/or water cooling (water-perfused suit). The latter has the advantage that it is suitable for simultaneous cooling and imaging inside the PET/CT scanner. Absolute temperatures for both thermoneutral and mild cold condition differ between both techniques because of the differences in thermal properties of the media. For example, environmental temperature for a thermoneutral measurement in healthy young men (dressed in sweatpants, T-shirt, and socks; 0.58 clothing-insulation value) positioned on an air-permeable stretcher was 24.3 ± 0.2 °C, whereas when wrapped in a water-perfused suit thermoneutral condition was obtained with a suit temperature (average of inlet and outlet) of 30.0 ± 2.3 °C. Stable non-shivering conditions were reached with an environmental temperature of 14.3 ± 1.1 °C during air-cooling, whereas the average suit temperature was 24.6 ± 0.9 °C. Nevertheless, these apparent different temperatures result in similar metabolic conditions, which is explained by the greater conductive heat loss during water-cooling. When using individualized cooling protocols, both air- and water-cooling protocols result in

high BAT detection (94–100%) in lean subjects (29, 39, 47).

In summary, temperatures for both thermoneutral and mild cold conditions differ among people and also depend on the cooling method used. This might result in differences in BAT activation and thus in the BAT activity values found. To study subjects in similar metabolic conditions (maximal NST), we advise the use of individualized cooling protocols. When obese subjects are studied, air-cooling down to 9°C is not sufficient to reach maximal NST conditions (34). Therefore, for obese subjects we recommend the use of water-cooling protocols. Moreover it is important, when the contribution of BAT on NST is investigated, to avoid ST completely. In Fig. 1 all dedicated studies on cold-activated BAT by means of PET/CT imaging are summarized.

BAT MEASUREMENTS

Nowadays, the assessment of [^{18}F]FDG uptake is the most commonly used method to visualize BAT activity in human adults. As indicated in Fig. 3, [^{18}F]FDG cannot be metabolized after phosphorylation by hexokinase and is therefore trapped inside the cell (3). Visualization of [^{18}F]FDG uptake therefore provides a measure of glucose uptake in the depots.

Static imaging. During static imaging protocols, subjects are exposed to (mild) cold for 1–2 h before [^{18}F]FDG administration. [^{18}F]FDG has a physical half-life of 110 min and is quickly taken up by tissues. Since tissues take up most of the tracer within 45 min, tracer administration is 1 h before the PET/CT scan. After injection subjects are exposed to cold for another hour. Subsequently, subjects are transported toward and positioned inside the PET/CT scanner. Imaging starts with a low-dose CT scan of the region of interest followed by a PET scan of the same region. The CT scan provides anatomical information, whereas with the PET scan tracer uptake sites are visualized (Fig. 2 visualizes a CT, a PET, and a fused PET/CT image of the supraclavicular region, showing [^{18}F]FDG uptake). The overall amount of [^{18}F]FDG accumulation in BAT can be expressed in Becquerel (Bq) or in standardized uptake values (SUV). The latter normalizes measured activity levels for body weight and the injected dose and is therefore recommended. SUV can be expressed in SUV mean (averaged activity values), SUV max (activity hotspot within the tissue), and/or SUV total (SUV mean times volume of the tissue). The use of SUV total is not recommended, because a ratio (SUV mean) is multiplied by a volume (an “exact value”).

Dynamic imaging. In contrast to static imaging protocols, where cooling, tracer administration, and uptake take place before the PET/CT scan, during dynamic scanning tracer administration is performed at start of PET acquisition. During dynamic acquisition, tracer distribution is obtained as a function of time, which results in time-activity curves (TACs). Therefore, cold stimulation is required during the actual scan. This can be achieved by cooling with a water-perfused suit (14, 39). Another reported method used to cool subjects during the scan is placement of the leg in ice water (6, 15, 25). The first method ensures whole body cooling that strictly regulates skin temperatures. It can be speculated that leg cooling results in less intense cold stimulation since only local body parts are actively cooled and that this might result in less BAT activation. This is consistent with the lower BAT prevalence (70%) reported by Orava et al. (15) compared with the higher BAT prevalence found by van der Lans et al. (39) and Ouellet et al. (14), 94% and 100% respectively. From the TACs, a quantitative measure of glucose uptake rate (expressed in $\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$) can be determined using Patlak curve fitting (53) using a lumped constant of 1.14 (54). The lumped constant accounts for the difference between [^{18}F]FDG and glucose with respect to tissue uptake and phosphorylation kinetics. Our research group performed both a dynamic and static PET scan in 16 young, healthy volunteers and we found a good correlation between glucose uptake rates and SUV mean values (Fig. 4, R^2 0.77; $P<0.001$).

BAT activity values obtained with static imaging (expressed in SUV) provide semi quantitative information on activity levels and represent overall tracer accumulation. A decreased total BAT activity can be observed when BAT activity decreases or when tracer availability is affected due to competitive tracer uptake by other tissue. For example, after a meal, ~80% of glucose is taken up by muscle tissue (55). This influences [^{18}F]FDG uptake in BAT and other tissues. Our research group showed a decreased [^{18}F]FDG uptake in the brain, which is anticipated to have a constant metabolism after a meal (47). By means of dynamic imaging a quantification of glucose uptake can be obtained due to the use of an arterial input function. This means that changes in tracer availability do not affect glucose metabolic rate values (56). So, depending on the research question, either a dynamic or a static scanning protocol should be performed, or both.

Figure 4. Correlation between glucose uptake rate in supraclavicular depot and standardized uptake values (SUV) mean. A significant correlation was found between cold-activated BAT activity values obtained by means of dynamic scanning (glucose uptake rate) and static scanning (SUV mean). 16 young, healthy subjects were included, of which were 8 females and 8 males, age 23 ± 3.2 yr, and body mass index 21.6 ± 2.2 . Data was obtained from BAT-positive subjects before cold acclimation (39).

Thresholds. To quantify BAT activity, accurate segmentation of BAT depots is essential. When a tracer is distributed homogeneously in a certain volume, a SUV of 1.0 is found. Since glucose metabolism is increased in metabolic active BAT, tracer uptake is elevated ($\text{SUV} > 1.0$) compared with surrounding tissue. Tissues that take up relatively high levels of glucose have high SUVs, e.g., brain has a SUV of > 5.0 (47). WAT, which is relatively metabolic inactive, has a $\text{SUV} < 0.5$ (29). Maximal SUV values for BAT range from 3.0 to 28.0 (31, 46). Therefore, BAT segmentation on [^{18}F]FDG-PET images is often performed using SUV thresholding techniques. Our research group uses a SUV threshold of 1.5 (29), whereas other research groups use SUV values of 1.0 SUV (14) or 2.0 SUV (27, 30, 40, 46). Obviously, with a lower threshold more activity and volume is included and assigned to BAT, in part, explaining the different outcomes between different research groups. Not only SUV thresholding provides information on BAT activity, CT Hounsfield units (HU) might give additional information. The HU of a certain tissue is defined as the difference in attenuation coefficient of this tissue normalized to water. Water has a HU of 0. Tissues denser than water have a

positive HU and tissues less dense than water have a negative HU. Ahmadi et al. (57) found that based on CT HU a distinction between BAT and WAT can be made. Voxels with HU -10 to -87 can be assessed as BAT, whereas voxels with HU -88 to -190 can be quantified as WAT. However, Ouellet et al. (14) showed that cold exposure increases HU of active BAT, indicating that active BAT oxidizes internally stored fatty acids. If HU can be used to assess BAT activity, the technique of Ahmadi et al. needs validation with PET imaging.

Figure 5. Transversal PET/CT fusion (top) and CT (bottom) slices of the supraclavicular (left) and paravertebral (right) region demonstrating [^{18}F]FDG uptake. Volumes of Interest drawn are PET based (threshold 1.5 SUV), visually demonstrating that PET-based “detectable” BAT volume overestimates true activate BAT volume.

BAT volume and activity. Despite the large number of dedicated studies investigating cold-activated BAT in humans, BAT volume levels are reported in only four studies. This is possibly due to the many difficulties concerning volume determination, which are discussed in this section. van Marken Lichtenbelt et al. (5) report an average BAT volume of 130 ± 98 ml in lean subjects and 77 ± 69 ml in overweight/obese subjects. Another study from our research group found an increase of BAT volume from 14 ± 22 to 85 ± 84 ml after gastric bypass-induced weight loss in obese subjects (26). Ouellet et al. (14) found an average BAT volume of 168 ± 56 ml, with a range of 31 to 329 ml. Ouellet et al. used both a SUV threshold (as mentioned above), in combination with a CT threshold. The

latter ensures inclusion of fat tissue only. Values within -200 and -10 HU on the CT scan represent fat tissue. A much higher BAT volume is reported by van der Lans et al. [“detectable” BAT volume 665 ± 451 ml (39)]; in this study BAT volume determination was PET based (only SUV threshold was used, CT HU were only used to verify the presence of fat tissue at the location of increased [18 F]FDG uptake). Because of the limited spatial resolution of the PET image, spillover of the PET signal from the high active BAT regions into the surrounding tissue occurs. Therefore, defining BAT regions based on an absolute, fixed SUV threshold only results in relatively large BAT volumes, this is visualized in Fig. 5. Thresholding based on SUV only can lead to BAT volume overestimation, whereas thresholding based on SUV and HU can on the other hand lead to an underestimation of total BAT activity. It is evident that when both information on BAT activity and BAT volume are needed, different analyzing techniques need to be used. We suggest the use of a SUVbased threshold technique to study total BAT activity. However, when BAT volume is needed the use of HU is an alternative (57). Additionally, the use of MRI can provide additional information on BAT anatomy (see *Alternative Imaging Techniques*).

In summary, based on both a SUV and a HU threshold, total BAT activity is underestimated, whereas based on an absolute, fixed SUV threshold, only BAT volume is overestimated. This only accounts when above-mentioned SUV thresholds (1.0–2.0 SUV) are used. Therefore, accurate determination of human BAT volume requires further investigation.

Fixed volume methodology. Upon an intervention, subjects are scanned repeatedly, and therefore comparisons need to be made between BAT activity values before and after the intervention. When a thresholding technique is used, problems arise when volumes of interest change. For example, “detectable” BAT volume after intervention increases due to addition of areas at the border of the region of interest with SUV values now reaching levels just above the threshold. Therefore, total BAT activity will increase only slightly, whereas volume changes can be relatively large. As a result BAT activity values expressed per volume ($\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$) remain equal or can even decrease. To overcome this issue, we propose the use of a methodology using fixed volumes. In the BAT depot confirmed as brown fat tissue based on both CT and PET images, a box with fixed dimensions ($8 \times 8 \times 8 = 152 \text{ mm}^3$) is centered at SUV max location of the PET image (47), without applying a threshold. In our opinion, when compared with SUV max, this technique is less affected by artifacts present in the image, since

the SUV max value is based on the value of a single voxel. For example, cold acclimation significantly increased BAT activity (39); however, with the “threshold” technique no increase in glucose uptake rate for total supraclavicular BAT was found [before: 6.9 ± 3.0 ; after: $7.6 \pm 2.5 \mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$ ($P>0.05$) (39)]. On the contrary, using the “fixed volume” methodology a significant increase in glucose uptake rate was found [before: 22.5 ± 12.5 ; after: $33.5 \pm 16.8 \mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$ ($P<0.05$)]. The “fixed volume” methodology can also be used to compare activity values between different tissues (e.g., white adipose tissue, liver, brain, skeletal muscle) (47). For these tissues, the location of the “fixed volume” can be chosen using anatomical information based on the CT image.

In summary, both dynamic and static imaging can be used to study BAT activity in humans. When BAT volume is PET based using a threshold of 1.5 SUV, BAT volume will in general be overestimated, and based on both PET and CT imaging, total BAT activity will be underestimated. The “fixed volume” method is proposed to compare tissue activity values upon an intervention and to compare activity values between different tissues.

ALTERNATIVE IMAGING TECHNIQUES

By means of [^{18}F]FDG-PET/CT imaging, tissue glucose uptake (rate) is measured. However, this tracer does not provide information about glucose oxidation or glycogen storage or brake down. With respect to the oxidative metabolism of active BAT, internal stores of fatty acids are likely important, which are also not measured by the glucose tracer. It is well known that BAT oxidizes fatty acids as well. A study in animals showed that fatty acid oxidation amounts to 90% of total oxidation (58). Thus studying BAT activity with a glucose tracer does not provide information on total BAT oxidative metabolism. The relative contribution of glucose and fat oxidation on total BAT oxidative metabolism is currently unknown in humans. In that perspective, fatty acid tracers provide important additional information. Ouellet et al. (14) used the fatty acid tracer [^{18}F]THA ([^{18}F]fluorothiaheptadecanoic acid) to study fat oxidation and [^{11}C]acetate to determine tissue oxidative activity. The results were compared with [^{18}F]FDG scans to study fat and glucose oxidation in cold-activated human BAT (14). The fatty acid tracers cannot identify the contribution of oxidation of internal stored fats, so the actual relative contribution of glucose and fatty acids on total BAT oxidation still awaits further investigation. The tracers [^{15}O]CO and [^{15}O]O₂ provide information on total BAT oxidation (obtained via oxygen consumption), and with the tracer [^{15}O]H₂O

additional information on BAT perfusion is obtained, therefore, Muzik et al. (28, 40) compared these tracers with [^{18}F]FDG scans. These studies showed that at least part of the glucose transported into BAT undergoes oxidative metabolism. The labeled water tracer [^{15}O]H₂O was also used by Orava et al. to define BAT perfusion (15, 25). These studies showed that upon cold exposure BAT perfusion doubles and that cold-stimulated glucose uptake rate and perfusion are correlated. Moreover, cold-activated BAT perfusion correlated with total energy expenditure. This confirms that BAT is highly vascularized and that BAT plays a role in human metabolism. Nowadays, the labeled glucose tracer [^{18}F]FDG is the most commonly used tracer to study BAT in human adults. Additional information on fatty acid and total BAT oxidation is obtained by the use of fatty acid and labeled oxygen tracers. It is currently unknown which proportion of total BAT oxidation is derived from glucose combustion. Therefore, further mechanistic studies on BAT oxidation are warranted.

All above-mentioned studies used radioactive tracers to investigate BAT in humans. Because of the ionizing radiation, the number of scans for each participant is limited. There is an urgent need for a technique that investigates BAT without radiation penalty. In that perspective, several research groups explored the feasibility of MRI for BAT quantification. BAT has higher mitochondrial content, multiple intracellular lipid droplets, and a greater vascularization than WAT. This results in different magnetic properties of BAT and WAT. These differences can be used by several MRI-scanning techniques to distinguish BAT from WAT. Mice studies revealed fat/water signal differences (13, 17, 59) and different relaxation times (60, 61). Moreover, functional MRI techniques can potentially be used to study blood perfusion (62) differences. All abovementioned studies support the capability of MRI to detect and anatomically characterize human BAT in vivo. Hu et al. (16) identified BAT in a human infant by means of chemical-shift MRI and Holstila et al. (63) used DUAL spectral presaturation inversion recovery (SPIR) MR to identify BAT in three adult humans. Our research group explored the feasibility of MRI to study BAT in 11 human adults, and we showed a lower fat fraction in BAT depots compared with subcutaneous WAT depots (62.5 ± 7 vs. 81.5 ± 5.4 %, $P < 0.05$). Additionally, dynamic T2*-imaging revealed signal fluctuations that were sensitive to BAT activation (17). The results so far are promising but need further development before they can be applied.

The main function of BAT is heat production. Therefore, measuring the

temperature in BAT could be a valid method to define whether it is active or not. BAT temperature can be assessed by placing temperature probes into BAT (64, 65) or by thermography of the skin overlaying BAT depots (66). Because of practical considerations, the first method is difficult to implement in humans. The use of infrared thermography was explored by several research groups (18, 19, 67). All showed a larger increase at BAT sites (supraclavicular, neck, and upper back) compared with non-BAT sites (mediastinum) upon cold stimulation (18, 19) or after ephedrine injection (67) in human adults. Therefore, the authors state that thermal imaging can be used to quantify thermogenesis in BAT regions. However, it should be noted that this technique measures skin temperature and not heat production in BAT. The heat produced in BAT is used to maintain core body temperature. Consequently, most of the produced heat will be transported by perfusion to core body sites. Some of the heat might nevertheless be transported toward the skin. The question is whether the change in supraclavicular skin temperature due to this heat leak is representative for BAT heat production. Symonds et al. (19) performed pilot experiments and no increase in supraclavicular skin temperature was found at higher environmental temperatures. In that perspective, thermography could potentially be used to show BAT activity in a qualitative way.

Another noninvasive method to measure BAT thermogenesis could be the use of near-infrared spectroscopy to measure regional blood oxygen saturation. Light from the near-infrared spectrum penetrates through several centimeters tissue and thereby oxygenated hemoglobin can be measured noninvasively (40). A significant correlation between blood oxygen saturation of BAT compared with muscle (not of BAT compared with abdominal WAT) and metabolic rate of oxygen fluxes (obtained by dynamic PET scanning) was found only in subjects with activated BAT. Therefore, the authors suggest that near-infrared spectroscopy can be used in subjects with activated BAT to measure oxidative metabolism noninvasively (40). However, no correlation was reported with the absolute blood oxygen saturation in BAT, only the correlation with the ratio of BAT to muscle is reported. Thus the ratio of BAT to muscle oxygen saturation might be used as an alternative method of BAT thermogenesis.

In summary, the use of labeled fatty acid, acetate, and oxygen tracers provide additional information to measure activity, metabolic pathways, and perfusion of BAT. The use of MRI and thermography is currently explored in humans. These techniques show promising results, however, further development is needed to

quantify human BAT metabolism.

In conclusion, the discovery of the relevance of BAT in human adults approximately seven years ago has forced research into the activators of BAT due to its promising effects on obesity and chronic diseases like Type 2 diabetes. The strongest physiological activator of BAT in humans is cold exposure. Males and females, but also lean and obese experience, a “fixed” cold environment differently. Therefore, when cold-activated BAT is studied the use of individualized cooling protocols is recommended. This is underscored by the fact that highest BAT prevalence is reported after the use of individualized cooling protocols that maximize NST. It is expected that standardization of cooling protocols to activate BAT by the different research groups might eliminate differences in BAT prevalence and activity observed between these groups. Besides differences due to cooling protocols, measurement and analysis methods are also different. This is illustrated by the different SUV thresholds used for BAT segmentation (ranging from 1.0 to 2.0 SUV), resulting in variation in BAT activity and volume values reported. Thresholding based on SUV only leads BAT volume overestimation, whereas thresholding based on SUV and HU results in total BAT activity underestimation. Both dynamic and static [^{18}F]FDG imaging can be used to quantify BAT presence, activity, and distribution. By means of the “fixed volume” methodology, SUV values before and after an intervention can be compared. Additionally, this methodology can be used to compare activity values between different tissues. A major drawback of [^{18}F]FDG-PET/CT imaging is that ionizing radiation is involved. The use of noninvasive methods like MRI and thermography is currently explored. Further studies are warranted to investigate techniques that can study the presence, activity, and distribution of BAT in adult humans relatively noninvasively.

PERSPECTIVES AND SIGNIFICANCE

There is an urgent need for standardization of BAT activating protocols and analyzing techniques. Based on the abovementioned studies, we advise the use of individualized cooling protocols to activate BAT and to maximally activate BAT in all subjects. In particular, when obese subjects are studied, the use of water-cooling is recommended. To compare BAT activity values using [^{18}F]FDG-PET/CT imaging between the different research groups, the same threshold values and analyzing techniques should be used. Therefore, we propose that all researchers studying BAT in humans report the results based on the using a threshold of 1.5

SUV. Besides using a threshold of 1.5 SUV, every research group, of course, can add their own BAT analyses. This would be beneficial to compare activity values between the several research groups investigating human BAT. To compare activity values upon an intervention and between different tissues we recommend the use of the “fixed volume” technique.

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CHAPTER 3

Imaging cold-activated brown adipose tissue using dynamic T2*-weighted magnetic resonance imaging and 2-deoxy-2-[¹⁸F]fluoro-D-glucose positron emission tomography

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ABSTRACT

Objectives. The objective of this study was to explore the use of magnetic resonance imaging (MRI) to identify and quantify active brown adipose tissue (BAT) in adult humans. 2-Deoxy-2-[^{18}F]fluoro-D-glucose (FDG) positron emission tomography (PET) combined with computed tomography was used as a reference method to identify active BAT depots and to guide the MRI data analysis.

Materials and Methods. The ethics committee of the institute approved the protocol, and all participants provided written informed consent before participation. Both PET combined with computed tomography and MRI of BAT were performed in 11 healthy volunteers. Brown adipose tissue was activated by cooling the participants using a dedicated water-perfused suit. For the MRI examination of BAT, water-fat imaging and dynamic T2^* imaging were performed at an effective temporal resolution of 2 minutes per volume. Water-fat images were derived from a multiecho MRI sequence using the Dixon technique.

Results. 2-Deoxy-2-[^{18}F]fluoro-D-glucose-PET identified active BAT in 8 of the 11 participants. Water-fat MRI showed that BAT depots had a fat fraction of $65.2 \pm 7.0\%$ compared with $81.5 \pm 5.4\%$ for the subcutaneous white adipose tissue (paired difference of $16.3 \pm 4.9\%$; $P < 0.05$). Dynamic T2^* imaging during cold stimulation revealed signal fluctuations that were sensitive to BAT activation. The presence of these components correlated with BAT activation quantified from FDG-PET ($r = 0.63$; $P < 0.05$).

Conclusions. Although FDG-PET has superior contrast for identifying active BAT, the MRI temporal resolution provides insight in activation dynamics. In addition, the flexibility of MRI allows for simultaneous mapping of tissue fat content and functional responses. The results indicate that MRI is a promising addition to PET for the identification of BAT and its activity responses to stimulation. An MRI-based methodology to quantify BAT activity is a highly desirable step in addressing the role of BAT in obesity disorders.

INTRODUCTION

Mammals have 2 distinct types of fat tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Brown adipose tissue is strongly innervated by the sympathetic nervous system and is much more densely vascularized than WAT (1). Brown adipose tissue is the main site for non-shivering thermogenesis (NST) in rodents (2, 3). Only recently, it was shown by positron emission tomography (PET) using 2-deoxy-2- ^{18}F fluoro-D-glucose (FDG) that adult humans possess functionally active BAT depots and that the presence of active BAT influences human metabolism (4). It has thus been postulated that pharmaceutical targeting of BAT is a possible strategy in the treatment of obesity.

The only noninvasive method to investigate the presence of BAT has been the use of FDG-PET combined with computed tomography (CT). Although this method offers excellent sensitivity in detecting active BAT, the use of ionizing radiation limits its application on healthy human participants in research. In addition, because FDG uptake is an irreversible process, obtaining information about the dynamics of BAT activation, such as on/off switching, is difficult. To complement these shortcomings, an additional noninvasive method is needed. Brown adipose tissue imaging using magnetic resonance imaging (MRI) has been mainly performed on rodents. Results from both fat imaging (5-7) and perfusion imaging (8, 9) suggest that MRI might be a valuable tool for identifying BAT depots and characterizing BAT activation. More recently, human BAT was identified using MRI in a human infant (10). The aim of this study was to investigate whether MRI can be used to characterize active BAT depots in adult humans for water-fat composition and activity response to cold conditions. On the basis of reported results, we hypothesized that, in MRI, both water-fat imaging and perfusion imaging are sensitive to the presence of active BAT. In this study, MRI was used to assess BAT fat content (as measured by a multiecho MRI acquisition (11)) and BAT activation using dynamic $T2^*$ imaging during cold exposure. In the same participants, FDG-PET/CT during cold stimulation was performed as the reference method for BAT activity.

MATERIALS AND METHODS

The ethics committee of the institute approved the protocol, and all participants provided written informed consent before participation.

Study Population. Healthy human participants with an age between 18 to 30 years and a body mass index between 18 to 30 kg/m^2 were eligible for the study.

Exclusion criteria were known diabetes mellitus, the use of β -blockers, and contraindications to MRI or ionizing radiation. Eleven young adults (7 women and 4 men) participated in the study. See Table 1 for the participants' characteristics.

Table 1. Characteristics of the study participants.

Characteristic	Value	Range
Men/women	4/7	
Age (y)	23.9 ± 2.2	21 – 28
Body mass (kg)	72.4 ± 13.4	50.7 – 99.0
Height (m)	1.80 ± 0.1	1.70 – 1.90
BMI (kg/m^2)	22.9 ± 3.2	18.2 – 27.7
Body fat (%)	27.0 ± 7.9	9.6 – 35.8

Values are reported as means $\pm$ SD. BMI indicates body mass index.

Study Design. All participants underwent a cooling trial experiment, a full MRI protocol, and an FDG-PET/CT. Within 5 days of the trial, MRI and, within 9 days, FDG-PET/CT were performed. For the trial experiment, MRI and PET/CT were performed after a fasting period of at least 5 hours.

Individualized Cooling Protocol. Cooling was performed by wrapping the participant in a water-perfused suit (Thermowrap, Universal MTRE System) connected to 2 temperature-controlled water baths (Blanketrol III, Cincinatti Sub-Zero) comparable with the study of Ouellet et al. (12). The cooling protocol was adjusted to the individual participant's response in the trial experiment. The participants were sequentially exposed to 30 minutes of thermoneutral conditions, cooling until shivering, warm conditions for 5 minutes, and mild cold conditions ($20.4^\circ\text{C} \pm 1.7^\circ\text{C}$) for 60 minutes. When the participant reported shivering during the mild cold condition, suit temperature was increased in steps of 1°C until no obvious muscle cramps or shivering was reported. When the participant did not report feeling cold, suit temperature was lowered in steps of 1°C . The optimal water temperature for NST resulted in suit temperatures in a range of 22.4 to 26.3°C ($24.1^\circ\text{C} \pm 1.4^\circ\text{C}$). This individual mild cold condition was used during the PET/CT and the dynamic T2^* imaging. In few cases, the individuals reported shivering under mild cold conditions while scanning; in response, suit temperature was raised by 1°C .

2-Deoxy-2- ^{18}F fluoro-D-glucose-PET/CT Acquisition. Before the PET/CT scan, the participants were kept in thermoneutral conditions for 45 minutes, followed by cooling until shivering and 5 minutes of warming. Subsequently, the participants were exposed to mild cold for 90 minutes, with injection of 74 MBq of FDG

intravenously after 30 minutes. Hereafter, the imaging started with a CT, followed by a PET scan (Gemini TF PET-CT; Philips, Best, The Netherlands). The resulting total effective radiation dose for the full examination was approximately 2.8 mSv. The PET scan covered the neck to the kidneys, requiring 6 to 7 bed positions (6 minutes per bed position). The CT scan was used for attenuation correction of the PET scan and localization of the FDG uptake sites. A tube voltage of 120 kV, 30mAs per slice, and 64 x 0.625 mm of collimation was used. Both image sets were reconstructed with a slice thickness of 4 mm (130 to 170 slices). The in-plane resolution was 4 mm for the PET and 1 mm for CT. The images were analyzed using PMOD (PMOD Technologies, Zürich, Switzerland). With this software, BAT activity was quantified by contouring the BAT areas with a set threshold. These regions of interest (ROIs) were verified by an experienced nuclear medicine physician.

Magnetic Resonance Imaging Acquisition. Magnetic resonance imaging acquisitions were conducted on a Philips Achieva 3.0T scanner (Philips, Best, The Netherlands). An 8-element neurovascular coil was used. The scans were positioned to include the supraclavicular and cervical adipose depots (14-cm field of view along the caudal-cranial axis). No respiratory compensation was used. The participants were wrapped in the water-perfused suit. All participants underwent the MRI protocol, which consisted of a T1 sequence, water-fat imaging, and followed by dynamic T2* imaging during which a cooling paradigm was performed. Before the cooling experiment, the participants were kept in thermoneutral conditions. The T1-weighted turbo gradient echo sequence had the following parameters: repetition time (TR), 10 milliseconds; echo time (TE), 2.3 milliseconds; flip angle (FA), 15 degrees; field of view (FOV), 246 mm anteriorposterior (AP), 450 mm right-left (RL); matrix, 320 x 320; slice thickness, 7 mm; slice gap, 1 mm; slices, 25; and acquisition time, 1 minute.

For water-fat imaging, a triple echo (for T2* correction (13)) gradient echo acquisition was performed. From the images containing either the sum (in-phase) or the difference (out of phase) of the water and fat signal, separated water and fat images were derived by the scanner software (11, 14). The method was validated by measurements on a phantom (see Supplemental Digital Content Fig. S1, Supplemental Digital Content 1) (15). The following parameters were used: TR, 289.9 milliseconds; TE1, 2.3 milliseconds (in-phase); TE2, 3.45 milliseconds (opposed phase); TE, 3 4.6 milliseconds (inphase); FA, 75 degrees; FOV, 372 x 465 mm (AP x RL); reconstruction matrix, 260 x 231; slice thickness, 6 mm; slice gap, 1

mm; slices, 20; and acquisition time, 2 minutes.

Dynamic T2*-weighted imaging was performed using gradient echo planar imaging with the following parameters: TR, 200 milliseconds; TE, 15 milliseconds; FA, 90 degrees; FOV, 204 x 450 mm (AP x RL); reconstruction matrix, 300 x 298; slice thickness, 2mm; slice gap, 5 mm; and 20 slices resulting in an acquisition time per single volume of 15 seconds. During the acquisition, no breath-hold, cardiac, or respiratory triggering was used. Two minutes after the start of the dynamic T2*-weighted acquisition, the participants were cooled until shivering and were subsequently warmed for 5 minutes. After this, the participants were exposed to mild cold for 10 minutes, followed by 15 minutes of thermoneutral condition. The whole paradigm was subsequently repeated to modulate the response with a characteristic temporal signature, which improves possible signal detection with respect to background fluctuations (Figs. 1A, B). The duration of the protocol varied between the participants (depending on the time in which the participants reported shivering) and was in the range of 46 to 86 minutes.

Time-Series Analysis. Preprocessing of the dynamic T2*-weighted acquisition was performed in MATLAB. Image noise was reduced by averaging 8 subsequent volumes. This results in an effective temporal resolution of approximately 2 minutes per volume. Motion correction was performed by using a 2-dimensional nonrigid b-spline registration (16).

Because there is limited a priori knowledge of the temporal characteristics of BAT activation, the expected tissue time response in the dynamic acquisition is not known. To identify possible responses without prior knowledge of the time signature, an independent component analysis (ICA) was performed using MELODIC (17-19). Independent component analysis is a mathematical data-driven method to decompose a signal into statistically independent temporal components (Fig. 1C). The response could, for instance, be delayed or distorted compared with the stimulus. In a direct regression with the stimulus, the contrast may then be lost. The images were masked with an ROI representing activated BAT found on PET/CT. The ROI was delineated on the T1-weighted scan on the basis of the corresponding PET/CT slice including supraclavicular and cervical depots. The ROIs were drawn by an experienced imaging researcher and under the supervision of a physician who has specialty in nuclear medicine. In addition, a model-based analysis was performed with the FEAT linear regression analysis tool from the FSL software package (18, 19). The response was modeled as a block wave derived from the time signature of the cooling protocol and has a value of 1

during cooling and 0 during heating and thermoneutral periods. Multiple regression with this model was performed on each voxel. The regression results are represented as z-maps containing the z-score for the probability that the model is an accurate representation of the corresponding voxel time trace.

Figure 1. Time-series analysis. A: The repeated cooling paradigm in the dynamic T2* MRI acquisition was repeated twice (solid line) to create four transitions in signal level. In the presence of noise and signal drift (dashed line), this improves the contrast to noise of the signal recovery. B: A single repetition of the paradigm would result in a poor contrast to noise ratio possibly more confounded by signal drifts. C: The ICA analysis is able to separate different independent components from a mixed signal.

Statistical Analysis. Descriptive results are reported as mean $\pm$ SD. Comparison of tissue fat content was performed using a (2-tailed) paired Student t test. The threshold in the analysis of the activation maps was set at a z-score of 1.65. The MRI and PET results in the supraclavicular and cervical depots were compared by calculating the Pearson correlation coefficient between the fraction of activated voxels in dynamic T2*-weighted imaging and the activity concentration measured by PET/CT. Correlations coefficients were tested for significance ($P < 0.05$) against the null hypothesis.

RESULTS

Positron Emission Tomography/Computed Tomography. On the PET images, 8 of the 11 participants showed a pronounced FDG uptake in BAT. For 3 participants, the FDG uptake was negligible, and these participants did not have common characteristics. A typical distribution of FDG uptake is shown in Figures 2B and C. Uptake in the supraclavicular, cervical, and paraspinal depots was observed.

These depots can be identified as adipose tissue on the fused PET/CT image because adipose tissue has Hounsfield units in the range of -150 to -50 (Fig. 2D).

Figure 2. 2-Deoxy-2- ^{18}F fluoro-D-glucose-PET/CT of activated BAT. A: Cooling protocol for BAT imaging using PET. The participants were cooled (dark grey) before FDG injection and exposed to NST (light grey) conditions during the imaging. Thermoneutral conditions are depicted with a white fill. B and C: Positron emission tomography of a BAT+ participant showing a typical FDG uptake distribution. B: The supraclavicular (sc), cervical (ce), and paraspinal (ps) depots are clearly observed in the coronal projection. Note that the heart and the liver also show displayed FDG uptake. C: Corresponding axial slices at different levels marked by the red lines. D: Axial slice of the low-dose CT. E: Overlay of the corresponding PET slice on the low-dose CT.

Water-Fat MRI. The fat depots identified on T1-weighted magnetic resonance images corresponded visually with the regions identified on the CT scan (Figs. 3A, B). On water-fat MRI (Fig. 3C), the adipose tissue could clearly be observed as regions with a high pixel intensity. Figure 3E shows the distribution of the voxel fat content of the subcutaneous WAT compared with that of the internal fat depots in the supraclavicular and cervical regions.

Figure 3. Water-fat MRI of BAT. A: Axial slice of a T1-weighted MRI scan. B: Corresponding axial slice of the low-dose CT scan. C: Axial slice of the fat content image of a BAT+ participant. The green contour denotes the complete supraclavicular fat depot, the red contour indicates the region showing FDG uptake on the PET, and the blue contour is the ROI used as the subcutaneous WAT. D: Axial PET/CT slice corresponding to the fat content image in C. The FDG uptake is not homogeneous throughout the adipose tissue depot. E: normalized histogram of the fat content of voxels at the different ROIs described in C, representing the subcutaneous WAT (blue line), the supraclavicular/cervical fat depot (green dashed line), and the region showing the FDG uptake (red dotted line).

The supraclavicular/cervical fat depots containing BAT showed a lower fat content compared with that of the subcutaneous WAT. The mean fat content in all participants was 65.2 ± 7.0 % for the supraclavicular/cervical fat depots compared with 81.5 ± 5.4 % for WAT with a paired difference of 16.3 ± 4.9 %; $P < 0.05$). The supraclavicular/cervical fat depots are morphologically different from the subcutaneous WAT. The subcutaneous WAT appeared homogeneous, whereas the supraclavicular/cervical depots were much more heterogeneous (Fig. 3C). This is also reflected in the width of their respective fat content distributions (Fig. 3E).

The PET/CT images (Fig. 3D) showed that the active BAT is only present in parts of the supraclavicular/cervical fat depots. Therefore, the fat distribution analysis was also performed including only regions that showed FDG uptake on the PET/CT images. The resulting fat content for BAT was 66.0 ± 9.3 %, which was similar to the complete supraclavicular/cervical depot. The mean (or median) of the fat distribution of the supraclavicular/cervical fat compartments did not correlate with the FDG uptake.

Brown Adipose Tissue Activation by Dynamic T2*-Weighted Imaging. Dynamic T2*-weighted imaging was used to measure physiological changes in BAT during cold exposure (Figs. 4A-C). The MRI time signal showed large fluctuations within the supraclavicular/ cervical depot (Fig. 4D). The signal responses had a large heterogeneity in time signature (Fig. 4E). In most participants, several voxel clusters could be found, in which fluctuations correlated with the cooling protocol. To independently validate the presence of such a component, the ICA was performed. The ICA identified a component with similar temporal characteristics (Fig. 5A). No other consistent components could be identified. Subsequently, regression with a modeled response of this component was performed (Fig. 5B). Some activating voxels were also found outside the ROI because the activation maps had a noisy appearance. Figure 5C shows that the fraction of activating voxels in the fat depot correlated with the activity concentration of FDG uptake of BAT in the PET image. This correlation existed both for voxels that showed an activation pattern similar to the model and for voxels that showed an activation pattern with the opposite sign. T2*-weighted contrast thus seems to be sensitive to local tissue reactions that correlate with BAT activation and deactivation. As a control, the activation in muscle and WAT was also assessed. The fraction of activating voxels in these tissues did not significantly correlate significantly with the FDG activity concentration in the participants (Fig. 5C).

DISCUSSION

Recent work has shown that water-fat imaging is useful to identify BAT in animal models (5, 6, 8, 9) and in human newborn (10). The applicability and validity of these techniques have not been shown in human adults. This study is an exploration of the use of MRI for identifying active BAT in adult humans. 2-Deoxy-2-[^{18}F]fluoro-D-glucose-PET was used as the reference standard because this technique has been shown to reliably detect active BAT depots (20). The MRI-derived fat fraction of the supraclavicular/cervical adipose depot was

approximately 66.0 %, which was considerably lower compared with the subcutaneous WAT with a fat fraction of 81.5 %. Using a similar MRI method for water-fat separation in rats, Hu et al. (6) found a fat fraction of approximately 50 % for juveniles increasing to 60 % at older age. Recent evidence suggests that the fat content in BAT of newborn humans is lower than that in WAT (10). The fat fraction in the supraclavicular/cervical adipose depots measured in our study is thus in a similar range as has been reported for BAT. However, our results may suggest that the fat fraction is a general property of these depots and may not be representative of the presence of active BAT.

Figure 4. T2*-weighted images of BAT activation. A: Design of the cooling protocol for dynamic T2* imaging. The duration and timing of thermoneutral (white), cold (dark grey), and mild cold conditions (light grey) are shown. B: T1-weighted (anatomical) axial MRI slice C: The T2*-weighted slice corresponding to the slice in B. D: Normalized signal fluctuations averaged from a 3 x 3 voxels large ROI in muscle (dotted line) and BAT (solid line). The location of the ROIs is shown in B. The grey areas represent the time points at which the participant was exposed to cold or mild cold conditions. The BAT T2* signal shown fluctuations with a characteristic temporal signature that correlates with the cooling paradigm. E: The individual time traces in the 3 x 3 BAT ROI shows the signal heterogeneity in the ROI.

Figure 5. Analysis of BAT activation during T2* imaging compared with PET. A: Example of a component identified by the ICA (black circles). The component is modeled as a square wave with a value of 1 during cold exposure and 0 during thermoneutral conditions (red line). B: Overlay of a color-coded activation map of the supraclavicular/cervical fat depot onto an axial slice of a T1-weighted MRI image. C: Scatterplot of BAT activation in all participants as measured by PET (FDG uptake in kilo Becquerel per milliliters) and MRI (the fraction of activating pixels in the supraclavicular/cervical ROI). The activation was also measured in muscle, WAT and BAT.

First, the fat fraction of the regions in the supraclavicular/cervical depots showed that FDG uptake was not significantly different from the adjacent adipose tissue. The regions showing FDG uptake on the PET were visually indiscernible from the adjacent adipose tissue on the MRI fat-content images. Second, the fat fraction of the supraclavicular/cervical fat depots did not correlate with the FDG uptake on the PET/CT. Whether this is related to cellular or tissue organizational differences cannot be determined at this spatial resolution. Recent studies suggest that the human supraclavicular and cervical adipose depot is a mix of brown and white adipocytes, in contrast to the distinct BAT depots found in rodents (21, 22). In humans, brown adipocytes were found to be organized in small islands and the depot contained, on average, 13 % brown adipocytes (22). Identification of active BAT based on tissue fat content alone might therefore be difficult in adult humans. Performing simultaneous PET-MRI could provide a more accurate characterization

of BAT properties. A more reliable delineation of active BAT with the water-fat MRI would provide a more accurate segmentation of the BAT depot and, thus, a better estimate of active BAT fat content.

Brown adipose tissue activation is associated with enhanced tissue perfusion (23). Increased blood flow would support the higher metabolic rate (24). In addition, for effective thermogenesis, the produced heat needs to be transported to the body core tissues. T2*-weighted imaging has been extensively used to map brain perfusion in functional MRI but has also been successfully applied to other tissues such as the skeletal muscle (25, 26) and the spinal cord (27, 28). Recent work by Khanna and Branca (9) using T2*-weighted imaging in mice has shown that image contrast was sensitive to pharmacological activation of BAT. During cold activation, we observed T2*-weighted signal fluctuations from BAT that coincided temporally with the cold stimulus. The presence of such a component suggests that BAT on/off switching is rapid and occurs at the scale of minutes. Such a temporal response corresponds to a sympathetic activation of BAT as found in rodents (3). The identification of this component was confirmed by the ICA, which has been proven a useful technique in identifying physiological responses in brain functional MRI (29). The presence of such signal components, as quantified by the model-based regression analysis, correlated with BAT activity. This means that the T2*-weighted signal is sensitive to BAT activation. However, the origin of this signal is not yet completely clear.

In the brain, the T2*-weighted signal is sensitive to perfusion through the blood oxygenation level-dependent (BOLD) effect, which influences the T2* decay. The decrease in the concentration of deoxyhemoglobin in well-perfused vessels causes a small signal increase in the order of 3 % to 5 % at 3 T. The magnitude of the signal changes observed in BAT was much larger and therefore cannot be attributed to the BOLD effect alone. Larger fluctuations in the T2*-weighted signal may have a different physiological origin as has been suggested previously in the spine and the muscle (30, 31). The T2*-weighted signal is sensitive to numerous other effects, such as blood volume, motion, and T1 relaxation time effects such as blood in/out flow. In addition, a priori, it remains unknown whether (absolute and/or relative) BOLD responses are similar in neuronal, muscle, and adipose tissue for reasons of likely differences between tissue background signal, metabolism, and coupling between blood vessels and tissue. Pinpointing the mechanisms underlying the sensitivity to BAT activation is challenging. First of all, the sign of the signal change was undetermined because both were present and

correlated with BAT activity. In addition, the image quality suffered from several artifacts. Breathing artifacts in the lung region and pulsation artifacts from the carotid arteries were visible on the T2*-weighted images. Compared with T2*-weighted imaging of the brain, movement artifacts are more difficult to correct because of the nonrigid nature of the anatomy under consideration. All of these effects contribute to some extent to the T2*-weighted signal and therefore might also explain the low specificity of measuring BAT activation with the proposed method. For instance, in the muscle, we found that the fraction of activating voxels showed a similar trend as that of BAT. However, this trend was weaker than that of BAT and not statistically significant. Whether this is a biological effect (for instance, through reduced muscle perfusion to reduce heat loss), an effect of movement artifacts or signal from BAT spreading through phase artifacts remains to be determined. Therefore, the use of MRI needs to be further refined before matching the specificity of FDGPET/CT for the identification of active BAT. However, the presence of signal components that correlate with FDG uptake is promising for the identification of BAT using perfusion-based MRI methods.

In summary, we have presented a functional and structural MRI exploration of BAT in humans combined with FDG-PET/CT. Water-fat imaging using MRI showed that active BAT cannot be discerned on the basis of tissue fat content. Dynamic imaging of BAT activation using T2*-weighted MRI during cold stimulation is promising for the identification of active BAT. During cold stimulation, BAT activation modulated the T2*-weighted signal. Although T2*-weighted image contrast seemed sensitive to activation, MRI of BAT is challenging because of the presence of susceptibility and physiological artifacts. Compared with T2*-weighted MRI, activated BAT can be identified by FDG-PET with a stronger contrast. However, in contrast to MRI, temporal and quantitative information on tissue fat content is lacking. Additional advanced MRI techniques, such as spin-echo echo-planar imaging and arterial spin labeling, can be used to obtain more detailed information on changes in blood flow, volume, and oxygenation of active BAT. Future work will also need to focus on the tailoring of an MRI method that is both sensitive and specific enough to make full use of the advantages of MRI over PET/CT and that stimulates research on combined PET/MRI systems (32, 33).

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SUPPLEMENTARY INFORMATION

Water-fat imaging. To check if the values for the fat and water signal calculated by the scanner software were correct, phantom measurements were performed (1). A phantom bottle was filled with a solution of 1mg/ml of $\text{CuSO}_4 \times 2 \text{H}_2\text{O}$ in demineralized water and mineral oil in a ratio of 1:1 (oil:water). The water and oil phase separation result in a clear interface between the water and oil. A multi-echo pulse sequence with similar settings as used on the subjects was performed on the phantom and images representing the fat content were calculated. The water-oil separation can be observed on the axial slice (Fig. S1a). A single oblique slice (slice thickness 5 mm) was subsequently positioned along the interface such that the water-fat ratio varies linearly with the lateral position in the slice (Fig. S1a/b). The variation of the calculated fat content with the lateral position is shown in Fig. 1c. The fat content shows the expected linear dependence of the fat content in a range of around 0.06 to 0.81. (The residual the fat content in the linear fit in this range was smaller than 0.01). Outside this range, noise contributions and the fact that the method wrongly assumes that the lipid protons only have a single spectral resonance frequency limit the accuracy (1). Therefore in practice tissues with a very high (close to 1) or low fat fraction (close to 0) will have a large and consistent erroneous estimation of their fat content. In addition to a phantom calibration rat adipose tissues were imaged as a reference standard. For this purpose ~1 ml of BAT and WAT excised from 3 rats, sacrificed at age 4-6 weeks was imaged using water-fat MRI. Figure 1d shows the resulting fat content image for a 10 mm thick axial slice. The fat content of BAT was found to be $65.9 \pm 16.0 \%$ whereas WAT had a fat content of $90.0 \pm 3.5 \%$.

FIGURE S1. Water fat imaging accuracy. A: Axial slice of the fat content image acquired by multi-echo MRI. The red contour shows the positioning of the oblique slice. The angle with the interface causes the fat content of the voxels to vary linearly with the position in the slice. B: The oblique slice that intersects the water-oil interface shows a gradient in the fat content. Along the red line, the fat content was obtained as function of position. C: Line profile of the fat content in the oblique slice. The red line shows a linear fit through the data in the range of fat content between 0.2 and 0.8. D: Axial slice (slice thickness 10 mm) through two tubes filled with BAT (red contour) and WAT (green contour).

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CHAPTER 4

Supraclavicular skin temperature and BAT activity in lean healthy adults

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The contents of this chapter are submitted

ABSTRACT

The 'gold standard' for measuring brown adipose tissue (BAT) in humans is [^{18}F]FDG-PET/CT-imaging. With this technique subjects are exposed to ionizing radiation and is therefore limited in the number of scans that can be performed. We investigated the relation between supraclavicular skin temperatures and BAT activity values using a strictly temperature-controlled air-cooling protocol. Data of 36 male subjects was analyzed. BAT activity was evaluated by [^{18}F]FDG-PET/CT-imaging and skin temperature was measured by means of iButtons. Supraclavicular skin temperature dropped less compared to skin temperatures at other sites (all P-values <0.01). A significant positive correlation was found between the change in supraclavicular skin temperature with BAT activity (R^2 0.23), and the change in supraclavicular skin temperature and non-shivering thermogenesis (R^2 0.18; both P-values <0.01). The correlations indicate that supraclavicular skin temperature (changes) can potentially be used as a qualitative measure of BAT activity and BAT thermogenesis.

INTRODUCTION

Mammals are homeotherms, which means that they are able to maintain stable internal body temperatures by physiological means (1). During cold exposure internal body temperatures can be maintained via two mechanisms; by decreasing heat loss to the environment via vasoconstriction and by increasing heat production (2). The extra heat can be produced by shivering thermogenesis or by non-shivering thermogenesis (NST). It has been known for many years that brown adipose tissue (BAT) is the main site for NST in small mammals and human infants (3, 4). In rodents, the largest BAT depot is located in between the scapula (i.e. interscapular BAT, iBAT); other BAT depots are the cervical, axillary, mediastinic, and perirenal depots (5). BAT contains many mitochondria with uncoupling protein-1 (UCP1). This protein uncouples ATP production from the respiratory chain and consequently heat is generated (3, 6). Besides the abundance of UCP1, BAT is densely innervated by the sympathetic nervous system and is highly vascularized (3, 6). These characteristics are all in support for BAT's main function: heat production to maintain core body temperature.

The occurrence of BAT in human adults was known for many years (7, 8). The findings of Huttunen et al. (9) who showed that outdoor workers have a higher occurrence of BAT compared to control subjects indicated that cold exposure might be important. It took until 2009 before a direct causal relationship between active BAT and cold exposure was found (10-12). Additionally, several studies show that cold-induced BAT activity and NST are related (13-17). This suggests that BAT is also important in maintaining core body temperatures in human adults. Human BAT is mainly located in the neck, acromial-clavicular, supraclavicular, para-aortic, axillary, paravertebral and in perirenal depots (5, 18). In order to localize BAT in human adults 2-deoxy-2- ^{18}F fluoro-D-glucose-positron-emission-tomography and computed-tomography (^{18}F FDG-PET/CT) is commonly used (19). This scanning technique involves ionizing radiation both by the CT-scan and the administration of a radioactive isotope (^{18}F FDG) for PET-imaging, and is therefore limited in the number of scans that can be performed. Therefore additional techniques are needed to study BAT without radiation penalty.

In that perspective, measuring BAT temperature could be a qualitative method to verify whether BAT is activated or not, and this could possibly be an indicator of BAT presence. Interscapular BAT can be measured directly in rodents by means of thermistors placed on the interscapular BAT depot (20). However, this technique is invasive and therefore not suitable for employment in humans. Instead,

measuring the temperature of the skin overlaying the BAT depots could be an attractive alternative. Indeed by means of infrared thermography, studies in rodents showed a smaller decrease in interscapular skin temperature upon cooling, compared to tail and back skin temperature (21). Moreover, skin temperature overlaying BAT-sites is significantly higher than non BAT-sites (22). In human adults similar results were found. Measured by means of infrared thermography supraclavicular skin temperature decreased less (BAT site) than skin temperature at mediastinum (non BAT-site) after cold exposure (17 °C for 30 minutes) (23). Besides infrared thermography, skin thermistors are frequently used to measure (changes in) skin temperature (24-26). Skin thermistors are small, relatively cheap and easy to use.

The studies published so far use different means of cold exposure to activate BAT (24-26), water- and air-cooling protocols are used, with either a fixed cooling protocol or an individualized cooling protocol. Different cooling protocols result in different BAT activity values (18). Therefore, in this study we investigated the relation between supraclavicular skin temperatures and BAT activity in human adults during strictly temperature-controlled cold stimulation.

MATERIALS AND METHODS

The ethics committee of Maastricht University Medical Centre+ approved the study protocols, and all study participants provided written informed consent. All procedures were conducted according to the principles of the Declaration of Helsinki.

Table 1. Subject characteristics

Characteristic	
Age (years)	23.4 ± 3.6
Height (m)	1.83 ± 0.1
Weight (kg)	73.5 ± 7.3
BMI (kg/m ²)	21.9 ± 1.75
Fat mass (%)	16.3 ± 3.9
BAT+/BAT-	34/2

Values are expressed as means ± SD. BAT+: number of BAT-positive subjects upon cold stimulation; BAT-: number of BAT-negative subject upon cold stimulation.

Subjects. In order to study the correlation between skin temperatures and BAT activity we studied the data of 36 male subjects undergoing an individualized cooling protocol by means of air-cooling. All were healthy, lean, young adults (see Table 1 for subject characteristics). Exclusion criteria were diabetes mellitus, use

of beta-blockers, and a history of cardiovascular diseases and asthma or other pulmonary obstructive disease.

Study design. Subjects were studied in the morning after an overnight fasting period. Subjects swallowed a telemetric pill (CoreTemp HT150002; HQ Inc.) for core temperature measurements and iButtons (Maxim Integrated Products) were placed for skin temperature measurements. A cannula was inserted in the antecubital vein for [^{18}F]FDG administration. Energy expenditure was measured continuously by means of a ventilated hood system (Omnical; Jaeger). Measurements started with a thermoneutral period of 45 minutes after this the individualized cooling protocol started. After 30 minutes of stable non-shivering condition the tracer was injected and mild cold stimulation remained for 60 minutes. Hereafter, all measurements were stopped and subjects were transported towards the scanner for PET/CT-imaging.

Figure 1. Visualization of the placement of iButtons on 17 sites for skin temperature measurements.

Skin temperatures. On 14 ISO-defined (27) sites iButtons were placed, three additional iButtons were placed at the underarm (ventral: middle in between base of the thumb and elbow) and fingertip (ventral side of the middle finger) and above the right clavicle (supraclavicular, BAT location) (Figure 1). Skin temperatures were measured by means of wireless temperature sensors

(Thermochron iButtons, Maxim, USA). The sensors were attached to the skin using Fixomull tape (BSN, Hamburg, Germany). Validation studies showed that mean accuracy is $-0.09\text{ }^{\circ}\text{C}$ with a precision of $0.05\text{ }^{\circ}\text{C}$ (27). Mean skin temperature was calculated as the average temperature of sites A-T, proximal temperature as the average of chest (C), abdomen (E), scapula (M) and lower back (N), distal temperature as the average of wrist (G) and foot (K). To gain more insight in body insulation, temperature gradients were calculated between core and mean skin, core and distal and between proximal and distal temperature.

Individualized cooling protocol by means of air-cooling. Measurements started with a thermoneutral period of 45 minutes, followed by an individualized cooling protocol. Subjects were cooled until maximal non-shivering thermogenesis (for detailed description of the cooling protocol see (18)). In short, subjects were cooled down until the onset of shivering, after this the environmental temperature was raised with $1\text{--}2\text{ }^{\circ}\text{C}$ in order to stop shivering. This was confirmed by EMG measurements and the absence of shivering was checked visually. After 30 minutes of stable non-shivering thermogenesis condition, [^{18}F]FDG was injected and mild cold exposure remained for 60 minutes. Subjects were laying semi supine on a nephrodialysis chair wearing sweatpants, a T-shirt and socks or on an air-permeable stretcher dressed in shorts and T-shirts. The chair or stretcher was placed in a specially equipped air-permeable tent (Colorado, Altitude Training) in which ambient temperature could be tightly controlled. After the cooling protocol, subjects were transferred to the PET/CT scanner (Gemini TF PET-CT, Philips).

PET/CT-scanning protocol. One hour after [^{18}F]FDG injection PET/CT-imaging was conducted. Imaging started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a PET scan. A total of six to seven bed positions were necessary to cover the area where BAT is usually found. The PET image was used to determine the [^{18}F]FDG uptake, and the CT-image was used for PET attenuation correction and localization of the [^{18}F]FDG uptake sites. The voxel size of reconstructed PET and CT image sets were $4 \times 4 \times 4\text{ mm}^3$ and $1.172 \times 1.172 \times 1.172\text{ mm}^3$, respectively.

PET analysis. The scans were analyzed using PMOD software (version 3.0 PMOD Technologies). The researcher (MJV) and an experienced nuclear medicine physician (BB) interpreted the PET/CT-images. BAT activity is defined as glucose uptake in fat tissue. This is expressed in mean standardized uptake value (SUV mean), with maximal SUV as the maximum value in that region. The regions of

interest were manually outlined in each slide (4 mm) in the fusion (PET and CT) image, in which SUV in each region was at least 1.5 and region localization was based on CT (Hounsfield units: -10 to -180).

Statistical analyses. For analyses, the last 20 minutes of thermoneutral condition were averaged, for mild cold condition a period of 30 minutes, starting 10 minutes after injection, was averaged. Percentage NST was calculated as the change in energy expenditure during mild cold condition compared to thermoneutral condition. Statistical analyses were performed using PASW Statistics 20.0 for Mac (SPSS). Pearson correlations were used to identify relationships between cold-induced body temperatures (temperatures at the end of cold stimulation) and BAT activity, and between the change in body temperatures (mild cold situation minus baseline) and BAT activity values. The effect of the cooling protocol was studied with a paired sample T test. $P < 0.05$ was considered statistically significant.

RESULTS

BAT activity and NST. In 94 % (34 of 36 BAT-positive) of the subjects active BAT depots were found. Average BAT activity was 2.3 ± 0.7 SUV mean and 10.1 ± 6.6 SUV max. The cooling protocol significantly increased energy expenditure (thermoneutral: 5.0 ± 0.4 , mild cold: 5.5 ± 0.5 kJ/min, $P < 0.01$). As a result, an average non-shivering thermogenesis (NST) of 10.9 ± 7.3 % was found.

Body temperatures. Upon cooling, mean skin temperature decreased from 33.1 ± 0.6 °C to 29.3 ± 0.8 °C ($P < 0.01$). Additionally, a significant drop in supraclavicular skin temperature was found (thermoneutral: 35.3 ± 0.5 °C; mild cold: 34.5 ± 0.7 °C, $P < 0.01$). Interestingly, supraclavicular skin temperature did not decrease to the same extent as mean skin temperature (-0.9 ± 0.6 drop in supraclavicular skin temperature and -3.8 ± 0.6 drop in mean skin temperature, $P < 0.01$). All but proximal and core temperature showed a greater drop than supraclavicular skin temperature upon the cooling protocol (all P-values < 0.01 , Table 2). Highly interesting in this respect is the greater drop in skin temperatures of skin sites surrounding the supraclavicular area (head: -1.8 ± 0.6 , chest: -2.2 ± 1.0 and deltoid: -4.0 ± 1.0 °C, all P-values < 0.01) compared to the drop in supraclavicular skin temperature (Table 2).

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Table 2. Body temperatures during thermoneutral and mild cold condition for air- and water-cooling protocols separately.

Variable	Air-cooling
Mean skin (°)	
Thermoneutral	33.1 ± 0.6
Mild cold	29.3 ± 0.8 ^A
Change upon cold stimulation	-3.8 ± 0.6 ^B
Core (°)	
Thermoneutral	36.8 ± 0.2
Mild cold	36.9 ± 0.3
Change upon cold stimulation	0.02 ± 0.22
Gradient underarm-fingertip (°)	
Thermoneutral	-0.10 ± 1.8
Mild cold	7.6 ± 2.4 ^A
Change upon cold stimulation	7.9 ± 2.9 ^B
Distal (°)	
Thermoneutral	30.8 ± 1.2
Mild cold	22.3 ± 1.3 ^A
Change upon cold stimulation	-8.5 ± 1.2 ^B
Proximal	
Thermoneutral	33.8 ± 1.2
Mild cold	31.6 ± 1.1 ^A
Change upon cold stimulation	-2.2 ± 0.7
Gradient core-mean skin (°)	
Thermoneutral	3.0 ± 1.4
Mild cold	7.6 ± 0.8 ^A
Change upon cold stimulation	3.8 ± 0.5 ^B
Forehead (°)	
Thermoneutral	35.0 ± 0.6
Mild cold	33.2 ± 0.8 ^A
Change upon cold stimulation	-1.8 ± 0.6 ^B
Left upper chest (°)	
Thermoneutral	34.2 ± 1.8
Mild cold	32.0 ± 1.4 ^A
Change upon cold stimulation	2.2 ± 1.0 ^B
Left upper arm (deltoid muscle) (°)	
Thermoneutral	33.4 ± 0.9
Mild cold	29.4 ± 1.5 ^A
Change upon cold stimulation	-4.0 ± 1.0 ^B
Supraclavicular (°)	
Thermoneutral	35.3 ± 0.5
Mild cold	34.5 ± 0.7 ^A
Change upon cold stimulation	-0.9 ± 0.6

Values are expressed as means ± SD. ^A P<0.01 mild cold vs. thermoneutral; ^B P<0.01 supraclavicular vs. other skin temperatures. Paired samples t test was used to test the effect of the cooling protocol and to test the difference with the change in supraclavicular skin temperature.

Correlations. Significant correlations were found between BAT activity and NST (R^2 0.40; $P<0.01$). Correlation analyses showed a significant positive relation between the change in temperature at the supraclavicular site and SUV mean ($R^2 = 0.23$; $P<0.01$) (Figure 2A). Thus subjects with a high BAT activity have a relative small decrease in supraclavicular skin temperatures. No correlations were found between change in supraclavicular skin temperature with SUV max, and for cold-induced skin temperatures. Additionally a significant correlation was found between NST and the change in supraclavicular skin temperature (R^2 0.19; $P<0.01$) (Figure 2B).

Figure 2. Correlations with change in supraclavicular skin temperature. A: Correlation between change in supraclavicular skin temperature and BAT activity (expressed in SUV mean), B: Correlation between change in supraclavicular skin temperature and NST (%).

DISCUSSION

In this study we showed a diminished decrease (-0.9 ± 0.6 °C) in supraclavicular skin temperature compared to the drop in mean skin temperature (-3.8 ± 0.6) and most other measured skin temperatures (Table 2). Additionally, we found significant positive correlations between the change in supraclavicular skin temperature with NST and BAT activity. Subjects with a higher NST and a high BAT activity have a relatively small decrease in supraclavicular skin temperature.

Several studies show that cold-induced BAT activity is related to NST. This indicates that BAT is important in maintaining core body temperatures upon cold exposure, by producing metabolic heat. Since the largest BAT depot in human

adults is located above the clavicle (i.e. supraclavicular), skin overlaying this depot is likely to be affected by BAT activation. This study confirms the findings of Lee et al. (23) who showed a steeper decrease in skin temperature at the mediastinum (BAT-negative site) compared to supraclavicular area (BAT-positive site) upon cold exposure (placement in a room of 17 °C for 30 min), whereby both sites were measured by infrared thermography. Thus skin temperature measured by means of infrared thermography and iButtons (this study) provide similar results. On the other hand, Symonds et al. (28) showed an increased supraclavicular skin temperature upon a cold challenge. Their cooling protocol consisted of placement of feet or hands in water of 20 °C. The discrepancy in supraclavicular skin temperature can be explained by the different cooling protocol, i.e. local cooling instead of whole body cooling.

This study confirms earlier findings that BAT activity and NST are related (17, 26). Subjects with high BAT activity values have a relatively large NST. These findings underscore the importance of BAT in maintaining core body temperatures. In this study, a significant correlation was found between the change in supraclavicular skin temperature and BAT activity. Subjects with a high BAT activity have a relative small decrease in supraclavicular skin temperatures. The explained variation is relatively low ($R^2 = 0.23$), however it indicates that the change in supraclavicular skin temperature gives an indication of BAT activity. Boon et al. (24) also studied supraclavicular skin temperature measured with iButtons in relation to BAT activity measured with [^{18}F]FDG-PET/CT-imaging. In contrast to our findings, they reported a significant increase in supraclavicular skin temperature, whereas other skin temperatures significantly decreased upon cooling. Moreover, significant positive correlations were found between absolute cold-induced supraclavicular skin temperature and total and clavicular BAT volume, and clavicular SUV max (24). No correlation between the changes in supraclavicular skin temperature and these variables was reported. The discrepancy between their results and ours might be explained by the differences in cooling protocol. Boon et al. used water-cooling with a suit, while we used air-cooling in this study. Different cooling protocols result in different BAT activity values (18). Different BAT analyzing techniques were used which influences activity values. This could be another reason for discrepancy of the results.

The main function of BAT is the regulated production of heat in order to maintain core body temperatures. Clearly, this heat is not intended to increase skin temperature. In that perspective, most of the produced heat needs to be

transported to core body sites. However, our results indicate that, most likely by means of perfusion or conduction, some of this heat ‘leaks’ towards the skin, resulting in relatively higher skin temperatures at sites covering BAT depots.

Several studies investigated (changes) in supraclavicular skin temperature upon a cold challenge using different cooling protocols. This study is the first using a strict temperature-controlled air-cooling protocol. It can be concluded that during cold stimulation the temperature decrease is less at skin overlaying supraclavicular BAT depots compared to other skin sites in human adults. Measuring changes in skin temperature could be useful to provide information on BAT activation upon repeated cold challenges. Additionally, this method could be used when information on BAT activity is needed in subjects that cannot undergo PET/CT-imaging. The correlation between supraclavicular temperature BAT activity and NST indicates that changes in supraclavicular skin temperature can be used as a qualitative measure of BAT activity and BAT thermogenesis.

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CHAPTER 5

Cold acclimation recruits human brown fat and increases non-shivering thermogenesis

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ABSTRACT

In recent years, it has been shown that humans have active brown adipose tissue (BAT) depots, raising the question of whether activation and recruitment of BAT can be a target to counterbalance the current obesity pandemic. Here, we show that a 10-day cold acclimation protocol in humans increases BAT activity in parallel with an increase in non-shivering thermogenesis (NST). No sex differences in BAT presence and activity were found either before or after cold acclimation. Respiration measurements in permeabilized fibers and isolated mitochondria revealed no significant contribution of skeletal muscle mitochondrial uncoupling to the increased NST. Based on cell-specific markers and on uncoupling protein-1 (characteristic of both BAT and beige/brite cells), this study did not show “browning” of abdominal subcutaneous white adipose tissue upon cold acclimation. The observed physiological acclimation is in line with the subjective changes in temperature sensation; upon cold acclimation, the subjects judged the environment warmer, felt more comfortable in the cold, and reported less shivering. The combined results suggest that a variable indoor environment with frequent cold exposures might be an acceptable and economic manner to increase energy expenditure and may contribute to counteracting the current obesity epidemic.

INTRODUCTION

Brown adipose tissue (BAT) is a thermogenic tissue, the main function of which is heat production (non-shivering thermogenesis [NST]) when activated by cold exposure. Nowadays, it is well recognized that BAT is present and active in human adults (1-3). Cold-induced BAT activity in humans has been shown by studies determining glucose uptake using 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) PET/CT scanning (4-6). It has also been well established that BAT activity is inversely related to BMI and body fat percentage, indicating its potential to counterbalance the current obesity pandemic (1, 4, 6). Several studies in humans indeed show that BAT presence and activity are related to NST (3-5, 7), confirming BAT as a target for increasing energy expenditure. When unacclimatized animals are placed in a cold environment, they will acutely defend their body temperature by means of shivering thermogenesis (muscle contractions), which increases heat production (energy expenditure). However, upon prolonged cold exposure, shivering will gradually decrease, but energy expenditure remains elevated, indicating increased NST (8). In rodents, the increase in NST can be fully attributed to BAT (9, 10). This metabolic adaptation over time is called adaptive thermogenesis (AT). Interestingly, an older study in humans already showed that prolonged cold exposure (12 °C, 8 h/d, 31 days) in healthy men also resulted in a gradual decrease of shivering, while heat production remained elevated (11). At that time, the tissue responsible for the observed increased NST in humans was enigmatic, as it was not clear whether human adults possessed sufficient amounts of active BAT. Nowadays, the presence of BAT in human adults is undisputed, but evidence that cold acclimation increases BAT presence and activity is lacking. Nevertheless, human cold acclimation studies show seasonal variation in NST (12), and interestingly, seasonal variation in cold-activated BAT has been reported in humans as well (6); the latter indicates the plasticity of BAT. We therefore investigated the effect of cold acclimation on both NST and BAT presence and activity. Following the “classical” study of Davis on cold acclimation in humans (11), under slightly less severe conditions, 17 healthy subjects (age, 23 ± 3.2 years; BMI, 21.6 ± 2.2 kg/m²; and fat percentage, 22.3 ± 8.1 %), of which 9 were female and 8 were male (Table 1), were acclimated to cold. For 10 consecutive days, subjects were exposed to an environmental temperature of 15–16 °C for 6 hours a day. As main outcome parameters we examined BAT activation by cold-induced glucose uptake using ^{18}F FDG-PET/CT (Gemini TF PET-CT; Philips) imaging and energy expenditure by indirect calorimetry before and after cold acclimation.

METHODS

Subjects. All subjects were studied between December 2011 and May 2012. No effect of the cold acclimation on the anthropometric parameters was found (Table 1). All females were on oral contraceptives with the active substances ethinyl estradiol and levonorgestrel and were not measured during their menstruation period to minimize hormonal effects on thermoregulation (13, 14). Exclusion criteria were diabetes mellitus, pregnancy, physical activity more than twice a week, use of beta blockers, and a history of cardiovascular diseases and asthma or other pulmonary obstructive diseases.

Table 1. Subject characteristics.

Characteristic	Group (n=17)	Females (n=9)	Males (n=8)
Age (yr)	23 ± 3.2	22 ± 3.4	23 ± 3.1
Body mass (kg)	68.4 ± 11.7	62.3 ± 9.4	75.2 ± 10.7 ^A
Height (m)	1.78 ± 0.09	1.72 ± 0.09	1.83 ± 0.07 ^A
BMI (kg/m ²)	21.6 ± 2.2	20.8 ± 1.7	22.4 ± 2.5
Body fat (%)	22.3 ± 8.1	28.3 ± 6.2	15.7 ± 3.2 ^B

Group values and values for females and males are displayed separately. Values are expressed as means ± SD. ^A P<0.05; ^B P<0.01. Independent sample t test were used for comparison between females and males.

Study design. Subjects were exposed to an environmental temperature of 15–16 °C for 10 consecutive days: 2 hours on the first day, 4 hours on the second day, and 6 hours per day for the remaining days. Before and after this period the following measurements were performed. In the morning, after an overnight fast, an abdominal subcutaneous fat biopsy (~1 g) was taken, 6–8 cm lateral from the umbilicus under local anesthesia (2 % lidocaine) by needle biopsy. Subsequently, a muscle biopsy from musculus vastus lateralis was taken according to the technique of Bergström (15). On a separate day, body composition was determined by means of dual x-ray absorptiometry (type discovery A; Hologic) and was followed by an individualized mild cold experiment. To define BAT activity, [¹⁸F]FDG-PET/CT-imaging was used. For this purpose, subjects were measured in the afternoon after a 4-hour fasting period and were asked to refrain from exercise 24 hours before the measurements. A cannula was inserted in the left antecubital vein for blood sampling during thermoneutral and mild cold conditions and injection of the tracer. On 14 ISO-defined (16) sites, iButtons (Maxim Integrated Products) were placed and subjects swallowed a telemetric pill (CoreTemp HT150002; HQ Inc.) for skin and core temperature measurements,

respectively. A pressure cuff (Cresta) to measure blood pressure and a chest strap (Polar T31; Polar) for measurement of heart rate were attached. Laser Doppler probes were attached for skin perfusion measurements at the ventral side of the hand at the base of the thumb, at the ventral side of the forearm halfway between the elbow and the wrist (Perimed PF4000; Perimed), at the ventral side of the hallux, and at the abdomen halfway between the umbilicus and the left lateral side of the body (Perimed PF5000; Perimed). Energy expenditure was measured continuously by means of a ventilated hood system (Omnical; Jaeger).

Cold acclimation. During the cold acclimation, subjects performed sedentary activities, such as studying and watching TV, and were instructed to refrain from physical activity. In the cold room, subjects were dressed in shorts and T-shirts. Every 2 hours, a cup with soup or tea was offered, and sandwiches were freely available.

Subjective responses during cold acclimation. On prescribed time points ($t = 0$, $t = 20$, $t = 40$, $t = 60$, $t = 90$, $t = 120$, $t = 180$, $t = 240$, $t = 300$, and $t = 360$) after entering the cold room, subjects completed VAS scales on sensation, thermal comfort and shivering (Supplemental Figure 1 displays an example of VAS scales completed). The responses of the third day (first time 6-hours cold exposure) were compared with the responses of the tenth day by means of a paired sample t test. The iAUC was calculated to gain more insight in the general effect of the cold acclimation. In total, 13 subjects (7 female/6 male) completed the VAS scales.

Individualized cooling protocol to define BAT presence and activity. Subjects were wrapped in a water-perfused suit, which is suitable for use inside the scanner. The protocol started with a thermoneutral period of 45 minutes, followed by the individualized cooling protocol. Each subject was cooled down (water temperature was lowered by 4°C every 15 minutes) until shivering occurred. After this, subjects were warmed up for 5 minutes so that shivering disappeared, and finally the suit temperature was set slightly above the temperature at which shivering occurred. An estimate of suit temperature was obtained by the average of the inlet and outlet. After 30 minutes of mild cold exposure, the subjects were transported toward the scanner for $[^{18}\text{F}]\text{FDG}$ -PET/CT imaging.

PET/CT scanning protocol. While subjects were inside the PET/CT scanner, mild cold stimulation continued. Imaging started with a low-dose CT scan (30 mAs, 120 kV), immediately followed by a one-hour dynamic PET scan. At the start of the PET-scanning protocol, subjects were injected with 74 MBq of $[^{18}\text{F}]\text{FDG}$

intravenously. Images were reconstructed according to the following time frames: 10×15 seconds, 5×30 seconds, 5×60 seconds, 5×120 seconds, and 8×300 seconds. After the dynamic scan, a static scan as described earlier (1) was performed. The PET image was used to determine the [^{18}F]FDG uptake, and the CT image was used for PET attenuation correction and localization of the [^{18}F]FDG uptake sites. The voxel size of reconstructed PET and CT image sets were $4 \times 4 \times 4 \text{ mm}^3$ and $1.172 \times 1.172 \times 4 \text{ mm}^3$, respectively.

PET analysis. The scans were analyzed using PMOD software (version 3.0; PMOD Technologies). Both the researcher (Anouk A.J.J. van der Lans) and an experienced nuclear medicine physician (Boudewijn Brans) interpreted the PET/ CT images. We define BAT activity as glucose uptake in fat tissue. The static scan expressed this as SUV mean, with maximal SUV as the maximum value in that region. BAT volume in this study was obtained from PET activity values and called detectable BAT volume. Besides the static scan, we also performed a dynamic scan to define glucose uptake rate in BAT. The latter was used to construct time activity curves (TACs) of the supraclavicular regions, and the aortic arch was used as an image-derived input function. Glucose uptake rates were calculated using Patlak curve fitting (17) and a lumped constant of 1.14 (18). For the static scan, the regions of interest were manually outlined, and we used a threshold of 1.5 SUV and Hounsfield units between -10 and -180 to define BAT, as described earlier (19).

SM respiration. After the biopsy, a portion of the muscle tissue was directly frozen in melting isopentane and stored at -80°C for determination of mitochondrial DNA (mtDNA) copy number (ratio ND1 to LPL), as described previously (20).

Permeabilization of muscle fibers. A small portion ($\sim 30 \text{ mg}$) of the muscle tissue was immediately placed in ice-cold preservation medium (BIOPS; OROBOROS Instruments). Muscle fibers were permeabilized with saponin according to the technique of Veksler et al. (21). After completion of the permeabilization protocol, oxygen consumption was quantified polarographically using a 2-chamber Oxygraph (OROBOROS Instruments) as described earlier (20, 22). Briefly, state 2 respiration was measured after the addition of malate plus glutamate or malate plus octanoyl-carnitine. Subsequently, ADP was added to evaluate state 3 respiration. Coupled respiration was then maximized via addition of succinate. Finally, the chemical uncoupler FCCP was titrated to evaluate the maximal capacity of the electron transport chain (state U) or oligomycin was added to determine respiration not coupled to ATP synthesis (state 4). The integrity of the

outer mitochondrial membrane was evaluated by addition of cytochrome c upon maximal coupled respiration. All measurements were performed in quadruplets. One muscle biopsy failed before cold acclimation; therefore the values presented are from 16 subjects in total (8 male/8 female).

Mitochondrial isolation. If sufficient biopsy material was obtained, SM mitochondria were isolated as previously described (23) with minor modifications. Briefly, biopsy samples (~200 mg) were placed in ice-cold isolation buffer (100 mmol/l sucrose, 100 mmol/l KCl, 50 mmol/l Tris HCl, 1 mmol/l KH_2PO_4 , 0.1 mmol/l EGTA, 0.2% FA-free BSA [wt/vol], pH 7.4) and finely minced with precooled scissors followed by an enzymatic digestion with protease (Subtilisin A; Sigma-Aldrich) on ice. The muscle pieces were then homogenized in a glass potter tube, and the homogenate was transferred to a centrifuge tube and centrifuged at 1000 g for 10 minutes at 4 °C. The resulting supernatant was filtered through cheesecloth, transferred to a centrifuge tube and centrifuged at 10000 g for 10 minutes at 4°C. The resulting mitochondrial pellet was resuspended in a small (~20 μl) amount of isolation buffer, and protein content was determined by Fluram assay as previously described (24). Subsequently, high-resolution respirometry was performed using 0.1 mg of mitochondria in a medium consisting of 100 mM sucrose, 20 mM K^+ -Tes (pH 7.2), 50 mM KCl, 2 mM MgCl_2 , 1 mM EDTA, 4 mM KH_2PO_4 , 3 mM malate and 0.1% of FA-free BSA. Three different protocols were performed. In protocol 1, mitochondria in the presence of 5 mM pyruvate as a substrate and oligomycin (1 $\mu\text{g}/\text{ml}$) to block ATP synthesis (state 4 respiration), were automatically titrated with increasing levels of palmitate (dissolved in 50% ethanol) by a titration pump (OROBOROS Instruments) while respiration was monitored. This protocol was performed in duplicate. The free concentrations of FAs were calculated using the equation described by Richieri et al. (25). Data for FA concentration response curves were analyzed with the 5-parameter logistic curve fit option of the Sigmaplot 8.0 application. In protocols 2 and 3, mitochondrial respiration was assessed upon carbohydrate-derived (5 mM pyruvate) and FA-derived (50 μM palmitoyl-CoA plus 2 mM carnitine) substrates, respectively. Subsequently, maximal coupled (state 3) respiration was initiated by the addition of 1 mM of ADP. State 4 respiration was measured as the leak respiration following addition of 1 $\mu\text{g}/\text{ml}$ oligomycin. Maximal oxygen flux rates (state U) were obtained by titration of the chemical uncoupler FCCP. From the muscle tissue taken, we managed to isolate mitochondria from 11 subjects (5 male/6 female) for the FA-induced uncoupling (protocol 1), and due to limitations

in available mitochondria, we remained with 9 subjects (3 male/6 female) in the remaining 2 protocols.

WAT browning. Abdominal subcutaneous adipose tissue was rinsed from blood, immediately snap frozen, and stored at -80°C for future analyses. Total RNA was isolated from human adipose and muscle biopsies by TRI Reagent (Sigma-Aldrich) extraction and purification using QIAGEN RNeasy Mini Columns according to the manufacturer's instructions. Then 900 ng of total RNA was reverse transcribed and analyzed using Applied Biosystems Real-time PCR Systems using the $\Delta\Delta\text{CT}$ method. Relative gene expression was normalized to TATA boxing-binding protein (tbp) mRNA levels. Similar results were obtained when relative expression levels were normalized to 2 additional housekeeping genes: β -2-microglobulin (B2M) and cyclophilin A (PPIA). Technical replicates were performed, and similar results were observed. Relative gene expression of UCP1, CIDEA, PRDM16, PGC1a, AP2, PPAR γ , adiponectin, adipisin, leptin, FNDC5, TMEM26, and CD137 both in WAT and in SM tissue was analyzed (real-time RT-PCR Primer Sequences in Supplemental Table 1 and Supplemental Methods).

Blood analysis. Blood was collected for analyses of several blood parameters. Plasma concentrations of glucose (ABX Glucose HK CP, Radiometer, Horiba ABX), free glycerol (Glycerol kit; R-Biopharm), and total glycerol (ABX Triglycerides CP, Radiometer, Horiba ABX) were determined on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica). Triglyceride levels were calculated by using the difference in total and free glycerol. Plasma catecholamines were determined using reagents from Recipe (Recipe Chemicals and Instruments) and analyzed on HPLC and by electrochemical detection. Serum insulin was analyzed with a Human Insulin Specific RIA Kit (Millipore) on a Gamma Counter (2470 Automatic Gamma Counter Wizard; Wallac, PerkinElmer). Serum TSH was measured by an Electrochemiluminescence Immunoassay Kit on a COBAS 6000 system (Roche Diagnostica), and free thyroxine (FT4) was analyzed by a solid-phase time-resolved fluoroimmunoassay FT4 kit on an AutoDELFIA system (PerkinElmer). Plasma inflammatory marker C-reactive protein (CRP) was measured with a particle-enhanced immunoturbidimetric assay on a COBAS c311 system (Roche Diagnostics GmbH), and IL-6 and IL-8 were measured with a chemiluminescent immunometric assay on an IMMULITE 1000 system (Siemens).

Statistics. Statistical analyses were performed with PASW Statistics 20.0 for Mac (SPSS). Two-sided paired sample t tests were used to compare findings between thermoneutral and mild cold conditions and to test the acclimation effects. Two-

sided independent sample t tests were used to compare findings between men and women. Pearson correlation was used to identify correlations between variables. $P < 0.05$ was considered statistically significant.

Study approval. The ethics committee of Maastricht University Medical Centre+ approved the study protocol, and all subjects provided written informed consent. All procedures were conducted according to the principles of the Declaration of Helsinki.

RESULTS

Increased NST upon cold acclimation. Before cold acclimation, resting metabolic rate (RMR) was 6.8 ± 1.0 mega joule (MJ) /24 h with females having a significantly lower RMR (6.2 ± 0.7 MJ/24 h) compared with males (7.6 ± 0.7 MJ/24 h) ($P < 0.05$). In order to determine NST, subjects were exposed to mild cold; for this purpose they were wrapped in a water-perfused suit (ThermaWrap Universal 3166 MTRE; Advanced Technologies Ltd.) connected to 2 water heating/cooling systems (Blanketroll III; SubZero). This setup is suitable for use inside the PET/CT scanner. The cooling protocol ensured that subjects were measured during stable non-shivering conditions. This mild cold condition caused a significant increase in energy expenditure, both in females and males (to 6.9 ± 1.0 and 8.5 ± 0.6 MJ/24 h respectively; $P < 0.05$; Table 2). Cold acclimation increased NST significantly from $10.8\% \pm 7.5\%$ before to $17.8\% \pm 11.1\%$ after ($P < 0.01$) (Figure 1), but no significant change in RMR occurred ($P > 0.05$) (Table 2). Despite the metabolic adaptation found after cold acclimation, no changes in the time until shivering were observed (50 ± 11 and 51 ± 12 minutes before versus after the cold acclimation period; $P > 0.05$), and similar suit temperatures during the mild cold conditions were used (25.4 ± 1.8 and $25.8 \pm 1.6^\circ\text{C}$ before versus after cold acclimation; $P > 0.05$). Therefore, the same mild cold stimulus (absolute suit temperature) resulted in a higher metabolic response after cold acclimation. The absence of changes in shivering threshold might be explained by the relatively short-term and intermittent cold challenge used in this protocol. Another explanation is that body temperature distribution might have changed. No sex differences in NST were observed; NST in females was $11.0 \pm 9.8\%$ before and $15.9 \pm 10.7\%$ after the cold acclimation, whereas in males an NST of $11.6 \pm 4.3\%$ before and $20.3 \pm 12.0\%$ after was found ($P > 0.05$).

Table 2. BAT activity and energy expenditure under thermoneutral conditions and during mild cold exposure, before and after cold acclimation.

Variable	Group values (n=17)		Females (n=9)		Males (n=8)	
	PRE	POST	PRE	POST	PRE	POST
Glucose uptake in BAT (SUVmean)	2.4 ± 0.7	2.8 ± 0.5 ^A	2.3 ± 0.9	2.8 ± 0.6 ^B	2.6 ± 0.3	2.9 ± 0.4 ^B
Glucose uptake in BAT (SUVmax)	14.1 ± 7.0	19.0 ± 8.1 ^A	12.5 ± 8.0	18.3 ± 9.8 ^B	15.9 ± 5.8	19.9 ± 6.3 ^B
Glucose uptake in BAT ('detectable' volume; cc)	665 ± 451	913 ± 458 ^B	556 ± 448	761 ± 544 ^B	788 ± 452	1085 ± 279 ^B
Glucose uptake rate in BAT (μmol/min/100g)	6.9 ± 3.0	7.6 ± 2.5	5.4 ± 3.1	6.9 ± 3.1	8.5 ± 2.0	8.4 ± 1.1
Energy expenditure (Kjoule/min)						
Thermoneutral	4.7 ± 0.7	4.7 ± 0.7	4.3 ± 0.4	4.3 ± 0.5	5.3 ± 0.5 ^C	5.1 ± 0.6 ^C
Mild cold	5.3 ± 0.8 ^D	5.5 ± 0.8 ^D	4.8 ± 0.7 ^S	5.0 ± 0.7 ^D	5.9 ± 0.4 ^{C,D}	6.1 ± 0.4 ^{C,D}
Resting metabolic rate; RMR (Mjoule/24h)	6.8 ± 1.0	6.8 ± 1.0	6.2 ± 0.6	6.2 ± 0.7	7.6 ± 0.7 ^C	7.3 ± 0.8 ^C
Non-shivering thermogenesis (%)	10.8 ± 7.5	17.8 ± 11.1 ^A	11.0 ± 9.8	15.9 ± 10.7 ^B	10.6 ± 3.2	20.3 ± 12.0 ^B

BAT activity was measured during and after mild cold exposure. Results are expressed as mean ± SD.

^A P<0.01, before vs. after, ^B P<0.05, before vs. after, ^C P<0.05 males vs. females, ^D P<0.05 thermoneutral vs. mild cold. The effect of cold acclimation was tested with a paired samples t test. Differences between males and females were tested with independent sample t test.

BAT recruitment after cold acclimation. Retrospective studies have suggested a higher prevalence of BAT in females (26); however, the results of the present study do not confirm this. Thus, cold-activated BAT activity before acclimation was 2.3 ± 0.9 mean standard uptake value (SUV mean; uptake kBq/ml/injected dose [kBq]/ patient weight [g]), "detectable" (PET-based) BAT volume was 556 ± 448 cc, and glucose uptake rate was 5.4 ± 3.1 μmol/min/100 g in females, whereas male values were not statistically different (2.6 ± 0.3 SUV mean, 788 ± 452 cc, and 8.5 ± 2.0 μmol/min/100 g, all P-values >0.05; Table 2). Since we did not observe sex differences in BAT activity values before cold acclimation, the combined results will be presented below. Before cold acclimation, 94 % (16 out of 17) of the subjects showed cold-activated BAT depots, and after cold acclimation, 100 % BAT prevalence was observed. Cold acclimation increased upper body BAT activity from 2.4 ± 0.7 to 2.8 ± 0.5 SUV mean (P<0.01) and detectable BAT volume expanded from 665 ± 451 cc before to 913 ± 458 cc (P<0.01) afterwards. Analysis of dynamic PET/CT imaging of the supraclavicular region revealed that upon cold acclimation, glucose uptake rate did not increase significantly (6.9 ± 3.0 vs. 7.6 ± 2.5 μmol/min/100 g; P>0.05). One subject showed a negative NST both before (−10.5 %) and after (−5.2 %) cold acclimation, which can be due to low or no NST in combination with lower temperatures of the extremities, causing a drop in metabolism in these regions (Arrhenius law) (27). Interestingly, this was also the subject in which no BAT could be detected before

the cold acclimation period and who only showed a small increase of BAT upon cold acclimation. Increase in NST was not related to increase in BAT. However, before cold acclimation, there was a significant positive relation between NST and BAT activity (R^2 0.78; $P < 0.01$) and NST and maximal BAT activity (R^2 0.55; $P < 0.01$). After acclimation, NST was still related to maximal BAT activity (R^2 0.32, $P < 0.05$; Figure 1B).

Figure 1. Individual data of BAT activity and NST before and after cold acclimation. 'Detectable' BAT volume and non-shivering thermogenesis increased significantly upon the cold acclimation period. A significant relation is found between non-shivering thermogenesis and BAT activity. A: Individual data on BAT activity; left panel 'Detectable' BAT volume (cc), middle panel Glucose uptake rate ($\mu\text{mol}/\text{min}/100\text{g}$) and right panel Non-shivering thermogenesis (%). B: Relation between non-shivering thermogenesis and BAT; left panel NST expressed as percentage and BAT activity as SUV mean, right panel NST expressed as percentage and BAT activity as SUV max. C: [^{18}F]FDG-PET images of the upper body after cold exposure in a female (top) and a male subject (bottom), before (PRE) and after (POST) cold acclimation. Main BAT locations are indicated with black arrows, additionally paravertebral BAT is activated. D: Transversal CT (top) and PET/CT fusion (bottom) slice of the supraclavicular region demonstrating [^{18}F]FDG-uptake in BAT locations (white arrows) after cold exposure, both before (PRE) and after (POST) the cold acclimation period. BAT, brown adipose tissue; CT, computed tomography; PET, positron emission tomography; SUV, standard uptake value.

Taken together, these results indicate that human BAT is recruited upon chronic cold stimulation, as in rodents. A significant increase in detectable BAT volume was found, which indicates that more white or brown adipocytes were activated. The increased maximal glucose uptake (SUV max) in BAT upon cold acclimation indicates an increased metabolic activity of existing BAT. In parallel, NST increased,

suggesting involvement of BAT. An individualized cooling protocol was used for the determination of NST and BAT activity; however, the cold acclimation was fixed at 16 °C, since this has previously been shown to increase cold-induced energy expenditure (28). No correlation was found between suit temperature and BAT activity and suit temperature and the subjective responses. Moreover, no correlations were found between sensation scores completed during cold acclimation and (the change in) BAT activity. Therefore, we were not able to show a correlation between the magnitude of the cold experience and BAT recruitment.

The role of skeletal muscle respiration in cold-induced thermogenesis. Besides BAT, skeletal muscle (SM) is an alternative putative tissue for NST. Particularly, studies in animals identified mitochondrial uncoupling (proton leak) in SM as a source of heat production in response to cold (29, 30). In humans, it has been shown that SM can be responsible for a 40 % increase in total body energy expenditure after epinephrine infusion (31). More recently, we showed in cold-exposed humans that NST is significantly related to estimates of mitochondrial uncoupling in SM, supporting a role for SM in human NST (32). To investigate the role of SM during cold acclimation, we first measured SM respiration in permeabilized muscle fibers using a multisubstrate protocol, similarly to our previous study (32). Samples were taken before and after the cold acclimation period. Cold acclimation did not affect oxygen consumption in any of the respiratory states analyzed. Particularly, state 4 respiration, reflecting mitochondrial proton leak, was similar before and after cold acclimation (125.9 ± 11.2 vs. 138.8 ± 11.8 pmol/(s*mg)/(MtDNA copy numbers) $\times 10.000$, respectively; Figure 2B). In addition, also the maximally coupled (state 3) and maximally uncoupled (state U, reflecting the capacity of the electron transport chain) respiration remained unchanged (Figure 2, C–E), indicating an unchanged SM oxidative capacity. For a more in-depth analysis of mitochondrial uncoupling upon cold acclimation, we also isolated SM mitochondria. In these mitochondria, “basal” mitochondrial oxygen consumption not related to ATP production, i.e., state 4 or leak respiration, was then analyzed on 2 different substrates (i.e., pyruvate and palmitoyl-CoA). In parallel with our findings in the permeabilized muscle fibers, also state 4 respiration fueled by single substrates in isolated mitochondria was unchanged upon cold acclimation (Figure 2F). Since it is well known that fatty acids (FAs) are capable of stimulating mitochondrial respiration that is not coupled to ATP production (i.e., FA-induced uncoupling), possibly due to activation of proteins that facilitate proton leak (33), we next tested the

sensitivity of the mitochondria for the uncoupling effects of FAs both before and after cold acclimation. As expected, FAs (palmitate) stimulated leak respiration in SM mitochondria in a dose-dependent manner (data not shown).

Figure 2. No effect of cold acclimation on skeletal muscle respiration. A: Mitochondrial density (n=16). Panels B-E represent the data from permeabilized muscle fibers (n=16, 8M/8F) corrected for mitochondrial density, while panels G and H (n=11, 5M/6F) and panels F-J (n=9, 3M/6F) reflect the data from isolated mitochondria obtained in a subset of subjects. B: Oxygen consumption not linked to ATP synthesis (State 4). C: Oxygen consumption upon substrates only (malate, malate + octanoylcarnitine, malate + glutamate State 2). D: ADP-stimulated respiration (State 3) fuelled by various complex-I and complex-II linked substrates. E: Maximally uncoupled respiration upon the chemical uncoupler FCCP. F: Mitochondrial oxygen consumption not linked to ATP synthesis (State 4) on pyruvate and palmitoyl-CoA + carnitine. G: Mean EC50 value of palmitate concentration-response curves. H: Mean Vmax of palmitate concentration-response curves. I: ADP-stimulated respiration (State 3). J: Maximally uncoupled respiration upon FCCP fuelled by pyruvate or palmitoyl-CoA + carnitine. The effects of cold acclimation on all skeletal muscle parameters were tested with paired samples t tests. Closed and open bars represent the values before and after cold acclimation, respectively. Values are expressed as means with SEM. M, malate; G, glutamate; S, succinate; O, octanoyl-carnitine.

Calculated half-maximal effective concentration (EC₅₀) values (163.1 ± 157.1 vs. 188.6 ± 114.6 nM free palmitate) and Vmax values (2999.5 ± 1072.4 before and 2866.1 ± 883.9 pmol O₂/s/mg mitochondrial protein after cold acclimation) were, however, similar, indicating a comparable sensitivity for FA-induced uncoupling before and after the cold acclimation period (Figure 2, G and H). Finally, also maximally coupled (state 3) and maximally uncoupled (state U) respiration

remained unchanged upon cold acclimation, both when fueled by either the carbohydrate-derived substrate pyruvate or the FA-derived substrate palmitoyl-CoA (plus carnitine) ($P>0.05$; Figure 2, I and J). Taken together, these results do not support the notion that an enhanced SM mitochondrial uncoupling underlies the increased NST upon cold acclimation, which is in line with the 2012 PET tracer study by Ouellet et al., who showed that SM does not contribute to NST (4).

“Browning” of white adipose tissue. Although BAT seems to be the main site for NST in rodents (10), recent findings indicate that within white adipose tissue (WAT), UCP1-positive, “inducible” cells can be found (34). These cells are called *beige* (35) or *brite* (36) adipocytes. Upon activation, these beige/brite cells resemble morphology (many small fat vacuoles and many mitochondria) and function (heat production) of brown adipocytes. Based on cell-specific markers, recent data, also from our group, support a mixed composition of white and beige/brite adipocytes in human depots that were identified as BAT (35). In accordance, Sharp et al. (37) recently confirmed that human BAT can be categorized as beige/brite and not “classical” BAT. In that perspective, recruiting beige/brite cells and transdifferentiation of WAT into beige/brite might be potential targets in body weight regulation. We therefore measured mRNA expression of several BAT, WAT and beige/brite markers in subcutaneous abdominal fat tissue collected before and after the cold acclimation period (real-time RT-PCR primer sequences in Supplemental Table 1 and Supplemental Methods). None of the measured browning markers were significantly altered in subcutaneous WAT after 10 days of cold acclimation ($P>0.05$; Supplemental Table 2). These results indicate that cold acclimation did not induce browning of cells within abdominal subcutaneous WAT. A stronger cold stimulation or a longer time period of acclimation might be required for the more general browning of the subcutaneous adipose tissue depot. We also examined gene expression of the same markers in SM tissue. A significant upregulation in PPAR γ (1.03 ± 0.25 before and 1.19 ± 0.34 AU after; $P<0.05$) and adiponectin was found (1.07 ± 0.71 before and 1.76 ± 1.42 AU after; $P<0.05$; Supplemental Table 2).

Changes in subjective responses during cold acclimation. During the cold acclimation period, subjects completed visual analogue scales (VAS scales) (38) on sensation, thermal comfort, and shivering at several time points after entering the cold room (Supplemental Figure 1 and Supplemental Methods). A lower incremental AUC (iAUC) indicates a sensation closer to neutral, lower self-reported shivering, and more comfort. The iAUC decreased with 57% for

sensation ($P<0.05$), 61% for self-reported shivering ($P<0.01$), and 72% ($P<0.01$) for comfort. This indicates that, after cold acclimation, the environment is judged as warmer and more comfortable and that self-reported shivering decreased during cold exposure (Figure 3).

Figure 3. Graphical reflection of VAS scales; a higher sensation and thermal comfort and lower self-reported shivering were reported on day 10, as compared to day 3. The incremental area under the curve decreased with 57 %, 72 % and 61 % for sensation, thermal comfort and self-reported shivering respectively (P -values; <0.05 , <0.01 and <0.01 respectively). This was tested with a paired samples t test. Values are expressed as means with SEM. Closed squares represent the 3rd day of acclimation (1st time 6 hours cold exposure), open squares represent the 10th day.

Chapter 5

Table 3. Skin perfusion, blood pressure, and body temperatures under thermoneutral conditions and during mild cold exposure, and blood value, before and after cold acclimation.

	Group values (n=17)	
	PRE	POST
Mean skin temperature (° C)		
Thermoneutral	35.2 ± 0.8	35.5 ± 0.5
Mild cold	31.8 ± 1.2 ^A	31.6 ± 1.1 ^A
Change upon cold stimulation	-3.5 ± 1.6	-3.9 ± 1.1
Core temperature (° C)		
Thermoneutral	37.2 ± 0.3	37.0 ± 0.8
Mild cold	37.2 ± 0.4	37.0 ± 0.7
Change upon cold stimulation	-0.1 ± 0.2	0.0 ± 0.3
Gradient core-mean skin (° C)		
Thermoneutral	2.0 ± 0.8	1.4 ± 0.8
Mild cold	5.1 ± 1.2 ^A	5.3 ± 1.3 ^A
Change upon cold stimulation	3.1 ± 1.7	3.6 ± 0.8
Normalized skin perfusion hand (%)		
Thermoneutral	100	100
Mild cold	21 ± 17 ^A	12 ± 9 ^{A,B}
Mean arterial pressure (mmHg)		
Thermoneutral	83 ± 7	80 ± 5 ^B
Mild cold	94 ± 9 ^A	94 ± 9 ^A
Change upon cold stimulation	11 ± 7	15 ± 8 ^B
Insulin (uU/ml)	9.8 ± 3.9	8.8 ± 3.7
Glucose (mM)	4.8 ± 0.4	4.8 ± 0.4
HOMA-index	2.1 ± 1.0	1.8 ± 0.8
Free fatty acids (uM)	619 ± 248	584 ± 296
Triglycerides (uM)	656 ± 327	530 ± 190
TSH (U/l)	1.5 ± 0.8	1.3 ± 0.6
FT4 (pmol/l)	12.9 ± 1.9	12.5 ± 2.4
CRP	<1	<1
IL-6	<2	<2
IL-8	<5	<5
Noradrenaline (ng/L)		
Thermoneutral	332.6 ± 123.6	355.4 ± 127.2
Mild cold	867.5 ± 237.4 ^C	916.7 ± 282.7 ^C
Change upon cold stimulation	528.6 ± 261.5	578.5 ± 274.4
Adrenaline (ng/L)		
Thermoneutral	41.7 ± 17.8	33.7 ± 16.9 ^A
Mild cold	34.6 ± 11.8	39.9 ± 18.6 ^D
Change upon cold stimulation	-9.1 ± 20.45	5.5 ± 15.2 ^D
Time until shivering (minutes)	50 ± 11	51 ± 12

Results are expressed as means ± SD. ^A P<0.05, thermoneutral vs. mild cold; ^B P<0.05 before vs. after; ^C P<0.01 before vs. after; ^D P<0.05 thermoneutral vs. mild cold. The effect of cold acclimation was tested with a paired samples t test. Differences between males and females were tested with independent sample t test.

The effects on body temperatures, skin perfusion, blood pressure, and blood values. The individualized cooling protocol used to activate BAT lowered mean skin temperature from 35.2 ± 0.8 °C (thermoneutral) to 31.8 ± 1.2 °C (mild cold). However, no changes in body temperatures (both skin and core) before and during cold exposure after cold acclimation were detected (Table 3). Hand skin blood flow during cold exposure was reduced to a larger extent after the cold acclimation (-78 ± 17 % before, and -88 ± 9 % afterwards; $P < 0.05$; Table 3). Systolic blood pressure and mean arterial pressure (MAP) during thermoneutral conditions decreased significantly after cold acclimation (MAP from 82.8 ± 6.8 to 79.5 ± 5.1 mmHg, $P < 0.05$). Before and after cold acclimation, the same values during cold exposure were found due to a stronger increase in MAP (Table 3). All values were within normal ranges of young adult humans (39). Blood values during the thermoneutral condition of insulin, glucose, free FAs, triglycerides, T4, thyroid-stimulating hormone (TSH), CRP, IL-6, and IL-8 did not change after cold acclimation (Table 3). Acute cold increased plasma noradrenaline significantly to the same extent both before and after cold acclimation (from 332.6 ± 123.6 during thermoneutral to 867.5 ± 237.4 ng/l during mild cold conditions before [$P < 0.01$] and from 355.4 ± 127.7 during thermoneutral to 916.7 ± 282.7 ng/l [$P < 0.01$] during mild cold conditions after the cold acclimation period; Table 3), as previously described (19, 40). There were no changes in adrenalin levels upon acute cold stimulation. However, cold acclimation resulted in lower adrenalin levels during thermoneutrality (41.7 ± 17.8 before and 33.7 ± 16.9 ng/l afterwards; $P < 0.05$) and higher levels during mild cold stimulation (34.6 ± 11.8 before and 39.9 ± 18.6 ng/l after cold acclimation; $P < 0.01$).

DISCUSSION

There has been revitalized interest in BAT after the discovery of functional BAT in human adults in 2009 (1, 2, 26), not in the least because BAT activation may be able to counteract obesity and type 2 diabetes (40, 41). Although extensive data are available on the plasticity of this tissue in rodents (10, 42), there is an urgent need for studies focusing on BAT recruitment in humans. The current study shows, for what we believe is the first time, highly significant BAT recruitment in human adults after a 10-day period of cold acclimation. Detectable BAT volume increased significantly, by 37 %, comparable to the changes occurring in rodents (36, 43). Cold-induced glucose uptake rate expressed in $\mu\text{mol}/\text{min}/100$ g did not increase significantly. However, the combination of both an increased maximal glucose

uptake (SUV max) and an increase in detectable volume points toward an increased activity and recruitment of brown adipocytes, as is supported by visual inspections of the PET images (Figure 1, C and D). It should be noted, however, that count spilling caused by the finite resolution of the PET-imaging process is responsible for an overestimation of true active BAT volume both before and after cold acclimation. This study shows that BAT activity and detectable BAT volume, before and after cold acclimation, are similar in females and males. An increase in NST was found after cold acclimation. Moreover, maximal BAT activity was related to NST before and after cold acclimation. Our results are in line with the study by Davis in 1961 (11). The increase in NST upon acute cold exposure in our study was 11 % before and 18 % after cold acclimation, whereas in the Davis study, using a somewhat longer cold acclimation period with lower temperatures, the average increase in NST after cold acclimation was 34%. The lower NST, both before and after acclimation, in our study may be explained by the use of different cold exposure protocols, as we used individualized mild cold exposure to ensure absence of shivering. These results therefore indicate that human BAT contributes to NST and to the increase of NST during acclimation. These results are in line with an acclimation experiment from our laboratory (44) of even shorter duration (3 days) and under continuous mild cold (16 °C). That study revealed a significant increase in total daily energy expenditure (TDEE) without shivering (TDEE increased from $11.5 \pm 0.0.1$ to 12.1 ± 0.2 MJ/day, corrected for fat-free mass [FFM]). No changes in RMR were found in the current study. The relatively short duration (6 hours/day) in combination with intermittent cold exposure may have been the reason for this. RMR increases are mediated by the thyroid hormone axis. Generally, an increase in thyroid hormone (T3) achieves its effect on RMR after a time span of 7–14 days of continuous cold exposure (45). In this study no changes in thyroid hormones (TSH, FT4) were found, probably due to the intermittent cold exposure and the short duration of the intervention. No changes in noradrenaline levels were found upon cold acclimation, and adrenaline levels were significantly lower in thermoneutral condition, which is in line with the trend found by Vybíral et al. (46), with winter swimmers having a nonsignificant (trend) lower adrenalin level during cold exposure when compared with control subjects. A possible explanation might be an increased sensibility of the receptor. Cold-induced BAT recruitment may be important in the regulation of body weight and prevention of obesity. However, in the current study, no significant changes in body weight and body composition were observed, which

may indicate that increased energy expenditure was compensated by increased food intake or reduced physical activity. Alternatively, for measurable changes in body composition, a longer cold acclimation period may be needed, and future studies with a similar setup, but with an increased duration and with inclusion of dietary instructions, are needed. Previously we have shown a positive relation between changes in SM mitochondrial uncoupling and the increase in 24-hour energy expenditure after 3 days of mild cold exposure (32, 47). In this study, we could not confirm a relation between changes in NST and SM uncoupling as assessed in permeabilized muscle fibers. Also, a more in-depth analysis of mitochondrial respiration in isolated mitochondria did not reveal changes in mitochondrial uncoupling. Although the type (continuous vs. intermittent) and duration (3 vs. 10 days) of cold exposure may contribute to this discrepancy in results, the present study shows that changes in mitochondrial uncoupling capacity in SM did not relate to the increased NST after cold acclimation in humans. From cell culture studies, it is now well established that there are 2 distinct types of BAT cells, one derived from myf5-positive lineage (48), called the classical BAT cells, and myf5-negative, called inducible BAT, or beige or brite cells (35, 36). Based on cell-specific markers and on UCP1 (characteristic of both BAT and beige/brite cells), this study did not show browning of abdominal subcutaneous WAT upon cold acclimation. Possible increases in beige/brite cells may have been under the detection level, or browning in deeper depots (visceral, deeper subcutaneous) may have occurred. Unfortunately, we are unable to take WAT biopsies from these areas in our lean, healthy subjects. Furthermore, it remains possible that a longer cold stimulation period may result in a more general browning of human WAT. MAP during thermoneutrality was reduced after cold acclimation, and a stronger reduction in skin perfusion upon acute cold exposure at the hand was found. The latter may indicate that next to a metabolic response to cold acclimation (increase in NST), also an insulative response has occurred. Together, these physiological adaptations are in line with the change in subjective reporting on cold sensation, as subjects judged the environment warmer, felt more comfortable in the cold, and reported less shivering, indicating that cold was better tolerated. These changes in comfort also indicate that daily mild cold exposure might be a feasible therapy against the obesity pandemic. Earlier studies have shown that temporal exposures to 17 °C are acceptable for both adults and the elderly (49). Introducing indoor temperature variations in

dwelling and offices may therefore activate BAT and NST and thus impose a healthier indoor environment.

In conclusion, the present study shows that repeated intermittent cold exposures recruited BAT in humans and that this was accompanied by an increase in NST. BAT presence and activity were not different between males and females, both before and after the cold acclimation period. SM mitochondrial uncoupling did not contribute to the increased NST. In addition, the subjective experience of cold shifted to a more comfortable experience. Although more prolonged studies are warranted, a variable indoor environment with frequent cold exposures might be an acceptable and economic manner of increasing energy expenditure and may contribute to counteracting the current obesity epidemic.

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SUPPLEMENTAL METHODS

On prescribed time points ($t=0$, $t=20$, $t=40$, $t=60$, $t=90$, $t=120$, $t=180$, $t=240$, $t=300$ and $t=360$) after entering the cold room, subjects completed visual analogue scales (VAS-scales), on sensation, thermal comfort and shivering (Supplemental Figure 1). On the scale they were asked to indicate their sensation (How are you feeling now?), their comfort (Do you think this is...?) and shivering was reported (Do you shiver?). The scores were transferred to numeric data (from 0-10 for sensation, from -3.1 to -0.1 and 0.1 to 3.1 for comfort and 0-10 for shivering), so that analyses could be done. Incremental area under the curve was calculated for day 3 (6 hours) and day 10 (6 hours) and these values were compared by means of a paired sample t test.

Supplemental Figure 1: Visual representation of the VAS-scales on sensation, overall comfort and shivering. The questionnaires were presented in either Dutch or English to the subjects through an Internet browser.

mRNA expression levels of several markers were measured both in subcutaneous white fat samples and in skeletal muscle material. No effect of the cold acclimation was found in fat tissue, however PPARgamma and adiponectin were up regulated in the skeletal muscle. The following Real-Time RT-PCR Primer Sequences were used;

Supplemental Table 1: Real-Time-RT-PCR Primer Sequences used for gene expression in both skeletal muscle and white adipose tissue.

Genes	Sequences
hUCP1	AGGTCCAAGGTGAATGCCC GCGGTGATTGTTCCAGGA TCCGGGTCTCCAACCATGA
hCIDEA	GGCATCCAGAGTCTTGCTGAT AGACTTCGGATGGGAGCAAAT
hPRDM16	TCCACGCAGAACTTCTCACTG TCTGAGTCTGTATGGAGTGACAT
hPGC1a	CCAAGTCGTTACATCTAGTTCA ACTGGGCCAGGAATTTGACG
hAP2	CCCCATCTAAGGTTATGGTGCTC ACCAAAGTGCAATCAAAGTGGA
hPPARgamma	AGGCTTATTGTAGAGCTGAGTCT TATCCCAACATGCCATTCTG
hAdiponectin	TAGGCAAAGTAGTACAGCCCA GACACCATCGACCACGACC
hAdipsin	GCCACGTCGCAGAGAGTTC TGCCTTCAGAAACGTGATCC
hLeptin	CTCTGTGGAGTAGCCTGAAGC AAGCACAAGGACTGACTCAAGC
hFNDC5	CATGTCCTTGATGGCTGGAT ATGGAGGGACTGGTCTTCCTT
hTMEM26	CTTCACCTCGGTCACTCGC AGCTGTTACAACATAGTAGCCAC
hCD137	TCCTGCAATGATCTTGTCTCT CCACTCACAGACTCTCACAAC
hTBP	CTGCGGTACAATCCAGAAGT TGGTGTTGGCAAAGTGAAA
hPPIA	TCGAGTTGTCCACAGTCAGC GTGCTCGCGCTACTCTCTCT
hB2M	TCTCTGCTGGATGACGTGAG

Supplemental Table 2: mRNA expression in skeletal muscle and white adipose tissue.

	SKELETAL MUSCLE		WAT	
	PRE	POST	PRE	POST
UCP1	1.14 ± 0.61	1.37 ± 0.83	0.79 ± 0.71	0.61 ± 0.56
CIDEA	1.12 ± 0.67	1.10 ± 0.79	1.13 ± 0.50	1.23 ± 0.66
PRDM16	1.14 ± 0.43	1.45 ± 1.01	1.06 ± 0.29	0.97 ± 0.27
PGC1a	1.03 ± 0.25	1.14 ± 0.37	1.11 ± 0.49	0.98 ± 0.33
AP2	1.03 ± 0.25	1.03 ± 0.39	1.06 ± 0.34	1.04 ± 0.32
PPARgamma	1.03 ± 0.25	1.19 ± 0.34*	1.06 ± 0.32	1.11 ± 0.23
ADIPONECTIN	1.07 ± 0.71	1.76 ± 1.42*	1.07 ± 0.38	0.98 ± 0.17
ADIPSIN	1.08 ± 0.40	1.00 ± 0.43	1.09 ± 0.37	1.10 ± 0.31
LEPTIN	1.08 ± 0.44	1.38 ± 0.91	1.16 ± 0.51	1.07 ± 0.73
FNDC5	1.04 ± 0.30	0.97 ± 0.35	1.04 ± 0.19	1.06 ± 0.34
TMEM26	1.10 ± 0.38	1.02 ± 0.40	1.12 ± 0.44	1.31 ± 0.46
CD137	1.20 ± 0.76	1.58 ± 1.08	1.12 ± 0.83	0.80 ± 0.45

Results are expressed as means ± SD. * P<0.05 PRE vs. POST. All expression levels are normalized to the average of all subjects PRE cold. The effect of the cold acclimation was tested with a paired samples t test.

CHAPTER 6

The effects of cold acclimation on brown adipose tissue in healthy lean and obese men

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The contents of this chapter are in preparation

ABSTRACT

Introduction. It is well known that brown adipose tissue (BAT) is an important site for non-shivering thermogenesis (NST) in human adults. Subjects with high BMI and body fat percentage have low BAT activity values and lower NST values. The aim of the current study was to investigate the effects of cold acclimation on BAT and NST in healthy overweight/obese male subjects.

Methods. Six healthy obese male subjects were included (mean BMI: 34.2 ± 4.0 kg/m²), and compared with 8 healthy lean male subjects (mean BMI: 22.4 ± 2.5 kg/m²). Cold acclimation was achieved by exposure to 14 °C (obese) or 15-16 °C (lean), 6 hours daily, for 10 consecutive days. Before and after cold acclimation, BAT activity was evaluated with [¹⁸F]FDG-PET/CT-imaging.

Results. Before cold acclimation 5 out of 6 overweight/obese subjects showed active BAT depots, whereas all lean subjects had active BAT depots. BAT activity was significantly lower in overweight/obese subjects compared to lean subjects both before (SUV max obese: 7.4 ± 5.2 ; lean: 15.9 ± 5.8) and after (SUV max obese: 15.4 ± 9.3 ; lean: 19.9 ± 6.3 , all P-values <0.05) cold acclimation. Before cold acclimation overweight/obese subjects had lower NST compared to lean subjects (obese: 4.7 ± 6.0 ; lean: 10.6 ± 3.2 %, P<0.01). After cold acclimation NST was not significantly different between both groups (obese: 17.4 ± 11.3 ; lean: 20.3 ± 12.0 , P>0.05). Cold acclimation increased BAT activity, volume and NST significantly in both groups (all P-values <0.01). Negative correlations were found between BAT activity values and body weight, BMI, body fat percentage and age (all P-values <0.05).

Conclusion. In both lean and obese subjects, cold acclimation results in BAT recruitment, in parallel with an increase in NST. Therefore, cold acclimation might be an interesting tool to increase energy expenditure in both lean and obese subjects. Future studies are needed to reveal whether this could be helpful to reduce body weight.

INTRODUCTION

It has long been known that brown adipose tissue (BAT) is the main site for non-shivering thermogenesis (NST) in rodents. In these animals BAT is important in maintaining internal body temperatures upon cold exposure (4). Nowadays it is well accepted that functional BAT is present in human adults as well. Cold-exposed subjects showed the presence of active BAT depots on 2-deoxy- ^{18}F -fluoro-D-glucose (^{18}F FDG) positron emission-tomography and computed-tomography (PET/CT) imaging (15, 19). Heat production in BAT is accomplished by the BAT-specific protein uncoupling protein 1 (UCP1). UCP1 bypasses the electron transport chain and thereby heat is produced (8). This heat is used to maintain internal body temperatures during cold exposure. The presence of UCP1 in human adults was confirmed by UCP1-positive stainings (15, 19, 23) from biopsy material.

Both retrospective studies and studies using an active cooling protocol showed that BAT activity correlates negatively with body mass index and body weight (11, 15, 19). Thus, subjects with a high body fat percentage and BMI have low BAT activity levels. The reduced heat-producing capacity of BAT in humans with a higher BMI, is reflected by the blunted NST in obese subjects that were exposed to 16 °C (25). Orava et al. (10) recently confirmed low BAT activity values in obese subjects. Moreover diet-induced weight loss caused a trend to an increased BAT activity in this study. In addition, our research group showed a significant increase in BAT activity upon weight loss induced by gastric surgery (21). More important, after weight loss BAT-positive subjects had a significantly higher NST compared to BAT-negative subjects. Thus, weight loss-induced BAT recruitment results in a higher NST. Besides weight loss, our research group and others demonstrated that prolonged intermittent cold exposure results in BAT recruitment in lean healthy subjects (3, 7, 16, 26). Interestingly, also a higher NST was found after the cold acclimation period (16, 26). Yoneshiro et al. (26) also showed that daily 2-hour cold exposure at 17 °C for 6 weeks significantly decreased fat mass. This was, at least partly, explained by BAT recruitment, because the changes in body fat mass correlate with the changes in BAT activity. The above-mentioned cold acclimation studies were conducted in lean subjects. To our knowledge, there is no information on the effect of cold acclimation on BAT and NST in overweight and obese subjects, which are characterized by low BAT activity and blunted NST response upon cold exposure.

Therefore, the aim of the current study was to investigate the effects of prolonged intermittent cold exposure on BAT activity values, NST and related parameters in healthy obese men. Additionally, we compared the effects of the cold acclimation period in obese healthy men with data from lean healthy men.

METHODS

Subjects. For this study 6 overweight/obese healthy male subjects between 19 and 51 years were included, this dataset was completed with data from 8 lean men from a previous study (16). The lean men were studied between December 2011 and May 2012, whereas the overweight/obese men were studied between March and June 2014. Exclusion criteria were diabetes mellitus, physical activity more than twice a week, use of beta-blockers, a history of cardiovascular diseases and asthma or other pulmonary obstructive diseases. The ethics committee of Maastricht University Medical Center+ approved the study protocol, and all subjects provided written informed consent. All procedures were conducted according to the principles of the Declaration of Helsinki.

Study design. The protocol of the measurements and the cold acclimation period were as previously published (16). Overweight/obese subjects have a thicker subcutaneous fat layer and therefore a higher insulation. Consequently they will experience the same environmental temperature as less cold (5, 22). To ensure the same metabolic condition for lean and overweight/obese subjects (shivering at the first day, that gradually diminished) during the cold acclimation period, room temperature was set at a lower temperature for the overweight/obese men. For that purpose, during the cold acclimation period overweight/obese subjects were exposed to 13-14 °C and lean subjects to 15-16 °C. The cold acclimation period lasted for 10 consecutive days, with cold exposure for 2 hours on the first day, 4 hours on the second day and 6 hours per day for the remaining 8 days. Before the cold acclimation period body composition was determined by means of dual X-ray absorptiometry (type Discovery A; Hologic). Body weight was measured before and after cold acclimation. Before and after the cold acclimation period a muscle biopsy from m. vastus lateralis was taken according to the technique of Bergström (2). In order to study BAT activity [¹⁸F]FDG-PET/CT-imaging was used both before and after the cold acclimation period. For this purpose, subjects were measured after a 4-hour fasting period and were asked to refrain from exercise 24h before the measurements. A cannula was inserted for blood sampling during the thermoneutral and mild cold period and for tracer

injection. iButtons (Maxim Integrated Products) were placed on 14 ISO-defined (18) sites and a telemetric pill (CoreTemp HT150002; HQ, Inc) was swallowed for measurement of skin and core temperatures, respectively. A chest strap (Polar T31; Polar) to measure heart rate and a pressure cuff (Cresta) for measurement of blood pressure were attached. Mean arterial blood pressure (MAP) was calculated as $MAP = 1/3 \text{ Systolic blood pressure} + 2/3 \text{ Diastolic blood pressure}$. Laser Doppler probes were attached for skin perfusion measurements at the ventral side of the hand at the base of the thumb, at the ventral side of the forearm halfway between the elbow and wrist (Perimed PF4000; Perimed), at the ventral side of the hallux and at the abdomen halfway between the umbilicus and the left lateral side of the body (Perimed PF5000; Perimed). Energy expenditure was measured continuously by means of a ventilated hood system with the use of a facemask (EZ call, IDEE Maastricht).

Cold acclimation. In the cold room subjects were dressed in shorts, T-shirts and flip-flops. During the cold acclimation subjects performed sedentary activities; studying, watching TV and were instructed to refrain from physical activity. A cup with soup or tea was offered frequently and sandwiches were freely available.

Subjective responses during cold acclimation. On prescribed time points ($t=0$, $t=20$, $t=40$, $t=60$, $t=90$, $t=120$, $t=180$, $t=240$, $t=300$ and $t=360$) after entering the cold room, subjects completed visual analogue scales (VAS-scales) on sensation, thermal comfort and shivering. The responses on the 10th day of the cold acclimation period were compared with the responses on the 3rd day (first time 6 hours cold exposure), by means of a paired sample t test. The incremental area under the curve was calculated to gain more insight in the general effect of the cold acclimation. In total 12 subjects (6 lean and 6 overweight/obese) completed the VAS-scales.

Individualized cooling protocol to study BAT presence and activity. Subjects were wrapped in a water-perfused suit (ThermoWrap Universal 3166 MTRE, Advanced Technologies Ltd.) connected to 2 water heating/cooling systems (Blanketroll III, SubZero Cincinnati, Ohio). The protocol started with a thermoneutral period of 45 minutes, followed by the individualized cooling protocol. Each subject was cooled down (tank temperature was lowered with 4 °C every 15 minutes) until shivering occurred. After this, subjects were warmed up for 5 minutes so that shivering disappeared and finally suit temperature was set slightly above the temperature at which shivering occurred. An estimate of suit temperature was obtained by the average of the in- and outlet. The cooling

protocol ensured that subjects were measured during stable non-shivering conditions. After 30 minutes of mild cold exposure [^{18}F]FDG was administered.

[^{18}F]FDG-PET/CT-imaging. A bolus of 74 MBq [^{18}F]FDG was injected one hour before the PET/CT-imaging protocol. During this hour mild cold stimulation remained. Hereafter, imaging started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a static PET-scan. A total of six to seven bed positions (4 minutes per bed position) were necessary to cover the area where BAT is usually found (i.e. upper abdominal, thoracic, and neck region). The PET-image was used to determine [^{18}F]FDG uptake and the CT-image was used for PET attenuation correction and localization of the [^{18}F]FDG uptake sites. Voxel size of reconstructed PET and CT image sets were $4 \times 4 \times 4 \text{ mm}^3$ and $1.172 \times 1.172 \times 4 \text{ mm}^3$, respectively.

PET analysis. The scans were analyzed using PMOD software (version 3.0, PMOD Technologies). Both the researcher (AAJvdL) and an experienced nuclear medicine physician (BB) interpreted the PET/CT-images. We define BAT activity as glucose uptake in fat tissue. BAT activity is expressed in SUV mean (averaged activity values) and SUV max as the maximum value in that region. BAT volume in this study was obtained from PET activity values and called detectable BAT volume. By using a threshold of 1.5 standard uptake value (SUV) and fat localization by means of Hounsfield Units (between -10 and -180), regions of interest were manually outlined.

Skeletal muscle respiration. After the biopsy, a portion of the muscle tissue was immediately frozen in melting isopentane and stored at -80°C for determination of mitochondrial DNA (mtDNA) copy number (ratio ND1 to LPL), as described previously (13). A small portion ($\sim 30 \text{ mg}$) of the muscle tissue was instantly placed in ice-cold preservation medium (BIOPS; OROBOROS Instruments). Muscle fibers were permeabilized with saponin according to the technique of Veksler et al. (20). After completion of the permeabilization protocol, oxygen consumption was quantified polarographically using a 2-chamber Oxygraph (OROBOROS Instruments). State 2 respiration was measured after the addition of malate plus octanoyl-carnitine. Subsequently, ADP was added to evaluate State 3 respiration. Coupled respiration was then maximized via addition of glutamate and succinate. Finally, the chemical uncoupler FCCP was titrated to evaluate the maximal capacity of the electron transport chain (State U). This trace evaluates the capacity of the skeletal muscle tissue to oxidize substrates. To study solely the effects of uncoupling a single substrate trace was developed. This trace was

performed in the muscle fibers of the overweight/obese subjects only. For that purpose, State 2 respiration was measured after the addition of malate plus pyruvate. State 3 was evaluated after the addition of ADP. Oligomycin was added to determine respiration not coupled to ATP synthesis (State 4), and FCCP was titrated to evaluate the maximal capacity of the electron transport chain (State U). For both traces, the integrity of the outer mitochondrial membrane was checked by addition of cytochrome c upon maximal coupled respiration. All measurements were performed in quadruplets.

Statistics. Statistical analyses were performed with PASW Statistics 20.0 for Mac (SPSS). Two-sided paired sample t tests were used to compare findings between thermoneutral and mild cold conditions and to test the acclimation effects. Two-sided independent sample t tests were performed to compare findings between lean and overweight/obese subjects. Pearson correlation was used to identify correlations between variables. $P < 0.05$ was considered statistically significant.

RESULTS

Subjects. By definition, overweight/obese subjects had a significantly higher weight, BMI and body fat percentage compared to lean subjects (all P-values < 0.01). Obese subjects tended to be older compared to lean subjects ($P = 0.08$). No effect of the cold acclimation period on anthropometric parameters was found in both groups (Table 1).

Table 1. Subject characteristics.

Characteristic	Lean (n=8)	Overweight/Obese (n=6)
Age (yr)	23.0 $\pm$ 3.1	31.8 $\pm$ 12.9
Body mass (kg)	75.2 $\pm$ 10.7	114.8 $\pm$ 15.9 ^A
Height (m)	1.83 $\pm$ 0.07	1.83 $\pm$ 0.06
BMI (kg/m ²)	22.4 $\pm$ 2.5	34.2 $\pm$ 4.0 ^A
Body fat (%)	15.7 $\pm$ 3.2	28.6 $\pm$ 6.5 ^A

Values for lean and overweight/obese men are displayed separately. Values are expressed as means $\pm$ SD. ^A $P < 0.01$. Independent sample t tests were used for comparisons between lean and overweight/obese men.

BAT activity and energy expenditure. Before cold acclimation 100% of the lean and 5 out of 6 of overweight/obese subjects showed cold-activated BAT depots (BAT activity was quantified as SUV > 1.5 ; Figure 1). Before the cold acclimation period, BAT activity values were significantly lower in overweight/obese men compared with lean men, (SUV max: 15.9 $\pm$ 5.8 in lean and 7.4 $\pm$ 5.2 in overweight/obese men, $P < 0.05$).

Figure 1. [^{18}F]FDG-PET images of the upper body after cold exposure in a lean (top) and overweight/obese (bottom) male subject, before (PRE) and after (POST) cold acclimation. Main supraclavicular BAT locations are indicated with black arrows; additionally paravertebral BAT is activated. Clearly visible is the lower BAT activity in the overweight/obese subject.

However, 'detectable' BAT volume did not significantly differ between the two groups ($P > 0.05$). This suggests that overweight/obese subjects have lower volume-specific BAT activity. Cold acclimation increased BAT activity in both groups significantly (for lean, SUV max: 15.9 ± 5.8 before and 19.9 ± 6.3 after, for overweight/obese, SUV max: 7.4 ± 5.2 before and 15.4 ± 9.3 SUV, all P -values < 0.05) (Table 2). After the cold acclimation period, BAT activity values in overweight/obese men were still significantly lower than in lean men (Table 2). The subject without BAT before cold acclimation was still BAT-negative after the cold acclimation period. However on average overweight/obese men showed

higher 'detectable' BAT volume after cold acclimation (lean: 1085 ± 279 vs. overweight: 1218 ± 879 cc respectively, $P < 0.05$).

Group values ($n=14$) revealed significant negative correlations between SUV mean and body weight, both before and after cold acclimation (pre: R^2 0.42; $P < 0.05$, post: R^2 0.29; $P < 0.05$, Figure 2A) and between SUV max and body weight (R^2 0.56; $P < 0.01$), only before the cold acclimation period. Next to body weight, also BMI was significantly correlated with SUV mean (R^2 0.39; $P < 0.05$) and SUV max (R^2 0.50; $P < 0.01$) before the cold acclimation and only with SUV mean (R^2 0.29; $P < 0.05$) after the cold acclimation period (data not shown). Additionally, a significant correlation was found between body fat percentage and SUV mean (R^2 0.37; $P < 0.05$) and SUV max (R^2 0.43; $P < 0.01$) (Body fat percentage was not measured after cold acclimation). Thus, subjects with a higher body weight, body fat percentage and/or BMI have lower BAT activity values both before and after cold acclimation.

Moreover, SUV mean and SUV max (data not shown) were negatively correlated with age, (R^2 0.71 and 0.47 respectively, both P -values < 0.01 , Figure 2B). No correlation was found between age and BAT recruitment values.

A significant positive correlation was found between SUV max and NST, when expressed in kJ/min (data not shown) and as percentage of resting metabolic rate (RMR) (R^2 0.33 and 0.36, both P -values < 0.05 , Figure 2C). This correlation was found only before the cold acclimation period; after cold acclimation no significant correlations with BAT activity values and (changes in) energy expenditure were found. When corrected for fat-free mass (FFM) (25), no significant difference in RMR between lean and overweight/obese men was found (lean: 7.6 ± 0.9 , obese: 8.6 ± 2.3 MJ/24h – corrected for FFM, $P > 0.05$). After cold acclimation no significant difference in RMR between both groups was detected. The cold acclimation period did not significantly change RMR in both groups. Before cold acclimation, energy expenditure (EE) during mild cold tended to be higher than during thermoneutral condition in lean subjects (thermoneutral: 5.3 ± 0.4 ; mild cold: 5.9 ± 0.4 KJ/min, $P = 0.064$). After cold acclimation, cold exposure increased EE significantly from 5.1 ± 0.6 KJ/min during thermoneutral to 6.1 ± 0.4 KJ/min ($P < 0.05$) during mild cold exposure. As a consequence NST was significantly higher after cold acclimation (before: 10.6 ± 3.2 ; after: 20.3 ± 12.0 %, $P < 0.05$).

Figure 2. Visual display of significant correlations found with BAT activity values. A: A significant positive correlation was found between BAT activity values and weight, both before (PRE) and after (POST) cold acclimation. B: A significant negative correlation was found between BAT activity (SUV max) and age. C: A significant positive correlation was found between BAT activity (SUV mean) and non-shivering thermogenesis (NST %).

There was no significant increase in EE in overweight/obese men before cold acclimation (thermoneutral: 6.0 ± 0.9 ; mild cold 6.3 ± 0.9 KJ/min, $P > 0.05$). As in the lean subjects, the cold acclimation period resulted in a significant higher EE during cold exposure in overweight/obese subjects (thermoneutral: 5.8 ± 0.9 ; mild cold: 6.7 ± 1.1 , $P < 0.05$). Thus, also overweight/obese subjects had a higher NST after cold acclimation (before: 4.7 ± 6.0 ; after: 17.4 ± 11.3 , $P < 0.05$). The lean men displayed a significantly higher NST before the cold acclimation period (lean: 10.6 ± 3.2 ; overweight/obese: 4.7 ± 6.0 % ($P < 0.05$). However, after cold acclimation no significant difference in NST was found between both groups (Table 2). The increased NST did not correlate with BAT recruitment values.

Skeletal muscle mitochondrial uncoupling. The biopsy procedure failed in 1 overweight/obese subject, therefore we here present data of 8 lean men and 5 overweight/obese men. The cold acclimation period did not affect mitochondrial copy number (MtDNA) in either group (all P -values > 0.05).

Table 2. BAT activity and energy expenditure under thermoneutral and during mild cold exposure, before and after cold acclimation.

Variable	Lean (n=8)		Overweight/Obese (n=6)	
	Before	After	Before	After
BAT activity (SUV mean)	2.6 ± 0.3	2.9 ± 0.4 ^A	1.9 ± 1.0	2.3 ± 1.2 ^{A,B}
BAT activity (SUV max)	15.9 ± 5.8	19.9 ± 6.3 ^A	7.4 ± 5.2 ^B	15.4 ± 9.3 ^{A,B}
BAT volume ('detectable' volume; cc)	788 ± 452	1085 ± 279 ^A	652 ± 549	1218 ± 879 ^{A,B}
Energy expenditure (kJ/min)				
Thermoneutral	5.3 ± 0.5	5.1 ± 0.6	6.0 ± 0.9	5.8 ± 0.9
Mild cold	5.9 ± 0.4	6.1 ± 0.4 ^A	6.3 ± 0.9	6.7 ± 1.1 ^A
RMR (MJ/24h)	7.6 ± 0.7		8.7 ± 1.3	
RMR (MJ/24h – corrected for FFM)	7.7 ± 0.9		8.6 ± 2.3	
NST (%)	10.6 ± 3.2	20.3 ± 12.0 ^A	4.7 ± 6.0 ^B	17.4 ± 11.3 ^A

Values for lean and overweight/obese men are displayed separately. Values are expressed as means ± SD. ^A P<0.05 pre vs. post, ^B P<0.05 lean vs. overweight/obese. The effect of cold acclimation was tested with a paired samples t test. Independent sample t tests were used for comparisons between lean and overweight/obese men. SUV; standardized uptake value, RMR; resting metabolic rate, FFM; fat-free mass, NST; non-shivering thermogenesis.

Before cold acclimation, State 3MO, State 3MOG, and State U respiration were significantly higher in lean compared to overweight/obese subjects (all P-values <0.05). After cold acclimation, State 3MO and State 3MOG were significantly higher in lean compared to overweight subjects (both P-values <0.01), with a similar trend for State U (P=0.074, Figure 3)). No effect of cold acclimation was found on oxygen consumption in any of the respiratory states. State 4 respiration was not significantly different before and after cold acclimation in both groups (lean: 143 ± 48 before and 126 ± 43 pmol/(s*mg) MtDNA copy numbers x 10.000 after cold acclimation (P>0.05), obese: 50 ± 48 before and 31 ± 17 pmol/(s*mg) MtDNA copy numbers x 10.000 after cold acclimation (P>0.05)). State 4 respiration in overweight/obese subjects was evaluated using a single substrate trace (pyruvate). As a result total oxygen consumption was lower.

Body temperatures, skin perfusion and blood pressure. During cooling, mean skin temperature significantly decreased, from 34.8 ± 1.8 to 31.4 ± 1.1 °C in lean and from 34.4 ± 1.4 to 30.2 ± 1.5 °C (both P-values <0.01) in overweight/obese subjects. No differences in mean skin temperature were found between both groups and the cold acclimation period did not alter mean skin temperature (all P-values >0.05; Table 3).

Figure 3. Visual display of SM respiration in lean and overweight/obese subjects, before and after cold acclimation. Left panel: Respiration in permeabilized muscle fibers before (PRE) cold acclimation in lean and overweight/obese subjects. Right panel: Respiration in permeabilized muscle fibers after (POST) cold acclimation in lean and overweight/obese subjects. Higher respiration for all states was found for lean subjects, this was tested by means of independent sample t test. * $P < 0.05$ lean vs. obese, ** $P < 0.01$ lean vs. obese. Black and white bars represent the values in lean and obese subjects, respectively. Values are expressed as means with SEM. 3MO, State 3 respiration after addition of malate and octanoyl-carnitine; 3MOG, State 3 respiration after addition of malate, octanoyl-carnitine and glutamate; U, State U reflects maximal respiration, which is fueled by addition of FCCP.

The cooling protocol did not significantly change core temperature in both groups before the cold acclimation period (P -values > 0.05). However, after the cold acclimation period, the lean men significantly reduce core temperature, from 37.1 ± 0.2 during thermoneutral condition to 36.9 ± 0.3 during mild cold stimulation ($P < 0.05$). The cold acclimation period did not affect core temperature in the overweight/obese men. The individualized cooling protocol significantly lowered skin blood perfusion in both groups, both before and after the cold acclimation period (Table 3). A trend was found in a stronger reduction in skin blood flow at the underarm in lean men, change in relative skin perfusion at underarm in lean - 65 ± 24 and -38 ± 12 in overweight/obese ($P = 0.068$). However after the cold acclimation period the overweight/obese men reduce skin perfusion at the underarm to the same extent as the lean subjects. Mean arterial pressure (MAP), was not significantly different during the thermoneutral period between both groups ($P > 0.05$). The cooling protocol significantly increased MAP in both groups (P -values < 0.01). Cold acclimation resulted in a lower MAP during thermoneutral condition only in lean subjects (87 ± 6 before and 82 ± 4 after the cold acclimation period, $P < 0.01$). The cold acclimation period did not alter MAP in overweight/obese subjects.

Table 3. Skin perfusion, blood pressure and body temperatures under thermoneutral conditions and during mild cold exposure, before and after cold acclimation.

Variable	Lean (n=8)		Overweight/Obese (n=6)	
	Before	After	Before	After
Mean skin temperature (° C)				
Thermoneutral	34.8 ± 1.8	35.5 ± 0.3	34.4 ± 1.4	35.2 ± 0.3
Mild cold	31.4 ± 1.1 ^A	30.8 ± 0.9 ^A	30.2 ± 1.5 ^A	30.2 ± 2.2 ^A
Change upon cold stimulation	-3.9 ± 1.5	-4.7 ± 1.0	-4.2 ± 1.8	-5.1 ± 2.5
Core temperature (° C)				
Thermoneutral	37.1 ± 0.4	37.1 ± 0.2	37.0 ± 0.3	36.9 ± 0.3
Mild cold	37.0 ± 0.4	36.9 ± 0.3 ^A	36.9 ± 0.4	36.9 ± 0.4
Change upon cold stimulation	-0.17 ± 0.2	-0.22 ± 0.17	-0.10 ± 0.2	-0.02 ± 0.2
Gradient core-mean skin (° C)				
Thermoneutral	1.9 ± 0.6	1.6 ± 0.2	2.5 ± 1.2	1.7 ± 0.2
Mild cold	5.3 ± 1.1 ^A	6.0 ± 1.0 ^A	6.7 ± 1.7 ^A	6.7 ± 2.5 ^A
Change upon cold stimulation	3.4 ± 1.5	4.5 ± 1.1	4.1 ± 1.8	5.1 ± 2.5
Normalized skin perfusion hand (%)				
Thermoneutral	100	100	100	100
Mild cold	16 ± 12 ^B	9 ± 8 ^B	22 ± 13 ^B	14 ± 4 ^B
Change upon cold stimulation	-84 ± 12	-91 ± 8	-78 ± 14	-86 ± 4
Normalized skin perfusion under arm (%)				
Thermoneutral	100	100	100	100
Mild cold	35 ± 24 ^B	35 ± 19 ^B	62 ± 12 ^B	43 ± 29 ^B
Change upon cold stimulation	-65 ± 24	-65 ± 19	-38 ± 12	-57 ± 29
Mean arterial pressure MAP (mm Hg)				
Thermoneutral	87 ± 6	82 ± 4 ^D	87 ± 4	87 ± 4 ^C
Mild cold	101 ± 7 ^B	101 ± 6 ^B	103 ± 7 ^B	102 ± 8 ^B
Change upon cold stimulation	14 ± 8	20 ± 5	16 ± 9	15 ± 5
Time until shivering (minutes)	61 ± 7	62 ± 8	77 ± 9 ^C	75 ± 22

Values for lean and overweight/obese men are displayed separately. Values are expressed as means ± SD. ^A P<0.05 thermoneutral vs. mild cold, ^B P<0.01 thermoneutral vs. mild cold, ^C P<0.05 lean vs. overweight/obese, ^D P<0.05 before vs. after cold acclimation. The effect of cold acclimation was tested with a paired samples t test. Independent sample t tests were used for comparisons between lean and overweight/obese men.

Before the cold acclimation period, time until shivering is significantly shorter for the lean subjects compared with the overweight/obese subjects (lean: 61 ± 7, overweight/obese: 77 ± 9 minutes, P<0.05). Time until shivering is not statistically different between both groups after the cold acclimation period (P>0.05).

Subjective responses during cold acclimation. The VAS-scales completed during the cold acclimation period provide an indication on cold tolerance as experienced by the subject. No significant effect of the cold acclimation period on thermal sensation was found in both groups (cold acclimation decreased iAUC in lean with 56 % and in overweight/obese with 24 %, P-values >0.05). A trend

towards a lower self-reported shivering was found, only in the lean subjects (cold acclimation decreased iAUC with 40 %, $P=0.08$). A significant change in comfort was found upon the cold acclimation period, again only in the lean subjects (iAUC decreased with 67 % upon the cold acclimation period, $P<0.01$) (Figure 4). iAUC for thermal comfort tended to be lower in lean subjects compared to overweight/obese subjects before the cold acclimation period (lean: -696 ± 322 ; overweight/obese: -327 ± 220 iAUC, $P=0.06$). After cold acclimation no significant differences on thermal comfort were found between the groups.

DISCUSSION

It is nowadays well accepted that functional BAT is present in the majority of lean healthy individuals. Activated BAT can increase energy expenditure, and it is therefore suggested that BAT is able to positively affect body weight and type 2 diabetes (1, 9). To our knowledge, this is the first study that shows BAT recruitment induced by cold acclimation in overweight/obese healthy men. BAT activity was recruited to the same extent as in lean healthy men. BAT activity values in overweight/obese were lower at baseline, and remained lower throughout the experiment. 'Detectable' BAT volume was not significantly different before acclimation, but after acclimation a higher volume was found in overweight/obese subjects.

However BAT volume is difficult to interpret, because in this study we used a PET-based threshold (> 1.5 SUV) to define BAT volume. The limited spatial resolution of the PET image results in spillover of the PET signal. Thus thresholding based on SUV only can lead to BAT volume overestimation (17). Only before cold acclimation a lower NST was found in overweight/obese subjects. The lower NST is in line with the findings of Wijers et al. (25), who showed that obese subjects had a lower NST after cold exposure to 16°C for 48h. It is important to note that in the current study NST was measured after an individualized cooling protocol, thus all subjects were exposed to conditions inducing maximal non-shivering thermogenesis. The cold acclimation period significantly increased NST in both groups to the same extent. This study confirms the outcomes of several retrospective and dedicated (using an active cooling protocol) studies, showing negative correlations of BAT activity with body weight, BMI and body fat percentage (10, 11, 19).

Figure 4. Graphical reflection of VAS scales. Cold acclimation did not alter the subjective responses in the overweight/obese subjects. A trend was found towards a lower self-reported shivering ($P=0.08$) in lean men, and a significant lower thermal comfort was reported on the 10th day of the cold acclimation period ($P<0.01$). Thermal comfort ($P=0.06$) tended to be lower in the lean vs. overweight/obese subjects before the cold acclimation period, however after cold acclimation this difference was no longer statistically different. The effect of cold acclimation was tested with a paired samples t test. Independent sample t tests were used for comparisons between lean and overweight/obese men. Values are expressed as means with SEM. Left panel displays the data of lean men ($n=6$) and right panel displays data of overweight/obese healthy men ($n=6$). White squares represent the third day of acclimation (first time 6 hours cold exposure); black squares represent the tenth day.

Additionally, a negative correlation between BAT activity and age was found. This is in line with retrospective studies (11, 12) showing lower BAT activity in older subjects. The only subject without active BAT depots was the oldest (51 years). This subject strongly affects the correlation between age and BAT activity. On the other hand, one subject of 60 years old that dropped out during the cold acclimation period, also had no active BAT. In order to draw conclusions on BAT

activity in relation to age, further research is needed including higher numbers of subjects with increasing age.

In the current study lean men show a decreased core temperature upon the cooling protocol after cold acclimation. This is in line with findings of Radomski and Boutelier (14), who found that cold adapted subjects (nine daily cold water (15 °C) immersions for 20-40 minutes a day) showed a declined rectal temperature. The authors state that this hypothermic response to chronic intermittent cold exposure might be explained by a diminished sympathetic response to cold exposure, because urinary norepinephrine excretion was lower than in control subjects (14).

The current study also confirms earlier findings that acute cold exposure significantly increases MAP (10, 24). The cold acclimation period resulted in a significantly lower MAP during thermoneutral condition in lean subjects, whereas during cold exposure MAP was not statistically different. Upon cold exposure vasoconstriction takes place, which decreases heat transfer to the skin. This results in diminished heat loss to the environment and as a consequence, MAP increases. Our research group showed in elderly that mild cold-induced increased systolic blood pressure is maintained even after a one-hour rewarming period (6). Since it is well known that obesity in itself is also a main risk factor for the development of hypertension and other related cardiovascular diseases, it is therefore highly important to monitor blood pressure when additionally imposing cold stress on obese subjects.

In this study the cold acclimation period did not change the subjective responses in the overweight/obese subjects. A possible reason for these findings is the low number of subjects that was included in this study. In 17 lean the environment was judged more comfortable, warmer and self-reported shivering decreased subjects after the cold acclimation period (16).

The major limitation of this study is the small number of subjects enrolled. Despite this low number several trends were found. Taken together, the results indicate that both in lean and obese subjects repeated intermittent cold exposure results in BAT recruitment, in parallel with an increased NST. No contribution of skeletal muscle mitochondrial uncoupling to the increased NST was found. Thus, frequent cold exposures might be a tool to increase energy expenditure both in lean and overweight/obese subjects. Future studies are needed to reveal if this could be helpful to reduce body weight and improve metabolic health.

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CHAPTER 7

Cold acclimation affects immune composition in skeletal muscle of healthy lean subjects

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ABSTRACT

Aim. Low environmental temperatures have a profound effect on biological processes in the body, including the immune system. Cold exposure coincides with hormonal changes, which may directly or indirectly alter the immune system, even in the skeletal muscle. The aim of the present study was to investigate the effect of cold acclimation on immune composition in skeletal muscle.

Methods. Skeletal muscle biopsies were obtained from 17 healthy lean subjects before and after 10 days of mild cold exposure (15 °C, 6 hours/day). Non-shivering thermogenesis was calculated by indirect calorimetry.

Results. Cold acclimation increased non-shivering thermogenesis from 10.8 ± 7.5 before to 17.8 ± 11.1 % after cold acclimation ($P < 0.01$), but did not affect plasma catecholamine nor cytokine levels. In contrast, cold acclimation affected mRNA expression of several immune cell markers in skeletal muscle. It downregulated expression of the Th17 markers RORC (-28%, $P < 0.01$) and NEDD4L (-15%, $P < 0.05$), as well as the regulatory T cell marker FOXP3 (-13%, $P < 0.05$). Furthermore, cold acclimation downregulated expression of the M2 macrophage markers CCL22 (-50%, $P < 0.05$), CXCL13 (-17%, $P < 0.05$) and CD209 (-15%, $P < 0.05$), while the M1 macrophage marker IL12B was upregulated (+141%, $P < 0.05$). Cold acclimation also enhanced several markers related to interferon (IFN) signaling, including TAP1 (+12%, $P < 0.01$), IFITM1/3 (+11%, $P < 0.05$), CD274 (+36%, $P < 0.05$) and STAT 2 (+10%, $P < 0.05$).

Conclusion. Ten days of intermittent cold exposure induces marked changes in the expression of immune cell markers in skeletal muscle of healthy lean subjects. The physiological consequences and therapeutic relevance of these changes remain to be determined.

INTRODUCTION

During cold exposure, the body attempts to maintain a constant internal body temperature by increasing heat production and minimizing heat loss, both of which are mediated by activation of the sympathetic nervous system (1). The increase in heat production is accomplished through shivering thermogenesis (*e.g.* involuntary muscle contractions) as well as non-shivering thermogenesis (NST) in which both muscle and brown adipose tissue are involved (2).

Low environmental temperatures have a profound effect on biological processes in the body, including the immune system. To date, only few studies have investigated the effect of acute (< 4 hours) cold exposure on plasma immune cell composition and cytokine production in humans. Immersion of healthy young men in cold (14 °C) water or exposure to air (5 °C) induced a rapid increase in neutrophils (3), B cells, and T cells as well as Natural Killer (NK) cell activity in the blood (4), while monocyte numbers had decreased (3), suggesting both pro- and anti-inflammatory effects of acute cold exposure. On the other hand, a recent study by Kox et al. (5) showed that acute activation of the sympathetic nervous system by, amongst others, acute cold attenuated the pro-inflammatory innate immune response upon LPS injection in humans, supporting a merely anti-inflammatory effect of acute cold exposure.

Several mechanisms may underlie the link between cold exposure and the immune system. Cold exposure coincides with hormonal changes, like catecholamine release, which either directly or indirectly may alter immune status (3, 4, 6). Indeed, several immune cell types including NK cells, B and T cells, contain β -adrenergic receptors that are targeted by catecholamines to induce a cascade of events resulting in immune cell mobilization (4). Furthermore, immune cells contain cold sensing transient receptor potential channels that affect intracellular signaling upon activation (7) and this may provide an additional mechanistic link between cold exposure and immune status. Besides plasma catecholamines, acute cold exposure also alters levels of several other hormones and cytokines in the plasma, including cortisol and interleukin-6 (IL-6) (4, 5).

As mentioned previously skeletal muscle is involved in shivering and non-shivering thermogenesis. Since immune cells, and in particular macrophages, serve an important role in skeletal muscle integrity (8) and cold exposure affects immune cells in plasma, we hypothesized that cold acclimation affects immune composition in skeletal muscle. Moreover, we anticipated that this may be

mediated by catecholamines as well as other hormones and cytokines that are released upon cold exposure.

To this end, we studied immune composition in skeletal muscle biopsies of 17 healthy lean subjects that were subjected to longer-term (10 days) mild cold exposure (15 °C, 6 hours/day) (9). Before and after cold acclimation, we investigated plasma cytokine levels and expression of several immune cell markers, including markers for macrophages, NK cells, T cells and pattern recognition receptors, in skeletal muscle biopsies.

MATERIALS AND METHODS

Subjects. Seventeen healthy lean ($\text{BMI} < 25 \text{ kg/m}^2$) subjects, of which 9 females and 8 males (age: 23 ± 2.3 years) participated in this study (9). Exclusion criteria were diabetes mellitus, pregnancy, physical activity more than twice a week, use of beta-blockers and a history of cardiovascular diseases and asthma or other pulmonary obstructive diseases. The ethics committee of Maastricht University Medical Centre+ approved the protocol, and all subjects provided written informed consent. All procedures were conducted according to the principles of the Declaration of Helsinki.

Study design. Cold acclimation was achieved by exposure of subjects to 15-16 °C for 10 consecutive days: 2 hours on the first day, 4 hours on the second day and 6 hours per day for the remaining 8 days. Before and after this period, muscle biopsies were taken. On a separate day, body composition was determined by means of dual x-ray absorptiometry (type A; Hologic), which was followed by an individualized cooling protocol during which energy expenditure (EE) was continuously assessed (indirect calorimetry) and venous blood samples were taken.

Cold acclimation. During the cold acclimation period, subjects were placed in a cold room that was air-cooled to an environmental temperature of 15-16 °C. Subjects wore shorts and T-shirts. During the cold acclimation period, the participants were instructed to refrain from physical activity and performed sedentary activities, such as studying and watching TV.

Skeletal muscle biopsy procedure. Before and after the cold acclimation period, muscle biopsies from the *m. vastus lateralis* (approx. 75-100 mg) were collected in fasted condition under localized anesthesia, according to the technique of Bergström (10). Muscle samples were directly frozen in melting isopentane and stored at -80 °C until further analysis.

Individualized cooling protocol and indirect calorimetry. To assess the effect of cold acclimation on heat production, during an individualized cooling protocol EE was measured continuously by means of a ventilated hood system (Omnical; Jaeger). To this end, subjects were wrapped in a water-perfused suit (ThermaWrap Universal 3166 MTRE; Advanced Technologies Ltd.) connected to 2 water heating/cooling systems (Blanketroll III; SubZero). The protocol started with a thermoneutral period of 45 minutes, followed by the individualized cooling protocol (11). In short, each subject was cooled down until shivering occurred. After this, subjects were warmed up for several minutes so that shivering disappeared and finally suit temperature was set slightly above the temperature at which shivering started for another 30 min. NST was calculated as the difference in EE between thermoneutrality and stable mild cold exposure (last 30 minutes of the cooling protocol).

Laboratory analysis. During the individualized cooling protocol, blood was collected at thermoneutrality and during mild cold exposure. Plasma concentrations of glucose (ABX Glucose HK CP, Radiometer, Horiba ABX), free glycerol (Glycerol kit; R-Biopharm), and total glycerol (ABX Triglycerides CP, Radiometer, Horiba ABX) were determined on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica). Triglyceride levels were calculated using the difference in total and free glycerol. Plasma catecholamines were determined using reagents from Recipe (Recipe Chemicals and Instruments) and analyzed on a HPLC and by electrochemical detection. Serum insulin was analyzed with a Human Insulin Specific RIA Kit (Millipore) on a Gamma Counter (2470 Automatic Gamma Counter Wizard; Wallac, PerkinElmer). The plasma inflammatory marker C-reactive protein (CRP) was measured with a particle-enhanced immunoturbidimetric assay on a COBAS c311 system (Roche Diagnostics GmbH), and IL-6 and IL-8 were measured with a chemiluminescent immunometric assay on an IMMULITE 1000 system (Siemens).

RNA isolation. Total RNA was isolated from skeletal muscle biopsies (approx. 25-30 mg) using the phenol-chloroform extraction method (Tripure RNA Isolation reagent, Roche, Germany) and treated with a DNase kit (TURBO DNase, Life Technologies, The Netherlands) according to the manufacturer's instruction. Amount of RNA was determined by NanoDrop.

dcRT-MLPA assay. A dual-color reverse transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) assay was performed as described previously (12). Briefly, for each target-specific sequence, a specific RT primer was

designed located immediately downstream of the left and right hand half-probe target sequence. Following reverse transcription, left and right hand half-probes were hybridized to the cDNA at 60°C overnight. Annealed half-probes were ligated and subsequently amplified by PCR (33 cycles of 30 s at 95°C, 30 s at 58°C and 60 s at 72°C, followed by 1 cycle of 20 min at 72°C). PCR amplification products were 1:10 diluted in HiDi formamide-containing 400HD ROX size standard and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (Applied Biosystems).

Trace data were analysed using the GeneMapper software package (Applied Biosystems). The areas of each assigned peak (in arbitrary units) were exported for further analysis in Microsoft Excel spread sheet software. Data were normalized to beta-2-microglobulin and signals below the threshold value for noise cutoff in GeneMapper (log2 transformed peak area 7.64) were assigned the threshold value for noise cutoff.

Immunohistochemical stainings. To examine if any putative changes in mRNA expression levels of macrophage markers originated from invasion of muscle tissue by macrophages, we performed immunofluorescence staining in muscle cross sections. In brief, 7 µm thick cryosections were thaw-mounted on glass slides and incubated (1:100) at room temperature with a monoclonal mouse antibody directed against human CD68 (M0178, Clone EBM11, DAKO), a generic macrophage marker protein. The primary antibody was visualized with an anti-mouseAlexaFluor488 conjugated antibody. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were captured with a Nikon E800 fluorescence microscope.

Statistics. Statistical analyses were performed with PASW Statistics 20.0 for Mac (SPSS). Two-sided paired sample t tests were used to compare findings between thermoneutral and mild cold conditions and to test the acclimation effects. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics. Of the 17 subjects who participated in the study, 9 were female and 8 were male. Mean age was 23 ± 2.3 years, mean BMI was 21.6 ± 2.2 kg/m² and mean fat mass $22.3 \pm 8.1\%$ (Table 1). Anthropometric values did not change upon cold acclimation (9).

Table 1. Subject characteristics.

Characteristic	Group (n=17)
Ratio males / females	8M / 9F
Age (yr)	23 ± 3.2
Body mass (kg)	68.4 ± 11.7
Height (m)	1.78 ± 0.09
BMI (kg/m ²)	21.6 ± 2.2
Body fat (%)	22.3 ± 8.1

Values are expressed as means ± SD.

Cold acclimation and energy expenditure. Cold acclimation did not affect resting metabolic rate (RMR) (Table 2). However, cold acclimation increased NST from 10.8 ± 7.5 % before to 17.8 ± 11.1 % after cold acclimation ($P < 0.01$) (Table 2). There was no difference between males and females, both before and after the cold acclimation (data not shown).

Cold acclimation and metabolic parameters. Thermoneutral plasma values of insulin, glucose, free fatty acids and triglycerides did not change after cold acclimation (Table 2).

Table 2. Energy expenditure and blood parameters under thermoneutral condition and during mild cold exposure, before and after cold acclimation.

Variable	Before	After
Energy expenditure (kJ/min)		
Thermoneutral	4.7 ± 0.7	4.7 ± 0.7
Mild cold	5.3 ± 0.8 ^C	5.5 ± 0.8 ^C
RMR (MJ/24h)	6.8 ± 1.0	6.8 ± 1.0
Non-shivering thermogenesis (NST; %)	10.8 ± 7.5	17.8 ± 11.1 ^A
Insulin (μU/ml)	9.8 ± 3.9	8.8 ± 3.7
Glucose (mM)	4.8 ± 0.4	4.8 ± 0.4
HOMA-index	2.1 ± 1.0	1.8 ± 0.8
Free fatty acids (μM)	619 ± 248	584 ± 296
Triglycerides (μM)	656 ± 327	530 ± 190
Noradrenalin (ng/l)		
Thermoneutral	332.6 ± 123.6	355.4 ± 127.2
Mild cold	867.5 ± 237.4 ^C	916.7 ± 282.7 ^C
Change upon cold stimulation	528.6 ± 261.5	578.5 ± 274.4
Adrenalin (ng/l)		
Thermoneutral	41.7 ± 17.8	33.7 ± 16.9 ^A
Mild cold	34.6 ± 11.8	39.9 ± 18.6 ^D
Change upon cold stimulation	-9.1 ± 20.5	5.5 ± 15.2 ^D
CRP	< 1	< 1
IL-6	< 2	< 2
IL-8	< 5	< 5

Results are expressed as mean ± SD. ^A $P < 0.01$ before vs. after, ^B $P < 0.05$ before vs. after, ^C $P < 0.01$ thermoneutral vs. mild cold, ^D $P < 0.05$ thermoneutral vs. mild cold. The effect of cold acclimation was tested with a paired samples t test.

The individualized cooling protocol significantly increased plasma noradrenalin levels to the same extent both before and after cold acclimation, as previously described (13, 14). The cooling protocol did not change plasma adrenalin levels. However, the cold acclimation period caused significantly lower adrenalin levels during thermoneutral condition (before: 41.7 ± 17.8 ng/l, after: 33.7 ± 16.9 ng/l, $P < 0.05$) and higher levels during mild cold stimulation (before: 34.6 ± 11.8 ng/l, after: 39.9 ± 18.6 ng/l, $P < 0.01$). Again, the response between males and females concerning the above-mentioned plasma values was not significantly different (data not shown). Therefore, we decided to pool the data for males and females in the following section.

Cold acclimation and markers of systemic and muscle inflammation. We assessed the thermoneutral plasma inflammatory factors CRP, IL-6 and IL-8 before and after the cold acclimation period, but these were below their respective detection limits (Table 2). Using the dcRT-MLPA assay, we measured mRNA expression of a large panel of inflammatory genes, including markers for innate and adaptive immune cells, pattern recognition receptors and cytokines in muscle biopsies before and after the cold acclimation (see Supplemental Table 1 for the complete list). Cold acclimation resulted in a significantly lower expression of RORC (-28%, $P < 0.01$) and NEDD4L (-15%, $P < 0.05$), both involved in Th17 response (Fig. 1A). In addition, the expression of the regulatory T cell marker FOXP3 was downregulated (-13%, $P < 0.05$).

Furthermore, cold acclimation affected several markers involved in innate immune response (Fig. 1B). More specifically, we found a downregulation of the M2 macrophage markers CCL22 (-50%, $P < 0.05$), CXCL13 (-17%, $P < 0.05$) and CD209 (-15%, $P < 0.05$). In contrast, the M1 macrophage marker IL12B was upregulated (+141%, $P < 0.05$). FLCN1, a marker involved in tumor-associated macrophages, was downregulated upon cold acclimation (-15%, $P < 0.05$). To investigate whether the changed expression of macrophage markers was accompanied by a change in recruitment of macrophages, we stained muscle biopsies for CD68 by immunofluorescence. Interestingly, the cold acclimation enhanced the amount of CD68-expressing cells pointing to infiltration of macrophages (stained in green, see Fig 1C for four representative subjects).

Of note, cold acclimation also enhanced several markers related to interferon (IFN) signaling, including TAP1 (+12%, $P < 0.01$), IFITM1/3 (+11%, $P < 0.05$), CD274 (+36%, $P < 0.05$) and STAT 2 (+10%, $P < 0.05$) (Fig 1D).

Figure 1. Expression of adaptive and innate immune markers in muscle biopsies before and after 10 days of cold acclimation in healthy subjects. mRNA expression levels of genes related to Th17 and Treg signaling (A), macrophage markers (B), and interferon signaling (D) were measured in skeletal muscle biopsies of healthy subjects ($n=17$) obtained before (white bars) and after (black bars) 10 days of cold acclimation. Expression levels are normalized to the housekeeping gene B2 microglobulin and expressed as fold change compared to baseline as mean $\pm$ SEM. (C) Immunofluorescence staining of C68 in skeletal muscle cross-sections. C68 positive macrophages are stained in green, nuclei are stained in blue. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ before vs. after cold acclimation.

DISCUSSION

Several studies have shown that short-term cold exposure affects the immune system in humans, an effect that may be mediated via sympathetic activation as well as by factors released by skeletal muscle. Up to date, no studies have investigated the effect of longer-term cold exposure on immune cell composition in skeletal muscle. In the present study we demonstrate that cold acclimation (10 days, 6h/day) affects the expression of several immune markers in skeletal muscle, in parallel with a significant increase in whole body NST. While markers involved in Th17 response and M2 macrophage markers were downregulated, markers for M1 macrophages and Th1 immunity were upregulated. This was accompanied by increase in the number of local CD68-positive cells. Furthermore,

immune markers related to IFN signaling were upregulated. Thus, cold acclimation markedly affects immune composition in skeletal muscle.

To the best of our knowledge, this is the first report describing the effect of a cold acclimation protocol on immune markers in skeletal muscle in humans. We have previously shown that markers of immune subsets in skeletal muscle can be markedly affected by dietary stimuli (15), as five days of high-fat high calorie diet upregulated several genes related to M1 macrophages. Interestingly, markers of M1 macrophages were also upregulated in the current study, while M2 macrophage markers were downregulated, suggesting that both high-fat diet feeding and cold elicit pro-inflammatory effects on macrophages. Moreover, we found increased CD68⁺ cells staining in skeletal muscle biopsies, suggesting influx of macrophages. This is in line with reports that show that acute cold exposure reduces circulating monocytes (3), which may thus be the consequence of extravasation of monocytes from the plasma and differentiation into macrophages in peripheral tissues, including skeletal muscle. Indeed, acute cold exposure also enhances MCP-1 levels, the attraction factor for monocytes, in plasma (3). As macrophages are the first line defense against many pathogens and tissue damage, the increase in M1 macrophages in muscle may thus point to an increased preparedness of the body against possible danger. Indeed, pro-inflammatory M1 macrophages are observed experimentally in the context of muscle repair, and are usually found at early stages after muscle injury (8). Furthermore, M1 macrophages are also thought to be involved in the development of muscle insulin resistance as pro-inflammatory cytokines including TNF- α hamper insulin signaling in skeletal muscle (16).

An interesting finding of the current study is the decrease in markers of Th17 signaling in skeletal muscle. Th17 cells are a subtype of T helper cells that have been shown to be present in skeletal muscle (17, 18) and are potent inducers of tissue inflammation (19). Furthermore, they are associated with the pathogenesis of several autoimmune diseases in humans, including rheumatoid arthritis (20), inflammatory bowel disease (21) and psoriasis (22). Thus, the reduction in markers of Th17 signaling upon cold acclimation may point to a beneficial effect on autoimmunity. This is also suggested by the recent paper of Kox et al. (5), who showed that acute activation of the sympathetic nervous system by, amongst others, acute cold attenuated the pro-inflammatory innate immune response upon LPS injection in humans. More specifically, this translated in lower release of TNF- α , an important pro-inflammatory mediator. On the other hand, Th17 cells

play an important role in orchestrating host defense against fungal infections and patients that exhibit a defect in Th17 function suffer from persistent fungal infections (23). So, balanced Th17 responses are of primary importance.

Several studies suggest that cold exposure is associated with an increased risk of viral infections (24-26). An important mediator in viral infections are interferons. In this respect, our results showing an increase in interferon inducible genes in skeletal muscle biopsies are particularly interesting as this suggests that cold acclimation enhances preparedness against viral infections. This finding is supported by previous literature showing that cold exposure enhances activity of NK cells in plasma, an important source of interferons (4). Thus, it may be questioned whether cold exposure is indeed disadvantageous for the immune system with respect to susceptibility for viral infections.

Alterations in sympathetic nervous system activity likely contribute to the link between cold exposure and changes in the immune system. When activated by cold stress, the adrenal medulla secretes catecholamines (*e.g.* adrenalin and noradrenalin) into the blood and catecholamines are known to mobilize leukocytes from several pools (4). More specifically, several immune cell types, including T and B cells and NK cells, exhibit β adrenergic receptors of which activation result in mobilization of these cell types. Previous studies have shown that short-term cold exposure enhances catecholamine release (4-6). Also in the current study, we found that the personalized cooling protocol enhanced noradrenalin release in the plasma. However, we could not find differences in catecholamine levels upon cold acclimation. This may be due to the fact that the blood samples were taken the day after the last acclimation day, *i.e.* perhaps catecholamine levels were already normalized. The changes we found on immune markers in skeletal muscle are thus likely the result of repeatedly enhanced catecholamine levels that occurred during the acclimation periods. Interestingly, cold acclimation did cause significantly lower adrenalin levels during thermoneutral condition and higher levels during mild cold stimulation. This is in line with the trend found by Vybíral et al. (27), who found that winter swimmers have non-significant (trend) lower adrenalin levels during cold exposure when compared with control subjects. A possible explanation might be an increased sensibility of the adrenergic receptor.

A potential limitation of the current study could be that we determined immune cell markers via MLPA-assay without performing flow cytometry analyses, which would have allowed the integration and correlation of RNA expression data with

changes in immune cell subsets in muscle tissue. However, performing flow cytometry analyses would require relatively large amounts of muscle tissues, the collection of which was not feasible in the current study. In addition, genetic expression assays more readily allow the assessment of differences in the quantitative expression levels of markers compared to cell surface staining techniques. Indeed, we found that, per immune group, several markers were consistently up- or downregulated, suggesting that our data are a reliable reflection of the effect of cold acclimation on immune cell composition in skeletal muscle.

In conclusion, the present study demonstrates that 10 days of cold acclimation not only results in enhanced non-shivering thermogenesis, but also induces marked changes in immune cell markers in skeletal muscle of healthy lean subjects. Markers involved in Th17 response and M2 macrophage markers were downregulated, and markers for M1 macrophages were upregulated. Furthermore, immune markers related to IFN signaling were upregulated. Future studies should be directed at unraveling the precise physiological consequences of these changes.

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SUPPLEMENTAL INFORMATION

Supplemental Table 1. Expression of adaptive and innate immune markers in muscle biopsies before and after 10 days of cold acclimation in healthy subjects.

Gene	Baseline	Cold (fold change vs baseline)	P-value
<i>Immune cell subset markers</i>			
BLR1 (B-cells)	1.00 ± 0.07	0.89 ± 0.04	0.16
CD19 (B-cells)	n.d.	n.d.	n.a.
NCAM1 (NK cells)	1.00 ± 0.16	1.04 ± 0.19	0.87
<i>T cell subsets</i>			
CD3E	1.00 ± 0.12	1.54 ± 0.59	0.38
CD4	1.00 ± 0.05	1.12 ± 0.09	0.076
CD8A [#]	1.00 ± 0.16	0.85 ± 0.08	0.19
CCR7	1.00 ± 0.08	0.83 ± 0.09	0.20
PTPRCv1	n.d.	n.d.	n.a.
PTPRCv2	1.00 ± 0.15	1.11 ± 0.25	0.33
AIRE	1.00 ± 0.14	0.87 ± 0.10	0.55
<i>Th1 response</i>			
CXCL10	1.00 ± 0.10	1.04 ± 0.10	0.74
IFNG	1.00 ± 0.29	0.47 ± 0.09	0.12
IL1B	1.00 ± 0.12	0.96 ± 0.10	0.79
IL2	1.00 ± 0.09	0.92 ± 0.07	0.48
IL15	1.00 ± 0.22	0.99 ± 0.13	0.95
TBX21	n.d.	n.d.	n.a.
TNF	1.00 ± 0.07	0.87 ± 0.03	0.13
<i>Th2 response</i>			
GATA3	1.00 ± 0.17	1.12 ± 0.40	0.78
IL4	n.d.	n.d.	n.a.
IL4d2 [#]	1.00 ± 0.59	0.79 ± 0.37	0.76
IL5	n.d.	n.d.	n.a.
IL6	1.00 ± 0.09	0.87 ± 0.05	0.17
IL9	1.00 ± 0.09	1.06 ± 0.11	0.66
IL13	1.00 ± 0.15	0.85 ± 0.14	0.36
<i>Th17 response</i>			
IL17A [#]	1.00 ± 0.22	0.79 ± 0.00	0.33
RORC	1.00 ± 0.06	0.72 ± 0.06	0.0038*
NEDD4L	1.00 ± 0.05	0.85 ± 0.03	0.04*
IL22RA1 [#]	1.00 ± 0.00	1.13 ± 0.08	0.12
<i>Treg markers</i>			
CTLA4	1.00 ± 0.65	0.53 ± 0.15	0.49
FOXP3	1.00 ± 0.06	0.87 ± 0.04	0.043*
IL10	1.00 ± 0.45	1.14 ± 0.34	0.81
IL2RA [#]	1.00 ± 0.16	1.15 ± 0.30	0.50
IL7R	1.00 ± 0.13	0.72 ± 0.05	0.08
LAG3	1.00 ± 0.13	0.75 ± 0.15	0.12
TGFB1	1.00 ± 0.05	1.06 ± 0.03	0.18
TNFRSF18	1.00 ± 0.05	0.94 ± 0.06	0.47

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Gene	Baseline	Cold (fold change vs baseline)	P-value
<i>Cytotoxicity markers</i>			
GNLY	1.00 ± 0.17	1.26 ± 0.30	0.41
GZMA	1.00 ± 0.06	0.91 ± 0.09	0.36
GZMB	1.00 ± 0.10	1.08 ± 0.08	0.52
PRF1	1.00 ± 0.08	0.93 ± 0.07	0.56
<i>Anti-microbial activity</i>			
LTF	1.00 ± 0.09	0.93 ± 0.12	0.46
<i>Macrophage markers</i>			
CD14	1.00 ± 0.11	0.95 ± 0.08	0.61
CD163	1.00 ± 0.23	0.90 ± 0.11	0.62
CD209	1.00 ± 0.05	0.85 ± 0.03	0.04*
CCL2	1.00 ± 0.16	1.11 ± 0.28	0.75
CCL3	1.00 ± 0.18	0.81 ± 0.06	0.35
CCL4	1.00 ± 0.06	0.95 ± 0.05	0.43
CCL5	1.00 ± 0.05	0.91 ± 0.06	0.33
CCL22	1.00 ± 0.17	0.50 ± 0.12	0.038*
CXCL13	1.00 ± 0.07	0.83 ± 0.05	0.049*
IL12A	1.00 ± 0.23	1.15 ± 0.39	0.75
IL12B [#]	1.00 ± 0.00	2.41 ± 0.60	0.029*
IL23A	1.00 ± 0.20	1.04 ± 0.16	0.86
<i>Scavenger receptors</i>			
MARCO	1.00 ± 0.23	2.78 ± 1.46	0.23
<i>Pattern recognition receptors</i>			
CLEC7A	1.00 ± 0.30	0.99 ± 0.33	0.99
MRC1	1.00 ± 0.25	1.17 ± 0.25	0.65
MRC2	1.00 ± 0.06	1.08 ± 0.08	0.36
NOD1	1.00 ± 0.06	0.82 ± 0.07	0.085
NOD2	1.00 ± 0.08	0.83 ± 0.07	0.068
TLR1 [#]	1.00 ± 0.00	2.01 ± 0.48	0.04*
TLR2	1.00 ± 0.10	0.88 ± 0.08	0.37
TLR3	1.00 ± 0.07	0.97 ± 0.06	0.75
TLR4	1.00 ± 0.17	1.15 ± 0.15	0.57
TLR5	1.00 ± 0.36	0.90 ± 0.31	0.75
TLR6 [#]	1.00 ± 0.22	0.83 ± 0.15	0.55
TLR7	1.00 ± 0.07	0.98 ± 0.05	0.82
TLR8	n.d.	n.d.	n.a.
TLR9	1.00 ± 0.05	0.89 ± 0.03	0.14
TLR10	n.d.	n.d.	n.a.
<i>Inflammasome components</i>			
NLR4	1.00 ± 0.04	0.89 ± 0.05	0.13
NLRP1	1.00 ± 0.24	0.82 ± 0.19	0.56
NLRP2	1.00 ± 0.06	0.89 ± 0.04	0.16
NLRP3	1.00 ± 0.33	1.55 ± 0.41	0.35
NLRP4	1.00 ± 0.09	0.92 ± 0.08	0.62
NLRP6	n.d.	n.d.	n.a.
NLRP7	n.d.	n.d.	n.a.

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Gene	Baseline	Cold (fold change vs baseline)	P-value
NLRP10	n.d.	n.d.	n.a.
NLRP11	n.d.	n.d.	n.a.
NLRP12	n.d.	n.d.	n.a.
NLRP13 [#]	1.00 ± 0.25	0.93 ± 0.20	0.83
<i>IFN signaling genes</i>			
CD274	1.00 ± 0.14	1.36 ± 0.14	0.049*
FCGR1A	1.00 ± 0.05	1.12 ± 0.08	0.12
GBP1	1.00 ± 0.12	1.15 ± 0.09	0.33
GBP2	1.00 ± 0.06	0.91 ± 0.06	0.22
GBP5	n.d.	n.d.	n.a.
IFI6	1.00 ± 0.12	0.84 ± 0.03	0.25
IFI16	1.00 ± 0.11	0.81 ± 0.07	0.13
IFI35 [#]	1.00 ± 0.87	0.11 ± 0.0	0.31
IFI44	1.00 ± 0.05	1.31 ± 0.25	0.22
IFI44L	1.00 ± 0.10	0.79 ± 0.10	0.08
IFIH1	1.00 ± 0.35	1.08 ± 0.31	0.84
IFIT2	1.00 ± 0.10	1.06 ± 0.11	0.7
IFIT3	1.00 ± 0.07	0.89 ± 0.04	0.11
IFIT5 [#]	1.00 ± 0.14	0.92 ± 0.07	0.59
IFITM1/3	1.00 ± 0.05	1.11 ± 0.07	0.026*
INDO	1.00 ± 0.11	0.90 ± 0.09	0.3
IRF7	n.d.	n.d.	n.a.
OAS1 [#]	1.00 ± 0.71	3.18 ± 1.52	0.098
OAS2	1.00 ± 0.10	1.09 ± 0.10	0.51
OAS3	1.00 ± 0.11	1.15 ± 0.09	0.4
SOCS1	1.00 ± 0.13	0.90 ± 0.06	0.52
STAT1	1.00 ± 0.02	0.97 ± 0.05	0.39
STAT2	1.00 ± 0.04	1.10 ± 0.04	0.028*
TAP1	1.00 ± 0.03	1.12 ± 0.05	0.0065*
TAP2	1.00 ± 0.08	0.94 ± 0.06	0.55
<i>Apoptosis / Survival</i>			
CASP8	1.00 ± 0.13	1.32 ± 0.11	0.054
BCL2	1.00 ± 0.08	0.83 ± 0.06	0.042*
FASLG	n.d.	n.d.	n.a.
FLCN1	1.00 ± 0.05	0.85 ± 0.06	0.048*
TNFRSF1A	1.00 ± 0.04	1.12 ± 0.09	0.14
TNFRSF1B	n.d.	n.d.	n.a.
<i>Small GTPases / (Rho)GTPase activating proteins</i>			
ASAP1	1.00 ± 0.80	1.67 ± 1.11	0.63
RAB13	1.00 ± 0.06	1.15 ± 0.11	0.12
RAB24	1.00 ± 0.07	1.05 ± 0.04	0.41
RAB33A [#]	1.00 ± 0.32	0.36 ± 0.12	0.08
TAGAP	1.00 ± 0.22	0.70 ± 0.14	0.088

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Gene	Baseline	Cold (fold change vs baseline)	P-value
<i>Chemokines</i>			
CCL11 [#]	1.00 ± 0.22	1.54 ± 0.39	0.18
CCL13	n.d.	n.d.	n.a.
CCL19	1.00 ± 0.09	1.01 ± 0.09	0.92
CXCL9	1.00 ± 0.22	1.03 ± 0.12	0.92
CX3CL1	1.00 ± 0.16	1.01 ± 0.17	0.95
<i>Cell growth / proliferation</i>			
BMP6	1.00 ± 0.18	0.76 ± 0.08	0.17
TGFBR2	1.00 ± 0.06	1.06 ± 0.05	0.39
AREG	n.d.	n.d.	n.a.
EGF	1.00 ± 0.13	0.96 ± 0.10	0.69
VEGF	1.00 ± 0.10	0.91 ± 0.08	1.0
<i>Cell activation</i>			
HCK	1.00 ± 0.19	0.80 ± 0.06	0.27
LYN	1.00 ± 0.09	0.91 ± 0.05	0.43
SLAMF7	1.00 ± 0.23	0.79 ± 0.07	0.41
<i>Transcriptional regulators / activators</i>			
CAMTA1	n.d.	n.d.	n.a.
TWIST1	n.d.	n.d.	n.a.
ZNF331	1.00 ± 0.19	0.72 ± 0.14	0.07
ZNF532	1.00 ± 0.07	0.89 ± 0.04	0.15
<i>Intracellular transport</i>			
SEC14L1	1.00 ± 0.04	0.94 ± 0.04	0.11
KIF1B	1.00 ± 0.08	0.92 ± 0.08	0.35
<i>Inflammation</i>			
DSE	1.00 ± 0.16	0.88 ± 0.08	0.43
MMP9	1.00 ± 0.07	1.01 ± 0.09	0.91
SPP1	n.d.	n.d.	n.a.
TIMP2	1.00 ± 0.05	1.03 ± 0.07	0.64
TNIP1	1.00 ± 0.10	0.91 ± 0.08	0.26
<i>Mitochondrial Stress / Proteasome</i>			
HPRT	n.d.	n.d.	n.a.

Data are presented as mean ± SEM and expressed relative to baseline. *P<0.05 vs. before cold acclimation. [#] Low expression of gene. n.d., not determined (e.g. due to no expression); n.a., not applicable.

CHAPTER 8

General Discussion

INTRODUCTION

The main function of brown adipose tissue (BAT) is the regulated production of heat (1). Uncoupling protein-1 (UCP1), in abundance present in BAT, bypasses the electron transport chain and as a consequence, instead of ATP, heat is produced (2). Upon cold exposure heat can be produced via shivering (involuntary muscle contractions) and non-shivering thermogenesis (NST). In rodents the main site for NST is BAT. This regulated heat production by BAT can increase energy expenditure (EE). Consequently, this can result in favorable effects on the energy balance. Nowadays it is well known that cold-exposed human adults have functional BAT. It is therefore speculated that BAT might be important for energy metabolism and body weight control in humans too (3). In that perspective in this thesis the effects of acute cold exposure and cold acclimation on BAT activation and NST in human adults were investigated. Additionally, the methodological aspects concerning BAT measurements in human adults were evaluated. In this final chapter the main findings of this thesis will be discussed and interpreted. In addition, the effects of cold exposure on other metabolic processes are described.

METHODOLOGICAL ASPECTS OF ACTIVATING AND MEASURING BAT IN HUMAN ADULTS

Cooling protocols. The strongest and most well known activator of BAT is cold exposure. As described in **Chapter 2** the research groups that study human BAT use different cooling protocols to activate BAT in human adults. The highest probability to detect BAT is found with an individualized cooling protocol (**Chapter 2**). With this protocol subjects are cooled until the onset of shivering and then temperature is raised slightly in order to reach maximal NST. Cooling can be achieved by means of air- (cold room or tent) or water-cooling (water-perfused suit) protocols (**Chapter 2**). When using an individualized cooling protocol the probability to detect BAT is nearly 100 % (94-100 %) in lean young subjects. Orava et al. (4) showed that when using 'fixed' cooling protocol only 31 % of obese subjects had cold-activated BAT depots. Our results show that by means of individualized cooling with water-cooling 83 % of overweight/obese healthy men show active BAT depots (**Chapter 2**). Therefore, we emphasize the use of individualized cooling protocols, especially when subject groups differing in for example body composition or physiological conditions are measured and compared. When overweight/obese subjects are studied, cooling by means of water-cooling is recommended (**Chapter 2**).

Measurement techniques. Currently, the ‘golden standard’ for the measurement of BAT in human adults is deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) positron emission tomography / computed tomography (PET/CT)-imaging. When using this technique subjects are exposed to ionizing radiation from radioactive isotope ^{18}F FDG and the CT-scan. Application of this technique is therefore limited in the number of scans that can be performed in the same subject. In that perspective, there is a need for techniques that investigate BAT without radiation penalty.

INTERMEZZO – BAT IN FEMALES AND MALES

*The study described in **Chapter 5** showed that cold-induced BAT activity levels were not significantly different between females and males. This is in contradiction with retrospective studies that report a higher BAT prevalence in females (5, 6). In those retrospective studies no active cooling protocol was applied, thus the appearance of BAT on those scans indicates that females were experiencing their environment cooler than males while waiting for their diagnostic scans. It is well known that females have a thermoneutral zone at higher ambient temperatures, and thus that physiological responses, i.e. ST and NST, are initiated at higher temperatures (7). Two dedicated (i.e. including a cooling protocol) studies found higher BAT values in females (8, 9), however it should be noted that those studies did not use an individualized cooling protocol. It is likely that male subjects undergoing a fixed temperature cooling protocol were experiencing less cold and thus less BAT activation was needed. In conclusion, by the use of an individualized cooling protocol we were able to show that BAT activity values are not significantly different between females and males.*

Therefore, in **Chapter 3** we investigated the feasibility of magnetic resonance imaging (MRI) to study BAT in human adults. Compared to white adipose tissue (WAT), BAT is highly vascularized and brown adipocytes contain more mitochondria and less fat, which is deposited in multiple intracellular lipid droplets. These differences in characteristics result in different magnetic properties of BAT and WAT. Several MRI-scanning techniques can be used to distinguish BAT from WAT. By means of the Dixon technique, the scanner software constructs images of the water and the fat signal. We showed that a clear distinction between subcutaneous fat (fat fraction: $81.5 \pm 5.4 \%$) and fat in

the supraclavicular depot (fat fraction: 65.2 ± 7.0 %) could be made (**Chapter 3**). These results are in line with finding by Hu et al. (10), who found a lower fat content in BAT compared to WAT in newborn humans. By means of chemical-shift water-fat MRI, a similar MRI method for water-fat separation Hu et al. (10) showed lower fat fractions in BAT than in WAT in a postmortem example and in seven out of eight patients. In addition to the anatomical differences of BAT and WAT studied by MRI, we used dynamic T2*-weighted MRI (fMRI) to study BAT activation during cold exposure. With this technique changes in local blood supply are studied. We found that fluctuations in the dynamic T2*-imaging signal correlated with BAT activation obtained by PET/CT-imaging. Moreover, we found that BAT on/off switching is rapid and occurs at the scale of minutes (**Chapter 3**). The specificity of fMRI to measure BAT activation was low. Therefore the use of MRI needs to be further refined before it can be implemented as measure for BAT activity in human adults.

Recent publications have focused mainly on fat-water differences in order to study human BAT (11, 12). In that respect, the availability of PET/MRI-scanners could be very helpful to gain more insight in human BAT. Such combined scanners enable to visualize metabolic processes in BAT with a high sensitivity (PET) in combination with the visualization of tissue perfusion and intracellular properties like lipid and/or water content or even mitochondrial activity (MRI) (13).

Besides the use of MRI to study human BAT without radiation penalty, supraclavicular skin temperature in relation to BAT activity was investigated. This depot is the largest in human adults and is located relatively close to the skin. In line with findings of Lee et al. (14) we found a smaller decrease in skin temperature at the supraclavicular site compared to decreases in skin temperature at other body sites (**Chapter 4**). Using infrared thermography Symonds et al. (15) even showed an increase in supraclavicular skin temperature upon a cold challenge. Boon et al. (16) also reported a rise in supraclavicular skin temperature, and significant positive correlations between cold-induced supraclavicular skin temperature and BAT volume and BAT activity (SUV max). Our results showed a significant correlation between the change in supraclavicular skin temperature and BAT activity (**Chapter 4**). The discrepancy in results between the different studies could be explained by the different cooling protocols used (we used air-cooling, whereas Boon et al. (16) used water-cooling). The correlation shows that (changes) in supraclavicular skin temperature can

potentially be used as a qualitative measure of BAT activity and BAT thermogenesis.

From **Chapters 3 and 4** that investigated alternative imaging techniques to study BAT in human adults it can be concluded that MRI and supraclavicular skin temperature measurements are promising in order to study BAT in human adults. However, they need further investigation before they can be applied.

THE EFFECTS OF COLD ACCLIMATION

A histological study in the eighties showed that BAT prevalence is higher in outdoor workers compared to office workers (17). These findings implicate that frequent cold exposure is able to recruit BAT, and this might have favorable effects on the energy balance. In that perspective, we studied the effects of frequent intermittent cold exposure on BAT activity values in relation to EE.

From **Chapters 5 and 6** it can be concluded that intermittent (6 hours a day for 10 consecutive days) cold exposure resulted in BAT recruitment in both females and males and in lean and obese subjects. Moreover, we showed that NST increased significantly in all groups. In addition, significant correlations were found between NST and BAT activity values. These results indicate that human BAT contributes to NST and to the increased NST during cold acclimation. During the cold acclimation period room temperature was 15-16 °C for the lean subjects and 13-14 °C for the overweight/obese males. The lower room temperature during cold acclimation was needed for overweight/obese subjects, because their higher fat mass results in higher insulation. To gain more insight in the subjective responses of the subjects during cold acclimation, visual analogue scales were completed. Thermal sensation, thermal comfort and self-reported shivering were studied. On the 10th day of the cold acclimation period the lean subjects reported temperature sensation closer to neutral, lower self-reported shivering and more comfort (**Chapter 5**). These results show that the cold was better tolerated. In contrast, the obese subject did not report significant changes in all subjective responses, which may indicate that despite the physiological adaptations the cold was not better tolerated. However, the number of overweight/obese subjects in this study was low, so a lack of statistical significance may also be caused by a lack of statistical power. Yoneshiro et al. (18) also showed an increased BAT activity after a cold acclimation period of 6 weeks, with a slightly higher environmental temperature (19 °C). Blondin et al. (19) showed a 45% increase in BAT activity after a 4-week cold acclimation period (5 days/week 2 hours exposure to 10 °C in

a water-perfused suit). In contrast to our findings, no change in shivering intensity was found. Using a 4-month crossover design (nightly exposure; 1st month 24 °C, 2nd month 19 °C, 3rd month 24 °C and 4th month 27 °C) Lee et al. (20) showed that human BAT is inducible and suppressible by mild cold and warm exposure. So far, these studies are the first ones investigating recruitment of BAT upon cold acclimation in human adults. All together it can be concluded that, like in rodents, repeated intermittent cold exposure results in BAT recruitment in human adults. The majority of the studies also report increased NST. In the long term this might have favorable effects on body energy metabolism and weight management.

BAT AND HEALTH

As discussed above BAT activation influences energy expenditure and this might have favorable effect on body weight. However, it is well known that cold-exposed rodents increase their food intake, and as a result body weight is not affected in cold-exposed animals compared to warm-housed animals. This is in line with the adipostat model for body weight control, that states that long-term alterations in the total amount of energy reserves will be compensated by alterations in food intake or thermogenesis, so that body weight is balanced (21). If compensation of increased energy expenditure by increased food intake also occurs in cold-exposed human adults remains to be investigated. Using a longer cold acclimation protocol (6 weeks instead of 10 days in our studies) Yoneshiro et al. (18) showed a significantly reduced fat mass after cold acclimation. This can, at least partly, be explained by BAT recruitment, because the changes in body fat mass correlate with the changes in BAT activity. On the other hand, nightly exposure to 19 °C did not change body composition (20). All together, these results indicate that in order to study the effect of cold exposure on body composition studies may have to be longer than 4 weeks. Whether compensation takes place can be further investigated in studies where food intake is strictly monitored. Still, in the cold our body spends more energy to maintain internal body temperatures and this might positively influence other metabolic processes; this is discussed below.

Glucose homeostasis. A study in cold-acclimated rats estimated that 90% of total BAT oxidation is fuelled by fatty acids (22). However active BAT also uses a relative large amount of glucose as substrate for oxidation (Figure 1). It is therefore speculated that active BAT can cause a significant clearance of glucose from the circulation. Rodent studies indicated that cold acclimation recruits BAT

and that this is associated with an increased insulin sensitivity (23, 24). In addition, another study in rodents showed that BAT transplantation improves glucose tolerance, increased insulin sensitivity and a complete reversal of diet-induced insulin resistance (25). To what extent these results are comparable in humans needs further investigation. In humans, a retrospective study in 4842 patients undergoing PET/CT-imaging showed that independent from the association with BMI, a reduced BAT activity was also associated with diabetic status (5). Blondin et al. (19) showed that cold acclimation resulted in significantly lower plasma glucose levels in healthy men. Additionally insulin levels were reduced, but this effect did not reach statistical significance. Recently, Lee et al. (20) confirmed these findings, showing an improved post-prandial whole body insulin sensitivity following cold acclimation. All together, the results in rodents and humans point towards the involvement of BAT in glucose homeostasis (Figure 1). Further research on cold acclimation and the underlying mechanisms in patients with type 2 diabetes is needed.

Fatty acid metabolism. Heat production in activated BAT is mainly fuelled by intracellular triglycerides (1). These internal stores are replenished by free fatty acids from plasma. Mice studies revealed that upon cold exposure, BAT internalizes triglycerides via whole particle uptake (3). In contrast, Khedoe et al. (26) found that BAT takes up triglyceride mostly after lipolysis; uptake of triglycerides does also occur, albeit to a low extent, and this uptake is higher for larger particles and upon cold exposure. Additionally, Boon et al. (27) showed that BAT is an important player in the plasma triglyceride lowering effect of metformin (first-line drug for the treatment of type 2 diabetes) in dyslipidemic mice. Moreover, they showed that a β 3-adrenergic receptor agonist (CL316243) lowered plasma triglyceride levels, attenuated plasma cholesterol levels and the development of atherosclerosis in the same dyslipidemic mice. Whether, targeting BAT could be used as a therapeutic strategy for the treatment of dyslipidemia in humans remains to be investigated. We do know that cold exposure increased plasma free fatty acids in humans (4, 28) (Figure 1). Ouellet et al. (29) indeed showed with the use of fatty acid tracer [^{18}F]THA ([^{18}F]fluorothiaheptadecanoic acid), that fatty acid uptake was enhanced in BAT of human adults. Additionally, the same authors showed that cooling also reduced radio density of BAT, pointing towards a reduced triglyceride content (29). If intracellular triglyceride storage of BAT is replenished by free fatty acids in humans needs further research. New tracer studies that can distinguish fatty acid

and glucose oxidation in BAT and that may provide an estimation of the contribution of BAT oxidation to total energy expenditure are warranted.

Figure 1. Overall effects of BAT activation. Upon activation brown adipocytes take up glucose from plasma. Rodent studies revealed that cold acclimation improves insulin sensitivity. These findings were recently confirmed in humans. All together, the findings point towards the involvement of BAT in glucose homeostasis. Besides that activated BAT also takes up free fatty acids and triglycerides from plasma. Rodent studies revealed that BAT is important in lowering plasma triglycerides after metformin administration. Whether BAT has the same function in humans remains to be investigated. It is furthermore hypothesized that, via cold exposure, BAT might play a role in body weight regulation via increased energy expenditure. Moreover, upon cold exposure TG are cleared and more vasoconstriction takes place, both effects can be beneficial for the cardiovascular system. Upon a 10-day intermittent cold exposure protocol, Th17 and M2 macrophage markers were increased whereas M1 macrophage markers were decreased. The role of BAT in the cardiovascular related responses and immune responses to cold exposure awaits further investigation.

Immune function. Immune cells contain cold sensing transient receptor potential channels (30), indicating that cold exposure might alter immune status. Indeed, acute cold exposure (2h) affected cells of the innate immune system in seven healthy men (31), which might be explained by increased levels of circulating norepinephrine levels. Interestingly, Kox et al. (32) concluded that activation of the sympathetic nervous system by cold, meditation and specialized breathing

techniques attenuated the innate immune response upon LPS (lipopolysaccharide) injection in humans. To verify the effects of chronic cold exposure on immune function, we investigated plasma cytokine levels and expression of immune function markers in skeletal muscle biopsies before and after cold acclimation (**Chapter 7**). Our study showed that cold acclimation (10 days, 6h/day) markedly affects the expression of immune markers in skeletal muscle. Markers involved in Th17 response and M2 macrophage markers were downregulated, while markers for M1 macrophages were upregulated (**Chapter 7**) (Figure 1). Future studies should gain more insight in the precise physiological consequences of these changes.

As described above, it is tempting to speculate that BAT activation via cold exposure has several health promoting effects. Furthermore, it is hypothesized that cold exposure may positively affect the cardiovascular system (vasoconstriction during cold and vasodilation during heat) (33). Besides that, cold-acclimated individuals are less vulnerable to the effects of sudden exposure to more extreme cold (increased blood pressure, lowered core temperature) (33). In that perspective, frequent cold exposures might be an important factor to ‘temperature’ train the body in order to stay healthy.

CONCLUDING REMARKS

The research presented in this thesis shows that frequent cold exposure results in BAT recruitment. The increased BAT activity indicates that existing BAT becomes more active and the increased BAT volume hints towards the recruitment of new brown adipocytes. As a result cold-induced non-shivering thermogenesis also increases. So far, the contribution of BAT on NST is still unresolved. Studies using tracers that are able to study tissue-specific metabolism, or heat production might be helpful. Besides that, frequent cold exposures seem to be beneficial to increase insulin sensitivity, control fatty acid metabolism and to improve immune function. Thus, frequent cold exposures could be an important way to remain healthy.

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Chapter 8

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Summary

SUMMARY

Worldwide the number of people with overweight and obesity has reached alarming numbers. A high body mass index is a risk factor for the development of cardiovascular diseases, diabetes and certain forms of cancer. Overweight and obesity only develop when an individual is in a long-lasting state of a positive energy balance. Research concerning obesity in humans has focused mainly on reducing energy intake and increasing physical activity. However, the majority of these studies have failed to be effective on the long term. Consequently, long-lasting positive health outcomes are rarely seen with this kind of interventions. It is well known that reductions in ambient temperature can increase energy expenditure. Rodent studies showed that the main site for the extra produced heat upon cold exposure without shivering is brown adipose tissue (BAT). This increased energy expenditure is called non-shivering thermogenesis (NST). In 2009, following the advent of deoxy-2- ^{18}F fluoro-D-glucose positron-emission-tomography / computed-tomography (^{18}F FDG-PET/CT) imaging it became clear that human adults possess cold-activated BAT depots. Interestingly, these first studies also showed that subjects with a high body mass index and high body fat percentage have relatively low BAT activity. In this thesis we studied the effects of acute and chronic cold exposure on BAT activity and recruitment in lean and obese subjects. Additionally, we evaluated two alternative techniques for BAT detection, magnetic resonance imaging (MRI) and supraclavicular skin temperature measurements. Finally, we appraised how mild cold exposure can be used as a new approach to increase energy expenditure.

The strongest and best-known activator of human BAT is cold exposure. The research groups that study human BAT use different cooling and analyzing protocols (**Chapter 2**), all affecting BAT activity values to a different extent. The highest probability to detect BAT is found when using individualized cooling protocols (**Chapter 2**). With individualized cooling protocols, subjects are cooled until the onset of shivering; after this temperature is increased slightly so that shivering just disappears. The measurements, including PET/CT-imaging are carried out during this 'maximal' non-shivering condition. We emphasize the use of this individualized cooling protocol, especially when different subject groups are compared.

The 'golden standard' for the measurement of human BAT is ^{18}F FDG-PET/CT-imaging. With this technique subjects are exposed to ionizing radiation from radioactive isotope ^{18}F FDG and the CT-scan. Therefore, this imaging technique is

limited in the number of scans that can be performed in the same subject. In that perspective, there is a need for techniques without radiation penalty. In **Chapter 3** we showed a significant distinction between subcutaneous fat and fat in the supraclavicular depot using the Dixon technique. Moreover, we found that the dynamic T2*-weighted MRI (fMRI) signal correlated with BAT activation obtained by PET/CT-imaging. The specificity of fMRI to measure BAT activation was however low. Therefore the use of MRI needs to be further refined before it can be implemented as a measure for BAT activity in human adults.

Additionally, we investigated supraclavicular skin temperature in relation with BAT activity. We found that supraclavicular skin temperature dropped less compared to skin temperatures at other body sites (**Chapter 4**) upon cold exposure. Our results also showed a significant correlation between the change in supraclavicular skin temperature and BAT activity. Furthermore, a significant correlation was found between the change in supraclavicular skin temperature and NST. The correlation shows that (changes) in supraclavicular skin temperature can potentially be used as a qualitative measure of BAT activity and BAT thermogenesis.

Rodent studies showed that chronic cold exposure resulted in BAT recruitment with a concomitant increase in non-shivering thermogenesis. We investigated the effects of an intermittent, 6 hours per day, 10-day cold exposure protocol in both lean and overweight/obese subjects (**Chapters 5 and 6**). The results showed that, like in rodents, cold acclimation results in BAT recruitment in both females and males and in lean and obese subjects. Both maximal BAT activity and BAT volume increased significantly in the lean subjects upon the cold acclimation period. BAT activity values were not significantly different between males and females, both before and after cold acclimation. Significantly lower BAT activity values were found in obese healthy men, both before and after the cold acclimation period. The cold acclimation resulted in significantly higher BAT activity values in both groups. In parallel, we showed an increased NST after the cold acclimation period in all groups. Interestingly, BAT activity and NST were significantly related both before and after the cold acclimation period in the lean subjects. Combined data from lean and obese men revealed significant correlations between BAT activity and body weight, BMI and fat percentage. The results indicate that human BAT contributes to NST and to the increased NST during cold acclimation. In the long term this may have favorable effects on body energy metabolism and weight management.

Summary

Besides the effects of cold exposure on BAT and energy expenditure, cold exposure can have other metabolic implications. Activated BAT takes up both glucose and fatty acids for oxidation. It is therefore speculated that cold exposure could be beneficial in order to regulate glucose metabolism and improve fatty acid metabolism. Besides that, we showed in **Chapter 7** that cold acclimation significantly affects the expression of immune markers in skeletal muscle. Markers involved in Th17 response and M2 macrophage markers were downregulated, while markers for M1 macrophages were upregulated. Future studies should gain more insight in the precise physiological consequences of these changes.

Thus frequent cold exposures might be an important factor to 'temperature' train the body in order to stay healthy.

Samenvatting

SAMENVATTING

Het aantal mensen met overgewicht en obesitas heeft wereldwijd alarmerende aantallen bereikt. Overgewicht en obesitas, oftewel een hoge body mass index, leiden tot een groter risico op het ontwikkelen van hart en vaatziekten, diabetes en bepaalde vormen van kanker. Overgewicht en obesitas ontstaan wanneer iemand voor een langere tijd een positieve energiebalans heeft. Onderzoek naar obesitas richt zich voornamelijk op het verminderen van de energie-inname en het verhogen van de fysieke activiteit. Echter, dit blijkt volgens een groot aantal studies op de lange termijn niet effectief te zijn en hierdoor worden positieve gezondheidsuitkomsten op de lange termijn zelden behaald.

Het is algemeen bekend dat verlagingen in de omgevingstemperatuur het energiegebruik van het lichaam kunnen verhogen. Studies in knaagdieren hebben laten zien dat bruin weefsel verantwoordelijk is voor deze extra warmteproductie, zonder dat er gerild wordt, ten gevolge van blootstelling aan kou. Dit verhoogde energiegebruik, zonder rillen, wordt niet-rillende warmteproductie genoemd (non-shivering thermogenesis; NST). In 2009, na de komst van deoxy-2- $[^{18}\text{F}]$ fluoro-D-glucose positron-emission-tomography / computed-tomography ($[^{18}\text{F}]$ FDG-PET/CT) beeldvorming werd duidelijk dat volwassen mensen ook bruin vetweefsel bezitten en dat dit bruine vet 'geactiveerd' kan worden door koude blootstelling. Deze eerste studies lieten zien dat mensen met een hoger lichaamsgewicht en vetpercentage een relatief lage bruin vetweefsel activiteit hebben. In dit proefschrift zijn de effecten van acute en langdurige koude blootstelling op bruin vetweefsel activiteit in slanke en obese proefpersonen onderzocht. Daarnaast zijn twee alternatieve technieken onderzocht om bruin vetweefsel te bestuderen; MRI en supraclaviculaire huidtemperaturen. Bovendien is bekeken hoe blootstelling aan milde kou kan worden gebruikt als nieuwe methode om het energiegebruik van de mens te verhogen.

De sterkste en bekendste activator van bruin vetweefsel in de mens is kou. De onderzoeksgroepen die bruin vetweefsel in de mens bestuderen gebruiken verschillende koel- en analyse-protocollen (**Hoofdstuk 2**), beide beïnvloeden bruin vetweefsel activiteit op een andere manier. De grootste kans om actief bruin vetweefsel te detecteren is door middel van een geïndividualiseerd koelprotocol (**Hoofdstuk 2**). Bij een geïndividualiseerd koelprotocol worden de proefpersonen afgekoeld totdat zij gaan rillen, waarna de temperatuur iets wordt verhoogd zodat het rillen verdwijnt. De metingen, waaronder PET/CT-beeldvorming worden verricht onder deze 'maximale' niet-rillende conditie. Aan

de hand van deze studie wordt aangeraden om geïndividualiseerde koelprotocollen te gebruiken, in het bijzonder wanneer verschillende groepen proefpersoon vergeleken worden.

De ‘gouden standaard’ om bruin vetweefsel in de mens te meten is door middel van [^{18}F]FDG-PET/CT-beeldvorming. Bij deze techniek worden de proefpersonen blootgesteld aan straling van de radioactieve isotoop [^{18}F]FDG en straling van de CT-scan. Hierdoor kunnen er met behulp van deze techniek niet veel scans uitgevoerd worden in dezelfde proefpersoon. Het is daarom van belang dat er technieken beschikbaar komen waarmee bruin vetweefsel in de mens bestudeerd kan worden zonder deze extra stralingsblootstelling. In **Hoofdstuk 3** is door gebruik te maken van de Dixon techniek een significant verschil aangetoond tussen het onderhuids vet en het vet in het supraclaviculaire depot, waar zich het voornaamste bruin vet gebied bevindt. Daarnaast lieten de resultaten zien dat dynamische T2*-gewogen MRI-signalen (fMRI) correleerden met de bruin vetweefsel activiteit die gemeten werd met PET/CT-beeldvorming. Echter, de specificiteit van fMRI om de activiteit van bruin vetweefsel te meten was laag. Daarom is het noodzakelijk om de MRI-technieken verder te ontwikkelen voordat deze gebruikt kunnen worden als methode om bruin vetweefsel activiteit in de mens te meten.

Er is ook gekeken naar de supraclaviculaire huidtemperatuur in relatie tot bruin vetweefsel activiteit. Resultaten laten zien dat na koude blootstelling de supraclaviculaire huidtemperatuur minder daalde dan de huidtemperaturen van andere lichaamsdelen (**Hoofdstuk 4**). De resultaten lieten ook een significante relatie zien tussen het verschil in de supraclaviculaire huidtemperatuur en bruin vetweefsel activiteit. Ook werd een significante correlatie gevonden tussen het verschil in supraclaviculaire huidtemperatuur en niet-rillende warmteproductie. De correlaties laten zien dat veranderingen in supraclaviculaire huidtemperatuur mogelijk kunnen worden gebruikt als kwalitatieve maat om bruin vetweefsel activiteit en warmteproductie te meten.

Studies in knaagdieren laten zien dat langdurige blootstelling aan kou zorgt voor extra aanmaak van bruin vetweefsel, met een gelijktijdige stijging in niet-rillende warmteproductie. In dit proefschrift is het effect van een onderbroken koude protocol bestudeerd. Tijdens dit protocol werden de proefpersonen, zowel slanke als proefpersonen met overgewicht/obesitas, 6 uur per dag, gedurende 10 achtereenvolgende dagen, blootgesteld aan milde kou (**Hoofdstuk 5 en 6**). De resultaten laten zien dat, net als in knaagdieren, deze koude acclimatisatie zorgt

Samenvatting

voor extra aanmaak van bruin vetweefsel in zowel vrouwen als mannen en in zowel slanke als obese proefpersonen. Door de koude acclimatisatie nam zowel de maximale bruin vet activiteit als het volume van het bruin vetweefsel in de slanke proefpersonen significant toe. De activiteit van het bruin vetweefsel was niet significant verschillend tussen de mannen en vrouwen, zowel voor als na de koude acclimatisatie periode. De mannen met overgewicht/obesitas hadden een significant lagere bruin vetweefsel activiteit, zowel voor als na de koude acclimatisatie. De koude acclimatisatie verhoogde het bruin vetweefsel activiteit in beide groepen. Parallel hieraan werd een verhoogde niet-rillende warmteproductie na de koude acclimatisatie gevonden in alle groepen. Ook is een significante relatie tussen de maximale bruin vetweefsel activiteit en de niet-rillende warmteproductie gevonden, zowel voor als na de koude acclimatisatie periode in de slanke proefpersonen. Gecombineerde waardes van de slanke en obese mannen lieten significante correlaties zien tussen bruin vetweefsel activiteit en het lichaamsgewicht, BMI en het vet percentage. Deze resultaten geven aan dat bruin vetweefsel activiteit bijdraagt aan niet-rillende warmteproductie in de mens, en aan de verhoogde niet-rillende warmteproductie na koude acclimatisatie. Voor de lange termijn zou dit gunstige effecten kunnen hebben op het energiemetabolisme en gewichtscontrole.

Naast de effecten van koude blootstelling op bruin vetweefsel activiteit en het energiegebruik kan kou ook andere metabole implicaties hebben. Geactiveerd bruin vetweefsel neemt zowel glucose als vetzuren op voor de verbranding. Daarom wordt verondersteld dat koude blootstelling gunstige effecten kan hebben op het glucosemetabolisme en dat het vetmetabolisme kan verbeteren. In **Hoofdstuk 7** is aangetoond dat koude acclimatisatie de expressie van verschillende immuun markers in de spier beïnvloed. Markers betrokken bij de Th17-reactie en de M2-macrofaag reactie gingen omlaag, terwijl markers voor de M1-macrofaag reactie omhoog gingen. Nieuwe studies moeten meer inzicht geven in de fysiologische consequenties van deze veranderingen.

Concluderend kan gesteld worden dat frequente blootstelling aan kou een belangrijke rol kan spelen bij het ‘temperatuur’ trainen van het lichaam, en zo kan bijdragen aan een goede gezondheid.

Valorization

SOCIAL RELEVANCE

Nowadays, the worldwide prevalence of people with obesity has reached alarming numbers, both in developed and developing countries. The World Health Organization estimated in 2008 that more than 1.4 billion adults were overweight, of which more than 200 million men and nearly 300 million women were obese. Most strikingly worldwide 42 million children under the age of 5 were overweight or obese in 2013. Overall, more than 10% of the world's population was obese. In the Netherlands in 2012 48.3% of the Dutch adult population was overweight, of which 12.7% were obese. Following the worldwide trend, of the Dutch youth aged 2 to 21 years 14% were overweight, of which 2% obese in 2009. Overweight and obesity are leading risks for global deaths. Around 3.4 million adults die each year as a result of being overweight or obese. Being overweight or obese increases the risk for the development of cardiovascular diseases, diabetes and certain forms cancer. It is therefore important to implement strategies that can counteract the current obesity pandemic. One strategy is the increase in energy expenditure. The research described in this thesis investigated cold-induced increases in energy expenditure and the activation of brown adipose tissue (BAT) in human adults.

SCIENTIFIC BACKGROUND

Body weight is a result of energy intake and energy expenditure. When intake equals expenditure, body weight remains stable. But when energy intake exceeds energy expenditure, body weight increases. Thus, obesity develops as a consequence of a long-lasting positive energy balance. To maintain a healthy energy balance and body weight, one can diminish energy intake or increase energy expenditure. Research focusing on human obesity mainly focused on food restriction via dieting. However the majority of these strategies have failed to be effective on the long term. As a consequence people cannot maintain their initial weight loss. On the other hand, increasing energy expenditure via stimulating physical activity could also be beneficial for a healthy energy balance. Increased exercise does promote health, but people often are struggling to incorporate regular exercise in their daily-life activities. Besides that, it is evident that some people are not able to perform physical exercise. It is therefore important to find alternative strategies that increase energy expenditure. It is well known that reductions in ambient temperature can increase energy expenditure. In human adults mild cold exposure can increase energy expenditure in a range of 5-30% in the absence of shivering. It is important to note that even slight increases in

energy expenditure can have effects on energy balance in the long run.

From rodent studies it became clear that BAT is the main site for the extra heat production upon cold exposure. BAT is important to maintain core body temperature and it is suggested that BAT is an important player in the energy balance regulation. This is illustrated by the fact that mice without BAT become obese in a thermoneutral environment when they can eat *ad libitum*. Approximately seven years ago it became clear that human adults also possess functional BAT depots, and that there is a negative relationship between BAT activity and adiposity. This shows that overweight and obese people have less active BAT. Consequently, it is suggested that BAT might play a role in body weight regulation in humans as well. The mission of the work described in this thesis was to gain more insight in the effects of acute (3h) and longer-term (10-day intermittent) cold exposure on BAT activity and energy expenditure. Work in this thesis confirmed that, like in rodents, BAT is a significant player in cold-induced energy expenditure. Moreover, research in this thesis showed that longer-term intermittent cold exposure recruits BAT, in parallel with increased cold-induced energy expenditure. These findings indicate that stimulating BAT could be a potential target in the current obesity problem.

Another goal of the project was to find alternative technique for measuring BAT in human adults. The methods described in this thesis, magnetic resonance imaging (MRI) and supraclavicular skin temperature measurements were promising, but more research is necessary. This means that the imaging technique MRI potentially can be developed as a non-invasive pre-clinical tool.

TARGET GROUPS

Being overweight or obese can lead to the above-mentioned comorbidities, treating people with such diseases has lead to extremely high healthcare costs around the world. In that perspective, finding strategies that can counteract obesity is important for the general public, in specific for those people that have an increased risk to develop overweight and obesity. Increasing energy expenditure via stimulation of BAT through cold exposure could be such a strategy. Not only people at risk need to be informed about strategies that are able to restore the energy balance, but also health institutions and health care professionals need to be informed. Translation of scientific knowledge to society is therefore essential. In this thesis we clearly demonstrate that cold exposure increases energy expenditure via BAT activation, and that longer-term

intermittent cold exposure results in BAT recruitment, in parallel with increased cold-induced energy expenditure. This occurs already at temperatures that are defined as mild cold, thus in the absence of shivering. Therefore, lowering temperatures in places people spend most of their time, houses and offices, might be a way to maintain energy balance. Clearly, more research is needed to identify if this intervention is successful in stimulating weight loss.

Besides this behavioral intervention to reduce overweight and obesity in the society, pharmaceutical interventions could be important as well. In order to activate BAT via pharmaceutical agents a precise understanding of BAT physiology and the molecular pathways that lead to BAT stimulation is required. A good collaboration between researchers that study human BAT and the pharmaceutical industry is therefore inevitable.

ACTIVITIES AND PRODUCTS

All research described in this thesis was conducted at the department of Human Biology in close collaboration with the department of Nuclear Medicine of Maastricht University Medical Center+. This collaboration between the hospital and the University is a good example of how basic science can be performed in a hospital setting. In this case, knowledge of scientists and medical specialists was combined at the site of the experiments. The 'gold standard' for measurement of BAT in human adults is 2-deoxy-2- ^{18}F fluoro-D-positron-emission-tomography and computed tomography (^{18}F FDG-PET/CT) imaging. In order to use this technique for BAT visualization and quantification knowledge of nuclear medical specialists is essential. This underscores the importance of a good collaboration between basic physiologists and medical specialists.

In order to increase energy expenditure via BAT activation and to use this as a possible treatment for overweight and obesity, further research is necessary. The strongest activator of BAT in humans is cold exposure. Recent studies have shown that adrenergic stimulation might be effective in human adults, but long-term use of adrenergic stimulation could have negative side effects. Further research in human adults that provide more information on other stimulation agencies and techniques are warranted.

The results described in this thesis are written in original research articles that have been published or submitted to scientific journals in the field of obesity or physiology. Moreover, these articles can be found online and are accessible to scientists who are interested in this topic. In addition to this, results have been

presented on several (inter) national conferences, and have raised public awareness through via public media, (journals and broadcast). Communicating the scientific observations to lay people and target groups organizations is important to stimulate translation of science to daily-life situations; this in order to obtain awareness and support from the society.

INNOVATION

The work described in this thesis is mostly new in the field of BAT research. Prof. dr. W.D. van Marken Lichtenbelt was among the first researchers to discover functional BAT in human adults in 2009. As a result, new researchers joined his research group from that moment. This thesis confirms the experiments of 2009 showing that acute cold exposure is an effective way to activate human BAT. The innovative part of this thesis was the exploration if longer-term intermittent cold exposures resulted in BAT recruitment in human adults. The experiment on the effects of cold acclimation in lean adults was the first to demonstrate that BAT can be recruited in lean healthy adults. Moreover, we showed increased cold-induced energy expenditure upon cold acclimation. Comparable results were found in the study that investigated the effects of cold acclimation in overweight/obese healthy men.

In this thesis we explored the feasibility of non-invasive measurement techniques to study human BAT. By using MRI, we confirmed earlier findings that BAT contains less fat than white adipose tissue. Additionally, we showed that fMRI signal was related to BAT activity values. Finally, we showed that supraclavicular skin temperature measurements could potentially be used as a qualitative measure of BAT activity and BAT thermogenesis. The findings of both studies were promising, but need further investigation before they can be applied.

From listing all studies that investigated cold-induced BAT in human adults with [^{18}F]FDG-PET/CT-imaging that were published since 2009, it became clear that the research groups that study human BAT use different cooling and analyzing protocols, both affecting BAT activity values. We contribute to developing standardized cooling techniques and PET/CT-analyzing techniques and communicate these to the scientific community in order to make the results from different research groups comparable.

PLANNING AND REALIZATION

In this thesis we have clearly demonstrated that BAT can be recruited upon cold acclimation in human adults, with a concomitant increase in cold-induced thermogenesis. The research team currently explores whether this approach has beneficial effects in type 2 diabetic patients. Finding alternative ways to stimulate and activate BAT are important. In that perspective, the effects of bile acids on human BAT are investigated at the department of Human Biology and Nuclear Medicine.

As described above, it is important to find alternative measurement techniques to investigate BAT in human adults. First, because [^{18}F]FDG-PET/CT-scanning is a very expensive technique, and second, because with this technique radioactive dose for the subjects is increased. This limits the amount of scans that can be done per subject. Furthermore, [^{18}F]FDG-imaging provides information on glucose uptake solely, no information is obtained on fatty acid oxidation or total BAT energy expenditure. In that perspective the alternative imaging techniques MRI and supraclavicular skin temperature measurements were explored, as described in this thesis. The results were promising, but more research is needed. Besides that, the PET/MRI-scanner could be a potential alternative. With this technique no additional radiation is required in order to study the anatomy (imaging with CT gives additional radiation). Moreover, alternative tracers can provide more information on fatty acid oxidation in BAT or total BAT oxidation.

An important final step is to gain more insight into the actual contribution of BAT thermogenesis on whole body thermogenesis. Therefore measuring BAT energy expenditure is important. This measurement provides more information on the contribution of BAT on total daily energy expenditure and to what extent BAT has an effect on body weight. This knowledge is crucial for the development of BAT-based health intervention strategies.

Dankwoord

DANKWOORD

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List of publications

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van der Lans AAJJ, Boon MR, Haks MC, Schaart G, Ottenhof TH, Quinten E, van Marken Lichtenbelt WD. *Cold acclimation affects immune composition in skeletal muscle of healthy lean subjects*. Physiol Rep. 2015 3(4), e12394.

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SUBMITTED PAPERS

van der Lans AAJJ, Vosselman MJ, Hanssen MJW, Brans B, van Marken Lichtenbelt WD. *Supraclavicular skin temperature and BAT activity in lean healthy adults*. Submitted.

Hanssen MJW, Hoeks J, Brans B, **van der Lans AAJJ**, Schaart G, van den Driessche JJ, Jörgensen JA, Boekschoten MV, Hesselink MK, Havekes B, Kersten S, Mottaghy FM, van Marken Lichtenbelt WD, Schrauwen P. *Cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus*. Submitted.

Vosselman MJ, Hoeks J, Brans B, Pallubinsky H, Nascimento EBM, **van der Lans AAJJ**, Broeders EPM, Mottaghy FM, Schrauwen P, van Marken Lichtenbelt WD. *Reduced brown adipose tissue activity in endurance trained compared to lean sedentary men*. Submitted.

Chen Y, Buyel JJ, Jansen F, Hanssen MJW, Siegel F, Pan R, Schmitz T, Wenzel D, Rieck S, **van der Lans AAJJ**, Fleischmann BK, Schlein C, Fröhlich, Nickenig G, Heeren J, van Marken Lichtenbelt WD & Pfeifer A. *Brown adipocyte-derived exosomal miRNAs promote vascular integrity and represent a serum biomarker for human brown fat activity*. Submitted.

Curriculum Vitae

Anouk van der Lans was born on the 8th of January 1985 in Leiden, the Netherlands. She completed secondary school at Rijnlands Lyceum in Sassenheim in 2004 and she started her study Health Sciences in the same year. After she received her Bachelor's degree in 2007, she continued with the Master Physical Activity and Health, focusing on European Metabolism and Nutrition and she obtained her Master's degree in October 2008. After this, she moved to Tanzania in East-Africa to perform voluntary work at Hedaru Secondary School for a period of 14 months. In May 2010 she started working as a PhD-student under the supervision of prof. W. van Marken Lichtenbelt and prof. P. Schrauwen at the Department of Human Biology at Maastricht University. During her PhD trajectory she studied the effects of cold acclimation on brown adipose tissue activity in lean and obese humans. Additionally, she investigated different techniques to measure human brown adipose tissue activity, as demonstrated in the present thesis and the numerous publications in scientific journals.