Human brown adipose tissue

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Metabolic effects and clinical implications

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Chapter 1
General introduction

General introduction

'Neither fat nor flesh'. That is how brown adipose tissue (BAT) was first described by Konrad Gessner in the 16th century (1). Brown adipose tissue resembles skeletal muscle tissue, due to the high perfusion and the large number of mitochondria in the cells, which give it its brownish colour. This resemblance with skeletal muscle cells is explained by the fact that both cell types are from a common progenitor cell type (2, 3). Brown adipocytes also resemble normal white adipocytes as both store lipids inside the cell. BAT is of vital importance in the mammalian pedigree, as it protects against cold by producing heat (4). To allow the production of heat, mitochondria in brown adipose tissue have a special characteristic, allowing a process called 'mitochondrial uncoupling'.

Mitochondria are cellular organelles equipped to produce energy in the form of ATP. To this end, mitochondria use the breakdown products from glucose and lipids, such as pyruvate and fatty acids, to transport protons across the mitochondrial inner membrane, leading to a proton gradient (4). The difference in proton levels provides potential energy. This energy can be used to form adenosine triphosphate (ATP), the main energy carrier for the body's metabolic processes, from ADP. In this way, the combustion of glucose and lipid is coupled to ATP production. Mitochondria in brown adipose tissue are equipped with a so-called uncoupling protein 1 (UCP1) that allows production of heat. Upon activation by cold exposure, UCP1 short-circuits the respiratory chain and lowers the proton gradient, thereby uncoupling substrate oxidation from ATP production, and dissipating the potential energy as heat (Figure 1) (4-6).

Cold is the most effective way to activate brown adipose tissue. Cold exposure stimulates thermoreceptors in the skin, which in turn activate neurons in the ventromedial nucleus of the hypothalamus, leading to the local release of norepinephrine. The norepinephrine leads to vasoconstriction in the extremities, which prevents heat loss. Brown adipose tissue is also strongly innervated by the sympathic nervous system, and the released norepinephrine binds the β3 adrenergic receptor on the brown adipocyte thereby activating an intracellular signalling cascade that, among others, activates UCP1 (7, 8). As a result, extra heat is produced as described above. This is referred to as non-shivering thermogenesis, in contrast to shivering thermogenesis, which refers to heat production through the contraction of skeletal muscles during shivering.

In early studies, depots of brown adipose tissue have been described in adult human cadavers. Anatomical studies in the 1970s showed that in adult humans, the main location of brown adipose tissue is in the neck, behind the clavicles, next to the vertebral column and next to the kidneys (Figure 2) (9-12). In small mammals these depots were mainly located around and between the scapulae, near the base of the hind legs and surrounding the visceral organs (13).

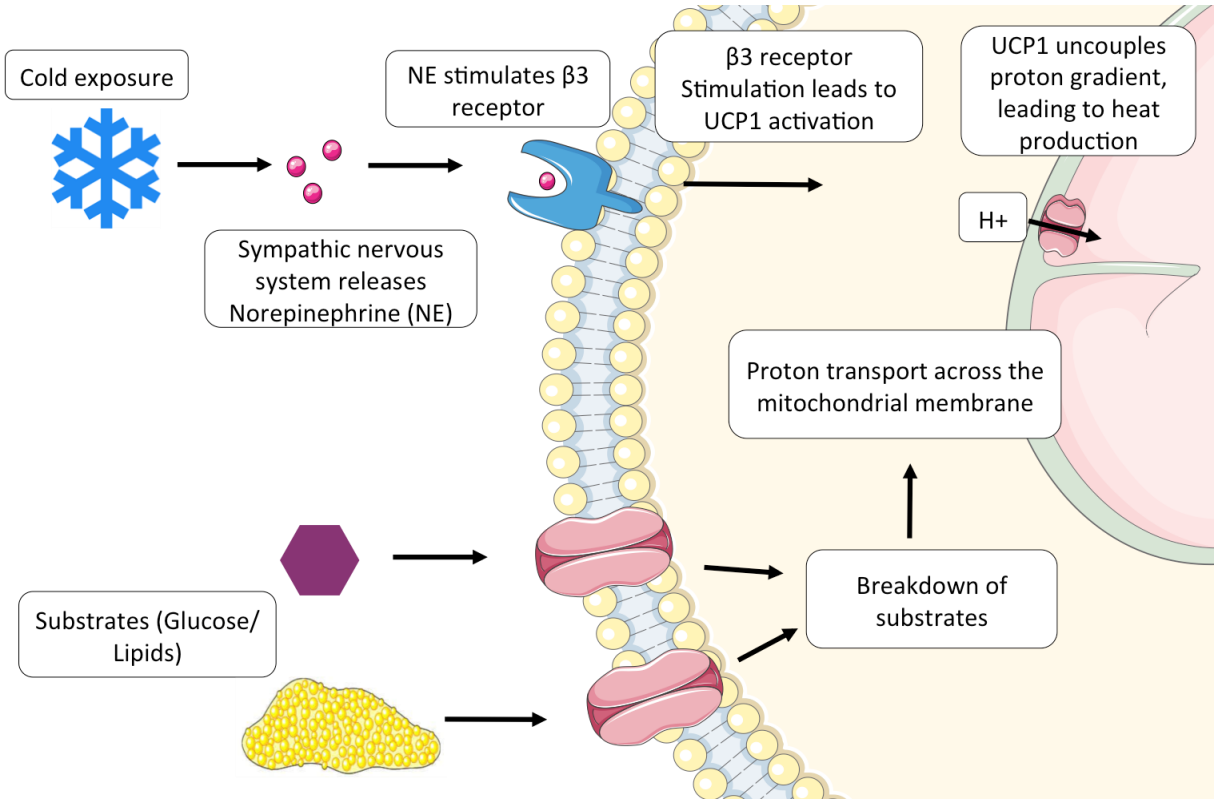

Figure 1. Activation of brown adipocytes by cold exposure, with the use of substrates, leads to heat production.

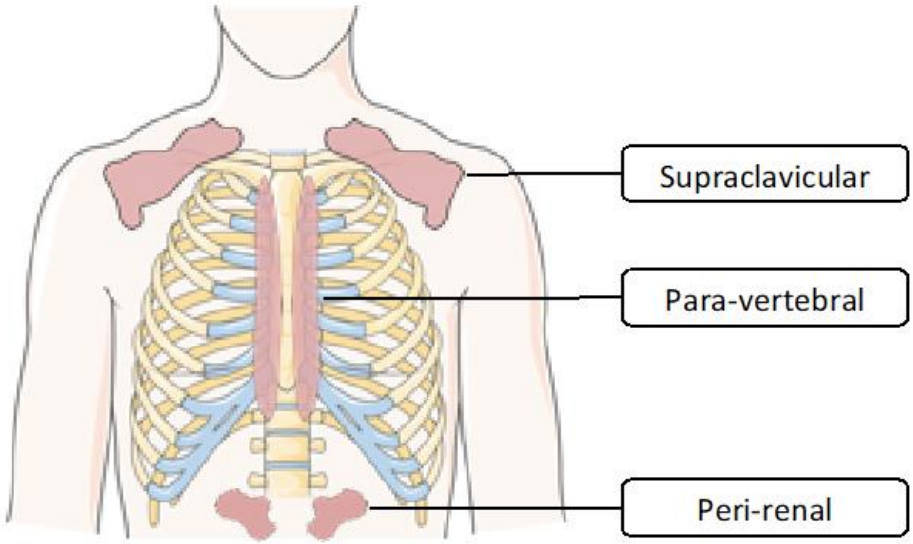

Figure 2. Distribution of brown adipose tissue in humans.

It had been shown that brown adipose tissue was able to induce mitochondrial uncoupling, but the mechanisms was only discovered in 1968 when UCP1 was found in these tissues (10, 11, 14).

In the early 2000s, techniques from nuclear medicine, involving radioactive tracers such as fluordeoxy-glucose (FDG) combined with PET/CT imaging, were used by researchers to study the metabolic activity of tissues in the body. Using PET/CT, it was observed that in humans, under certain conditions, symmetric glucose uptake in the neck region could be observed. However, it took until 2009 before three research groups independently described the presence of brown adipose tissue in human adults and showed that the activity was enhanced by cold exposing the human volunteers (15-17).

In homoeothermic small animals, such as rodents but also in babies, brown adipose tissue plays an important role in maintaining a stable body core temperature without shivering. Also in hibernators, brown adipose tissue can generate extra heat for raising the body temperature (4, 18). As humans do not hibernate and have, because of the large body size a relatively small body surface to volume ratio, there is less need for active brown adipose tissue. It is therefore not unexpected that brown adipose tissue is found in smaller quantities compared to rodents and other small mammals. Nevertheless, the discovery of functional BAT in adults sparked new research into the role of brown adipose tissue in human energy and substrate metabolism.

Energy balance and brown adipose tissue

When body weight is stable, on the long term energy expenditure is balanced by energy intake (19). However, our modern society is generally characterised by a relatively high dietary intake, while energy expenditure is not increased to match the increased intake; in fact energy expenditure due to physical activity is low and has been decreasing at the population levels. This combination will lead to a gain in body weight and increased fat mass (20, 21). Lifestyle programs mainly focus on dieting (decrease EI) and increased physical activity (increase EE), but long-term adherence to these interventions are disappointing. Cold exposure and brown adipose tissue activation could, in addition to other lifestyle factors, contribute to counteract obesity by increasing energy expenditure.

Obesity can affect our metabolic health. After a meal, the body will produce insulin to store nutrients, such as fats and sugars. The excess energy from dietary intake is stored in several tissues, depending on the dietary component. Lipids are mainly stored in adipose tissue in the form of triglycerides, while glucose is mainly stored in liver and skeletal muscle in the form of glycogen. In obesity the amount of adipose tissue is increased, with spill over of triglycerides

in to the rest of the body. These excess lipids can be stored in metabolically active tissues, such as liver and muscle and accumulation of fat in these tissues leads to a disturbed response to insulin (22). This means that insulin released from the pancreas does lead to a diminished uptake of glucose from the meal, which is referred to as insulin resistance. Clinically this results in an increased plasma glucose level and insulin resistance, which are the hallmarks of type 2 diabetes mellitus (22). Type 2 diabetes leads to the development of symptoms such as polyuria and polydipsia, as well as the development of long-term complications such as nerve and retinal damage.

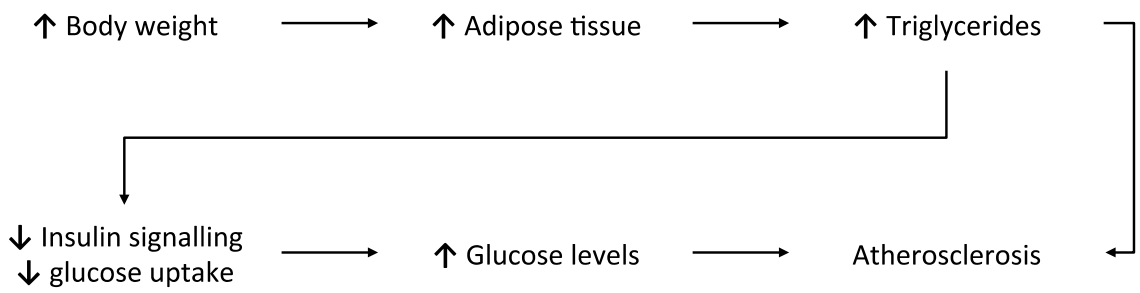

Figure 3. The process from increased body weight to atherosclerosis.

In addition to these glycemia-induced damages, the excess lipids and glucose contribute to the development of inflammation in wall of blood vessels, leading to the development of atherosclerosis as shown in Figure 3. This process affects both small and larger blood vessels and can lead to complications such as myocardial infarctions and renal failure (23).

Brown adipose tissue and substrate metabolism

The activation of brown adipose tissue is accompanied by an increased uptake of substrates such as glucose and fatty acids that fuel the mitochondria. In mice, it is has been shown that brown adipose tissue takes up huge amounts of substrates during cold exposure, effectively clearing these substrates from the bloodstream (24). Notably, even the development of atherosclerosis was reduced in mice after repeated cold exposure (25). It is therefore interesting to investigate whether cold exposure could be used in obese humans to reduce levels of glucose and lipids during acute cold exposure. To this end, in chapter 5 we investigated the changes in postprandial lipid levels during acute non-shivering cold exposure.

Additionally, it has been shown that repeated cold exposure (e.g. for several days up to several weeks) can lead to adaptations in the human body; this is referred to as cold acclimation. Especially interesting is the fact that the cold-induced non-shivering

thermogenesis and brown adipose tissue volume and activity are all increased after 10 days of cold acclimation (26, 27). Besides, cold acclimation has been shown to lead to reduced glucose levels and increased oxidative capacity in young healthy volunteers (28). In addition, Hanssen et al. showed a significant improvement in insulin sensitivity after 10 days of cold acclimation in patients with type 2 diabetes mellitus (29). Interestingly, the improvement in insulin sensitivity was not accompanied by an increased brown adipose tissue activity, but with increased translocation of glucose transporters, such as GLUT4, and increased glucose uptake in skeletal muscle. This would indicate that skeletal muscle could play an important role in adult humans during non-shivering cold acclimation. We further examined the role of skeletal muscle tissue in non-shivering cold acclimation in chapter 4.

While our focus is on the contribution of brown adipose tissue to the effect of non-shivering cold exposure and acclimation, research also indicates a role for skeletal muscles. This especially during shivering, however a overlap between BAT and skeletal muscles is suggested in more severe cold acclimation (30). Therefore a potential link may exist between these two tissues during cold exposure. Therefore we aimed at elucidating the role of both tissues in cold exposure. The information from this thesis could be helpful in improving human energy and substrate metabolism, which can be used to combat diabetes mellitus type 2 and atherosclerosis. We therefore studied the role of both brown adipose tissue and skeletal muscle during acute (non-)shivering cold exposure, as described in chapter 5, and after non-shivering cold acclimation as described in chapter 4.

Thesis outline

The aim of this thesis was to evaluate the role of brown adipose tissue activation in human glucose- and lipid metabolism. The focus is on insulin sensitivity, postprandial lipid metabolism and brown adipose tissue activity. In order to achieve this goal, several intervention studies were performed.

A review on the role of brown adipose tissue in human energy metabolism is described in Chapter 2. As described in Chapter 3, brown adipose tissue activity was investigated in a placebo-controlled intervention study with nicotinamide riboside. This is a vitamin B3 precursor aimed at improving mitochondrial function and brown adipose tissue activation. In this study, the effects of a 40-day supplementation of nicotinamide riboside on brown adipose tissue activity and volume were studied in a population of elderly volunteers with obesity. Additionally, these effects were also studied in, *in vitro* brown adipocytes, and mice. In Chapter 4 we investigated the effects of cold acclimation in a population of elderly patients diagnosed with obesity and type 2 diabetes mellitus. The main focus was on cold exposure without shivering, as to solely evaluate the role of brown adipose tissue activity and non-shivering thermogenesis. The main outcomes were insulin sensitivity, postprandial lipid levels

and arterial stiffness. The study described in Chapter 5 focuses on the effects of acute cold exposure on post-prandial lipid metabolism in young healthy adult humans. The main outcomes in this study were the postprandial levels of circulating substrates and the correlation with skeletal muscle oxidative capacity and brown adipose tissue activity. As described in Chapter 6, the potential link between brown adipose tissue and Madelung's disease is investigated. In this study, a single patient diagnosed with Madelung's disease was investigated. The main outcome was cold-induced brown adipose tissue activity and histological material taken from adipose tissue depots in this patient. In the final chapter, Chapter 7, the main outcomes of the major studies are discussed in a broader perspective.

References

1. Gessner K. Conradi Gesneri medici Tiguine Historiae Animalium Lin I de Quadripedibus uiuparis. Zurich, Switzerland: Froschaver; 1551.
2. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature. 2008;454(7207):961-7.
3. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. Nat Med. 2015;21(4):389-94.
4. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
5. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. FASEB J. 2009;23(9):3113-20.
6. Betz MJ, Enerback S. Human Brown Adipose Tissue: What We Have Learned So Far. Diabetes. 2015;64(7):2352-60.
7. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. Proc Natl Acad Sci U S A. 2012;109(25):10001-5.
8. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. Cell Metab. 2015;21(1):33-8.
9. Astrup A. Thermogenesis in human brown adipose tissue and skeletal muscle induced by sympathomimetic stimulation. Acta Endocrinol Suppl (Copenh). 1986;278:1-32.
10. Heaton JM. The distribution of brown adipose tissue in the human. J Anat. 1972;112(Pt 1):35-9.
11. Huttunen P, Hirvonen J, Kinnula V. The occurrence of brown adipose tissue in outdoor workers. Eur J Appl Physiol Occup Physiol. 1981;46(4):339-45.
12. Sacks H, Symonds ME. Anatomical locations of human brown adipose tissue: functional relevance and implications in obesity and type 2 diabetes. Diabetes. 2013;62(6):1783-90.
13. Frontini A, Cinti S. Distribution and development of brown adipocytes in the murine and human adipose organ. Cell Metab. 2010;11(4):253-6.
14. Lean ME, James WP, Jennings G, Trayhurn P. Brown adipose tissue uncoupling protein content in human infants, children and adults. Clin Sci (Lond). 1986;71(3):291-7.
15. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.
16. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.

17. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
18. Himms-Hagen J. Nonshivering thermogenesis. Brain Res Bull. 1984;12(2):151-60.
19. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R285-96.
20. Yoneshiro T, Aita S, Matsushita M, Kameya T, Nakada K, Kawai Y, et al. Brown adipose tissue, whole-body energy expenditure, and thermogenesis in healthy adult men. Obesity (Silver Spring). 2011;19(1):13-6.
21. Organization WH. Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016. Geneva; 2018.
22. Guilherme A, Henriques F, Bedard AH, Czech MP. Molecular pathways linking adipose innervation to insulin action in obesity and diabetes mellitus. Nat Rev Endocrinol. 2019;15(4):207-25.
23. Nordestgaard BG, Varbo A. Triglycerides and cardiovascular disease. Lancet. 2014;384(9943):626-35.
24. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
25. Berbee JF, Boon MR, Khedoe PP, Bartelt A, Schlein C, Worthmann A, et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. Nature communications. 2015;6:6356.
26. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
27. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest. 2013;123(8):3404-8.
28. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. J Clin Endocrinol Metab. 2014;99(3):E438-46.
29. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
30. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. J Physiol. 2017;595(6):2099-113.

Chapter 2
Human brown adipose tissue: Underestimated target in metabolic disease?

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Abstract

Active brown adipose tissue (BAT) has, since it rediscovery in adult humans in 2009, received much attention for its ability to increase energy expenditure when activated. By means of mitochondrial uncoupling activity BAT's main function is to produce heat instead of storing energy such as in white adipose tissue [1]. Therefore, BAT is considered a new potential target to treat obesity and the metabolic syndrome. However, the contribution of this thermogenic tissue is still a matter of debate among researchers.

The aim of this review is to give an overview of the differences between classical brown adipocytes and inducible beige adipocytes in humans, and the potential activators of BAT in humans. Furthermore newly described genetic markers for identification of these two types of brown adipocytes are examined. Finally, the potential of the current measurement techniques, and the contribution of BAT activity to whole body energy expenditure are discussed.

Highlights

1. Human BAT can be divided in two types: classical and recruitable, although functional distinction is not evident
2. Recent identified cell markers for thermogenic adipocytes are presented that potentially can be used to distinguish different BAT types
3. The potential of different measurement techniques for the determination of BAT activity are discussed
4. The contribution of BAT activity to human energy metabolism is potentially underestimated

Introduction

In modern society, energy intake often exceeds energy expenditure. This in turn leads to obesity, in which excess energy is stored in white adipose tissue [1]. Under a microscope, WAT can be recognized by the presence of a single large lipid droplet and few mitochondria. In contrast brown adipose tissue (BAT) is characterized by large amounts of mitochondria and a relatively small capacity of fat storage, and most importantly is responsible for non-shivering thermogenesis [2]. At least in animals BAT plays a dominant role in maintaining body temperature in the cold. The first description of BAT was by Konrad Gessner in 1551, who described it as 'as nor flesh nor fat'. In 1985, Himms-Hagen described BAT as a tissue containing multilocular adipocytes with multiple lipid-containing droplets and many mitochondria [3]. The presence of uncoupling protein 1 (UCP1 aka thermogenin) in the mitochondria of BAT enables heat production. UCP1 uncouples the proton gradient of the electron transport chain that would normally be used to generate ATP, thus resulting in heat production [2].

In newborns of precocial species such as deer and lamb BAT is predominantly found in the perirenal fat deposit, while in rodents, and human newborns, it is mainly found in the interscapular area, as well as in the cervical, para-aortal and subcostal area [3]. In early studies by Huttunen et al in 1981 and by Heaton in 1972 brown adipocytes were seen in multiple adipose tissue depots in human subjects, in several age categories [4, 5]. Nevertheless, the assumption was that in humans BAT dissipates with age and interest in BAT was diminished. However in 2007, Nedergaard et al. reviewed nuclear imaging studies in humans that showed that the uptake of $^{18}F^{-}$FDG (glucose) in the neck and shoulder region, was by brown adipose tissue [6]. Then in 2009, three independent research groups identified functional cold activated BAT in adult humans [7-9] igniting the discussion of its importance in human physiology and metabolism. Since then studies on BAT increased dramatically, although the amount of well-controlled intervention studies in humans is relatively scarce compared to the number of animal studies, cell studies and retrospective studies. The importance and contribution of human BAT in whole body metabolism is still not understood despite this strong revival of studies on BAT [10, 11]. In this review we will first discuss different types of thermogenic and UCP1-positive adipocytes. Next, we will discuss different ways to activate and measure BAT activity. Finally, we will examine the potential role of human BAT activity in relation to whole body metabolism and energy expenditure to prevent and/or treat obesity and the metabolic syndrome.

Defining the brown adipocyte

Brown adipocytes vs. beige/brite adipocytes

In order to understand the role of BAT at the whole body level, we first need to examine what defines brown adipocytes. The presence and activation of uncoupling protein 1 (UCP1) in BAT is crucial for its function to produce heat. UCP1 is located in the inner membrane of mitochondria where it influences the proton gradient in oxidative phosphorylation. Normally oxidative phosphorylation generates ATP via the proton gradient that is present between the mitochondrial matrix and the mitochondrial intermembrane space. However, when activated, UCP1 uncouples the proton gradient from ATP-synthase thereby generating heat instead of ATP. Cold is the most effective way to activate BAT. Cold exposure stimulates thermoreceptors in the skin, which in turn activate neurons in the ventromedial nucleus of the hypothalamus, leading to the release of norepinephrine [2, 12]. The importance of the sympathetic nervous system is shown in studies with pharmacological blockade of adrenergic receptors, or lesions in the ventromedial nucleus in animals [13, 14]. In one such study, injections with glutamate into the ventromedial nucleus in mice induced a dose-dependent stimulation of interscapular BAT [13], while another study showed that β-blockade via propranolol results in reduced BAT activity [14].

Norepinephrine binds the β3 adrenergic receptor on the brown adipocyte thereby activating the intracellular signalling cascade. Via second messenger cyclic AMP, activated protein kinase A (PKA) binds cAMP responsive binding element (CREB) stimulating gene transcription of UCP1. Secondly, PKA can directly stimulate lipolysis by activating hormone sensitive lipase (HSL) leading to the release of fatty acids from the intracellular lipid droplet. These intracellular released fatty acids also stimulate UCP1 [12]. Besides the essential sympathetic activity, other factors influence BAT activity as well. CREB stimulates transcription of iodothyronine deiodinase 2 (DIO2), which stimulates intracellular conversion of inactive thyroid hormone T4 to active thyroid hormone T3 [12]. Optimal BAT thermogenesis depends on intracellular levels of T3 [15, 16], which explains the association between hypothyroidism and lower body temperature and the feeling of cold [17].

UCP1 is a requisite for uncoupling in adipocytes, however UCP1 expression is not always similar in thermogenic adipocytes found in different adipose tissue depots. In rodents, the presence and activity of BAT is a well-known and studied fact [18]. In these animals, the interscapular depot is most recognized for the presence of brown adipocytes [3]. These brown adipocytes are usually referred to as constitutive or classical brown adipocytes (cBAT). These cBAT cells are UCP1 positive, even without cold stimulation. Furthermore, classical brown adipocytes are myogenic factor 5 (myf5) positive indicating a similar lineage as skeletal muscle [19]. Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC1a) is known as an important regulator of mitochondrial biogenesis and plays a crucial role in adipogenesis

of thermogenic adipocytes. Next to that PGC1a is able to activate the UCP1 gene in BAT [20].

PRDM16 is a key element in differentiation of brown adipocytes in mice and humans. Indeed, knock down of PRDM16 in precursor cells for brown adipocytes, redirected differentiation in the direction of muscle cells [21]. In addition, in PRDM16 knockout mice, the BAT depots showed clear signs of whitening [22]. Upon cold exposure, the white adipose tissue depots showed no sign of compensatory browning. The adipose cells in knockout mice contained very few mitochondria, with less mitochondrial organisation then in wild type mice [22]. During cold exposure the knockout mice had a larger drop in core temperature then wild type mice, and total body energy consumption upon NE exposure was clearly depressed in the knockout mice. All in all, these results show the importance of PRDM16 in the regulation of BAT in mice [22].

Cold exposure in rodents can transform the adipocytes in WAT. The discovery of recruitable adipocytes was already reported in 1984 in rodent WAT following cold [23]. These changes include, among others, increased mitochondrial biosynthesis and expression of UCP1, enabling a shift towards a brown adipocyte phenotype [24]. These recruitable adipocytes appeared to be from a myf5 negative lineage and are referred to as beige or brite (=brown in white) adipocytes [21, 24]. Histology clearly shows presence of these recruitable adipocytes in WAT following prolonged cold exposure [25]. Though originally from different lineages, classical brown adipocytes and recruitable adipocytes have similar cellular structure. Both possess paucilocular fat droplets and multiple mitochondria [25, 26]. Both also show strong activation upon cold exposure [25] as both types use UCP1 to uncouple ATP production to produce heat.

In mice, the development of these recruitable brown adipocytes is a genetically controlled process because certain mouse strains display major increases in UCP1 expression in WAT following cold stimulation while others do not [27]. Furthermore in a knockout model, mice without cBAT compensate this loss by developing recruitable BAT [18]. There are also strong indications that britening and whitening occurs through a direct interconversion of a white adipocyte into a brown adipocyte phenotype, a process referred to as transdifferentiation, in which the earlier mentioned PDRM16 plays an important role [22, 28]. The main difference between cBAT and recruitable adipocytes is the presence of UCP1. In cBAT, UCP1 is always present, while in recruitable adipocytes UCP1 is upregulated following appropriate stimulation like cold exposure.

In adult humans, biopsy material does not always resemble cBAT as seen in rodents. Biopsies of human BAT are UCP1 positive in adults and infants [7-9, 29], however it has been suggested that adipocytes in adult human BAT are more of a recruitable nature compared to classical brown adipocytes [25, 30]. Furthermore it is not known whether myf5 positive UCP1 positive adipocytes are present in humans. The suggestion that murine recruitable adipocytes are

derived from a smooth-muscle like origin [31] might also hold true for human brown adipocytes.

While cBAT in rodents persists with ageing [3], BAT in humans seems to dissipate in the elderly [32]. BAT activity is also lower in obese subjects, with an improvement after weight loss [33]. However recruiting BAT is a proven concept, both in obese and older human subjects [34-36]. These studies lead to the conclusion that recruiting BAT is a distinct possibility to influence energy metabolism in humans. We will discuss measuring BAT activity in humans below in section 3.

Markers for BAT

Although the composition and origin of BAT in humans is still under debate, it is evident that the adipocytes in human BAT are UCP1 positive and that these cells have the potential of norepinephrine and cold-stimulated thermogenesis [37]. It should be noted that BAT in mouse and man cannot simply be compared. Many publications are dedicated to identify molecular markers in order to distinguish thermogenic adipocytes [25, 38-41]. Although UCP1 is the right marker of choice when examining brown and recruited adipocytes, it might not be the most practical marker, because it is located inside the mitochondria. Therefore, the scientific community has been looking for other markers aimed to identify UCP1 positive adipocytes that are preferentially expressed at the cell surface, in order to easily extract these cells via cell sorting experiments that could aid to further elucidate the origin of recruitable adipocytes in humans. Also in general, the discovery of novel markers of UCP1 positive adipocytes could help in drug development to enhance thermogenesis. It is not our goal to review all markers associated with BAT identity (for review see [42]). Here we present a selection of the most recently discovered molecular markers with high potential aimed to identify UCP1 positive adipocytes in humans, as shown in Figure 1. The discussed markers can either be targeted by pharmacological means or they provide novel insights into BAT origin and physiology.

As mentioned earlier BAT is dependent on norepinephrine release from the sympathetic neuron. Following norepinephrine release, it binds β3 adrenergic receptor thereby activating BAT. The uptake of norepinephrine in adipocytes is inhibited by cell membrane transporter organic cation transporter 3 (OCT3). This transporter is able to shuttle various hormones across the cell membrane. Although ubiquitously expressed [43], higher expression can be detected in deep neck adipose tissue compared to human subcutaneous WAT [44]. OCT3 function can be inhibited by corticosterone [44]. This opens pathways for future venues to develop pharmacological intervention directed to OCT3 that could stimulate human BAT activity.

The PAT2 gene encodes a pH dependent proton/amino acid transporter that is selective for small chain amino acids [45]. The transcript for this gene was detected primarily in skeletal

muscle and kidney [46]. When it comes to adipose tissue, PAT2 expression is specific for human BAT compared to subcutaneous WAT [47], however PAT2 was not detected in brown-like/recruitable adipocytes from children in WAT [48] although these samples were UCP1 positive. With an eye towards the future, PAT2 is a valuable marker to detect adipocytes that express UCP1. Because PAT2 is a cell surface expressed protein, this gives openings to isolate cells via cell sorting.

Purinergic receptor P2X, ligand-gated ion channel 5 (P2RX5) belongs to a family of extracellular ATP-gated ion channels. The ion channel localizes to the cell membrane and its expression is high in immune system and brain [49]. P2RX5 is sensitive to changes in extracellular concentrations of calcium [50]. In adipose tissue, P2RX5 shows specificity towards human BAT over subcutaneous WAT [47]. In mice, P2RX5 is expressed in murine brown preadipocytes and its expression is further increased during adipocyte differentiation. Furthermore, expression of P2RX5 was increased following cold stimulation in mice in both BAT and subcutaneous WAT [47] similar to the response as UCP1.

Mitochondrial tumor suppressor 1 (MTUS1 also known as ATIP) is a mitochondrial protein that controls cellular proliferation [51]. MTUS1 expression is higher in human BAT compared to WAT. MTUS1 expression can be regulated via cold, because human BAT biopsies after prolonged cold exposure showed an increase in MTUS1 [52]. Knockdown of MTUS1 in human cultured brown adipocytes resulted in reduced UCP1 expression and decreased mitochondrial uncoupling suggesting a role in BAT thermogenesis [52]. Interestingly in mice, overexpression of MTUS1 resulted in reduced inflammation, less recruitment of macrophages and increased insulin sensitivity in WAT [53]. These findings in WAT might suggest that BAT might handle an inflammatory insult better resulting in normal metabolism.

KCNK3 (also known as TASK) encodes a pH sensitive potassium channel, subfamily K, member 3 [54]. Expression of KCNK3 is specific to human BAT compared to human WAT [52, 55], and KCNK3 expression shows a negative correlation to age or BMI in human BAT [56]. The amount of KCNK3 is correlated with PRDM16 levels [52, 57]. KCNK3 negatively affects second messenger signalling of the beta-adrenergic receptor via cAMP [57]. KNCK3$^{-/-}$ mice are obese and their brown adipocytes show resistance to activation of the beta-adrenergic receptor [57]. Furthermore, the explanation for this phenotype was through enhanced mineralocorticoid receptor signalling, which could be targeted by the inhibitor eplerenone [57]. KCNK3 is an interesting marker to identify BAT, which in the future could result in novel methods to stimulate BAT in humans.

Adenosine is a product of ATP breakdown; however it can also act as a signalling molecule via interaction with adenosine and purinergic receptors. Adenosine stimulates in vitro BAT activity and this stimulatory effect is further enhanced by adrenergic stimulation. More specifically, an agonist for the A_{2A} receptor markedly increased in vitro BAT activity. In

murine experiments, stimulation of BAT with norepinephrine results in release of adenosine further linking adenosine to BAT activity [58]. Novel ligands have been designed to target the A_{2A} receptor (for review see [59]), however it remains to be seen how these compounds can affect human BAT activity.

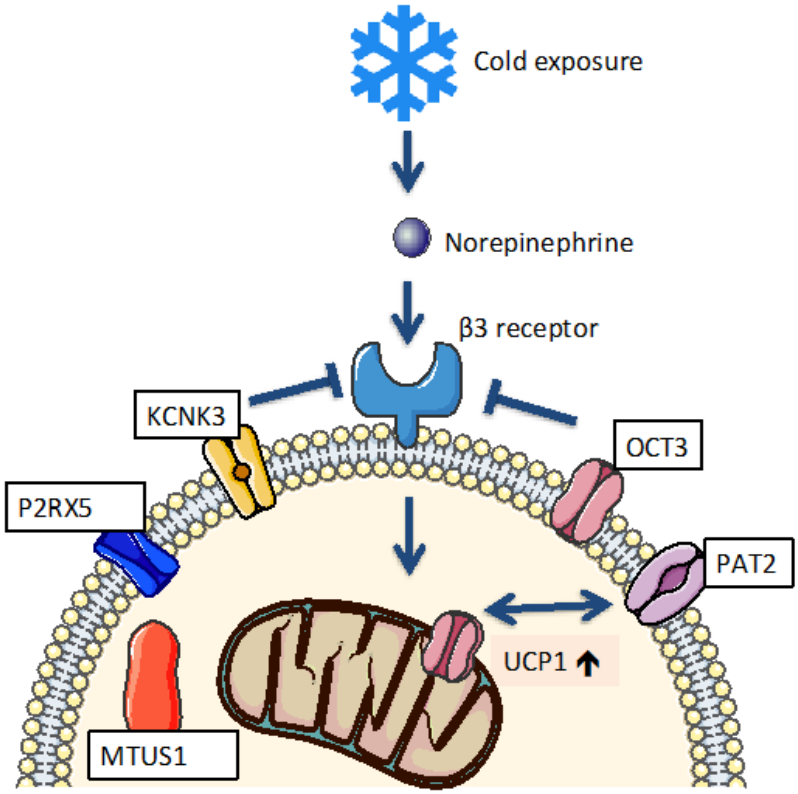

Figure 1. Overview of discussed recently identified cellular markers for human brown adipocytes. In the central pathway, cold exposure acting through norepinephrine on the β3 receptor is shown, resulting in increased UCP1 expression. UCP1 (uncoupling protein 1) ; OCT3 (organic cation transporter 3) ; PAT2 (proton/amino acid transporter 2) ; P2RX5 (purinergic receptor P2X, ligand-gated ion channel 5) ; MTUS1 (mitochondrial tumor suppressor 1) ; KCNK3 (pH sensitive potassium channel, subfamily K, member 3).

Summary/take home message

Contrary to many rodent species, adipocytes detected in adult human BAT biopsies are mostly of a recruitable nature (beige or brite adipocytes) [25]. Characterisation of these recruitable brown adipocytes can be useful to better understand the origin of these adipocytes and to ultimately devise new strategies to stimulate thermogenesis in humans. Earlier attempts to characterize these recruitable BAT cells were built upon the use of genetic and intracellular markers, which often gave inconclusive results. Several new markers with high potential have been discovered in the recent past, especially to detect these BAT cells using cell membrane specific markers. These new markers focus on activation of BAT cells using different interventions.

Brown adipose tissue activation and detection in humans

BAT activity stimulation besides cold exposure

Besides cold exposure, pharmacological stimulants have also been tested in humans. A non-selective systemic beta-adrenergic stimulation, by isoprenaline [60] and ephedrine [61] did not show significant activation in BAT glucose uptake and resulted in negative cardiovascular side effects [60]. More promising results are found in the use of a selective β3 stimulant, mirabregon. This showed an elevated glucose uptake in BAT upon stimulation [62] comparable to cold activation. However, the required dosage and potential side effects are still a matter of debate. Systemic administration of pharmacological stimulants is not selective for BAT only, as it also affects other tissues, such as white adipose tissue.

The suspected relationship between BAT activity in humans and diet-induced thermogenesis (DIT) has also been an interesting topic. As reviewed by Kozak [63], the theory that excessive energy with food intake is expended as heat through BAT activation has not been proven. Vosselman et al [64] compared BAT activity after meal consumption with cold exposure. They found that the amount of BAT activity did increase after large meal consumption; however, there was no relationship with DIT.

BAT may still be stimulated through certain dietary components such as compounds in green tea (cathechin-polyphenols and caffeine), peppers (capsinoids) and menthol [65, 66]. Green tea extract contains cathechin-polyphenols, which are able to inhibit catechol-O-methyl-transferase, the enzyme responsible for the breakdown of sympathetically released norepinephrine. The caffeine, present in green tea extract, is believed to inhibit phosphodiesterase, which in turn breaks down the cAMP second messenger system. Furthermore, caffeine also acts as an antagonist on adenosine receptors (see above). Green tea extract could thus be used to potentiate the adrenergic and/or adenosine pathways and thus lead to more BAT activation [66].

The pungent capsaicinoids, found in peppers are able to stimulate BAT activity via the transient receptor potential channels (TRPV) [65]. Stimulation of TRPV channels in the brain would lead to more sympathetic activity and in turn to increased BAT activity. However the strong pungency, may lead to reduced intake or gastrointestinal side effects [67]. The non-pungent forms, known as capsinoids, have been studied for their potential to stimulate BAT [68]. However, this study only showed a higher increase in energy expenditure upon cold exposure in BAT positive subjects compared to BAT negative subjects using capsinoids.

Another recently discovered potential stimulant in humans is sildenafil. Sildenafil is a well-known phosphodiesterase type 5 (PDE5) inhibitor, commonly used to treat erectile dysfunction in men. By inhibiting PDE5, it can elevate levels of cGMP via guanylyl cyclase,

which in turn can further stimulate lipolysis in BAT cells. Sildenafil was shown to induce browning in human WAT [69], and could thus be an interesting target for study in humans.

As seen in earlier studies, the direct stimulation of soluble guanylyl cyclase has already been shown to increase whole-body energy expenditure and lipid uptake in BAT in mice, as well as inducing browning of murine white adipocytes [70, 71]. This makes increasing cGMP an interesting potential target for BAT stimulation.

Measurement of BAT activity in humans

In order to determine the role and the contribution of BAT to whole body metabolism in adult humans, quantitative measurements of BAT activity are needed. The rediscovery of the presence of active BAT in humans was accomplished by positron emission tomography/computed tomography (PET/CT) scans with a radioactive labelled glucose tracer, 18-Fluoro-Deoxy-Glucose (^{18}F-FDG) [7-9]. Ever since, the ^{18}F-FDG-PET/CT technique has been the most used test for detecting the presence of BAT in humans. Alternatively, some studies use other substrate tracers such as 18-Fluoro 6-thia-heptadecanoidic acid (^{18}F-FTHA) as a marker for fatty acid uptake.

Besides substrate uptake, several other aspects of BAT activity can be measured such as oxidative metabolism, local blood perfusion and sympathetic innervation. In the following, we will discuss the usefulness of some of these techniques to quantify the contribution of BAT to whole body metabolism. For a more exhaustive review of these techniques and tracers we would like to refer to Bauwens et al [72] and Chondronikola et al [73]
In figure 2, we provide a visual overview of these methods.

Table 1. Overview of different measurements of BAT activity

Measurement of BAT aspect		**Quantitative analysis possible?**
Temperature		
Skin temperature	[74-78]	No quantitative analysis, only surface temperature Indirect and imprecise
Substrate uptake tracers		
18-Fluoro 6 deoxyglucose	[7-9, 36, 62, 79-81]	Yes, glucose uptake rate
18-Fluoro 6-thia-heptadecanoidic acid	[81-84]	Yes, fatty acid uptake rate
Adrenergic innervation		
123-Iodine meta-iodo-benzylguanidine	[85-88]	No quantitative analysis, Sympathetic innervation
18-Fluoro-L-dihydroxyphenylalanine	[89]	No quantitative analysis, Sympathetic innervation
Oxidative metabolism tracers		
Acetate	[81, 83]	No quantitative analysis, Oxidative metabolism
15-Water	[90, 91]	Yes, Blood perfusion
15-Oxygen	[90, 92]	Yes, oxygen consumption
Additional techniques		
CT scan (Hounsfield Units)	[82]	No quantitative analysis, Changes in BAT density
MRI scan	[93, 94]	Changes in BAT water/fat fractions, potential of measuring metabolic processes
Exosomal miRNA	[95, 96]	Associated with BAT activity in healthy subjects

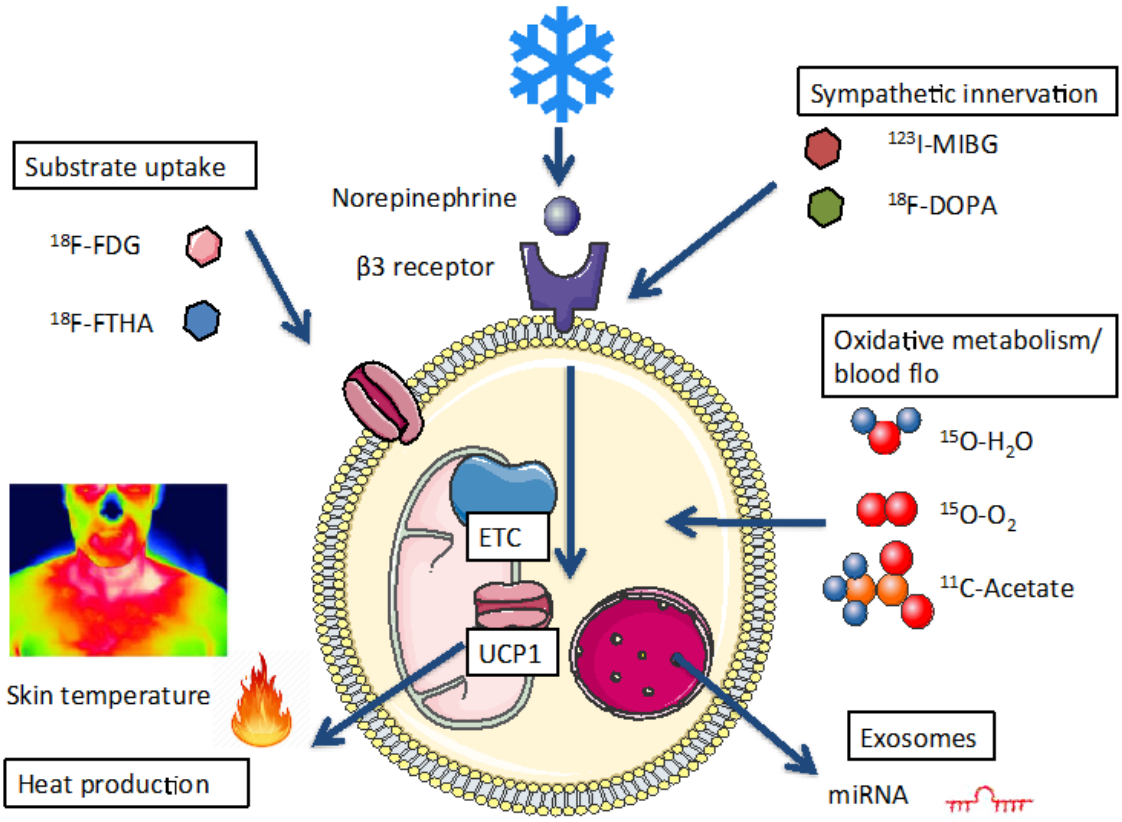

Figure 2. Overview of the different measurement techniques for brown adipose tissue activity, as discussed in the text. Centrally shown is cold exposure acting via norepinephrine on the β3 receptor. This in turn leads to increased UCP1 expression, resulting in heat production. ETC (electron transport chain); UCP1 (uncoupling protein 1) ; ^{18}F-FDG (18-Fluoro-Deoxy-Glucose) ; ^{18}F-FTHA (18-Fluoro 6-thia-heptadecanoidic acid) ; ^{123}I-MIBG (123-Iodine meta-iodo-benzylguanidine) ; ^{18}F-DOPA (18-Fluoro-L-dihydroxyphenylalanine).

Skin temperature

Since active BAT releases energy in the form of heat, one way of measuring this energy is by looking at the temperature around the BAT depots. As BAT is active and releases heat, one can expect the temperature around the depot to increase. Measuring the skin temperature via thermometers or wireless sensors has been used before, while using the ^{18}F-FDG PET/CT as a benchmark [77]. This study by Boon et al showed a positive correlation between supraclavicular skin temperature upon cold exposure and BAT volume and activity.

Another option is the use of thermal imaging. Via an infrared thermal camera, it is possible to measure the temperature of a larger part of the skin's surface.It is a quick and easy technique, without radiation exposure or invasive actions[76]. Several studies showed a significant correlation between BAT activity on ^{18}F-FDG PET/CT and the changes in supraclavicular skin temperature [74, 77, 78]. The changes in the supraclavicular skin temperature are not

consistent, which may be because of the different cooling techniques, air-cooled vs. water-cooled respectively affecting the skin temperature differently [74].

The downside is that the increase in skin temperature at the cervical depots is offset by the cold exposure used to stimulate BAT. It is therefore difficult to estimate the amount of heat produced by BAT. Furthermore, the infrared thermal imaging and skin temperature do not measure the heat production in the deeper depots [76]. The amount of subcutaneous fat at the cervical region may also hinder the adequate measurement of the effect of BAT on the skin temperature [97, 98]. In conclusion, while this technique is non-invasive and is without radiation exposure, it is an imprecise and indirect measurement. At best it is a qualitative approach.

Measuring substrate uptake

The details behind positron emission tomography are well discussed in the technical literature [99, 100]. In short, a so-called tracer is injected into the subject, after which the tracer will distribute inside the body. Tracers usually consist of a substrate with an attached radioactive component. The most well known tracer is $^{18}F^{-}$FDG (18-fluoro deoxy-glucose). This consists of glucose, with an attached 18-fluor atom. Like normal glucose, this $^{18}F^{-}$FDG undergoes uptake by glucose transporters [101, 102]. However, $^{18}F^{-}$FDG is then only metabolized to ^{18}F-FDG-6-phosphate, as the next step in glycolysis does not happen to FDG. This is due to the fact that the enzyme responsible for the next step, glucose-6-phosphate isomerase is unable to convert FDG-6 phosphate. The reverse conversion of ^{18}F-FDG-6-phosphate to $^{18}F^{-}$FDG is not possible due to low levels of the necessary enzymes. The $^{18}F^{-}$FDG remains therefore trapped inside the cell, and is only slowly broken down by other pathways [101, 102]. As the PET image only shows tissue tracer uptake, another technique is necessary to correlate the positron emission with an anatomical image.A regular computed tomography, is often performed together with the PET capture. This will provide a detailed anatomical scan, allowing a correlated image. The ^{18}F is an unstable isotope of the Fluor atom, and will decay into ^{18}O, with concomitant release of energy. This released energy will be in the form of positrons, which is measured on a sensor outside the body. The $^{18}F^{-}$FDG is mainly trapped and visualized in those tissues of the body that take up glucose, such as the brain, the heart under specific conditions, tumours, and activated BAT.

In the case of brown adipose tissue, the tracer is often administered after and during cold exposure. During the distribution phase, the tracer will localize to the active BAT, if present in the subject. Most tracers are usually used in a static scan, while BAT is a highly dynamic tissue. To measure tissue substrate uptake rate, a dynamic scan method can be used. A dynamic PET/CT, using Patlak curve [103, 104] fitting reveals a measure of glucose uptake rate [36].

For comparison between studies the procedures of the combined PET/CT (or PET/MR) requires clear descriptions and preferably standardization. In the so-called BARCIST (Brown Adipose Reporting Criteria in Imaging Studies) publication, an expert panel, assembled by the National Institutes of Diabetes and Digestive and Kidney diseases on November 4, 2014, wrote guidelines and suggestions for determining BAT activity in $^{18}F^{-}$FDG PET/CT scans [105]. A main guideline states that determining the activity of BAT should be presented using standardized uptake values (SUV). The SUV is calculated as the ratio between the measured radioactivity of the tracer within a region and the dosage of the administered tracer relative to the body weight [105]. This makes comparing the results in repeated measurements or across different subjects possible. However, the subject characteristics, such as BMI (body fat percentage) influence the distribution of the tracer and therefore the SUV in the target tissue [105]. For instance, in obese subjects less $^{18}F^{-}$FDG uptake is seen in BAT depots, which may be related to competition with the WAT depots. It may therefore be more applicable to calculate the SUV value as the tracer dosage relative to lean body mass. Other subject characteristics to take into account are medications, which influence the sympathetic nervous system, smoking habits and dietary components such as capsinoids [62]. There is still some uncertainty regarding the fate of the $^{18}F^{-}$FDG once taken up and trapped by the BAT cells. It is unknown if it undergoes intracellular metabolism or if it is excreted after a certain time period. In diabetic subjects the insulin resistance in these subjects, may lead to less uptake and trapping of $^{18}F^{-}$FDG in the cells.

Besides the methodological variation, there is also the biological variation of BAT to take in to account. The outdoor temperature and climate also influence the analysis of BAT, as a cold climate or outdoor temperature may stimulate BAT activity and lead to increased BAT volume. Studies regarding cold acclimation and weight loss have shown to increase BAT volume and activity [14, 64, 105].

FDG accurately reflects the capacity of tissue to take up glucose and is used as a standard diagnostic tool in cancer patients. However, FDG does not directly reflect the energy expenditure of BAT, where fatty acids (external and internal stored) are the main source of energy [2]. This can have important consequences for the interpretation of results, potentially leading to an underestimation of BAT energy metabolism. Indeed, use of different tracers show that depending on the type of tracer, the interpretation of the results (for instance the contribution of BAT to whole body energy expenditure) is different [90, 92].

Beyond FDG several alternative tracers - such as 99mTc-sestamibi, 123Imetaiodobenzylguanidine (MIBG), 18F-fluorodopa, perfusion and oxygen consumption studies and also ^{18}F-14(R, S)-FTHA - have been employed for BAT imaging [72]. Fatty acid metabolism (exemplified by FTHA uptake) may be less hampered by associated disturbances in glucose metabolism than FDG uptake in metabolic syndrome. Still for quantification, the

use of FTHA may be difficult, because internal brown adipocyte fat stores are also used as fuel during BAT activation.

Besides anatomical information the CT scan can also be used to register BAT activity. CT measures the density of the tissues, which is in the first place used to discriminate between BAT and WAT. However, during BAT activation, internal fat stores may be used which affects the density of the BAT. If the density is measured before and after an intervention (for instance cooling), the decrease in density can be used to estimate the lipids combusted by active BAT [82].

Alternatively, magnetic resonance (MR) scans can be used to make an anatomical scan, instead of a CT scan. This technique allows for highly detailed anatomical information, without the extra radiation exposure a CT scan requires. PET/MRI systems have become more widely available. Moreover MRI (Dixon technique) can be used to determine the changes of the fat fraction in tissues that may reflect metabolic activity. Next to monitoring the fat fraction by Dixon MRI, the use of ^{1}H Magnetic Resonance Spectroscopy (^{1}H-MRS) can be used to follow metabolic changes in activated BAT (for instance NAD+ precursor nicotinamide riboside) [94]. Future studies are needed to reveal the applicability.

Adrenergic innervation

Another option is to measure the adrenergic innervation using 123-I meta-iodo-benzyl guanidine (^{123}I-MIBG) as a tracer. This tracer binds to the adrenergic receptors present on cells, and could thus be used to determine the amount of adrenergic stimulation in BAT. This tracer has been used in diagnosing pheochromocytomas and other tumours of adrenergic origin [87, 88]. One study combined ^{18}F-FDG-PET/CT with MIBG-SPECT, which showed that both tracers show uptake in the same cervical regions [85], which would then be determined as BAT tissue. Another option is to use 18-Fluoro-L-dihydroxyphenylalanine (^{18}F-DOPA) as a tracer. This tracer is used in the same capacity as ^{123}I-MIBG, and only a case report describes the use of ^{18}F-DOPA in measuring BAT tissue [89]. Although capable of measuring the sympathetic innervation of BAT tissue, for the quantification of the contribution of BAT to whole body metabolism these tracers are not useful.

Oxidative metabolism tracers

The amount of blood flow to BAT can be measured using ^{15}O labelled water (^{15}O-H_2O), as well as oxygen consumption via an oxygen-tracer (^{15}O-O_2). Only a few studies used these techniques and found that subjects with FDG uptake in the cervical region did have increased blood flow and oxygen consumption [90, 91]. However the estimated contribution of BAT to the total energy expenditure is small, about 5% of the total energy expenditure [90]. The major issue is that ^{15}O has an extremely short half-life of about 2 minutes [93]. This in combination with the rapidly diffusing isotope and the limited field of view that can be studied with this technique may lead to an underestimation of BAT activity. The currently available

studies also show the need for well attuned cooling procedures and the PET/CT procedure [90, 92].

Another option to measure BAT oxidative metabolism is to use 11-Carbon Acetate (^{11}C-Acetate) during dynamic scanning. This tracer is labelled with the 11-C isotope, and undergoes uptake in active tissues. Studies using ^{11}C-Acetate have shown a significant oxygen metabolism of human BAT, [81, 83]. The use of these tracers might actually give the most direct measurement of BAT activity, as they measure the oxidative capacity of BAT. The short half-life of tracers such as ^{15}O-O_2, are issues that would need to be resolved, before they could be used to accurately quantify BAT energy consumption.

Exosomal miRNA

As published by Chen et al. in 2016 and Ng et al. in 2017, several micro RNA sequences (miRNA's) have been found in association with BAT activity [95, 96]. Chen et al. analysed serum from both murine brown adipocytes as well as from cultured human brown adipocytes. They found several miRNA's to be expressed differently. One of these miRNA's, miRNA-92 inversely correlated with BAT activity in young healthy subjects, as measured via PET/CT [95]. Ng et al. describe another miRNA, miRNA-32, to be involved in the response of adipocytes to cold stress. After cold stress increased levels of miRNA-32 were associated with browning of white adipocytes, and increased levels of browning markers such as PGC1α and increased expression of UCP1 [96]. Further evaluation of the use of miRNA's is needed, but the results are promising. The great advantage of this method would be in measuring BAT activity via blood sampling, and would possibly render the use of nuclear imaging and radiation exposure superfluous.

Contribution of BAT to total body energy expenditure

In a normal human, the total body energy expenditure (TEE) can be divided into different components: 1) energy expenditure during sleep (sleeping metabolic rate, SMR), 2) resting energy expenditure during arousal (Basic metabolic rate, BMR), 3) diet-induced energy expenditure (DEE) and the energy expenditure during activity (AEE) [106, 107]. Diet-induced thermogenesis (DIT) can be divided into an obligatory and a facultative component [108]. The obligatory component is the energy expended to consume, digest and metabolize food, while the facultative component depends on the composition and amount of food consumed [106, 109].

Energy expenditure also increases during cold exposure, as the body is stimulated to maintain a stable core temperature. This thermogenesis can in turn be divided in two parts: shivering and non-shivering [110]. Shivering thermogenesis refers to the involuntary muscle

contractions, which organisms show upon cold exposure. These contractions occur quickly upon cold exposure and generate heat in an effort to maintain a stable core temperature. Non-shivering occurs mainly in BAT, and is a process requiring adaptation [110].

In order to measure the NST and potentially BAT activity, indirect calorimetry is normally used: the analyses of inhaled and exhaled air. By this means O_2 consumption and CO_2 production can be determined and the amount of energy expended in time can be calculated [111]. When using this technique during both resting and cold exposure, the increase in energy expenditure upon cold exposure, the NST can be calculated. It appears in many studies that NST strongly correlates with BAT activity measured via ^{18}F-FDG PET/CT [8, 36].

However, the contribution of activated BAT to whole body energy expenditure is still not established. In animals, especially rodents, the role of BAT is more clear-cut than in humans. The studies using tracers ^{15}O-O_2, which actually measure oxygen usage have all shown that the overall contribution of BAT to total energy expenditure comes down to about 1-5% of resting metabolic rate (RMR) [90, 92]. Though very interesting experiments, these measurements are hampered by the short half life of the isotopes of 2 minutes, the rapidly diffusing isotope and the use of a limited field of view (only a small part of the body is actually scanned). The authors therefore conclude that the activity of BAT may be underestimated. Their results are lower than based on an earlier estimation of BAT contribution using BAT metabolic rate from rodents and allometric comparison, as done by Van Marken Lichtenbelt and Schrauwen [108]. Their calculation revealed an estimation of 5 % of RMR. The data of the study of van der Lans et al [36], who performed a dynamic ^{18}F-FDG PET/CT scan, show that glucose uptake rate varied substantially between subjects, from almost zero to more than 25 umol/g BAT. When using these numbers maximal BAT activity comes up to 16 % of RMR and almost 90 % of NST.

Although the current contribution of BAT is still a matter of dispute in humans, the potential of BAT can be significant. All current studies to increase BAT focus on a short period of exposure to cold (cold acclimation) or pharmacological stimulation.
Cold acclimation, even as short as 10 days has been shown to increase BAT activity and volume [36]. As seen in different acclimation studies, the energy expenditure of BAT increases after acclimation, as well as the oxidative capacity [36, 81, 112]. A more recent study by Leitner et al. showed that FDG-uptake upon cold exposure also occurs outside of classical BAT depots [11]. They also show the ^{18}F-FDG uptake in a patient with a paraganglioma with a long exposure to high levels of adrenergic hormones. This shows ^{18}F-FDG uptake in the perirenal and subcostal WAT depots. This would mean that potentially these regions could be stimulated to develop BAT-like properties. Estimated by Leitner et al. a maximum of 115.5 kcal/d could be produced by the entire BAT volume [11].

This estimation would lead to the conclusion that most studies thus far, have only shown an under appreciation of brown adipose tissue. A stronger or longer exposure to cold or pharmacological stimulants could lead to a much larger contribution of BAT to energy expenditure. The effect on metabolic health in that regard could be significant. Earlier studies have shown some effects on lipid and glucose metabolism, [79, 82] but a much more profound effect on metabolic health would be a welcome sight.

In order to maximize the effect of BAT, more exposure to cold would be desirable. However, most studies have used a temperature of around 14-16 degrees Celsius, which is often not appreciated by subjects. A prolonged exposure to a less cold environment, may be a potential solution. New research should not only focus on finding new stimulants for BAT, but also on increasing the potential of BAT in humans.

Although increasing energy expenditure can influence energy balance, potential compensation can occur by increased food intake. This could lead to attenuation of beneficial effects of increased energy expenditure on health and metabolism. There are indications that not all of the increase in energy expenditure is compensated [113].

Another issue is the potential negative consequence of a prolonged increase in energy expenditure in obese subjects. Several observational studies showed that obese subjects with heart failure might have a better prognosis then lean subjects with heart failure [114, 115]. This so-called obesity paradox indicates that increasing energy expenditure to combat obesity should be closely observed for long-term cardiovascular side effects. In this case, the goal should be weight maintenance and not decrease of body weight.

To conclude, brown adipose tissue remains an interesting and possibly underestimated target in metabolic health. Further exploration is needed to determine to what extent BAT activation can become a new treatment for obesity and its complications.

References

1. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes. 2009;58(7):1526-31.
2. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
3. Himms-Hagen J. Brown adipose tissue metabolism and thermogenesis. Annu Rev Nutr. 1985;5:69-94.
4. Heaton JM. The distribution of brown adipose tissue in the human. J Anat. 1972;112(Pt 1):35-9.
5. Huttunen P, Hirvonen J, Kinnula V. The occurrence of brown adipose tissue in outdoor workers. Eur J Appl Physiol Occup Physiol. 1981;46(4):339-45.
6. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. Am J Physiol Endocrinol Metab. 2007;293(2):E444-52.
7. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.
8. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
9. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
10. Smith RE. Thermoregulatory and Adaptive Behavior of Brown Adipose Tissue. Science. 1964;146(3652):1686-9.
11. Leitner BP, Huang S, Brychta RJ, Duckworth CJ, Baskin AS, McGehee S, et al. Mapping of human brown adipose tissue in lean and obese young men. Proceedings of the National Academy of Sciences of the United States of America. 2017;114(32):8649-54.
12. Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. Nature. 2000;404(6778):652-60.
13. Amir S. Intra-ventromedial hypothalamic injection of glutamate stimulates brown adipose tissue thermogenesis in the rat. Brain Res. 1990;511(2):341-4.
14. Parysow O, Mollerach AM, Jager V, Racioppi S, San Roman J, Gerbaudo VH. Low-dose oral propranolol could reduce brown adipose tissue F-18 FDG uptake in patients undergoing PET scans. Clin Nucl Med. 2007;32(5):351-7.
15. Bianco AC, Silva JE. Intracellular conversion of thyroxine to triiodothyronine is required for the optimal thermogenic function of brown adipose tissue. J Clin Invest. 1987;79(1):295-300.
16. Bianco AC, Silva JE. Nuclear 3,5,3'-triiodothyronine (T3) in brown adipose tissue: receptor occupancy and sources of T3 as determined by in vivo techniques. Endocrinology. 1987;120(1):55-62.

17. Silva JE. Thyroid hormone control of thermogenesis and energy balance. Thyroid. 1995;5(6):481-92.
18. Schulz TJ, Huang P, Huang TL, Xue R, McDougall LE, Townsend KL, et al. Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. Nature. 2013;495(7441):379-83.
19. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proc Natl Acad Sci U S A. 2007;104(11):4401-6.
20. Barbera MJ, Schluter A, Pedraza N, Iglesias R, Villarroya F, Giralt M. Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. The Journal of biological chemistry. 2001;276(2):1486-93.
21. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature. 2008;454(7207):961-7.
22. Harms MJ, Ishibashi J, Wang W, Lim HW, Goyama S, Sato T, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. Cell Metab. 2014;19(4):593-604.
23. Young P, Arch JR, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. FEBS letters. 1984;167(1):10-4.
24. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. The Journal of biological chemistry. 2010;285(10):7153-64.
25. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell. 2012;150(2):366-76.
26. Giralt M, Villarroya F. White, brown, beige/brite: different adipose cells for different functions? Endocrinology. 2013;154(9):2992-3000.
27. Guerra C, Koza RA, Yamashita H, Walsh K, Kozak LP. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. J Clin Invest. 1998;102(2):412-20.
28. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. Nat Cell Biol. 2013;15(6):659-67.
29. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, et al. Evidence for two types of brown adipose tissue in humans. Nat Med. 2013;19(5):631-4.
30. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. PLoS One. 2012;7(11):e49452.
31. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, et al. A smooth muscle-like origin for beige adipocytes. Cell Metab. 2014;19(5):810-20.

32. Yoneshiro T, Aita S, Matsushita M, Okamatsu-Ogura Y, Kameya T, Kawai Y, et al. Age-related decrease in cold-activated brown adipose tissue and accumulation of body fat in healthy humans. Obesity (Silver Spring). 2011;19(9):1755-60.
33. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, van Marken Lichtenbelt WD. Brown adipose tissue in morbidly obese subjects. PLoS One. 2011;6(2):e17247.
34. Saito M, Yoneshiro T, Matsushita M. Activation and recruitment of brown adipose tissue by cold exposure and food ingredients in humans. Best Pract Res Clin Endocrinol Metab. 2016;30(4):537-47.
35. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest. 2013;123(8):3404-8.
36. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
37. Broeders EP, Nascimento EB, Havekes B, Brans B, Roumans KH, Tailleux A, et al. The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity. Cell metabolism. 2015;22(3):418-26.
38. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. Nat Med. 2013;19(5):635-9.
39. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. Cell Metab. 2013;17(5):798-805.
40. de Jong JM, Larsson O, Cannon B, Nedergaard J. A stringent validation of mouse adipose tissue identity markers. Am J Physiol Endocrinol Metab. 2015;308(12):E1085-105.
41. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nat Med. 2013;19(10):1252-63.
42. Wang W, Seale P. Control of brown and beige fat development. Nature reviews. Molecular cell biology. 2016;17(11):691-702.
43. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharmaceutical research. 2007;24(7):1227-51.
44. Breining P, Pedersen SB, Pikelis A, Rolighed L, Sundelin EI, Jessen N, et al. High expression of organic cation transporter 3 in human BAT-like adipocytes. Implications for extraneuronal norepinephrine uptake. Mol Cell Endocrinol. 2017;443:15-22.
45. Boll M, Foltz M, Rubio-Aliaga I, Daniel H. A cluster of proton/amino acid transporter genes in the human and mouse genomes. Genomics. 2003;82(1):47-56.
46. Bermingham JR, Jr., Pennington J. Organization and expression of the SLC36 cluster of amino acid transporter genes. Mammalian genome : official journal of the International Mammalian Genome Society. 2004;15(2):114-25.

47. Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. Science translational medicine. 2014;6(247):247ra103.
48. Rockstroh D, Landgraf K, Wagner IV, Gesing J, Tauscher R, Lakowa N, et al. Direct evidence of brown adipocytes in different fat depots in children. PLoS One. 2015;10(2):e0117841.
49. Le KT, Paquet M, Nouel D, Babinski K, Seguela P. Primary structure and expression of a naturally truncated human P2X ATP receptor subunit from brain and immune system. FEBS letters. 1997;418(1-2):195-9.
50. Haines WR, Torres GE, Voigt MM, Egan TM. Properties of the novel ATP-gated ionotropic receptor composed of the P2X(1) and P2X(5) isoforms. Molecular pharmacology. 1999;56(4):720-7.
51. Seibold S, Rudroff C, Weber M, Galle J, Wanner C, Marx M. Identification of a new tumor suppressor gene located at chromosome 8p21.3-22. FASEB J. 2003;17(9):1180-2.
52. Shinoda K, Luijten IH, Hasegawa Y, Hong H, Sonne SB, Kim M, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes. Nat Med. 2015;21(4):389-94.
53. Jing F, Mogi M, Min LJ, Ohshima K, Nakaoka H, Tsukuda K, et al. Effect of angiotensin II type 2 receptor-interacting protein on adipose tissue function via modulation of macrophage polarization. PLoS One. 2013;8(4):e60067.
54. Duprat F, Lesage F, Fink M, Reyes R, Heurteaux C, Lazdunski M. TASK, a human background K+ channel to sense external pH variations near physiological pH. The EMBO journal. 1997;16(17):5464-71.
55. Svensson PA, Jernas M, Sjoholm K, Hoffmann JM, Nilsson BE, Hansson M, et al. Gene expression in human brown adipose tissue. International journal of molecular medicine. 2011;27(2):227-32.
56. Nascimento EB, Sparks LM, Divoux A, Gisbergen van MW, Broeders EP, Jorgensen JA, et al. Genetic markers of brown adipose tissue identity and in vitro brown adipose tissue activity in humans. Obesity (Silver Spring). In Press.
57. Chen Y, Zeng X, Huang X, Serag S, Woolf CJ, Spiegelman BM. Crosstalk between KCNK3-Mediated Ion Current and Adrenergic Signaling Regulates Adipose Thermogenesis and Obesity. Cell. 2017.
58. Gnad T, Scheibler S, von Kugelgen I, Scheele C, Kilic A, Glode A, et al. Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature. 2014;516(7531):395-9.
59. Sousa JB, Diniz C. The Adenosinergic System as a Therapeutic Target in the Vasculature: New Ligands and Challenges. Molecules. 2017;22(5).
60. Vosselman MJ, van der Lans AA, Brans B, Wierts R, van Baak MA, Schrauwen P, et al. Systemic beta-adrenergic stimulation of thermogenesis is not accompanied by brown adipose tissue activity in humans. Diabetes. 2012;61(12):3106-13.

61. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. Proc Natl Acad Sci U S A. 2012;109(25):10001-5.
62. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. Cell Metab. 2015;21(1):33-8.
63. Kozak LP. Brown fat and the myth of diet-induced thermogenesis. Cell Metab. 2010;11(4):263-7.
64. Vosselman MJ, Brans B, van der Lans AA, Wierts R, van Baak MA, Mottaghy FM, et al. Brown adipose tissue activity after a high-calorie meal in humans. Am J Clin Nutr. 2013;98(1):57-64.
65. Saito M. Brown adipose tissue as a therapeutic target for human obesity. Obes Res Clin Pract. 2013;7(6):e432-8.
66. Dulloo AG, Seydoux J, Girardier L, Chantre P, Vandermander J. Green tea and thermogenesis: interactions between catechin-polyphenols, caffeine and sympathetic activity. Int J Obes Relat Metab Disord. 2000;24(2):252-8.
67. Westerterp-Plantenga M, Diepvens K, Joosen AM, Berube-Parent S, Tremblay A. Metabolic effects of spices, teas, and caffeine. Physiol Behav. 2006;89(1):85-91.
68. Yoneshiro T, Aita S, Kawai Y, Iwanaga T, Saito M. Nonpungent capsaicin analogs (capsinoids) increase energy expenditure through the activation of brown adipose tissue in humans. Am J Clin Nutr. 2012;95(4):845-50.
69. Li S, Li Y, Xiang L, Dong J, Liu M, Xiang G. Sildenafil Induces Browning of Subcutaneous White Adipose Tissue in Overweight Adults. Metabolism. 2017.
70. Hoffmann LS, Etzrodt J, Willkomm L, Sanyal A, Scheja L, Fischer AW, et al. Stimulation of soluble guanylyl cyclase protects against obesity by recruiting brown adipose tissue. Nature communications. 2015;6:7235.
71. Pfeifer A, Kilic A, Hoffmann LS. Regulation of metabolism by cGMP. Pharmacol Ther. 2013;140(1):81-91.
72. Bauwens M, Wierts R, van Royen B, Bucerius J, Backes W, Mottaghy F, et al. Molecular imaging of brown adipose tissue in health and disease. Eur J Nucl Med Mol Imaging. 2014;41(4):776-91.
73. Chondronikola M, Beeman SC, Wahl RL. Non-invasive methods for the assessment of brown adipose tissue in humans. J Physiol. 2018;596(3):363-78.
74. van der Lans AA, Vosselman MJ, Hanssen MJ, Brans B, van Marken Lichtenbelt WD. Supraclavicular skin temperature and BAT activity in lean healthy adults. J Physiol Sci. 2016;66(1):77-83.
75. Mccafferty DJ. The value of infrared thermography for research on mammals: previous applications and future directions. Mammal Rev. 2007;37(3):207-23.
76. Jang C, Jalapu S, Thuzar M, Law PW, Jeavons S, Barclay JL, et al. Infrared thermography in the detection of brown adipose tissue in humans. Physiol Rep. 2014;2(11).

77. Boon MR, Bakker LE, van der Linden RA, Pereira Arias-Bouda L, Smit F, Verberne HJ, et al. Supraclavicular skin temperature as a measure of 18F-FDG uptake by BAT in human subjects. PLoS One. 2014;9(6):e98822.
78. Symonds ME, Henderson K, Elvidge L, Bosman C, Sharkey D, Perkins AC, et al. Thermal imaging to assess age-related changes of skin temperature within the supraclavicular region co-locating with brown adipose tissue in healthy children. J Pediatr. 2012;161(5):892-8.
79. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
80. Hong TS, Shammas A, Charron M, Zukotynski KA, Drubach LA, Lim R. Brown adipose tissue 18F-FDG uptake in pediatric PET/CT imaging. Pediatr Radiol. 2011;41(6):759-68.
81. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. J Clin Invest. 2012;122(2):545-52.
82. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
83. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. J Clin Endocrinol Metab. 2014;99(3):E438-46.
84. Labbe SM, Caron A, Bakan I, Laplante M, Carpentier AC, Lecomte R, et al. In vivo measurement of energy substrate contribution to cold-induced brown adipose tissue thermogenesis. FASEB J. 2015;29(5):2046-58.
85. Admiraal WM, Holleman F, Bahler L, Soeters MR, Hoekstra JB, Verberne HJ. Combining 123I-metaiodobenzylguanidine SPECT/CT and 18F-FDG PET/CT for the assessment of brown adipose tissue activity in humans during cold exposure. J Nucl Med. 2013;54(2):208-12.
86. Admiraal WM, Verberne HJ, Karamat FA, Soeters MR, Hoekstra JB, Holleman F. Cold-induced activity of brown adipose tissue in young lean men of South-Asian and European origin. Diabetologia. 2013;56(10):2231-7.
87. Gelfand MJ. 123I-MIBG uptake in the neck and shoulders of a neuroblastoma patient: damage to sympathetic innervation blocks uptake in brown adipose tissue. Pediatr Radiol. 2004;34(7):577-9.
88. Okuyama C, Sakane N, Yoshida T, Shima K, Kurosawa H, Kumamoto K, et al. (123)I- or (125)I-metaiodobenzylguanidine visualization of brown adipose tissue. J Nucl Med. 2002;43(9):1234-40.
89. Sondergaard E, Gormsen LC, Christensen MH, Pedersen SB, Christiansen P, Nielsen S, et al. Chronic adrenergic stimulation induces brown adipose tissue differentiation in visceral adipose tissue. Diabet Med. 2015;32(2):e4-8.
90. Muzik O, Mangner TJ, Granneman JG. Assessment of oxidative metabolism in brown fat using PET imaging. Front Endocrinol (Lausanne). 2012;3:15.

91. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, et al. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. Cell Metab. 2011;14(2):272-9.
92. M UD, Raiko J, Saari T, Kudomi N, Tolvanen T, Oikonen V, et al. Human brown adipose tissue [(15)O]O2 PET imaging in the presence and absence of cold stimulus. Eur J Nucl Med Mol Imaging. 2016;43(10):1878-86.
93. Borga M, Virtanen KA, Romu T, Leinhard OD, Persson A, Nuutila P, et al. Brown adipose tissue in humans: detection and functional analysis using PET (positron emission tomography), MRI (magnetic resonance imaging), and DECT (dual energy computed tomography). Methods Enzymol. 2014;537:141-59.
94. Canto C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, et al. The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. Cell Metab. 2012;15(6):838-47.
95. Chen Y, Buyel JJ, Hanssen MJ, Siegel F, Pan R, Naumann J, et al. Exosomal microRNA miR-92a concentration in serum reflects human brown fat activity. Nature communications. 2016;7:11420.
96. Ng R, Hussain NA, Zhang Q, Chang C, Li H, Fu Y, et al. miRNA-32 Drives Brown Fat Thermogenesis and Trans-activates Subcutaneous White Fat Browning in Mice. Cell Rep. 2017;19(6):1229-46.
97. Haq T, Crane JD, Kanji S, Gunn E, Tarnopolsky MA, Gerstein HC, et al. Optimizing the methodology for measuring supraclavicular skin temperature using infrared thermography; implications for measuring brown adipose tissue activity in humans. Sci Rep. 2017;7(1):11934.
98. Gatidis S, Schmidt H, Pfannenberg CA, Nikolaou K, Schick F, Schwenzer NF. Is It Possible to Detect Activated Brown Adipose Tissue in Humans Using Single-Time-Point Infrared Thermography under Thermoneutral Conditions? Impact of BMI and Subcutaneous Adipose Tissue Thickness. PLoS One. 2016;11(3):e0151152.
99. Muehllehner G, Karp JS. Positron emission tomography. Phys Med Biol. 2006;51(13):R117-37.
100. Raichle ME. Positron emission tomography. Annu Rev Neurosci. 1983;6:249-67.
101. Gallagher BM, Fowler JS, Gutterson NI, MacGregor RR, Wan CN, Wolf AP. Metabolic trapping as a principle of oradiopharmaceutical design: some factors resposible for the biodistribution of [18F] 2-deoxy-2-fluoro-D-glucose. J Nucl Med. 1978;19(10):1154-61.
102. Rigo P, Paulus P, Kaschten BJ, Hustinx R, Bury T, Jerusalem G, et al. Oncological applications of positron emission tomography with fluorine-18 fluorodeoxyglucose. Eur J Nucl Med. 1996;23(12):1641-74.
103. Graham MM, Peterson LM, Hayward RM. Comparison of simplified quantitative analyses of FDG uptake. Nucl Med Biol. 2000;27(7):647-55.
104. Patlak CS, Blasberg RG. Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data. Generalizations. J Cereb Blood Flow Metab. 1985;5(4):584-90.

105. Chen KY, Cypess AM, Laughlin MR, Haft CR, Hu HH, Bredella MA, et al. Brown Adipose Reporting Criteria in Imaging STudies (BARCIST 1.0): Recommendations for Standardized FDG-PET/CT Experiments in Humans. Cell Metab. 2016;24(2):210-22.
106. Westerterp KR. Physical activity and physical activity induced energy expenditure in humans: measurement, determinants, and effects. Front Physiol. 2013;4:90.
107. Westerterp KR, Verboeket-van de Venne WP, Bouten CV, de Graaf C, van het Hof KH, Weststrate JA. Energy expenditure and physical activity in subjects consuming full-or reduced-fat products as part of their normal diet. Br J Nutr. 1996;76(6):785-95.
108. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R285-96.
109. Westerterp KR. Diet induced thermogenesis. Nutr Metab (Lond). 2004;1(1):5.
110. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol. 2011;214(Pt 2):242-53.
111. Ferrannini E. The theoretical bases of indirect calorimetry: a review. Metabolism. 1988;37(3):287-301.
112. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.
113. Cannon B, Nedergaard J. Thermogenesis challenges the adipostat hypothesis for body-weight control. Proc Nutr Soc. 2009;68(4):401-7.
114. Fonarow GC, Srikanthan P, Costanzo MR, Cintron GB, Lopatin M, Committee ASA, et al. An obesity paradox in acute heart failure: analysis of body mass index and inhospital mortality for 108,927 patients in the Acute Decompensated Heart Failure National Registry. Am Heart J. 2007;153(1):74-81.
115. Lavie CJ, Milani RV, Artham SM, Patel DA, Ventura HO. The obesity paradox, weight loss, and coronary disease. Am J Med. 2009;122(12):1106-14.

Chapter 3
Nicotinamide riboside enhances in vitro brown adipose tissue in humans, but not in vivo

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Abstract

Context

Elevating NAD^+ levels systemically improves metabolic health, which can be accomplished via nicotinamide riboside (NR). Previously, it was demonstrated that NR supplementation in high fat diet (HFD)-fed mice decreased weight gain, normalized glucose metabolism and enhanced cold tolerance.

Objective

As brown adipose tissue (BAT) is a major source of thermogenesis, we hypothesize that NR stimulates BAT in mice and humans.

Design and intervention

HFD-fed C56BL/6J mice were supplemented with 400 mg/kg/day NR for 4 weeks and subsequently exposed to cold. In vitro primary adipocytes derived from human BAT biopsies were pretreated with 50 µM or 500 µM NR prior to measuring mitochondrial uncoupling. Human volunteers (45-65 years, BMI: 27-35 kg/m^2) were supplemented with 1000 mg/day NR for 6 weeks to determine whether BAT activity increased, as measured by [18F]FDG uptake via PET-CT (randomized, double blinded, placebo-controlled, cross-over study with NR supplementation).

Results

NR supplementation in HFD-fed mice decreased adipocyte cell size in BAT. Cold exposure further decreased adipocyte cell size on top of that achieved by NR alone independent of ex vivo lipolysis. In adipocytes derived from human BAT, NR enhanced in vitro norepinephrine-stimulated mitochondrial uncoupling. However, NR supplementation in human volunteers did not alter BAT activity or cold induced thermogenesis.

Conclusions

NR stimulates in vitro human BAT, however not in vivo BAT in humans. Our research demonstrates the need for further translational research to better understand the differences in NAD^+ metabolism in mouse and human.

Introduction

Brown adipose tissue (BAT) has been proposed as a promising target to stimulate energy expenditure in humans with obesity or type 2 diabetes. The presence of uncoupling protein 1 (UCP1) in mitochondria of BAT enables heat production instead of ATP synthesis while using glucose and fatty acids as fuel (1). The interest in human BAT was sparked by the discovery of active BAT in adult humans (2-5) and high BAT activity is associated with healthy whole body metabolism. Cold exposure is the most effective way to stimulate BAT activity in humans (6). Prolonged cold exposure may, however, not be an attractive treatment option for many people and thus pharmacological alternatives are actively explored. Numerous potential ways to activate BAT have been investigated for example vitamin A, FGF21, thyroid hormones and bile acids (7). In this context, we and others have shown that bile acids stimulate BAT activity in mice (8) and healthy humans (9), however, it is not known whether this approach is effective in human metabolic disease. Another approach to activate BAT involves activation of the beta-adrenergic receptor via administration of agonists, like mirabegron. However, beta-adregenergic stimulation at doses high enough to activate BAT also affect cardiac function by increasing heart rate and blood pressure, due to beta-adrenergic receptors in the heart (10). Therefore, other more specific and safer pharmacological strategies are warranted that stimulate BAT activity in humans that do not need to rely on the activation of the beta-adrenergic receptor.

NAD^+ is an important mediator of cellular metabolism. NAD^+ demand is high in order to carry out metabolic redox reactions yielding energy like glycolysis and oxidative phosphorylation. However besides these redox reactions, NAD^+ is also consumed by NAD^+-dependent enzymes like sirtuins, poly-ADP-ribose polymerases and cADP-ribose synthases (11). Low NAD^+ is associated with ageing and decreased metabolic health (12), therefore increasing NAD^+ concentrations is an interesting approach to boost metabolism. The vitamin B3 analogue nicotinamide ribose (NR) is a NAD^+ precursor that increases activity of sirtuin (SIRT) 1 and 3 (13). In vitro, NR boosts NAD^+ levels (14) also resulting in elevated SIRT1/3 activity in vivo (13). In obesity models, NR protected high fat diet (HFD)-fed mice from weight gain and glucose intolerance (13,15). Furthermore, NR supplementation in various rodent models was able to counteract harmful effects of eg. brain damage (16), Alzheimer's disease (17), autism spectrum disorder (18), age-related ovarian infertility (19), senescence (20) and muscular dystrophy through increased mitochondrial function (21).

NR supplementation also stimulated mitochondrial biogenesis in muscle and BAT in a mouse model of mitochondrial myopathy (22). Interestingly, in mice NR safeguarded body temperature following a cold challenge (13) thus implicating the involvement of BAT due to the thermogenic properties of BAT. Combined these findings generated two additional questions: (1.) Is beta-adrenergic stimulated BAT metabolism altered in murine adipocytes and in cultured adipocytes derived from human BAT following NR? (2.) Does NR enhance cold-

stimulated BAT activity in humans? Therefore, we here assessed how NR affected BAT morphology in a in vivo HFD-fed mouse model. Since murine BAT is different from human BAT and the effect of NAD^+ boosting on human BAT remain unclear, we also investigated the effects of NR on human BAT by using in vitro primary cultured adipocytes derived from human BAT biopsies. Finally, we performed a first human clinical trial in which we supplemented human volunteers with 1000 mg/day NR in order to examine the effects on in vivo BAT activity in humans.

Materials and Methods

Animal experiments

Male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed under a 14 hour light, 10 hour dark cycle at 21 °C and had *ad libitum* access to water and food throughout the experiment. From the age of 8 weeks, mice were split into 4 groups of 10 animals. All animals received a high fat diet (D12492) for 4 weeks from Research Diets Inc. (New Brunswick, NJ). Half of animals were fed with pellets containing vehicle (double-distilled water; ddH_20) or NR-supplemented by providing NR (400 mg/kg/day) for 4 weeks as previously described (23). Mouse body weight was assessed weekly. Cold test was performed as described (24) in 7 animals on HF diet alone and in 7 animals receiving HF and NR. The rest of the animals was maintained at room temperature. In cold-test groups, mice were anesthetized with sodium pentobarbital after 6 hours of cold exposure (i.p. injection, 50 mg/kg body weight). In the RT group, mice were anesthetized at the same time with sodium pentobarbital (i.p. injection, 50 mg/kg body weight). BAT was stored in 4% formaldehyde or used for lipolysis experiments. All animal experiments were carried according to national Swiss and European Union ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license #2868.

Lipolysis assay

Murine BAT was isolated and tissue explants were minced and incubated with lipolysis medium at 37 °C and 5% CO_2. After 2 hours, media was collected and incubated for of 5 minutes at 37 °C with free glycerol reagent from Sigma-Aldrich (St. Louis, MO). Following absorption was measured at 540 nm as previously described (25). In cultured adipocytes derived from human BAT and WAT, glycerol release was determined using the enzyChrom™ adipolysis assay kit from BioAssay systems (Hayward, CA) according to the manufacturers instructions.

Histology

Murine BAT samples were fixed overnight in buffered 4% formaldehyde and embedded in paraffin. 5 µm thick serial sections were made from paraffin embedded tissue which were subsequently stained with hematoxylin and eosin (HE).

Culture of human primary adipocytes

The collection of BAT and WAT biopsies in patients was reviewed and approved by the ethics committee of Maastricht University Medical Center (METC 10-3-012, NL31367.068.10, NCT03111719). Isolation of the stromal vascular fraction and differentiation of cultured adipocytes derived from human BAT and WAT have been described previously (9). In short, collected cells from the stromal vascular fraction were grown to confluence. Differentiation was initiated by a cocktail containing biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (100 nM), IBMX (250 µM), rosiglitazone (5 µM), T3 (2 nM) and transferrin (10 µg/ml). Cells were transferred to maintenance medium consisting of biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (10 nM), T3 (2 nM) and transferrin (10 µg/ml) until lipid-accumulating adipocytes had formed.

Mitochondrial respiration

Mitochondrial respiration in cultured adipocytes derived from human BAT and WAT has been described before (9). In short, adipocytes derived from human BAT and WAT were differentiated in XF96 well plates. Oxygen consumption rates were measured using the XF96 extracellular flux analyzer from Seahorse Biosciences (North Billerica, MA). Cells were incubated for 1 hour at 37 °C in unbuffered DMEM (2 mM GlutaMAX, 1 mM sodium pyruvate and 25 mM glucose). Basal oxygen consumption was measured followed by injection of 2 µM oligomycin subsequently followed by injection of the compounds of interest (1 µM NE, 0.3 µM FCCP, 1 µM antimycin A+rotenone). When indicated cells were preincubated with NR for 24 hours prior to the start of the experiment. Data is plotted as a percentage compared to uncoupled respiration following oligomycin.

RNA isolation and gene expression analysis

Total RNA was extracted from cultured adipocytes derived from human BAT and WAT using the miRNEasy kit from Qiagen (Hilden, Germany) according to the manufacturer. cDNA was created using the high capacity RNA-to-cDNA-kit from Applied Biosystems (Foster City, CA). Gene expression data was normalized to TATA box-binding protein (TBP) and further analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers for SIRT3 (Hs00953477_m1), SOD2 (Hs00167309_m1) and UCP1 (Hs00222453_m1) were from Applied Biosystems (Foster City, CA). SYBR-green qPCR primers for PGC1A: forward primer 5'-TGCTGAAGAGGGAAAGTGAGCGATTAGTTGA-3', reverse primer 5'-AGGTGAAAGTGTAATACTGTTGGTTGA-3'; SIRT1: forward primer 5'-AGAGCCTCACATGCAAGCTCTAG-3', reverse primer 5'- GCCAATCATAAGATGTTGCTGAAC-3'; TFAM: forward primer 5'- TTCCCAAGACTTCATTTCATTGTC-3', reverse primer 5'-GATGATTCGGCTCAGGGAAA-3'. Primers for TBP have been described previously (26).

Western blot analysis

Protein was extracted using lysis buffer (50 mM Tris, 1 mM EDTA, 1% NP40, 5 mM nicotinamide, 1 mM sodium butyrate, 150 mM KCl, protease inhibitors [pH 7.4]). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blocking and antibody incubations were performed in 5% BSA. Proteins were detected and quantified using the Odyssey from LI-COR Biosciences. Antibodies against OXPHOS were purchased from Abcam (Cambridge, United Kingdom). Beta-actin (A5316) was detected using an antibody from Sigma-Aldrich (St. Louis, MO).

NAD^+ determination

NAD^+ concentrations were determined using the enzyChrom™ NAD^+/NADH assay kit from BioAssay Systems (Hayward, CA) according to the manufacturers instructions.

ATP determination

ATP concentrations were determined using the CellTiter-Glo® Luminescent cell viability assay from Promega (Madison, WI) according to the manufacturer's instructions.

Clinical trials in humans

The ethics committee of Maastricht University Medical Center approved the study protocol (METC 16-30-19, NL58119.068.16, NCT02835664) and all volunteers provided written informed consent. Eight healthy overweight and obese men and postmenopausal women were recruited. Inclusion criteria were 45-65 years of age, BMI 27-35 kg/m^2, sedentary lifestyle (<3 h exercise per week), non-smoking for at least 6 months, no alcohol use of >2 servings per day, stable body weight for at least 6 months and no active diseases. A randomized controlled, double blinded, placebo controlled, cross-over study with NR supplementation was performed. Volunteers underwent 2 times a 6-week period with oral supplementation of either NR 1000 mg daily (NIAGEN, Chromadex) or placebo (capsules identical to NR in external appearance and number). Supplements were consumed with breakfast (500 mg) and lunch (500 mg). Measurements were performed on the last day of each supplementation period. Participants were fasted for at least 11 hours when entering the test facilities.

For the determination of cold-induced BAT activity, volunteers were wrapped in a water-perfused suit (ThermaWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne, Israel). First, volunteers remained at thermoneutral conditions (32 °C water) for 30 minutes, during which basal metabolic rate was measured by indirect calorimetry (IDEE, Maastricht Instruments, Maastricht, The Netherlands). Thereafter an individualized cooling procedure was started to determine (non-shivering thermogenesis) NST as described before (27). In brief, volunteers were cooled down in order to maximize NST after which energy expenditure was measured for 30 minutes. Following, 75 MBq of [18F]FDG was injected via the intravenous canula. Cold exposure was continued for 60 minutes, while volunteers were instructed to remain lying still. Next, the volunteers were unwrapped and underwent a static [18F]FDG-

PET/CT scan (Gemini TF PET-CT, Philips, The Netherlands). This consisted of a low-dose CT scan (120 kV, 30 mAs) followed by a PET scan. Six to seven bed positions (5 minutes per bed position) were used, to cover te area from the skull to the iliac crest. Tracer uptake was determined with the PET scan while the CT was used for attenuation correction and anatomical localization of the active BAT.

The scans were analyzed with PMOD software (version 3.0; PMOD Technologies). The regions of interest were manually outlined, while a threshold of 1.5 SUV (standardized uptake value) and Hounsfield units between -10 en -180 were used, as described previously by our group (27). Additionally fixed volumes (10 mm by 10 mm) were placed in the cervical adipose tissue behind the clavicula to measure general uptake values as described before (28). BAT activity was expressed in SUV ([18F]FDG uptake (kBq/ml/ (injected dose (kBq/patient weight (g)). The activity was determined as average SUV (SUVmean) and as total SUV (SUVmean times the volume of interest).

Statistics

Two means were compared using student's t-test, or with the Wilcoxon-signed rank test in case of non-parametric data. Comparison of multiple means was assessed by ANOVA. $p<0.05$ was considered statistically significant. Data is expressed as mean ± SEM. Analyses were performed using Graph Pad Prism (San Diego, CA).

Results

NR combined with cold exposure decreases adipocyte cell size in murine BAT without affecting lipolysis

NR supplementated mice were able to maintain a higher body temperature when faced with a a cold tolerance test (13) thus suggesting the involvement of BAT. Therefore, we here specifically investigated BAT by examining morphology and adipocyte cell size following NR supplementation and cold exposure (4 °C) in high fat diet-fed mice. As expected, acute cold decreased brown adipocyte cell size (Figure 1A and B). Brown adipocyte cell size also decreased following NR supplementation to HFD (Figure 1A and B). NR supplementation and cold combined resulted in the smallest adipocyte cell size (Figure 1A and B), thus potentially hinting towards increased lipolysis. In order to examine whether NR supplementation affected lipolysis in BAT in mouse, we performed ex vivo lipolysis experiments in mouse BAT harvested at room temperature and after acute cold exposure. At room temperature, supplementation of NR to a HFD did not alter lipolysis (Figure 1C). Also under conditions of acute cold exposure, supplementation of NR did not significantly change lipolysis in murine BAT (Figure 1C).

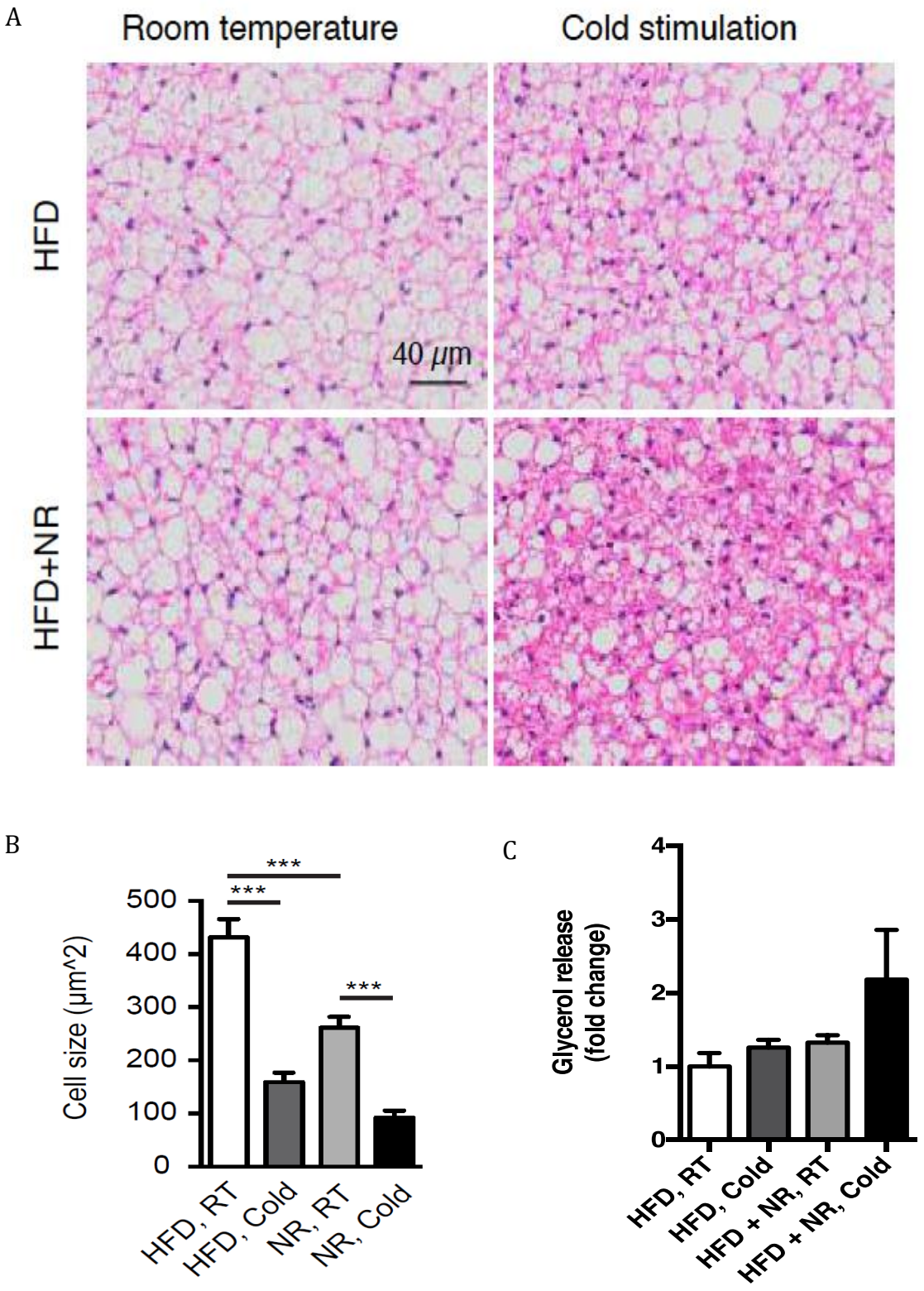


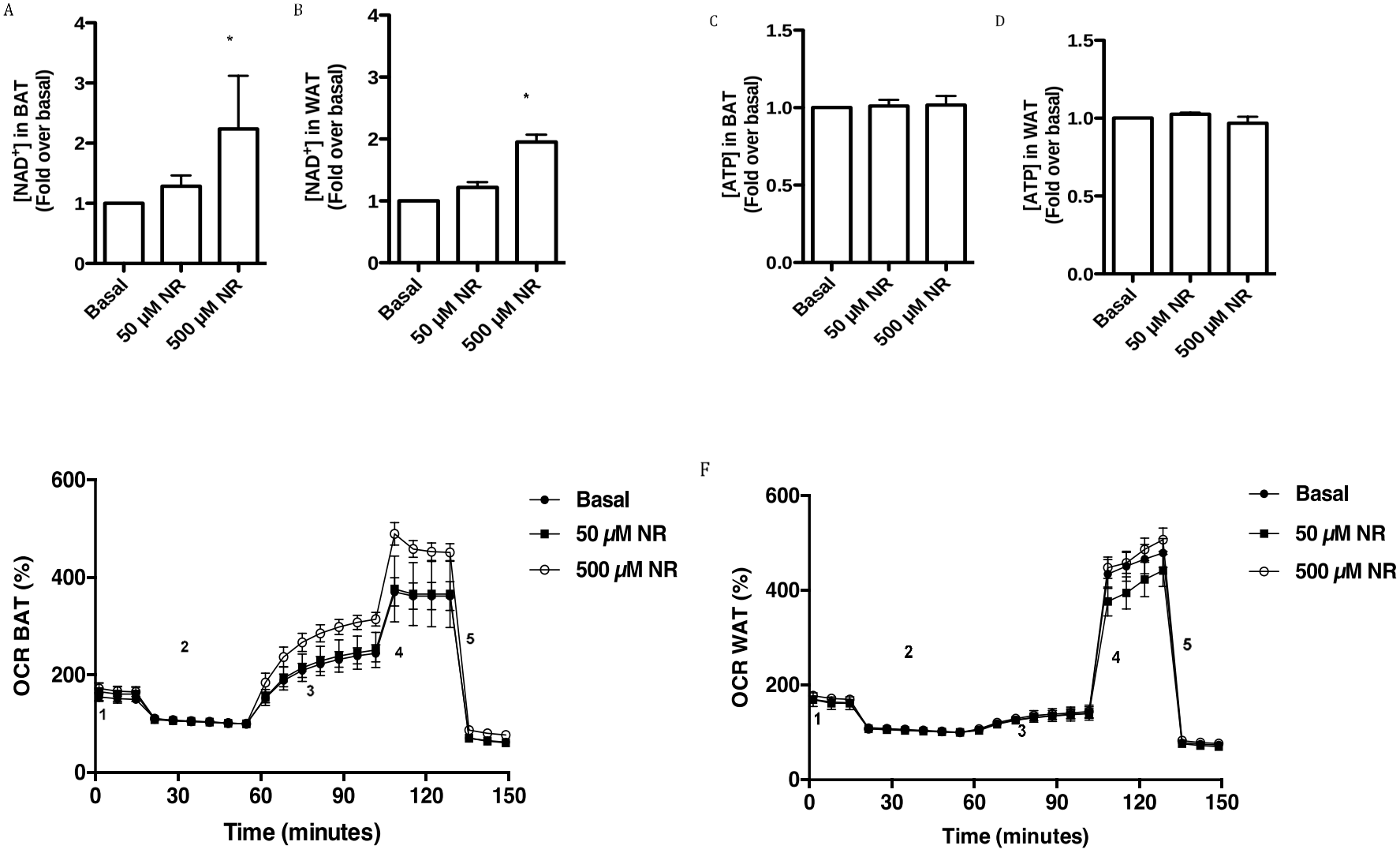
Figure 1. NR supplementation alters adipocyte cell size in mouse BAT following a high fat diet. Mice were fed a high fat diet (HFD) or a high fat diet supplemented with NR (HFD+NR). Following the 4 week diet, animals were acutely exposed to cold (cold) or remained at room temperature (RT). A. HE staining of BAT. B. Cell size quantification of results obtained in panel A. *: $p<0.05$ (n=5-7). C. Ex vivo lipolysis was performed on BAT explants (n=2-3). Data is expressed as mean ± SEM.

NR stimulates NE-stimulated mitochondrial uncoupling only in adipocytes derived from human BAT

To investigate if the positive effects of NR on BAT morphology in mouse can be translated to humans, we incubated cultured adipocytes derived from human BAT and WAT with 50 or 500 µM NR to examine effects on NE-induced mitochondrial uncoupling. Whereas 50 µM NR did not increase NAD^+ levels, 500 µM NR significantly increased NAD^+ levels in adipocytes derived from human BAT and WAT (Figure 2A and B). Both doses of NR were without effect on ATP concentrations in adipocytes derived from human BAT or WAT (Figure 2C and D). To further investigate the effect on mitochondrial uncoupling in adipocytes derived from BAT and WAT, cultured adipocytes were treated with oligomycin to block ATPase and subsequently stimulated with NE. NE enhanced mitochondrial uncoupling in adipocytes derived from BAT, while this response was negligible in adipocytes derived from human WAT (Figure 2E and F). 50 µM and 500 µM NR were unable to potentiate NE-stimulated mitochondrial uncoupling in WAT (Figure 2F and H). In human primary brown adipocytes however, 500 µM NR increased NE-stimulated mitochondrial uncoupling (Figure 2E and G), as well as maximal FCCP-induced mitochondrial respiration (Figure 2E and G). At 500 µM NR, antimycin A and rotenone resulted in higher OCR compared to 0 µM NR and 50 µM NR.

NR does not affect lipolysis in cultured adipocytes derived from human BAT and WAT

To investigate whether lipolysis plays a pivotal role in NR-stimulated mitochondrial uncoupling in human BAT cells, we examined lipolysis following NR treatment in cultured adipocytes derived from human BAT and WAT. Both basal and beta-adrenergic NE-stimulated lipolysis was unchanged following NR treatment in adipocytes derived from human BAT and WAT (Figure 2I and J).


A
[NAD+] in BAT (Fold over basal)
Basal
50 μM NR
500 μM NR
B
[NAD+] in WAT (Fold over basal)
C
[ATP] in BAT (Fold over basal)
D
[ATP] in WAT (Fold over basal)
E
OCR BAT (%)
Time (minutes)
Basal
50 μM NR
500 μM NR
F
OCR WAT (%)

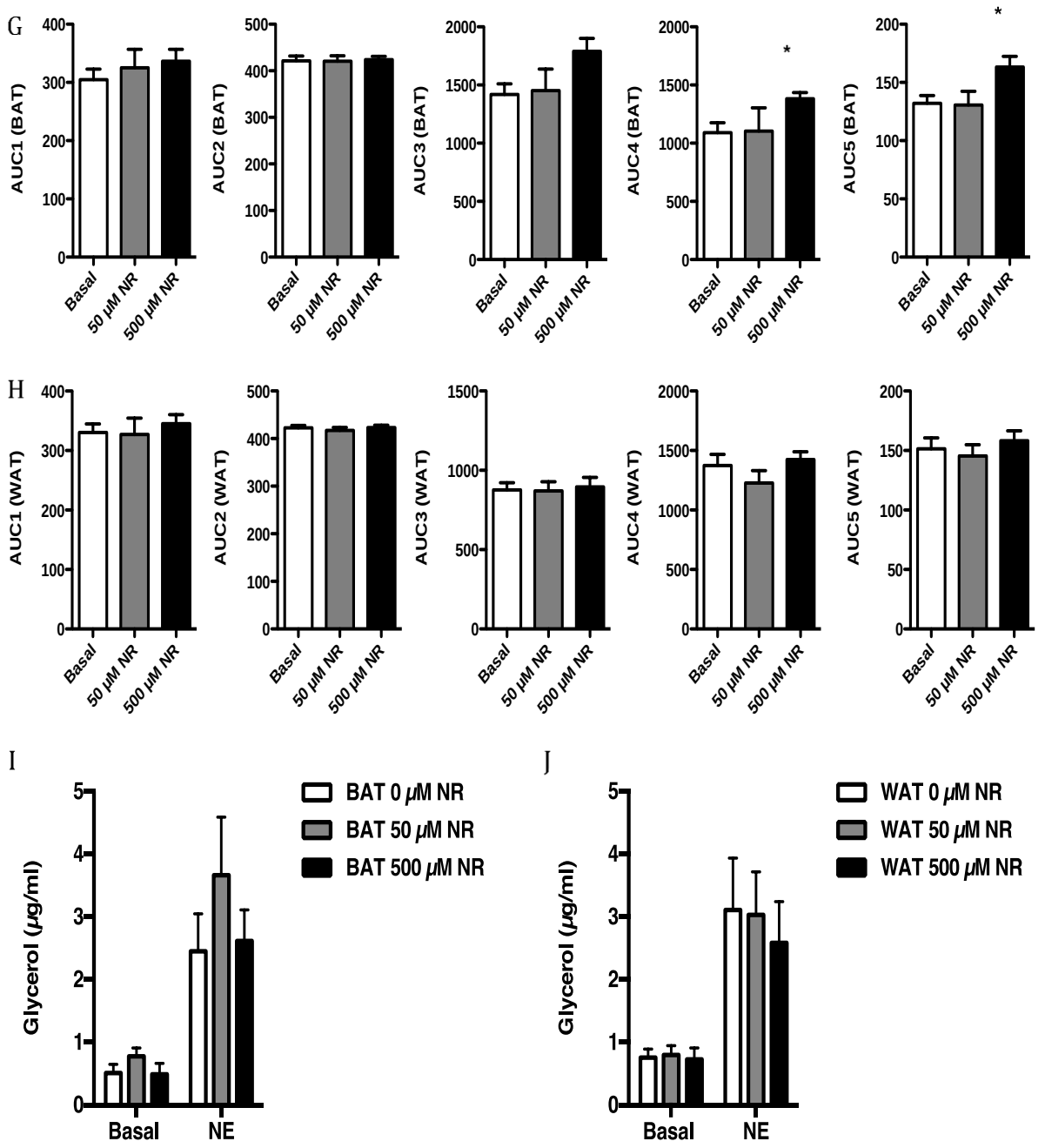
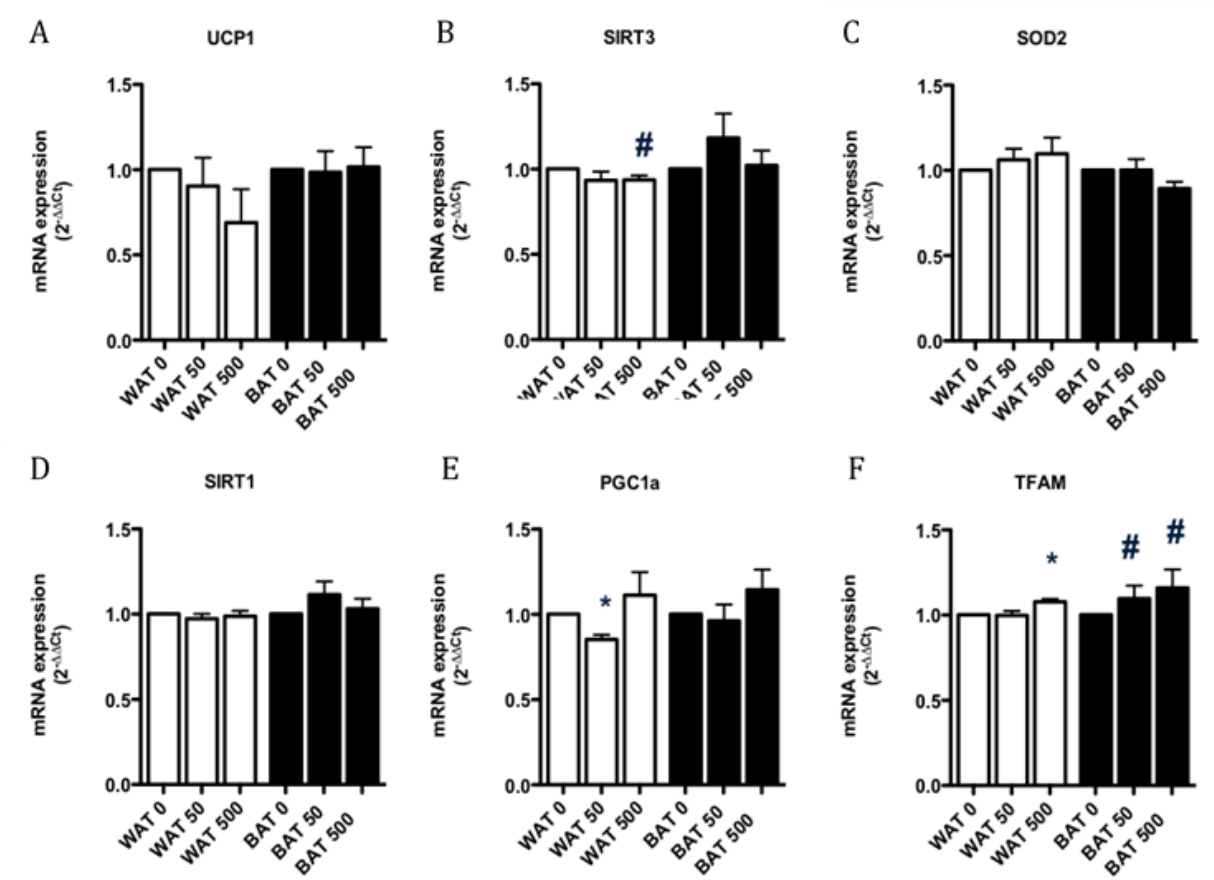


Figure 2. NR stimulates mitochondrial uncoupling in cultured primary adipocytes derived from human BAT. NAD^+ (A, B) and ATP (C, D) levels were determined in cultured adipocytes derived from human BAT or WAT after 24 hours with 0 µM, 50 µM or 500 µM NR. Data is expressed as a fold increase compared to 0 µM NR. Cellular respiration was measured using the Seahorse bioanalyzer in cultured adipocytes derived from human BAT (E) and WAT (F) following 24 hour incubation with 0 µM, 50 µM or 500 µM NR. Mitochondrial respiration was measured following injections with oligomycin, NE, FCCP and antimycin A+rotenone. G. Quantification of AUC from results in panel E. H. Quantification of AUC from results in panel F. NR does not alter lipolysis in cultured adipocytes derived from human BAT. Cultured adipocytes derived from human BAT (I) and WAT (J) were incubated for 24 hours with 0 µM, 50 µM or 500 µM NR. Following adipocytes were stimulated with NE in order to measure glycerol release as a marker of lipolysis. Data is expressed as mean ± SEM. *: $p<0.05$ (n=4-6)

NR does not alter SIRT-associated genes or OXPHOS protein expression in adipocytes derived from human BAT

SIRTs are NAD^+-dependent deacetylases and NR stimulates SIRT activity by increasing the level of its obligatory co-substrate NAD^+ (29). Next we assessed whether NR was able to alter gene expression of SIRT-related pathways. Adipocytes derived from human BAT and WAT were incubated with vehicle, 50 µM NR or 500 µM NR. In cultured adipocytes derived from human WAT, 50 µM NR decreased PGC1A gene expression (Figure 3E) and 500 µM NR increased transcript levels of TFAM (Figure 3F) compared to vehicle. In cultured adipocytes derived from human BAT, NR did not significantly change the transcript levels of UCP1, SIRT3, SOD2, PGC1A or TFAM (Figure 3A-F). We also determined protein expression of OXPHOS by western blot analysis examining the individual complexes in adipocytes derived from human BAT and WAT. The highest concentration of 500 µM NR decreased the amount of complex I (Figure 3G) in adipocytes derived from human BAT, while 50 µM NR increased protein abundance of complex I in adipocytes derived from human WAT (Figure 3H).



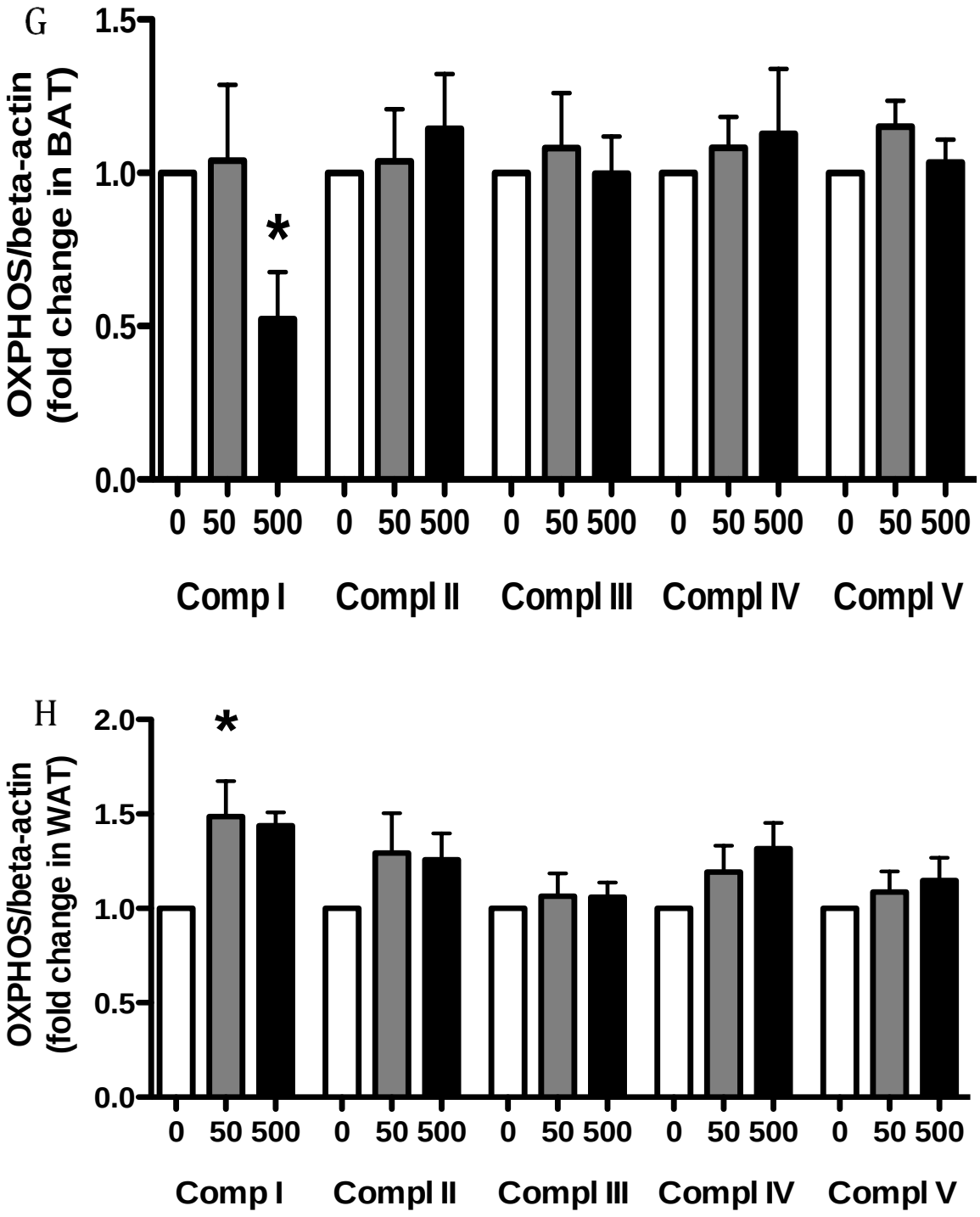


Figure 3. NR does not alter expression of SIRT targets in cultured primary adipocytes derived from human BAT. Cultured adipocytes derived from human BAT (black bars) and WAT (white bars) were stimulated for 24 hours with 0 µM (0), 50 µM (50) or 500 µM (500) NR. Gene expression of UCP1 (A), SIRT3 (B), SOD2 (C), SIRT1 (D), PGC1A (E) and TFAM (F) were determined using qPCR techniques. Cultured adipocytes derived from human BAT (G) and WAT (H) were stimulated for 24 hours with 0 µM (0), 50 µM (50) or 500 µM (500) NR. OXPHOS complexes were analyzed by Western blot. Data is expressed as mean ± SEM. *: $p<0.05$ compared to matching control (n=4).

NR does not affect BAT activity or energy expenditure in human volunteers

Our results show that NR can enhance BAT activity in human primary brown adipocytes. These promising results urged us to examine the true translational potential of NR on human BAT and therefore we performed a first double-blind placebo-controlled cross-over design clinical trial in a small cohort of volunteers with obesity. During 6 weeks, 1000 mg/day NR or placebo was supplemented. Cold-stimulated BAT activity was determined via uptake of [18F]FDG using PET-CT and whole body energy expenditure was also measured. However, NR supplementation for 6 weeks had no effect on [18F]FDG mediated glucose uptake accessed as SUVmean (Figure 4A) and SUVmax (Figure 4B) in fixed volumes. Cold exposure was able to significantly increase energy expenditure in human volunteers (Figure 4C and D). However the increase in energy expenditure following cold was similar in human volunteers receiving placebo or human volunteers receiving NR (Figure 4C and D). Also non-shivering thermogenesis was unchanged following NR supplementation (Figure 4E). The respiratory quotient, reflecting substrate oxidation did lower during cold exposure, both in the placebo (from 0.81 to 0.79) and NR (from 0.79 to 0.78) supplementation periods. There was no significant difference between the hange in RQ value between these periods.

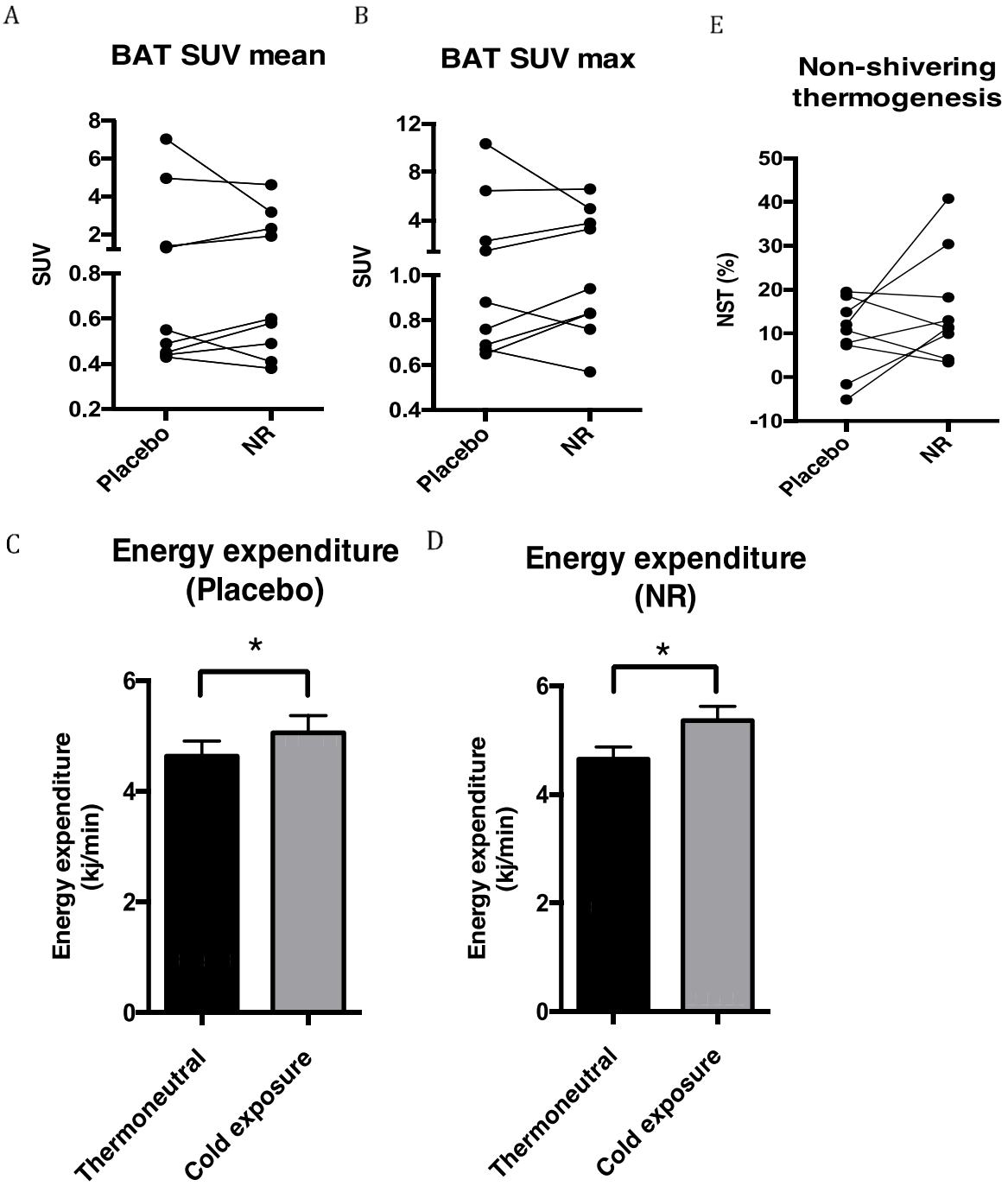


Figure 4. NR does not stimulate energy expenditure or BAT activity in humans. Human volunteers were supplemented with 1000 mg/day NR for 6 weeks in a placebo-controlled cross over design. BAT SUV mean (A) and BAT SUV max in fixed volumes (B) following cold-exposure. Energy expenditure at thermoneutrality and after cold exposure following placebo (C) or NR (D). Non-shivering thermogenesis (NST) following NR supplementation. Data is expressed as mean ± SEM. *: $p<0.05$ (n=9)

Discussion

Preclinical data has demonstrated that increasing NAD^+ levels can counteract harmfull effects of metabolic disease, and NR supplementation is a powerful intervention to do so. Here, we specifically assessed the effect of NR, on mouse BAT morphology and on adipocytes derived from human BAT and WAT in vitro and in vivo in human volunteers. In mice, NR supplementation combined with beta-adrenergic cold-exposure decreased brown adipocyte cell size (Figure 1). Similarly, in cultured adipocytes derived from human BAT, elevating NAD^+ levels via NR enhanced beta-adrenergic NE-mediated mitochondrial uncoupling (Figure 2). This stimulatory effect was specific for adipocytes derived from human BAT, which is in line with earlier observation in animals where NAD^+ supplemention increased NAD^+ concentrations in BAT and stimulated BAT metabolism following a cold challenge (13). However, 6 weeks of supplementation with 1000 mg/day NR did not increase cold-stimulated BAT activity in humans. These observations together show that NR and beta-adrenergic signaling can work together to enhance BAT activity, however the current dose of NR was unable to increase BAT activity in humans.

We examined lipolysis ex vivo in murine BAT and in vitro in human BAT in the context of NR stimulation. There are obvious differences when comparing murine adipose tissue explants to in vitro differentiated human adipocytes. However, in vitro lipolysis in adipocytes derived from human BAT was unaltered following NR (Figure 2I) and also ex vivo lipolysis in mouse BAT following NR exposure was unaffected (Figure 1C). This indicates that the direct mode of action of NR is most likely independent of lipolysis in mice and humans, however further measures of intracellular lipolysis should be explored. A strong link has been suggested between BAT and WAT as BAT volumes correlate with whole body lipolysis (30) which is mostly mediated by WAT. In our study we were unable to assess WAT metabolism in human volunteers, however this could provide explanations in the future why we were unable to detect an increase in BAT activity in human volunteers. Previously, it has been reported that NR increases NAD^+ content in cells and mitochondria (13) and mitochondria are pivotal players in providing energy for lipolysis. In line with unaltered lipolysis in human and murine BAT, we also observed no change in OXPHOS protein, ATP levels or gene expression related to mitochondrial biogenesis in cultured adipocytes derived from human BAT.

mRNA abundance of the classical mitochondrial marker for BAT, UCP1, was unchanged following NR treatment in cultured adipocytes derived from human BAT. However, when it comes to UCP1 expression and NR, different results have been obtained depending on the experimental setup. In young lean mice on a chow diet supplemented for 5 weeks with NR, UCP1 protein content was increased in BAT, however, in this study there was no mention of total BAT mass (31). In another study, NR was administered directly after birth in mice which was subsequently followed by a HFD-challenge for 10 weeks. UCP1 mRNA expression was unaffected in animals on a HFD in BAT, but interestingly the animals on a control diet showed

decreased expression of UCP1 mRNA expression following NR in BAT (32). These findings together indicate that gene and/or protein expression alone might not be the best indicator for BAT metabolic activity thus therefore we purposely included metabolic readouts for BAT activity in vitro and in vivo. Next to UCP1-dependent pathways, several UCP1-independent pathways have been described. First UCP1-independent pathway revolves around insulin. Beta-adrenergic cold stimulation triggers insulin release resulting in lipolysis in order to refuel the activated BAT in mice (33) however whether this mechanism is present in humans warrants further investigation. Second UCP1-independent pathways is based on calcium cycling. Specifically in beige adipocytes calcium cycling is regulated through SERCA2B and RYR2 (34) resulting in fuel for thermogenesis. The third UCP1-independent driven pathway is creatine-dependent ADP/ATP substrate cycling (35) resulting in increased thermogenesis. These UCP1-independent pathways can be of importance when examining human NR-stimulated (in vitro) BAT activity, especially since human BAT resembles murine beige adipose tissue (26,36).

Stimulating BAT activity by other means than cold in humans could have beneficial effects on whole body metabolism. Human BAT activity shows a negative correlation with obesity (4,37), and stimulation of BAT activity through cold acclimatization increases energy expenditure that potentially could lead to loss of fat mass (38). Furthermore, cold-induced BAT activity is related to increased insulin sensitivity in humans (39,40). Cold exposure is the most effective method to stimulate BAT activity; however, not the most practical one. Therefore, alternative routes to stimulate BAT in humans are warranted. In the current study, NR was able to stimulate BAT activity in human primary brown adipocytes. However, we could not confirm these results in a clinical trial in humans which is in line with other clinical trials using NR (41-43). The reason why NR had beneficial effects in human primary brown adipocytes but not in vivo in humans, can not be deduced from the current study. Based on our research, 6 weeks of NR supplementation in healthy volunteers with obesity did not increase NAD or mitochondrial function in skeletal muscle, however the NAD^+ metabolites nicotinic acid adenine dinucleotide and methylnicotinamide were increased thus demonstrating enhanced NAD^+ metabolism following NR (44). SIRTs are targets for NR/NAD^+, however metabolic disease can alter SIRT expression. For example, NAFLD is associated with lower SIRT expression in human liver (45) and obesity is associated with lower SIRT expression in human WAT (46). This could potentially mean that higher concentrations or longer duration of NR supplementation are needed when examining a human cohort with obesity to see beneficial effects on human BAT. In the future, translational research will be crucial when employing other NAD^+ boosting strategies (eg. nicotinamide mononucleotide (47) and dihydronicotinamide riboside (48)) to increase BAT activity. Therefore, further translational research is needed in order to better understand the differences in NAD^+ metabolism between mouse and man and in vitro and in vivo.

References

1. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiological reviews. 2004;84(1):277-359.
2. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR. Identification and importance of brown adipose tissue in adult humans. The New England journal of medicine. 2009;360(15):1509-1517.
3. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, Iwanaga T, Miyagawa M, Kameya T, Nakada K, Kawai Y, Tsujisaki M. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes. 2009;58(7):1526-1531.
4. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJ. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-1508.
5. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P. Functional brown adipose tissue in healthy adults. The New England journal of medicine. 2009;360(15):1518-1525.
6. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, Scheinin M, Taittonen M, Niemi T, Enerback S, Virtanen KA. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. Cell metabolism. 2011;14(2):272-279.
7. Villarroya F, Vidal-Puig A. Beyond the sympathetic tone: the new brown fat activators. Cell Metab. 2013;17(5):638-643.
8. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature. 2006;439(7075):484-489.
9. Broeders EP, Nascimento EB, Havekes B, Brans B, Roumans KH, Tailleux A, Schaart G, Kouach M, Charton J, Deprez B, Bouvy ND, Mottaghy F, Staels B, van Marken Lichtenbelt WD, Schrauwen P. The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity. Cell metabolism. 2015;22(3):418-426.
10. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, English J, Chatman K, Trauger SA, Doria A, Kolodny GM. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. Cell metabolism. 2015;21(1):33-38.
11. Canto C, Menzies KJ, Auwerx J. NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus. Cell Metab. 2015;22(1):31-53.
12. Verdin E. NAD(+) in aging, metabolism, and neurodegeneration. Science. 2015;350(6265):1208-1213.
13. Canto C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, Fernandez-Marcos PJ, Yamamoto H, Andreux PA, Cettour-Rose P, Gademann K, Rinsch C, Schoonjans K, Sauve AA, Auwerx J. The NAD(+) precursor nicotinamide riboside enhances oxidative

metabolism and protects against high-fat diet-induced obesity. Cell Metab. 2012;15(6):838-847.
14. Yang T, Chan NY, Sauve AA. Syntheses of nicotinamide riboside and derivatives: effective agents for increasing nicotinamide adenine dinucleotide concentrations in mammalian cells. Journal of medicinal chemistry. 2007;50(26):6458-6461.
15. Trammell SA, Weidemann BJ, Chadda A, Yorek MS, Holmes A, Coppey LJ, Obrosov A, Kardon RH, Yorek MA, Brenner C. Nicotinamide Riboside Opposes Type 2 Diabetes and Neuropathy in Mice. Scientific reports. 2016;6:26933.
16. Vaur P, Brugg B, Mericskay M, Li Z, Schmidt MS, Vivien D, Orset C, Jacotot E, Brenner C, Duplus E. Nicotinamide riboside, a form of vitamin B3, protects against excitotoxicity-induced axonal degeneration. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017.
17. Sorrentino V, Romani M, Mouchiroud L, Beck JS, Zhang H, D'Amico D, Moullan N, Potenza F, Schmid AW, Rietsch S, Counts SE, Auwerx J. Enhancing mitochondrial proteostasis reduces amyloid-beta proteotoxicity. Nature. 2017;552(7684):187-193.
18. Gerasimenko M, Cherepanov SM, Furuhara K, Lopatina O, Salmina AB, Shabalova AA, Tsuji C, Yokoyama S, Ishihara K, Brenner C, Higashida H. Nicotinamide riboside supplementation corrects deficits in oxytocin, sociability and anxiety of CD157 mutants in a mouse model of autism spectrum disorder. Sci Rep. 2020;10(1):10035.
19. Yang Q, Cong L, Wang Y, Luo X, Li H, Wang H, Zhu J, Dai S, Jin H, Yao G, Shi S, Hsueh AJ, Sun Y. Increasing ovarian NAD(+) levels improve mitochondrial functions and reverse ovarian aging. Free Radic Biol Med. 2020;156:1-10.
20. Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, D'Amico D, Ropelle ER, Lutolf MP, Aebersold R, Schoonjans K, Menzies KJ, Auwerx J. NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. Science. 2016;352(6292):1436-1443.
21. Ryu D, Zhang H, Ropelle ER, Sorrentino V, Mazala DA, Mouchiroud L, Marshall PL, Campbell MD, Ali AS, Knowels GM, Bellemin S, Iyer SR, Wang X, Gariani K, Sauve AA, Canto C, Conley KE, Walter L, Lovering RM, Chin ER, Jasmin BJ, Marcinek DJ, Menzies KJ, Auwerx J. NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation. Science translational medicine. 2016;8(361):361ra139.
22. Khan NA, Auranen M, Paetau I, Pirinen E, Euro L, Forsstrom S, Pasila L, Velagapudi V, Carroll CJ, Auwerx J, Suomalainen A. Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3. EMBO molecular medicine. 2014;6(6):721-731.
23. Gariani K, Menzies KJ, Ryu D, Wegner CJ, Wang X, Ropelle ER, Moullan N, Zhang H, Perino A, Lemos V, Kim B, Park YK, Piersigilli A, Pham TX, Yang Y, Ku CS, Koo SI, Fomitchova A, Canto C, Schoonjans K, Sauve AA, Lee JY, Auwerx J. Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice. Hepatology. 2016;63(4):1190-1204.
24. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J. Resveratrol improves

mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell. 2006;127(6):1109-1122.
25. Gnad T, Scheibler S, von Kugelgen I, Scheele C, Kilic A, Glode A, Hoffmann LS, Reverte-Salisa L, Horn P, Mutlu S, El-Tayeb A, Kranz M, Deuther-Conrad W, Brust P, Lidell ME, Betz MJ, Enerback S, Schrader J, Yegutkin GG, Muller CE, Pfeifer A. Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature. 2014;516(7531):395-399.
26. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerback S, Schrauwen P, Spiegelman BM. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell. 2012;150(2):366-376.
27. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, Hansen J, Jorgensen JA, Wu J, Mottaghy FM, Schrauwen P, van Marken Lichtenbelt WD. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. The Journal of clinical investigation. 2013;123(8):3395-3403.
28. Vosselman MJ, Brans B, van der Lans AA, Wierts R, van Baak MA, Mottaghy FM, Schrauwen P, van Marken Lichtenbelt WD. Brown adipose tissue activity after a high-calorie meal in humans. The American journal of clinical nutrition. 2013;98(1):57-64.
29. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nature reviews Molecular cell biology. 2012;13(4):225-238.
30. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, Yfanti C, Chao T, Wong D, Shinoda K, Labbe SM, Hurren NM, Cesani F, Kajimura S, Sidossis LS. Brown Adipose Tissue Activation Is Linked to Distinct Systemic Effects on Lipid Metabolism in Humans. Cell Metab. 2016;23(6):1200-1206.
31. Crisol BM, Veiga CB, Lenhare L, Braga RR, Silva VRR, da Silva ASR, Cintra DE, Moura LP, Pauli JR, Ropelle ER. Nicotinamide riboside induces a thermogenic response in lean mice. Life sciences. 2018.
32. Serrano A, Asnani-Kishnani M, Rodriguez AM, Palou A, Ribot J, Bonet ML. Programming of the Beige Phenotype in White Adipose Tissue of Adult Mice by Mild Resveratrol and Nicotinamide Riboside Supplementations in Early Postnatal Life. Molecular nutrition & food research. 2018:e1800463.
33. Heine M, Fischer AW, Schlein C, Jung C, Straub LG, Gottschling K, Mangels N, Yuan Y, Nilsson SK, Liebscher G, Chen O, Schreiber R, Zechner R, Scheja L, Heeren J. Lipolysis Triggers a Systemic Insulin Response Essential for Efficient Energy Replenishment of Activated Brown Adipose Tissue in Mice. Cell Metab. 2018;28(4):644-655 e644.
34. Ikeda K, Kang Q, Yoneshiro T, Camporez JP, Maki H, Homma M, Shinoda K, Chen Y, Lu X, Maretich P, Tajima K, Ajuwon KM, Soga T, Kajimura S. UCP1-independent signaling involving SERCA2b-mediated calcium cycling regulates beige fat thermogenesis and systemic glucose homeostasis. Nat Med. 2017;23(12):1454-1465.
35. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, Vetrivelan R, Lu GZ, Laznik-Bogoslavski D, Hasenfuss SC, Kajimura S, Gygi SP, Spiegelman BM. A

creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. Cell. 2015;163(3):643-655.
36. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, de Jong J, Mathur N, Cannon B, Nedergaard J, Pedersen BK, Moller K, Scheele C. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. Cell Metab. 2013;17(5):798-805.
37. Nascimento EBM, Sparks LM, Divoux A, van Gisbergen MW, Broeders EPM, Jorgensen JA, Schaart G, Bouvy ND, van Marken Lichtenbelt WD, Schrauwen P. Genetic Markers of Brown Adipose Tissue Identity and In Vitro Brown Adipose Tissue Activity in Humans. Obesity. 2018;26(1):135-140.
38. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, Iwanaga T, Saito M. Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest. 2013;123(8):3404-3408.
39. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, Mottaghy FM, Schrauwen P, van Marken Lichtenbelt WD. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-1189.
40. Iwen KA, Backhaus J, Cassens M, Waltl M, Hedesan OC, Merkel M, Heeren J, Sina C, Rademacher L, Windjager A, Haug AR, Kiefer FW, Lehnert H, Schmid SM. Cold-Induced Brown Adipose Tissue Activity Alters Plasma Fatty Acids and Improves Glucose Metabolism in Men. The Journal of clinical endocrinology and metabolism. 2017;102(11):4226-4234.
41. Dollerup OL, Trammell SAJ, Hartmann B, Holst JJ, Christensen B, Moller N, Gillum MP, Treebak JT, Jessen N. Effects of Nicotinamide Riboside on Endocrine Pancreatic Function and Incretin Hormones in Nondiabetic Men With Obesity. J Clin Endocrinol Metab. 2019;104(11):5703-5714.
42. Dollerup OL, Christensen B, Svart M, Schmidt MS, Sulek K, Ringgaard S, Stodkilde-Jorgensen H, Moller N, Brenner C, Treebak JT, Jessen N. A randomized placebo-controlled clinical trial of nicotinamide riboside in obese men: safety, insulin-sensitivity, and lipid-mobilizing effects. Am J Clin Nutr. 2018;108(2):343-353.
43. Dollerup OL, Chubanava S, Agerholm M, Sondergard SD, Altintas A, Moller AB, Hoyer KF, Ringgaard S, Stodkilde-Jorgensen H, Lavery GG, Barres R, Larsen S, Prats C, Jessen N, Treebak JT. Nicotinamide riboside does not alter mitochondrial respiration, content or morphology in skeletal muscle from obese and insulin-resistant men. J Physiol. 2020;598(4):731-754.
44. Remie CME, Roumans KHM, Moonen MPB, Connell NJ, Havekes B, Mevenkamp J, Lindeboom L, de Wit VHW, van de Weijer T, Aarts S, Lutgens E, Schomakers BV, Elfrink HL, Zapata-Perez R, Houtkooper RH, Auwerx J, Hoeks J, Schrauwen-Hinderling VB, Phielix E, Schrauwen P. Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans. Am J Clin Nutr. 2020.
45. Wu T, Liu YH, Fu YC, Liu XM, Zhou XH. Direct evidence of sirtuin downregulation in the liver of non-alcoholic fatty liver disease patients. Ann Clin Lab Sci. 2014;44(4):410-418.

46. Martinez-Jimenez V, Cortez-Espinosa N, Rodriguez-Varela E, Vega-Cardenas M, Briones-Espinoza M, Ruiz-Rodriguez VM, Lopez-Lopez N, Briseno-Medina A, Turijan-Espinoza E, Portales-Perez DP. Altered levels of sirtuin genes (SIRT1, SIRT2, SIRT3 and SIRT6) and their target genes in adipose tissue from individual with obesity. Diabetes Metab Syndr. 2019;13(1):582-589.
47. Yoshino J, Baur JA, Imai SI. NAD(+) Intermediates: The Biology and Therapeutic Potential of NMN and NR. Cell Metab. 2018;27(3):513-528.
48. Yang Y, Mohammed FS, Zhang N, Sauve AA. Dihydronicotinamide riboside is a potent NAD(+) concentration enhancer in vitro and in vivo. J Biol Chem. 2019;294(23):9295-9307.

Chapter 4
Non-shivering cold acclimation does not improve postprandial metabolism or insulin sensitivity

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Abstract

Background & Aims

Type 2 diabetes is a major health problem and is accompanied with increased cardiovascular disease risk. We showed that ten days of cold acclimation with non-shivering thermogenesis (NST) improved skeletal muscle insulin sensitivity in type 2 diabetes. Here, we aimed to investigate whether cold acclimation with NST beneficially affected postprandial metabolism and cardiovascular risk markers, and if the effect on insulin sensitivity was sustained for a longer time.

Materials & Methods

Nine obese participants diagnosed with non-insulin dependent type 2 diabetes were exposed to mild cold (16-17 °C) for ten days. Before and immediately after the cold exposure postprandial glucose and lipid metabolism, vascular function measurements, insulin sensitivity and intrahepatic lipid content were determined. Furthermore skeletal muscle biopsies were obtained. Lastly, insulin sensitivity was measured again ten days later after acclimation to assess the sustained effect of cold acclimation.

Results

Based on direct observations, questionnaire results and gene expression analyses in muscle biopsies, no shivering occured during the ten-day cold acclimation. Cold acclimation with NST had no effect on postprandial glucose and lipid metabolism, intrahepatic lipid content or other vascular function markers. Surprisingly, insulin sensitivity was unaffected both when measured directly (one day) or ten days after the last cold exposure.

Conclusion

A ten-day cold acclimation period at 16-17 °C under non-shivering conditions resulted in mild effects on substrate oxidation and cardiovascular risk markers. The lack of marked effects in the current study may be attributed to the absence of shivering compared to previous studies.

Introduction

Mortality rates in type 2 diabetes mellitus (T2DM) patients are approximately twice as high compared to individuals without T2DM and can mostly be attributed to an increased risk of coronary heart diseases (1, 2). In the last decade, cold exposure as a tool to alleviate insulin resistance has attracted lots of scientific interest. Nowadays, there is little demand for the human body to adjust to colder temperatures, since humans nowadays spend most of their time in a well-controlled indoor environment with optimal temperatures within the body's thermoneutral zone. The physiological reaction of the human body to cold exposure includes simultaneously 1) insulative responses by peripheral vasoconstriction and 2) an increase in metabolic rate by shivering thermogenesis (ST) and/or non-shivering thermogenesis (NST). It has been shown that during daily cold exposure in humans, shivering gradually decreases within 10-20 days, while the related increase in metabolic rate remains at a stable level (3-5). This indicates that acclimation to cold occurs and that ST can be replaced by NST. Both brown adipose tissue (BAT) (6-9) and skeletal muscle (4, 10) have been identified as contributors to NST.

Cold acclimation has been shown to promote insulin sensitivity in humans, originally attributed to increased BAT activity (11, 12). Research performed within our group, however, has shown that ten days of cold acclimation (14-15 °C) markedly improved skeletal muscle insulin sensitivity by 43% in patients with type 2 diabetes (13), an improvement that is comparable to the effect seen after long term exercise training (14). Interestingly, the cold induced improvement of insulin sensitivity did not originate form BAT activation but was associated with increased GLUT4 translocation in skeletal muscle (13).

Cold exposure may also impact other aspects of metabolic health, such as postprandial metabolism. Besides hyperglycemia, dyslipidemia is a common metabolic abnormality in patients with T2DM (15). Interestingly, animal studies have shown reduced postprandial lipids upon prolonged cold exposure (16). Moreover, a postprandial reduction in hypertriglyceridaemia in human individuals was associated with BAT (17). Lowering postprandial hyperglycemia and hypertriglyceridemia is clinically relevant as high plasma glucose and triglyeride levels can cause damage to the vascular wall inducing an impaired vascular function (18), which causes artherosclerotic plaque development that may ultimately lead cardiovascular disease events. However, the effect of cold exposure on these cardiovascular risk markers has not yet been investigated in humans.

Therefore, we primarily aimed to investigate the effect of ten days of cold acclimation with NST in overweight and obese patients with T2DM on postprandial glucose and lipid metabolism and cardiovascular risk markers. Secondly, we investigated whether a ten-day cold acclimation period with NST affects insulin sensitivity, and if so, if this effect is sustained

for another ten days at room temperature. Given the previous results on skeletal muscle (13), we also aimed to test the hypothesis that beneficial effects of cold exposure can be truly achieved by stimulating NST, and hence took specific care to prevent shivering in our participants.

Results

Subject characteristics

Nine obese men and women (age 65 ± 5 years; BMI 32.1 ± 2.8 kg/m^2; four women) participated in the study (see Table 1). In line with the inclusion criteria, participants were diagnosed for at least one year with type 2 diabetes, and were treated with oral medication only (see Supplementary Table 1). Participants were non-smokers, had no other active diseases and had a sedentary lifestyle according to the Baecke questionnaire score (7.94 ± 2.00, Table 1).

Table 1. Participant characteristics

Parameter	**Mean ± SD**
Gender F/M	4/5
Age (years)	65 ± 5
Body weight (kg)	93.7 ± 17.3
Height (m)	1.70 ± 0.10
BMI (kg/m^2)	32.1 ± 2.8
HbA1c (%)	7.3 ± 0.7
TG (mmol/L)	1.54 ± 0.34
ASAT (U/L)	24 ± 8
ALAT (U/L)	37 ± 19
GGT (U/L)	34 ± 13
eGFR (ml/min/1.73mm^2)	79 ± 9
Physical activity level (Baecke score)	7.51 ± 1.16

Abbreviations: BMI, body mass index; HbA1c, hemoglobin A1c; TG, triglycerides; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma-glutamyl transferase; eGFR, estimated glomerular filtration rate according CKD-EPI method.

Supplementary table 1. Subject characteristics regarding diabetes

Participant	Diabetes duration (years)	Diabetes medication
1	15	Metformin 850mg, 3x/day Gliclazide 80mg, 2x/day
2	14	Metformin 500mg, 2x/day
3	15	Metformin 1000mg, 2x/day Gliclazide 30mg, 2x/day
4	6	Metformin 1000mg, 3x/day
5	8	Metformin 850mg 1x/day Glimepiride 2mg, 1x/day Liraglutide 6mg, 18eh, 1x/day
6	5	Metformin 850mg, 2x/day
7	9	Metformin 500mg, 2x/day
8	7	Metformin 500mg, 3x/day Gliclazide 30mg, 1x/day
9	1	Gliclazide 80mg, 1x/day

Body and room temperature, thermal comfort and shivering during cold acclimation

Room temperature during cold acclimation was on average 16.4 ± 0.30 °C, which was approximately 1.4 °C higher compared to the previous study by Hanssen et al (13). Average skin temperature dropped from 27.5 ± 0.33 °C to 26.3 ± 0.97 °C during day 3. This was similar to the drop in average skin temperature from 27.4 ± 0.80 °C to 26.4 ± 1.26 °C on day 10. Thermal sensation and thermal comfort, assessed via VAS scales, was not significantly different between day 3 and day 10 of cold acclimation, as shown in Supplementary Figure 1. The temperature was progressively perceived colder and more uncomfortable over time. The shivering questionnaires revealed that participants experienced no shivering and only occasionally reported tense muscles. Self-reported shivering intensity was less than described in the study from Hanssen et al (13) and were not significant different between day 3 and day 10 of cold acclimation (Figure 6A).

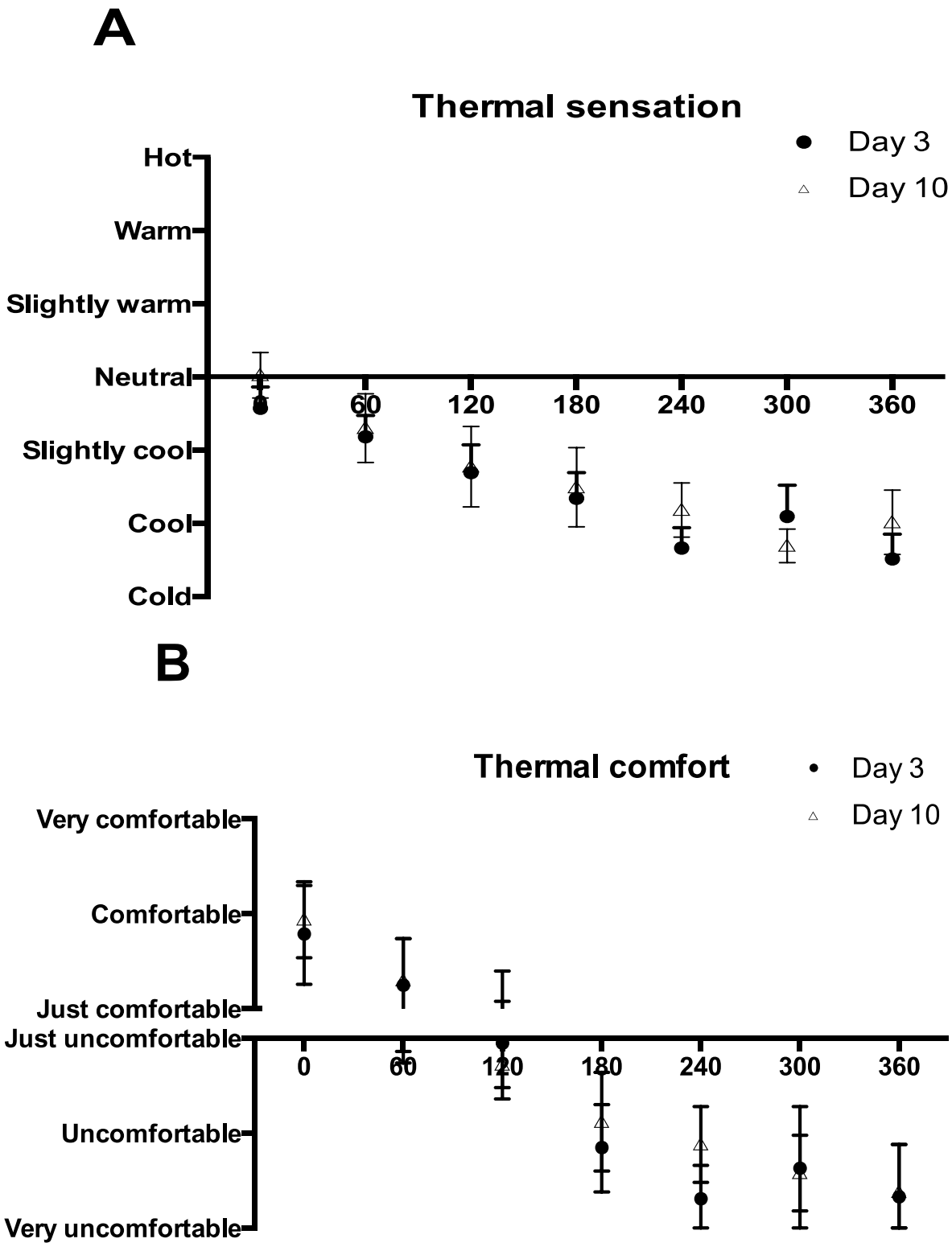


Figure 1. Subjective responses to cold acclimation
Self-reported thermal sensation (A) and thermal comfort (B) measured with VAS scales at selected timepoints (from t=0 min until t=360 min) during day 3 and day 10 of the cold acclimation period, shown as AUC. Data are expressed as mean ± SE. n=9. Data was analyzed with a Wilcoxon matched-pairs signed rank test.

Postprandial metabolism and substrate kinetics

Meal test total area-under-the-curve values (AUC) for glucose, insulin and triglycerides were not significantly different before and after cold acclimation (p=0.43, p=0.65 and p=0.50 respectively, Figure 2A-C and Supplementary Table 4). In addition, no significant differences were observed in AUC when the 1st or 2nd meal of the meal test were analysed separately (Supplementary Table 4). Area under the curve for plasma free fatty acids was also not significantly different before and after ten days of cold acclimation (p= 0.16, Figure 2D and Supplementary Table 4). However, when the 1st and 2nd meal of the meal test were analysed separately, the total AUC for FFA during the 1st meal was significantly lower after cold acclimation (pre: 94228 ± 5115 mmol/l; post: 86014 ± 4545 mmol/l, p=0.039) as shown in Supplementary Table 4.

Total energy expenditure during the meal tests, calculated as AUC, was significantly higher after the cold acclimation (pre: 2620 ± 146; post: 2752 ± 168 kJ, p=0.03, Figure 3A and Supplementary Table 4). Although this elevated energy expenditure seemed to be mainly due to higher glucose oxidation, the differences in glucose and fat oxidation during the meal tests were not statistical significant (p=0.44 and p>0.99 respectively, Figure 3B-C, Supplementary Table 4).

The results of the meal test suggest that cold acclimation may increase energy expenditure, probably due to higher carbohydrate oxidation. To further test this, we analysed substrate oxidation measured in the morning of the clamps. Overnight fasted energy expenditure at the start of the clamp (baseline) was not affected by cold acclimation (Supplementary Table 3). However, energy expenditure during the high insulin phase was significantly higher after cold acclimation compared to before (pre 4.67 ± 0.30 vs. post 4.89 ± 0.27 kJ/min, p=0.03) and this elevation tended to return to pre-intervention levels on the longterm (post 4.89 ± 0.27 vs. long term 4.67 ± 0.18 kJ/min, p=0.10, Supplementary Table 3). Carbohydrate oxidation after an overnight fast was significantly higher after ten days of cold acclimation compared to before (pre 3.76 ± 0.47 vs. post 4.97 ± 0.68 μmol/kg/min, p<0.01, Supplementary Table 3). In addition, fat oxidation after an overnight fast was significantly lower directly after cold acclimation compared to before (pre 3.80 ± 0.17 vs. post 3.57 ± 0.17 μmol/kg/min, p<0.01, Supplementary Table 3). These effects on carbohydrate and fat oxidation did not sustain on the long term (Supplementary Table 3). The changes in substrate oxidation after cold acclimation were not observed during insulin infusion (Supplementary Table 3).

There was no significant difference after cold acclimation for baseline HDL (pre 1.12 ± 0.12 vs. post 1.21 ± 0.08 mmol/l, p=0.16), LDL (pre 1.80 ± 0.24 vs. post 1.84 ± 0.24 mmol/l, p=0.82) or total cholesterol (pre 3.57 ± 0.31 vs. post 3.76 ± 0.31 mmol/l, p=0.16)

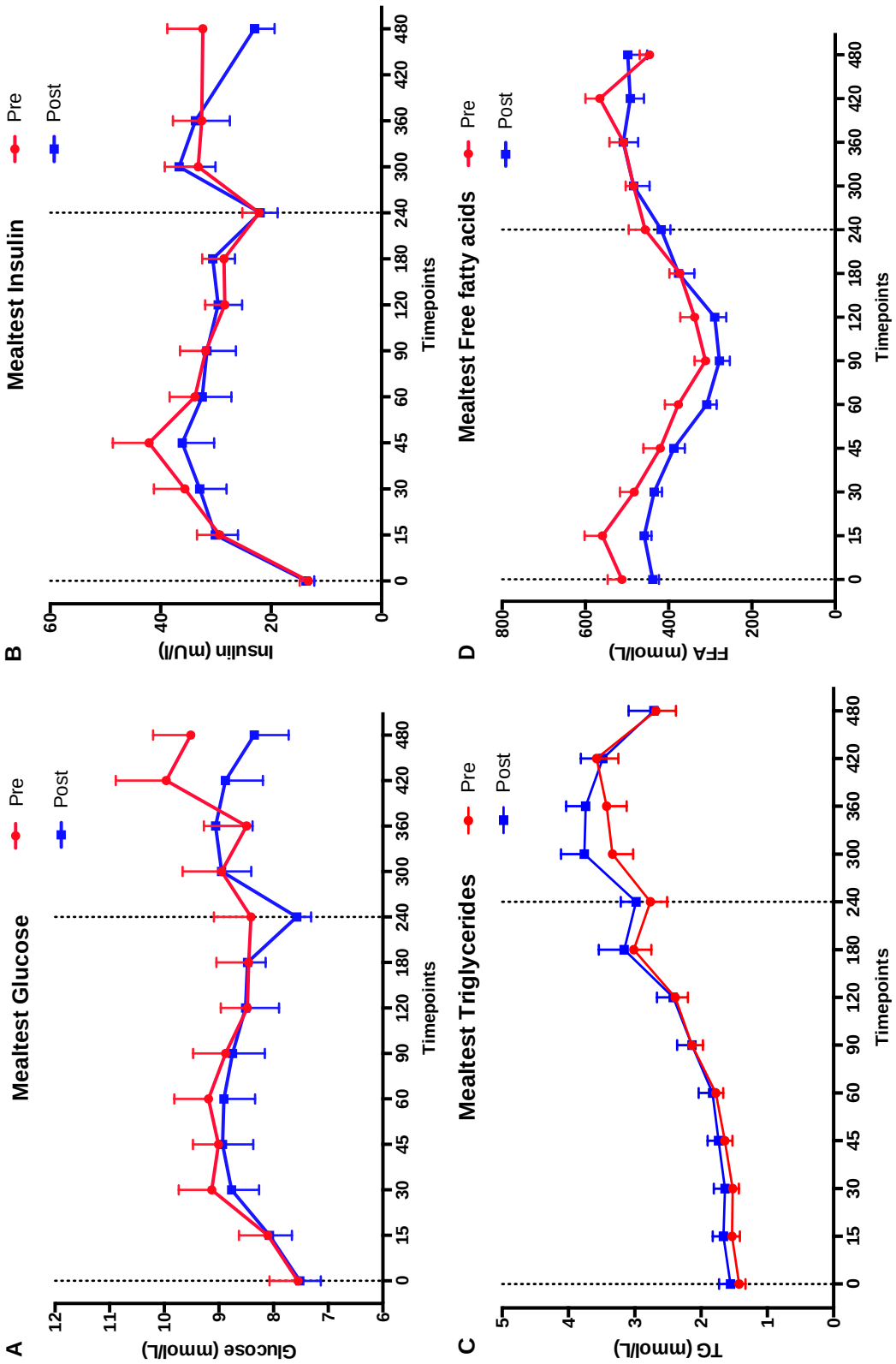


Figure 2. Plasma levels during the meal tests

A: plasma levels of glucose presented, B: Insulin, C: Triglycerides and D: Free fatty acids during the meal test. Pre cold acclimation is presented as the red line, post cold acclimation as the blue line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=9. Data was analyzed with a Wilcoxon matched-pairs signed rank test. Data is presented as mean ± SE.

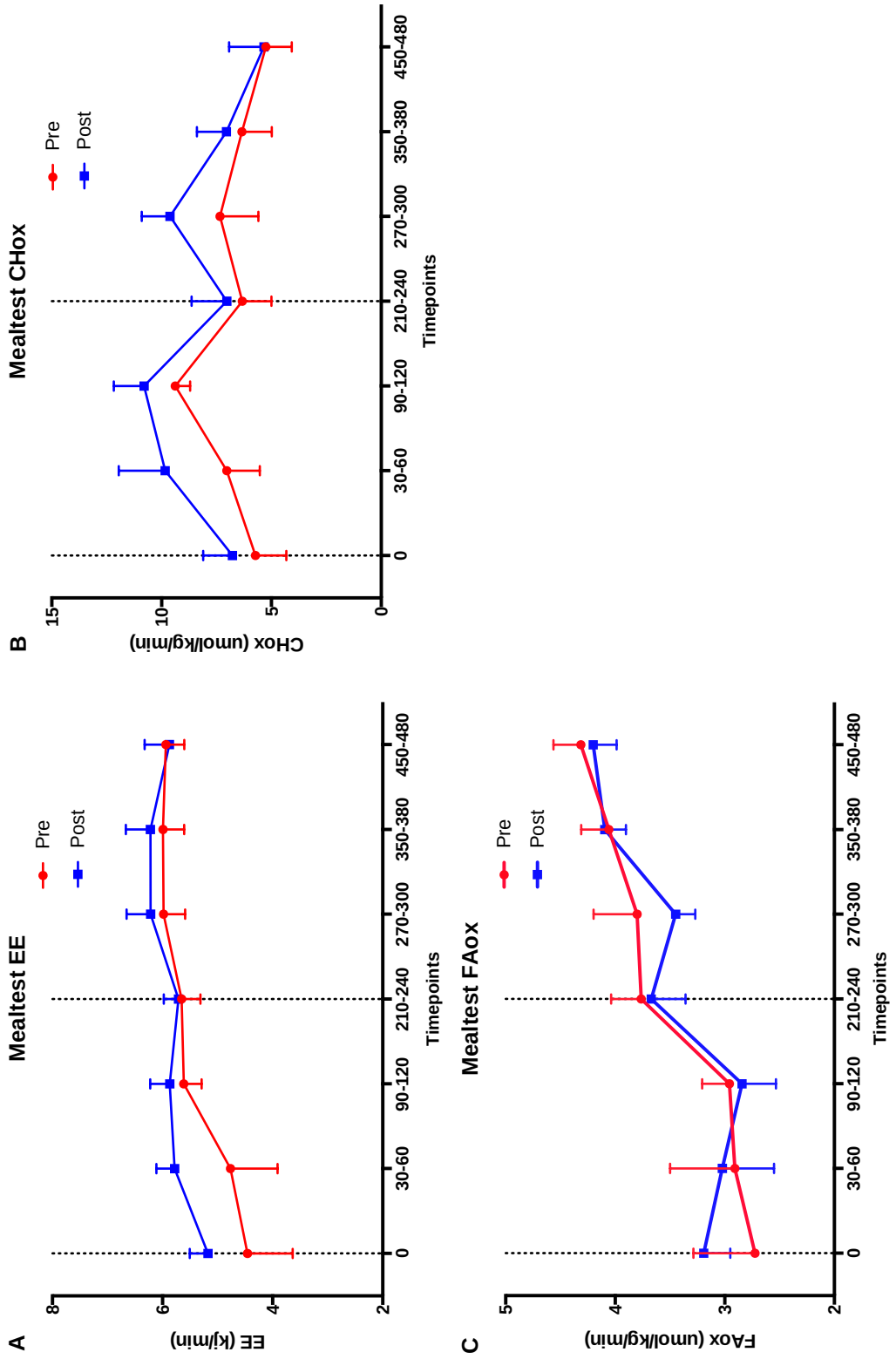


Figure 3. Energy expenditure and substrate oxidation during the meal tests
A: Energy expenditure, B: Carbohydrate oxidation (CHox), and C: Fatty acid oxidation (FAox) during the meal test . Pre cold acclimation is presented as the red line, post acclimation as the blue line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=9. Data was analyzed with a Wilcoxon matched-pairs signed rank test. Data is presented as mean ± SE.

Table 3. Plasma levels and substrate kinetics measured during the clamps

	Clamp pre	Clamp post	Clamp longterm	p-value
Plasma Glucose (mmol/l)				
Baseline	7.77 ± 0.54	7.98 ± 0.45	7.91 ± 0.54	0.99
Low insulin	5.99 ± 0.25	5.78 ± 0.31	5.63 ± 0.17	0.21
High insulin	5.21 ± 0.12	5.11 ± 0.11	5.04 ± 0.08	0.69
Plasma Insulin (mU/l)				
Baseline	6.32 ± 1.992	6.99 ± 1.48	6.56 ± 1.71	0.69
Low insulin	14.09 ± 1.87	12.64 ± 1.56	12.62 ± 0.88	0.57
High insulin	62.50 ± 3.24[C]	59.34 ± 4.13	56.03 ± 2.68[C]	**0.02***
Energy expenditure (kJ/min)				
Baseline	4.82 ± 0.25	4.91 ± 0.27	4.86 ± 0.20	0.79
Low insulin	4.86 ± 0.34	4.92 ± 0.28	4.75 ± 0.24	0.24
High insulin	4.67 ± 0.30[B]	4.89 ± 0.27[B,D]	4.67 ± 0.18[D]	**0.02***
Carbohydrate oxidation (μmol/kg/min)				
Baseline	3.76 ± 0.47[A]	4.97 ± 0.68[A]	4.68 ± 0.68	**<0.01****
Low insulin	7.27 ± 0.79	7.83 ± 0.84	7.15 ± 0.77	0.79
High insulin	10.36 ± 1.15	11.07 ± 1.21	9.73 ± 1.41	0.30
Fatty acid oxidation (μmol/kg/min)				
Baseline	3.80 ± 0.17[A]	3.57 ± 0.17[A,D]	3.71 ± 0.19[D]	**<0.01****
Low insulin	2.81 ± 0.23	2.86 ± 0.24	2.96 ± 0.16	0.87
High insulin	2.14 ± 0.24	2.16 ± 0.21	2.39 ± 0.26	0.99

Abbreviations: Ra, rate of appearance; Rd, rate of disappearance; S_i, whole body insulin sensitivity; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acids.

Data are expressed as mean ± SE. n=9. Data was analyzed with a Friedman test. **p<0.01, *p<0.05, [A]significant difference (p<0.01) between Clamp pre and Clamp post, [B]significant difference (p<0.05) between Clamp pre and Clamp post, [C]significant difference (p<0.05) between Clamp pre and Clamp post, [D]trend in difference (p<0.10) between Clamp post and Clamp longterm.

Table 4. Plasma levels and substrate kinetics measured during the meal tests

	Meal test pre	Meal test post	p-value
Plasma Glucose			
AUC	4433 ± 236	4285 ± 170	0.43
iAUC	860 ± 171	747 ± 159	0.25
Plasma Insulin			
AUC	15826 ± 1550	15640 ± 1458	0.65
iAUC	9457 ± 1097	9060 ± 1119	0.36
Plasma Triglycerides			
AUC	1339 ± 109	1411 ± 123	0.50
iAUC	655 ± 89	664 ± 67	0.36
Plasma Free fatty acids			
AUC	214852 ± 9104	202701 ± 10011	0.16
iAUC	13182 ± 6077	23031 ± 7650	0.20
AUC first meal	94228 ± 5115	86014 ± 4545	**0.04***
AUC second meal	120624 ± 5448	106486 ± 11976	0.30
AUC Energy expenditure			
AUC	2620 ± 145	2752 ± 168	**0.03***
iAUC	316 ± 37	355 ± 22	0.69
AUC Carbohydrate oxidation			
AUC	3328 ± 371	3866 ± 550	0.44
iAUC	726 ± 152	998 ± 103	0.22
AUC Fatty acid oxidation			
AUC	1688 ± 92	1667 ± 78	>0.99
iAUC	611 ± 159	526 ± 63	0.94

Abbreviations: AUC, area under the curve; iAUC, incremental area under the curve.
Data are expressed as mean ± SE, n=9. Data was analyzed with a Mann-Whitney test. *p<0.05

Vascular function

AIxHR75, an indirect marker of arterial stiffness, was significantly improved after ten days of cold acclimation measured in the overnight fasted state (pre T0 22.57 ± 1.36 % vs. post T0 19.84 ± 1.96 %, p=0.03, Figure 4A). Before cold acclimation, AIxHR75 tended to decrease upon the first meal ingestion (T120) and this meal-induced effect became significant after the second meal (T300). No statistically significant meal-effects on AIxHR75 were observed after cold acclimation (pre T120 15.63 ± 2.80 % vs. post T120 18.26 ± 1.67 % p=0.50 and pre T300 14.94 ± 2.55 % vs. post T300 16.33 ± 2.70 % p=0.82, Figure 4A). Postprandial changes in AIxHR75 were also not significant different before and after cold acclimation (pre delta 120 -6.94 ± 2.31 vs. post delta 120 -1.58 ± 1.96, p=0.16, pre delta 300 -7.63 ± 1.83 vs. post delta 300 -3.51 ± 1.91, p=0.30, Figure 4B).

The current non-invasive gold standard technique to measure arterial stiffness ($PWV_{c\text{-}f}$), was however not affected by cold acclimation in the overnight fasted state $PWV_{c\text{-}f}$ (pre T0 12.36 ± 0.61 vs. post T0 11.99 ± 0.57, p=0.36, Figure 4C). As expected, no meal-induced effects were observed (see Figure 4D).

Finally, ten days of cold acclimation did not significantly affect fasting retinal vessel diameter, as the arteriolar width (pre 120.83 ± 7.48 μm vs. post 120.52 ± 7.89 μm, p=0.95), venular width (pre 209.19 ± 10.73 μm vs. post 207.17 ± 10.07 μm, p=0.23) and the arteriolar-to-venular ratio (pre 0.58 ± 0.02 vs. post 0.58 ± 0.02, p=0.84, Figure 4E) did not change.

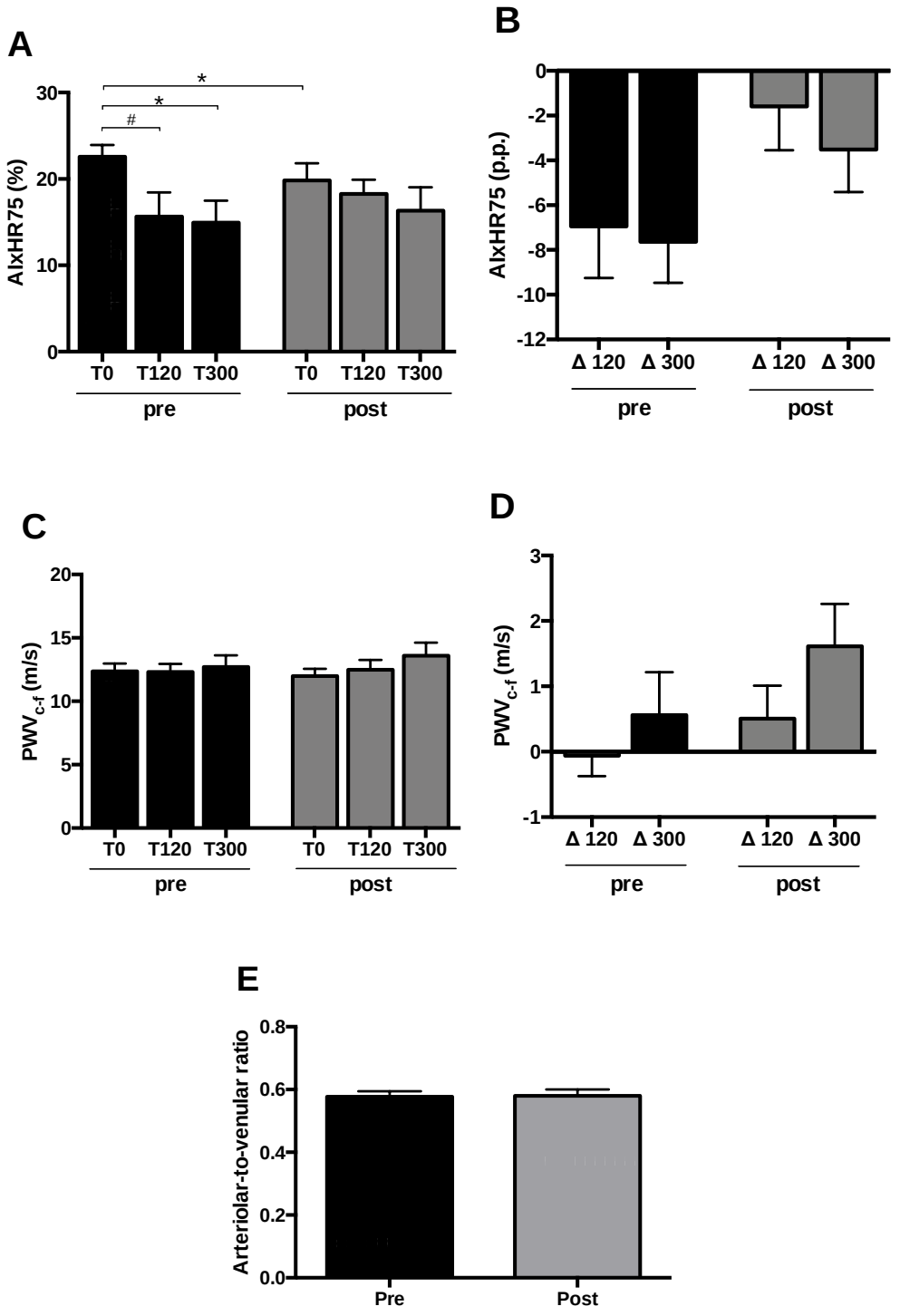


Figure 4. Vascular function markers

A: Aortic augmentation index (AIxHR75) at timepoints T0, T120, T300. B: AIxHR75 delta's T0-120 and T0-300 expressed as percentage points. C: Pulse wave velocity (PWV_{c-f}) at timepoints T0, T120, T300 (n=8 at T300). D: PWV_{c-f} delta's T0-120 and T0-300 (n=8 at delta T0-300). E: Arteriolar-to-venular ratio of the retinal vessels in the right eye (n=8). Black bars represent data from the meal test before cold acclimation, grey bars represent data from the meal test after cold acclimation. Data was analyzed with a Wilcoxon matched-pairs signed rank test. Data shown are shown as mean ± SE. *$p<0.05$, $^{\#}p<0.10$.

Liver fat content

As we hypothesized that cold acclimation could affect postprandial lipid metabolism, we also investigated if cold acclimation affects liver fat content. However, liver fat content was not different before and directly after cold acclimation (pre 6.1 ± 4.3 vs. post 7.0 ± 4.0 %, p=0.22, n=8, Figure 5D).

Insulin sensitivity

We previously demonstrated that cold acclimation improved insulin sensitivity when measured directly after ten days of cold acclimation. Here, we aimed to investigate if this increase in insulin sensitivity would be sustained for ten days after cessation of the cold acclimation intervention. Plasma insulin levels during the high insulin phase of the clamp were significantly different between test days (p=0.02) with significantly higher values directly after the cold acclimation compared to before cold acclimation (p=0.02, Supplementary Table 3), and therefore we calculated Si as a measure of insulin sensitivity. However, whole body insulin stimulated glucose uptake (Rd glucose high insulin minus baseline) corrected for plasma insulin levels (Si), was not different before and directly after cold acclimation nor on the longterm (p=0.53, Figure 5A). Hepatic insulin sensitivity during the low insulin phase was also not affected by cold acclimation (p=0.28, Figure 5B), however during the high insulin phase, EGP suppression was significantly lower directly after cold acclimation compared to before and on the longterm (pre 88.06 ± 7.00 % vs. post 77.60 ± 7.45 % vs. longterm 88.39 ± 3.44 %, p=0.04). HbA1c, a marker of long term glucose homeostasis, was not different before cold acclimation compared to two months after cold acclimation (pre 7.3 ± 0.3 % vs. two months later 7.2 ± 0.3 %, p=0.71, Figure 5C).

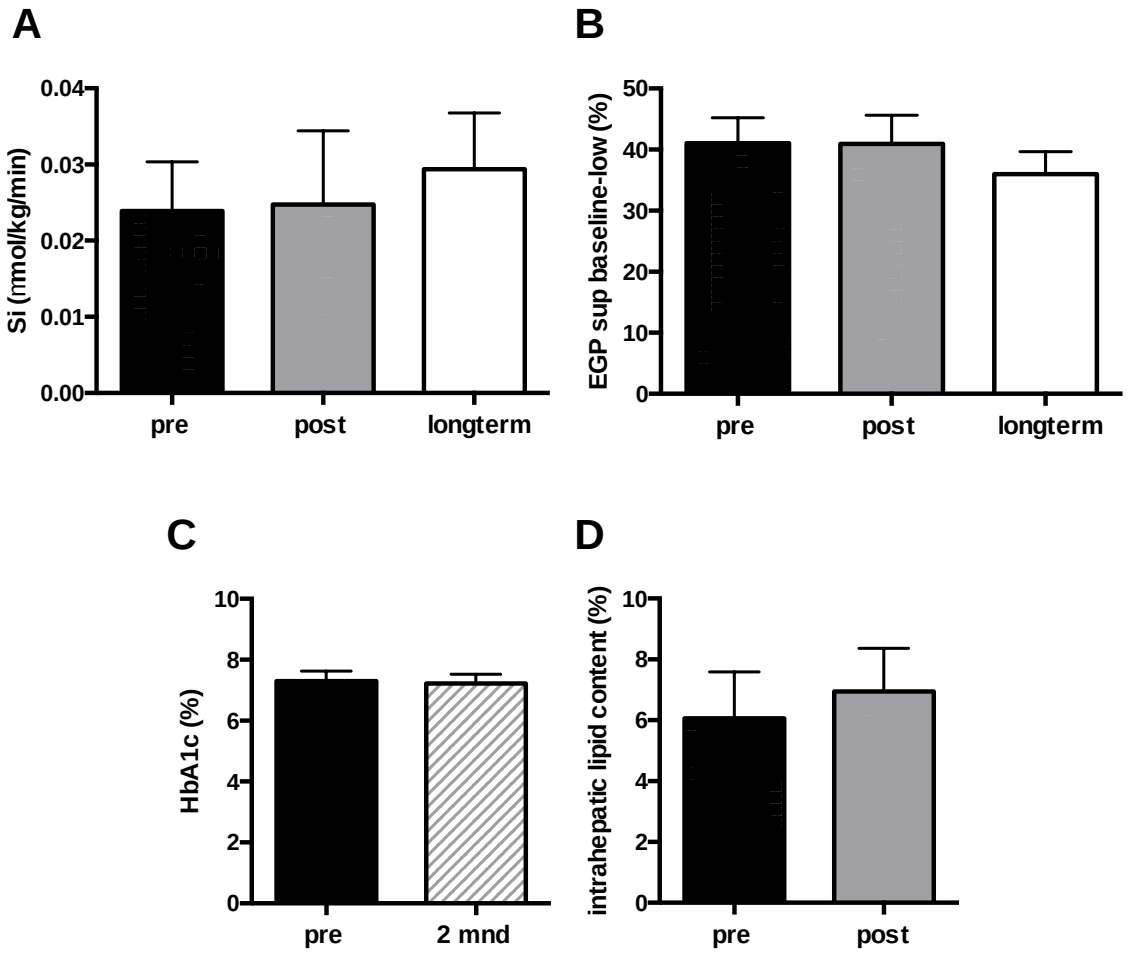


Figure 5. Insulin sensitivity and intrahepatic lipid content

A: Whole body insulin sensitivity. B: Suppression of hepatic endogenous glucose production. C: HbA1c before the cold acclimation and 2 months after the cold acclimation. D: Intrahepatic lipid content.

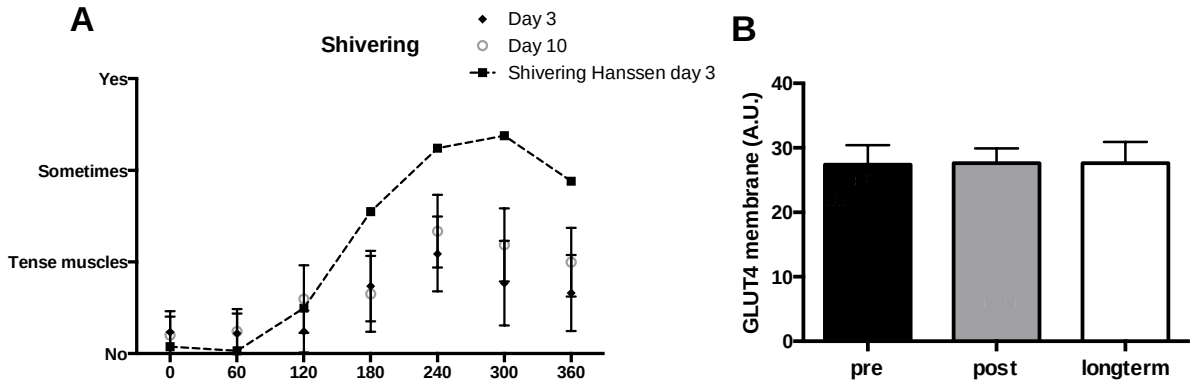
Black bars represent before cold acclimation, grey bars represent directly after cold acclimation, white bars represent ten days after the cold acclimation, dashed bar represents two months after the cold acclimation. n=9. Data was analyzed with a Friedman test for Si and EGP. Data was analyzed with a Wilcoxon matched-pairs signed rank test for HbA1c and intrahepatic lipid content. Data are shown as mean ± SE.

Skeletal muscle GLUT4 translocation

The lack of effect of cold induced non-shivering thermogenesis on insulin sensitivity contrasts our previous study, in which we found that the increased insulin sensitivity was due to enhanced GLUT4 translocation in skeletal muscle (13). Consistent with a lack of effect of cold induced NST on insulin sensitivity, GLUT4 intensity at the membrane measured in muscle biopsies taken in the non-insulin stimulated condition, was also not affected by cold induced NST (pre 27.37 ± 3.05 vs. post 27.63 ± 2.28 vs. longterm 27.61 ± 3.30 A.U., p=0.77, n=7, Figure 6B). Cytosolic GLUT4 intensity was not changed either (pre 20.21 ± 1.89 vs. post 20.71 ± 1.33 vs. long term 20.46 ± 1.90 A.U., p=0.96).

Skeletal muscle shivering markers

Because we could not replicate our previously reported positive effects of cold acclimation on insulin sensitivity and GLUT4 translocation (13), we decided to investigate if the absence of shivering in the current study could underly this phenomenon. Compared to our previous study (13), we see a distinct lack of self-reported shivering, as can be seen in Figure 6A. To further test the hypothesis that lack of shivering/muscle contraction in the current study may underly the lack of beneficial effects on insulin sensitivity, we measured mRNA expression of genes based on microarray analysis from our previous cold acclimation study (13) and an exercise intervention study (14). We have selected genes related to muscle contraction and the extracellular matrix (unpublished data) that demonstrate the importance of muscle contraction on peripheral insulin sensitivity in the cold acclimation study performed by Hanssen et al (13). Based on the heatmap there is a clear upregulation of the selected genes in original cold acclimation cohort from Hanssen et al, while such an upregulation was not observed in the current study (Figure 6C). In more detail, smooth muscle actin (ACTC1), the alpha and delta subunit of muscle acetylcholine receptor (CHRNA1, CHRND), alpha-1 type-1 collagen (COL1A1), skeletal muscle myosin heavy chain 3 (MYH3) and myosin binding protein H (MYBPH) were higher in the cold acclimation study of Hanssen *et al*. compared to our current study. Also coiled coil domain containing protein 80 (CCDC80) and immunoglobulin-like and fibronectin type III domains-containing protein 1 (IGFN1) showed tendancies to be increased in the cold acclimation study from Hanssen *et al*. compared to our current study.



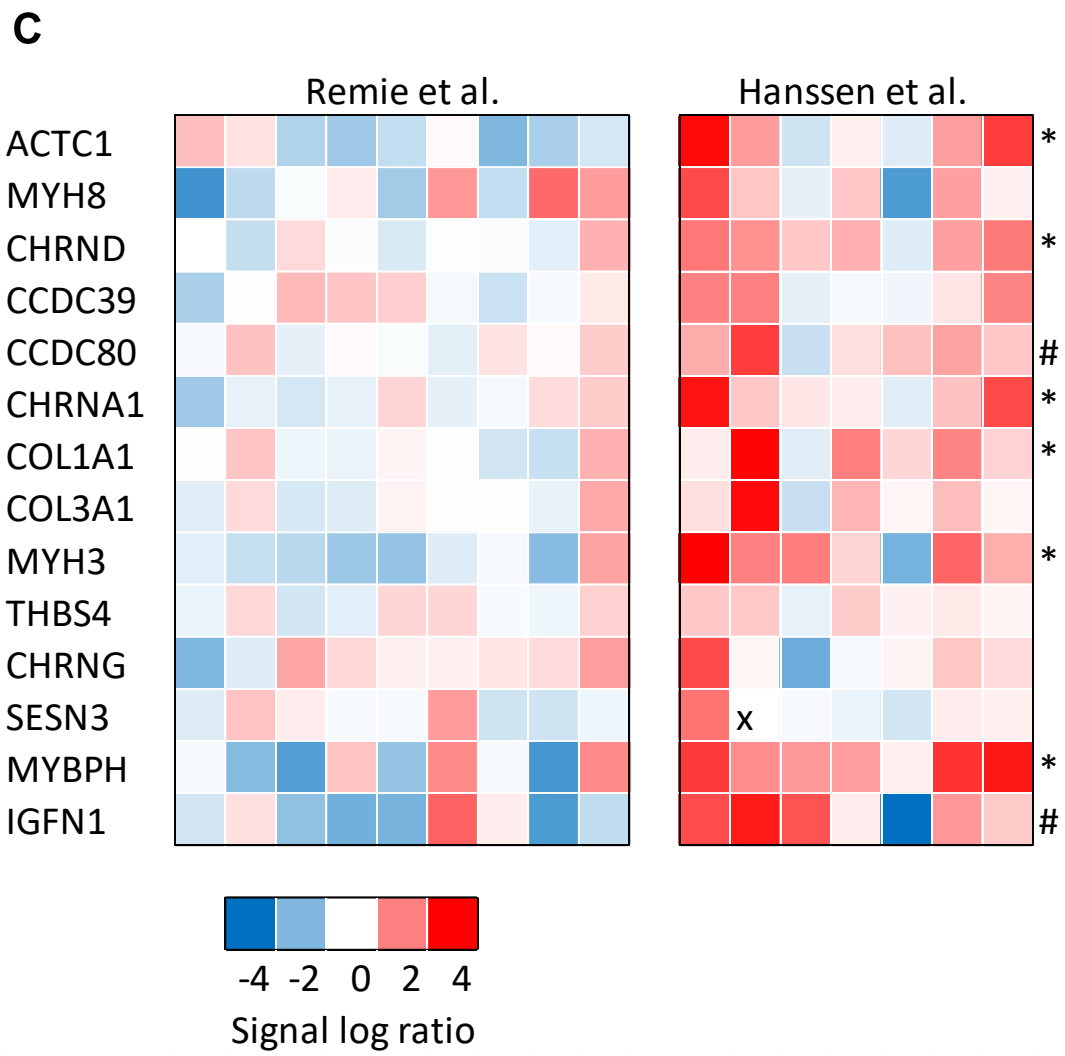


Figure 6. Skeletal muscle shivering markers
A: Self-reported shivering measured with VAS scales at selected timepoints (from T=0 minutes until T=360 minutes) during day 3 and day 10 of the cold acclimation period, shown as AUC. The dashed line indicates the self-reported shivering AUC from Hanssen et al. (13) at day 3 B: GLUT4 intensity at the skeletal muscle membrane, expressed as arbitrary units (A.U.). C: Relative gene expression analysis in skeletal muscle biopsy samples obtained from the current study (Remie et al.) (n=9) and from Hanssen et al. (13) (n=7) before and after cold acclimation. qPCR data is expressed as heatmap. Amplification failed for one sample and has been identified by "x" in the heat map. Data was analyzed with a Wilcoxon matched-pairs signed rank test for shivering. Data was analyzed with a Friedman test for GLUT4. Data was analyzed with a Mann-Whitney test for relative gene expression. Data are shown as mean ± SE. *$p<0.05$, $^{\#}p<0.10$.

Discussion

Previous studies investigating cold acclimation in humans have shown a potential to treat obesity and T2DM via an increase in energy expenditure (4-7, 12, 13, 20-22) and insulin sensitivity (13), acting through NST in brown adipose tissue and skeletal muscle. Therefore we hypothesized that cold acclimation with NST could also be beneficial for postprandial metabolism and reduce cardiovascular risk. To this end, we primarily investigated the effects of ten-day cold acclimation without shivering on cardiovascular risk markers, including postprandial glucose and lipid metabolism and markers for arterial stiffness. Moreover, we investigated the long-term effects of cold acclimation without shivering on insulin sensitivity.

Acute cold exposure with and without shivering has frequently been shown to increase energy expenditure, reported as an increase in basal metabolic rate (4, 5, 10, 11, 21-26). Yet, consistent with other previous reports (4, 6, 7, 12, 13, 20, 22, 27), we did not observe an increase in basal metabolic rate, measured under thermoneutral conditions after cold acclimation with NST. However, our results show a change in fasting substrate selection after cold acclimation with NST, with a decrease in fat oxidation and an increase in carbohydrate oxidation. Furthermore, as also reported before (12), postprandial energy expenditure, which reflects diet induced thermogenesis, was higher in the meal test after cold acclimation compared to the meal test prior cold acclimation. Other evidence suggests that acute cold exposure (5, 11, 16, 23, 25), but not cold acclimation (5), increases glucose oxidation, fat oxidation and lipid clearance. In agreement with previous work (5, 26), we did not observe changes in fasting plasma lipid levels, postprandial substrate oxidation or postprandial triglyceride response. We only observed a small, albeit significant decrease in plasma fatty acid levels during the first step of the meal test after cold acclimation. Furthermore, no effects of cold acclimation with NST were observed on fasted plasma glucose and insulin levels, consistent with findings after acute cold exposure (26). These results indicate that cold acclimation under non-shivering conditions only had marginal effects on postprandial glucose and lipid metabolism.

Furthermore, we observed a significant improvement in the augmentation index, indicating an indirect reduction in arterial stiffness. However, we did not see any change in $PWV_{c\text{-}f}$. This indicates that the effects of ten days of cold acclimation with NST might only affect peripheral resistance arteries and not stiffness of the large elastic arteries, such as the aorta. It is likely that the study period of ten days was too short to induced pronounced effects on arterial vessel wall properties. Alternatively, cold exposure can increase blood pressure – a main determinant of the $PWV_{c\text{-}f}$ – through vasoconstriction (28), and an increased blood pressure during cold exposure could mitigate potential beneficial effects that could be expected from the cold acclimation.

We previously showed marked effects of cold acclimation on insulin sensitivity in T2DM patients (13). Here we aimed to investigate if these effects would be retained for a longer period of time after the last cold exposure under non-shivering conditions. Thus, we performed a hyperinsulinemic euglycemic clamp before cold acclimation, directly (one day) after cold acclimation and ten days after the last cold exposure. Surprisingly, we could not replicate the increase in insulin sensitivity following the ten-day cold acclimation period, and also no effects on the long term were observed.

Since the increase in insulin sensitivity in our previous study (13) was very marked (40% increase), observed in all subjects, and was accompanied by marked increases in muscle GLUT4 translocation, we carefully evaluated the differences between the two studies. Thus, in both studies patients with T2DM were investigated before and after ten days of cold exposure, following a similar design. One difference in the current study design was the inclusion of a meal test as the primary outcome parameter. This meal test was performed on the day following the ten day cold exposure.

Consequently, the clamp in the current study was performed three days after the meal test, with two days of cold exposure in between. We can hence not exclude a carry-over effect of the meal test on the results of the clamp. It should be noted though, that postprandial glucose metabolism measured during the meal tests, which can be seen as a marker of insulin sensitivity, was also not affected by cold acclimation. Another difference between the two studies was that in the current study we specifically aimed to investigate the effect of NST and aimed to prevent shivering. To this end, the room temperature during cold exposure was approximately 1.4 °C higher compared to our previous study (13).

Furthermore, subjects were provided with extra clothing when shivering occurred to prevent shivering thermogenesis. While this strategy was more effective in preventing shivering and/or tense muscle compared to our previous study (13), as indicated by the self-reported shivering questionnaires taken during the cold acclimation, it may also have affected the clamp outcome. We previously reported a marked increase in GLUT4 translocation in the overnight fasted state (so in the absence of elevated insulin levels) (7, 13). Classically, this increase in GLUT4 in the celmembrane in the absence of insulin stimulation is attributed to muscle contraction, which is required for shivering or to increase muscle tension (29). Consistently, no effect of cold acclimation on GLUT4 translocation was found in the current study.

To further investigate the possible effects of shivering that we may have missed in our previous study (13), we performed gene-expression analyses in skeletal muscle biopsies obtained before and directly after the cold acclimation in both studies. Results of these analyses show upregulation of genes related to muscle contraction and remodeling of the extracecellular matrix in our previous study (13) but no change in the current study. Those

genes were selected from micro-array data obtained after cold exposure (13) and compared with an exercise training study (14). This comparison revealed overlap in skeletal muscle gene expression related to muscle contraction pathways (unpublished data). Interestingly, all previous published studies that observed improvements in insulin sensitivity after cold acclimation, included at least a few days of (mild) shivering before NST occurred (4, 5, 7, 13, 22). Taken together, the findings reported here and in literature (4, 10, 30), suggest that some mild form of cold-induced shivering is needed to trigger beneficial effects on skeletal muscle insulin sensitivity.

In conclusion, we here show that a ten-day cold acclimation period at 16-17 °C with NST does not induce metabolic improvements nor reduce cardiovascular risk markers in obese men and women with T2DM. The lack of effects in the current study are probably due to the absence of shivering compared to previous studies. Therefore, further research could focus on the potential of (mild) shivering thermogenesis on metabolic health, as ten days of cold acclimation without shivering at 16-17 °C in obese men and women with T2DM is insufficient to induce positive effects on human metabolism.

Methods

Participants

Ten obese men and women were included in the study with one volunteer dropping out because of personal reasons. Hence, nine participants completed the study. All participants underwent a screening including assessment of blood parameters, electrocardiography, anthropometric measurements and a questionnaire to evaluate eligibility. Inclusion criteria were: 45-70 years of age, BMI 27-35 kg/m^2, diagnosed with type 2 diabetes for at least one year (relatively well-controlled HbA1c <8.5%), use of oral glucose lowering drugs (metformin and/or sulfonylurea agents), a sedentary lifestyle (<3 h exercise per week), non-smoking for at least six months, no alcohol use of >2 servings per day and a stable body weight for at least six months. Other medication without known effects on the primary outcome measurement was allowed. Data were collected between March 2016 and August 2017.

Ethical approval

The study was conducted according to the declaration of Helsinki and was approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at https://trialregister.nl (NL4469/NTR5711). All participants provided written informed consent before screening.

Study design

Figure 1 provides an overview of the study design. A ten-day cold acclimation intervention was performed as previously described (13), except for the temperature, which was set approximately 1-2°C higher, at 16-17 °C to avoid shivering. Before the onset of the cold acclimation period a high-fat meal test (mealtest pre) was combined with several vascular function measurements. Three days later, liver fat content was measured followed by a muscle biopsy and a hypersinsulinemic-euglycemic 2-step clamp (clamp pre). Subsequently, a ten-day cold acclimation intervention started (Day 1-10). One day after the last cold exposure, (Day 11), the first test day was repeated (mealtest post). The following two days (Day 12-13), additional cold exposure was maintained to ensure sustainment of the intervention effect for the re-tests on Day 14 (clamp post). Upon ten days without intervention, another muscle biopsy was taken followed by a hypersinsulinemic-euglycemic 2-step clamp (clamp long term) to assess the sustained long-term effect of cold acclimation on whole body insulin sensitivity. Two months after the last test day, fasting blood samples were taken to measure markers for glucose homeostasis. All tests were executed at room temperature. At the evening preceding all test days, participants consumed a standardized meal and remained fasted from 20:00 h onwards. In addition, participants were asked to refrain for at least 48 hours from any physical activity different from their daily routine. Participants arrived at the testing facility by car. Blood glucose lowering medication was discontinued on the morning of the clamps.

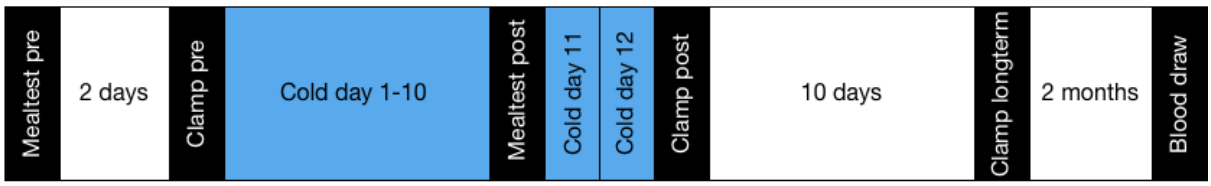


Figure 1. Study design

A meal test and clamp were performed before cold acclimation, separated by two days. After ten days of cold exposure a second meal test was performed, followed by 2 additional days of cold exposure and followed by a second clamp. Ten days after the last intervention day, a third clamp was performed. Two months after the last clamp another blood sample was collected. Blue boxes represent cold acclimation intervention (16-17°C). White boxes represent no intervention. Black boxes represent test days.

Cold acclimation

During cold acclimation, participants were progressively exposed to an environmental temperature of 16-17 °C for ten consecutive days: 2-hours on day 1, 4-hours on day 2 and 6-hours on days 3 to 10. On Day 11-12, participants were again cold-exposed for six hours. Participants were dressed in short-sleeved T-shirts and shorts and remained sedentary while staying in the cold room and instructed not to change their habitual diet during the entire study period. Food intake while staying in the cold room was kept constant. Wireless temperature sensors (iButtons, Maxim Integrated, San Jose, CA, USA) were placed on 15 ISO-defined sites on days 3 and 10 of the cold acclimation period to measure skin temperature. Average skin temperature as well as proximal and distal temperature was calculated as described before (31). Participants were not allowed to shiver. In case shivering started, extra clothing was immediately provided to ensure that shivering stopped. At selected timepoints during day 3 and 10 VAS scales on thermal sensation, thermal comfort and shivering were completed. Incremental AUCs (iAUC) were calculated to determine subjective responses during the cold acclimation period, as described before (8).

High-fat meal test

After placing an intravenous cannula, a fasted blood sample was drawn (T=0) and energy expenditure and substrate oxidation was measured via indirect calorimetry. At 09:00 h, participants were asked to consume a high-fat shake, within ten minutes. The nutritional content of the shake is shown in Supplementary Table 2. Subsequently, blood samples were drawn after 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes. After the blood sampling at T=240, at 13:00 h, the participants consumed a second high-fat shake with the exact same contents as in the morning to investigate the second-meal effect, as described before (32). At T30-60, T90-120, T210-240, T270-300, T350-380 and T450-480 indirect calorimetry was performed to measure energy expenditure and substrate oxidation. Participants were not allowed to eat or drink anything else throughout the test day, except for water.

Supplementary Table 2. Nutritional information of meal test shake

	Content	**Energy %**
Energy	755 kcal	100
Fat	50.8 gr	60.6
Saturated fat	26.1 gr	31.1
Unsaturated fat	23.8 gr	28.4
Cholesterol	0.9 gr	1.1
Carbohydrate	62.3 gr	33.0
Protein	10.9 gr	5.8

Vascular function measurements

On the morning of the meal tests, radial artery pulse wave analysis (PWA) was performed with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia), from which the aortic augmentation index corrected for heart rate (AIxHR75) was calculated as described before (33). Using the same tonometer, carotid-to-femoral pulse wave velocity ($PWV_{c\text{-}f}$) was also determined as described before (33). The vasculare stiffness measurements were performed before (T0) and during the high-fat meal tests at two different time points (T120 and T300).

Retinal vascular images were obtained to measure microvascular effects. Retinal images were obtained as described before using a nonmydriatic retinal camera (Topcon TRC-NW-300; Topcon Co.) (34). At least two arteriolar and two venular segments were measured and summarized by using the Parr-Hubbard formulas (35). These segments differed between study participants, however, they had to be exactly the same segments for each participant at all measurements.

Hyperinsulinemic euglycemic clamp

To determine insulin sensitivity, a two-step hyperinsulinemic euglycemic clamp (36) with co-infusion of D-[6.6-$^2H_2$] glucose tracer (0.04 mg/kg/min) was performed, as described before (37). In short, insulin-suppressed endogenous glucose production (EGP) during low insulin infusion (10 mU/m^2/min) was measured as a reflection of hepatic insulin sensitivity. This was followed by a high insulin infusion (40 mU/m^2/min) to mesaure whole body glucose disposal (Rd). Indirect calorimetry was performed during baseline, low insulin and high insulin to measure energy expenditure and substrate oxidation. Steele's single pool non-steady state equations were used to calculate glucose appearance (Ra) and glucose disposal (Rd) (38). Volume of distribution was assumed to be 0.160 l/kg for glucose. Whole-body insulin sensitivity (S_i) was calculated according to Bergman et al. (39), taking differences in insulin and glucose levels into account: $S_i = \Delta Rd / (\Delta insulin * clamping\ glucose)$, where Δ represents the change from the basal state to the insulin-stimulated condition. EGP was calculated as Ra minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation.

Indirect calorimetry

Whole body oxygen consumption and carbon dioxide production were measured during specific timepoints in the meal tests and the clamps using an automated respiratory gas analyzer with a ventilated hood system (Omnical; Maastricht Instruments, Maastricht, The Netherlands). Participants were measured in supine position for 30 minutes each time. Energy expenditure, glucose oxidation and fat oxidation rates were calculated using equations based on the measured averaged oxygen and carbon dioxide concentrations with the assumption that protein oxidation was negligible (40, 41).

Intrahepatic lipid quantification by MR spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) was used to quantify intrahepatic lipid content (IHL) at 07:00 h in the morning of Clamp pre and Clamp post. All measurements were performed on a 3.0T whole body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands). Spectra were acquired as described before (42). Values are given as T2 corrected ratios of the CH_2 peak relative to the unsuppressed water peak, expressed as percentage.

Skeletal muscle biopsies

On each clamp day, a muscle biopsy was taken from the *m. vastus lateralis* under local anesthesia (2% lidocaine, without epinephrine) using the Bergström technique (43). The muscle biopsy was taken before the start of the insulin infusion on the morning of the clamp, at 08:30 h after an overnight fast. The biopsy was divided in several parts. One part was immediately frozen in melting isopentane for biochemical analyses. Other parts were embedded in Tissue-Tek and frozen in melting isopentane for immunohistochemical analyses.

Histochemical analysis of GLUT4 in skeletal muscle biopsies

Muscle biopsies taken in the overnight fasted state, prior to all three clamps, were analysed for GLUT4 translocation. Sections were stained for GLUT4 as described previously (13), with primary antibodies against GLUT4 (sc-1608, Santa Cruz, Dallas, USA) and Laminin (L-9393, Sigma, St. Louis, USA), and appropriate conjugated secondary antibodies AlexaFluor488 (AF488, Invitrogen, Life Technologies Europe, Bleiswijk, The Netherlands) and AlexaFluor555 (AF555, Invitrogen, Life Technologies Europe). Images were acquired with a Nikon E800 fluorescent microscopy (Nikon, Amsterdam, The Netherlands) as described previously (13). Images were analyzed using ImageJ (NIH, Bethesda, USA) (44). Mean intensity of GLUT4 was measured on the cell membranes and in the cytosol.

Shivering markers in skeletal muscle biopsies

RNA extraction and cDNA synthesis from skeletal muscle was performed as previously described (45, 46). In short, RNA was isolated from skeletal muscle biopsies using Trizol followed by purification using the RNeasy kit from Qiagen (Hildenberg, Germany). cDNA was created by using the high-capacity RNA-to-cDNA kit from Applied Biosystems (Foster City, USA). mRNA expression was determined using a CFX384 Touch Real-Time PCR Detection System from BioRad Laboratories (Hercules, CA) using the following Taqman assays: CHRNA1 (Hs00909664), CCDC39 (Hs00977326), MYH8 (Hs00267293), CCDC80 (Hs00277341), CHRND (Hs00897937), ACTC1 (Hs01109515), COL1A1 (Hs00164004), COL3A1 (Hs00943809), MYH3 (Hs01074230), THBS4 (Hs00170261), CHRNG (Hs00183228), SESN3 (Hs00914870), MYBPH (Hs00192226) and IGFN1 (Hs01567410). Gene expression was normalized to RPLPO (fwd: CCATTCTATCATCAACGGGTACAA, rev: AGCAAGTGGGAAGGTGTAATCC) and expression was analyzed via the standard curve method. Data is expressed in heatmap showing changes over basal using signal log ratios.

Blood sampling and analyses

Blood collected in EDTA-coated tubes were immediately stored on ice, centrifuged and plasma was stored at -80 °C until analyses. Blood collected in serum-tubes was stored at room temperature for at least 30 minutes to allow coagulation, followed by centrifugation and storage at -80 °C until analyses. Glucose (Hk-CP, Axonlab, Amsterdam, The Netherlands) and FFA (NEFA-HR, WAKO chemicals, Neuss, Germany) in meal test samples were analyzed enzymatically in EDTA plasma using a Pentra 400 (Horiba, Montpellier, France). Insulin during the meal test was analyzed using RIA, and insulin during the clamp was analyzed using ELISA. Triglycerides (Sigma, Zwijndrecht, The Netherlands), cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) and HDL-cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim,Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, were analyzed in serum also using a Pentra 400. LDL-cholesterol was calculated according the Friedewald equation (47).

Statistical analyses

Participant characteristics are reported as mean ± SD. Other results are reported as mean ± SE. Data are presented for n=9, unless otherwise indicated. Differences between Clamp pre, Clamp post and Clamp longterm were analyzed with a Friedman test. Posthoc analyses were performed with Dunn's multiple comparison test. Differences between Mealtest pre and Mealtest post were analyzed with a Wilcoxon matched-pairs signed rank test. Differences between muscle genexpression in the current and previous study were analyzed with a Mann-Whitney test. All statistical tests were performed two sided, with the statistical significance was set at $p<0.05$. Statistical analyses were performed using IBM SPSS version 23.0 for MacOSx (IBM, Armonk, NY, USA).

Reference list

1. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. JAMA. 1979;241(19):2035-8.
2. Emerging Risk Factors C, Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. 2010;375(9733):2215-22.
3. Davis TRA. Chamber cold acclimatization in man. Journal of Applied Physiology. 1961.
4. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. J Physiol-London. 2017;595(6):2099-113.
5. Gordon K, Blondin DP, Friesen BJ, Tingelstad HC, Kenny GP, Haman F. Seven days of cold acclimation substantially reduces shivering intensity and increases nonshivering thermogenesis in adult humans. J Appl Physiol. 2019;126(6):1598-606.
6. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased Brown Adipose Tissue Oxidative Capacity in Cold-Acclimated Humans. J Clin Endocr Metab. 2014;99(3):E438-E46.
7. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.
8. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
9. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
10. Wijers SLJ, Schrauwen P, Saris WHM, Lichtenbelt WDV. Human Skeletal Muscle Mitochondrial Uncoupling Is Associated with Cold Induced Adaptive Thermogenesis. Plos One. 2008;3(3).
11. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. Diabetes. 2014;63(12):4089-99.
12. Lee P, Smith S, Linderman J, Courville AB, Brychta RJ, Dieckmann W, et al. Temperature-Acclimated Brown Adipose Tissue Modulates Insulin Sensitivity in Humans. Diabetes. 2014;63(11):3686-98.
13. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
14. Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. Diabetes. 2010;59(3):572-9.

15. Arca M, Pigna G, Favoccia C. Mechanisms of diabetic dyslipidemia: relevance for atherogenesis. Curr Vasc Pharmacol. 2012;10(6):684-6.
16. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
17. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, et al. Brown Adipose Tissue Activation Is Linked to Distinct Systemic Effects on Lipid Metabolism in Humans. Cell Metab. 2016;23(6):1200-6.
18. Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, et al. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment. Circulation. 2002;106(10):1211-8.
19. Kersten ENRHGHJHWDvMLMHCHPSS. Comparative transcriptome analysis of human skeletal muscle in response to cold acclimation and exercise training in human volunteers. Under submission. 2020.
20. van der Lans AAJJ, Hoeks J, Brans B, Vijgen GHEJ, Visser MGW, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. Journal of Clinical Investigation. 2013;123(8):3395-403.
21. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. Journal of Clinical Investigation. 2013;123(8):3404-8.
22. Blondin DP, Tingelstad HC, Noll C, Frisch F, Phoenix S, Guerin B, et al. Dietary fatty acid metabolism of brown adipose tissue in cold-acclimated men. Nat Commun. 2017;8.
23. Vallerand AL, Zamecnik J, Jones PJH, Jacobs I. Cold stress increases lipolysis, FFA R-a and TG/FFA cycling in humans. Aviat Space Envir Md. 1999;70(1):42-50.
24. van Ooijen AMJ, Lichtenbelt WDVM, van Steenhoven AA, Westerterp KR. Seasonal changes in metabolic and temperature responses to cold air in humans. Physiol Behav. 2004;82(2-3):545-53.
25. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, et al. Brown Adipose Tissue Activation Is Linked to Distinct Systemic Effects on Lipid Metabolism in Humans. Cell Metab. 2016;23(6):1200-6.
26. Iwen KA, Backhaus J, Cassens M, Waltl M, Hedesan OC, Merkel M, et al. Cold-Induced Brown Adipose Tissue Activity Alters Plasma Fatty Acids and Improves Glucose Metabolism in Men. J Clin Endocr Metab. 2017;102(11):4226-34.
27. Plasqui G, Kester AD, Westerterp KR. Seasonal variation in sleeping metabolic rate, thyroid activity, and leptin. Am J Physiol Endocrinol Metab. 2003;285(2):E338-43.
28. Kingma BR, Frijns AJ, Saris WH, van Steenhoven AA, Lichtenbelt WD. Increased systolic blood pressure after mild cold and rewarming: relation to cold-induced thermogenesis and age. Acta Physiol (Oxf). 2011;203(4):419-27.
29. Douen AG, Ramlal T, Rastogi S, Bilan PJ, Cartee GD, Vranic M, et al. Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct

intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. J Biol Chem. 1990;265(23):13427-30.
30. Bal NC, Maurya SK, Singh S, Wehrens XHT, Periasamy M. Increased Reliance on Muscle-based Thermogenesis upon Acute Minimization of Brown Adipose Tissue Function. Journal of Biological Chemistry. 2016;291(33):17247-57.
31. Vosselman MJ, Hoeks J, Brans B, Pallubinsky H, Nascimento EB, van der Lans AA, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. Int J Obes (Lond). 2015;39(12):1696-702.
32. Baumgartner S, Mensink RP, Plat J. Effects of a Plant Sterol or Stanol Enriched Mixed Meal on Postprandial Lipid Metabolism in Healthy Subjects. PLoS One. 2016;11(9):e0160396.
33. Joris PJ, Plat J, Bakker SJ, Mensink RP. Long-term magnesium supplementation improves arterial stiffness in overweight and obese adults: results of a randomized, double-blind, placebo-controlled intervention trial. Am J Clin Nutr. 2016;103(5):1260-6.
34. Joris PJ, Plat J, Kusters YH, Houben AJ, Stehouwer CD, Schalkwijk CG, et al. Diet-induced weight loss improves not only cardiometabolic risk markers but also markers of vascular function: a randomized controlled trial in abdominally obese men. Am J Clin Nutr. 2017;105(1):23-31.
35. Hubbard LD, Brothers RJ, King WN, Clegg LX, Klein R, Cooper LS, et al. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. Ophthalmology. 1999;106(12):2269-80.
36. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214-23.
37. de Ligt M, Bruls YMH, Hansen J, Habets MF, Havekes B, Nascimento EBM, et al. Resveratrol improves ex vivo mitochondrial function but does not affect insulin sensitivity or brown adipose tissue in first degree relatives of patients with type 2 diabetes. Mol Metab. 2018;12:39-47.
38. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci. 1959;82:420-30.
39. Bergman RN, Finegood DT, Ader M. Assessment of insulin sensitivity in vivo. Endocr Rev. 1985;6(1):45-86.
40. Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. Canadian journal of sport sciences = Journal canadien des sciences du sport. 1991;16(1):23-9.
41. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. The Journal of physiology. 1949;109(1-2):1-9.
42. Lindeboom L, Nabuurs CI, Hesselink MK, Wildberger JE, Schrauwen P, Schrauwen-Hinderling VB. Proton magnetic resonance spectroscopy reveals increased hepatic lipid content after a single high-fat meal with no additional modulation by added protein. Am J Clin Nutr. 2015;101(1):65-71.
43. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. Acta Physiol Scand. 1967;71(2):140-50.

44. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 2012;9:671-5
.
45. van Moorsel D, Hansen J, Havekes B, Scheer F, Jorgensen JA, Hoeks J, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. Mol Metab. 2016;5(8):635-45.
46. Wefers J, van Moorsel D, Hansen J, Connell NJ, Havekes B, Hoeks J, et al. Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle. Proc Natl Acad Sci U S A. 2018;115(30):7789-94.
47. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry. 1972;18(6):499-502.

Chapter 5
Acute cold exposure leads to increased levels of triglycerides during mixed-meal tests in young healthy subjects

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In preperation

Abstract

Background & Aims

Atherosclerosis and elevated lipid levels are important factors contributing to the growing mortality and morbidity due to cardiovascular disease in the modern world. In order to reduce lipid levels, animal studies revealed that brown adipose tissue (BAT) is able to combust large amounts of circulating triglycerides. Here, we aim to investigate if stimulation of non-shivering thermogenesis via acute cold exposure could reduce lipid levels in humans.

Materials & Methods

14 healthy young volunteers were included. All volunteers underwent three separate mixed meal tests lasting 8 hours, under different thermal conditions: a) at thermoneutrality, b) during acute non-shivering cold exposure and c) at thermoneutrality but after three hours of non-shivering cold exposure. Postprandial substrate and lipid metabolism was investigated by regular blood sampling, and skin temperatures and energy expenditure were measured. Muscle biopsies were obtained during acute cold exposure and at thermoneutrality to measure skeletal muscle mitochondrial function. On a separate day, cold induced brown adipose tissue activity was measured using an FDG-PET/CT scan.

Results

Acute cold exposure increased resting and diet-induced energy expenditure (17.4±3.4 % vs. 10.5±1.2%) with a concomitant increase in fat oxidation (8.6±1.6 g vs. 5.1±1.0 g; p=0.0785). Contrary to our hypothesis, postprandial triglyceride levels were increased during cold exposure compared to thermoneutral (8.2±0.1 mmol/l/min vs. 6.2±0.1 mmol/l/min; p=0.0134), or compared to pre-cooling (8.2±0.1 mmol/l/min vs. 4.6±0.1 mmol/l/min; p=0.0295). Acute cold exposure did not affect skeletal muscle mitochondrial function (maximum coupled respiration, p=0.15; leak respiration, p=0.74). BAT activity and volume did not correlate with postprandial changes in plasma lipid levels. Conclusion: Opposite to findings in animals, acute non-shivering thermogenesis does not decrease postprandial lipids levels in young healthy volunteers, despite increased whole body energy expenditure and fat oxidation.

Introduction

In our modern Western society cardiovascular disease (CVD) has become one of the foremost causes of death, with up to 31.4% of all deaths worldwide (1). One of the most important risk factors for CVD is hyperlipidaemia (2), which is classified as elevated levels of cholesterol and/or triglycerides. Indeed, lifestyle and pharmacological therapies are aimed at lowering levels of low-density lipoprotein cholesterol, known as the lipoprotein that carries the major risk for CVD (2) and on the lowering of circulating triglyceride levels. Indeed, hypertriglyceridemia is also considered as an important risk factor for CVD (3), even independent of high levels of LDL (4, 5).

Preclinical research from the last decade has provided strong evidence that brown adipose tissue (BAT) is an interesting tissue in the clearance of circulating triglycerides. BAT has the unique feature of having a special mitochondrial protein, uncoupling protein 1 (UCP-1) (6), which is used to uncouple the mitochondrial proton gradient from ATP production.. Therefore, this mitochondrial uncoupling leads to futile use of substrates with fatty acids as main substrate (6), thereby producing heat. It has been shown that activation of BAT through cold increases the density of BAT tissue on CT scan images in humans (7, 8), indicating that intracellular lipids are combusted (7, 8). As a result, BAT activation would eventually lead to a depletion of the intracellular lipid stores, and circulatory lipids are needed to replenish Bat lipid stores. Indeed, it was shown in mice that active brown adipose tissue might be able to take up a large amount of particles containing triglycerides upon cold exposure (9). It is suggested that activated BAT will first utilize intracellular lipid stores, and upon continued activation will start to increase uptake of fatty acids from lipoprotein particles to maintain the heat production. Indeed, in a recent study with transgenic mice overexpressing UCP1, it was shown that cold exposure increases lipoprotein lipase activity in BAT (10). Furthermore it was shown that activation of brown fat in *APOE*3-Leiden.CETP* mice leads to reduced atherosclerosis (11). Additionally, in ApoE-/- and LDLR-/- mice, the effect of cold exposure in preventing atherosclerosis is absent (12). These results suggest that the clearance of fatty acids from lipoprotein particles by activated BAT plays an important role in atherosclerosis development in mice.

In humans, the exact role of BAT is still under debate. We and others have shown that brown adipose tissue is functionally present in human adults and activated by acute cold and cold acclimation (13-15), in both obese human subjects (16) and patients with T2DM (17). Next to the presence of active BAT in adult humans, there is also evidence that human BAT is involved in cold-induced metabolism (18, 19). More recently Chondronikolas et al (20) confirmed that short-term cold exposure results in an increased density of BAT tissue suggesting the use of intracellular lipid droplets, and a subsequent activation of white adipose tissue lipolysis to refuel the BAT. Additionally, Blondin et al have shown that, comparable to mice, BAT is

involved in uptake of dietary fatty acids (21).In their study, they used only a single meal and a predetermined temperature on each participant. This could mean that they missed a so-called second meal effect and interindividual differences in the response to cold exposure. However, Blondin et al also concluded that the net contribution of BAT to the clearance of dietary fatty acids is small Indeed, relative to rodents, in humans the amount of BAT is small (6, 22). Hence, in addition to BAT, skeletal muscle can play a role in cold induced energy and substrate metabolism. Indeed, it has previously been shown that the uncoupling process in muscle mitochondria is related to an increase in non-shivering thermogenesis (23, 24), and muscle tissue could thus be important in the clearance of plasma triglycerides.

Therefore, the aim of the current study was to investigate if acute cold exposure in humans can impact postprandial substrate metabolism and enhance triglyceride clearance.

Methods

Volunteers

14 healthy young volunteers, nine male and four female, completed the study. Eligibility was evaluated via a screening including assessment of blood parameters, cardiac function (electrocardiography), anthropometric measurements and a questionnaire about lifestyle. Inclusion criteria were: 20-50 years of age, BMI 20-30 kg/m^2, a normal physically active lifestyle (<3 h exercise per week), non-smoking for at least 6 months at inclusion, no alcohol use of >2 servings per day and stable dietary habits (no weight loss or gain of more than 5 kg in the past 3 months). No medication use was allowed, except for the use of any form of hormonal anticonception in female volunteers

Volunteers were excluded if they had any active metabolic or cardiovascular diseases, were pregnant or if they had a haemoglobin content below 8.4 mmol/L. All data were collected between April 2018 and November 2019.

Ethical approval

The study was conducted according to the declaration of Helsinki and was approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at https://trialregister.nl (NTR6573). All volunteers provided their written informed consent before screening.

Study design

The volunteers participated in three meal tests, each characterized by different thermal conditions: thermoneutral (at thermoneutrality), thermoneutral with three hours of preceding cold exposure (pre-cooling) and during acute cold exposure, as shown in figure 1.

On a separate day all volunteers underwent an 18F-FDG PET/CT-scan directly after four hours of acute non-shivering cold exposure to assess the individuals capacity to activate brown adipose tissue, as described previously (25). Between each test day (meal test or PET/CT scan) there were at least two days to prevent any effect of the previous test on the next test day.

All test days were randomized, as to prevent any effect of the order of tests on the measurements.

Cold exposure

To investigate the effect of BAT activity on lipid metabolism, we cooled volunteers down to maximize non-shivering thermogenesis, as described before (25). In short, Volunteers were wrapped in a water-perfused suit (ThermoWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne, Israel), while the water temperature was controlled using a two temperature management units (Blanketrol M3; Cincinnati Sub Zero, OH, USA). First, volunteers remained at thermoneutral conditions (32 °C water temperature), after which the water in the suit was cooled in a stepwise fashion, 4°C per 15 minutes. This was done until visible shivering occurred, at which point the subjects were heated to 34 °C for 10 minutes to minimize shivering. After these 10 minutes, the water temperature was set back to 2 °C above the shivering onset temperature. For the remainder of each cooling period, temperature was increased by 1°C degree if visible shivering occurred again.

Meal test

Volunteers arrived at our research centrum the evening before each meal test, around 6.00PM. Upon arrival they consumed a standardized meal, after which they were asked to remain fasted. The volunteers stayed overnight at the lab, and were asked to sleep from 10.00PM onwards. The following morning volunteers were woken at 5.30AM, after which they moved to a separate room and were asked to lie down on a bed in our research lab.

After placing an intravenous cannula, a fasted blood sample was drawn (T=0) and energy expenditure and substrate selection was measured by indirect calorimetry with a ventilated hood system (Omnical; Maastricht Instruments, Maastricht, The Netherlands). Between 6.00 and 9.00AM the volunteers were instructed to remain on the bed, and remain fasted. At 9.00AM, volunteers were asked to consume a high-fat shake within ten minutes. The shake had a mean temperature of 43.5±0.8 °C, as measured with a thermocouple (PL-125-T4USB VS, Voltcraft, Conrad Electronic SE, Hirschau, Germany). The nutritional content of the shake is shown in supplementary table S2. Subsequently, blood samples were drawn after 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes. After the blood sampling at T=240, at 1.00PM, the volunteers consumed a second milkshake with the same composition as in the morning to investigate the so-called second-meal effect, as described before (26). At T30-60, T90-120, T210-240, T270-300, T350-380 and T450-480 indirect calorimetry was performed to

measure energy expenditure and substrate oxidation by ventilated hood. Volunteers were not allowed to eat or drink anything else throughout the meal test, except for water.

The difference between the meal tests was the temperature to which the volunteers were exposed, as shown in figure 1. The first meal test was performed at thermoneutrality. The second meal test was performed during cold exposure via the water-perfused suit to maximize non-shivering thermogenesis during the meal test. The third meal test was performed after cold exposure: here volunteers were exposed to cold from 6.00AM until 9.00AM, again to maximize non-shivering thermogenesis (pre-cooling). After 9.00AM, the volunteers were exposed to a thermoneutral temperature. The pre-cooling was meant to deplete the intracellular lipid storages of BAT tissue.

18F-FDG PET/CT scan

For the determination of cold-induced BAT activity, volunteers first remained at thermoneutral conditions (32 °C water) for 30 minutes, during which basal metabolic rate was measured with a ventilated hood system (Omnical; Maastricht Instruments, Maastricht, The Netherlands). Thereafter an individualized cooling procedure was started to determine non-shivering thermogenesis (NST) as described above, after which energy expenditure was measured for 30 minutes. Following this, 75 MBq of 18F-FDG was injected via an intravenous cannula. Cold exposure was continued for 60 minutes, while volunteers were instructed to remain lying still. Next, the water-perfused suit was removed and the volunteers underwent a static 18F-FDG-PET/CT scan. For the first five volunteers a Gemini TF PET-CT, Philips, The Netherlands was used (120 kV, 30 mAs, six to seven bed positions (5 minutes per bed position)) were used, to cover the area from the skull to the iliac crest. For the other nine volunteers a Discovery MI 5R, GE Healthcare, Chicago, Ill, USA, was used (120 kV, 30 mAs, a maximum of four bed positions was used (4 minutes per bed position). A PET scan followed the low-dose CT scan. Tracer uptake was determined with the PET scan while the CT was used for attenuation correction and anatomical localization of the active BAT. With the use of an EARL reconstruction, the scans from the nine volunteers were made comparable to the scans from the five volunteers, as described by Boellaard et al (27). This ensured that we were able to compare results from both scanners in this study.

The scans were analyzed with PMOD software (version 3.0; PMOD Technologies). The regions of interest were manually outlined, while thresholds of 1.5 SUV (standardized uptake value) and Hounsfield units between -10 en -180 were used, as described previously by our group (25). Additionally fixed volumes (10mm by 10 mm) were placed in the cervical adipose tissue behind the clavicle to measure general uptake values as described before (28). BAT activity was expressed in SUV (18F-FDG uptake (kBq/mL/ (injected dose (kBq/volunteer weight (g)). The activity was determined as average SUV (SUVmean) and as total SUV (SUVmean times the volume of interest).

Indirect calorimetry

Whole body oxygen consumption and carbon dioxide production were measured during fixed time points in the meal tests with the use of an automated respiratory gas analyzer with a ventilated hood system (Omnical; Maastricht Instruments, Maastricht, The Netherlands). Volunteers were measured in supine position for 30 minutes each time. The measured averaged oxygen and carbon dioxide concentrations were used to calculate energy expenditure, glucose and fat oxidation with the assumption that protein oxidation was negligible (29, 30). These fixed time points are indicated in figure 1.

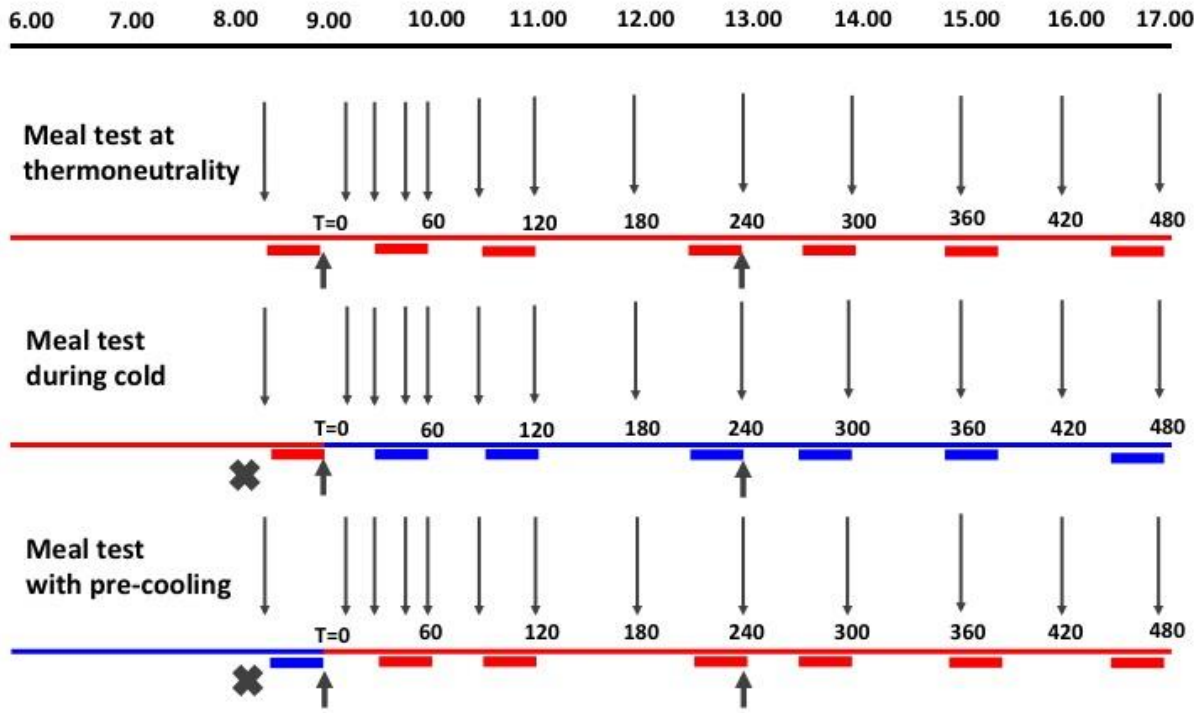


Figure 1. Study design

Three meal tests. Colour of horizontal lines indicates cooling (blue) or thermoneutral (red) conditions. Bold arrows indicate meal consumption. Crosses show muscle biopsies. Horizontal boxes indicate the indirect calorimetric measurements. Thin arrows show blood drawings.

Skin temperature

During all measurements, volunteers were asked to wear shorts and t-shirt (overall clo value 0.49), and were instructed to remain supine and resting. Wireless temperature sensors (iButtons DS1922L, Maxim Integrated, San Jose, CA, USA) were placed on 15 ISO-defined sites (ISO-standard 9886:200) during all measurement days to measure skin temperature (31). Average skin temperature as well as proximal and distal temperature was calculated as described before (32).

Skeletal muscle biopsies and mitochondrial respiration

To assess the effect of acute cold exposure on mitochondrial respiration, two muscle biopsies were taken under local anaesthesia using the Bergström technique (33). These biopsies were taken after an overnight fast before the consumption of the first shake on the morning of the meal test at 8.30AM. One biopsy was taken during cold exposure (under stable non-shivering conditions) and one biopsy was taken under thermoneutral conditions. After the incision was made, a sterile golden temperature probe (BAT-10 Multipurpose Thermometer, Physitemp Instruments LLC, Clifton, NJ, USA) was inserted into the muscle to measure both muscle temperature and skin temperature. Subsequently the biopsy was taken and the obtained muscle tissue was immediately placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria). Intact muscle fibers were permeabilized, after which the muscle fibers were transferred into ice-cold mitochondrial respiration buffer (MiR05, OROBOROS Instruments, Innsbruck, Austria). Next, ex vivo mitochondrial respiration was determined by measuring oxygen consumption rate upon several substrates using high-resolution respirometry (Oxygraph, OROBOROS Instruments, Innsbruck, Austria) as described previously (34). All measurements were performed in triplicate or quadruplicate, depending on the reliability of the respirometric data, as assessed by NvP and JH. Data is presented per mg wet muscle weight.

Blood sampling and analyses

Blood collected in EDTA-coated tubes was immediately stored on ice, centrifuged and plasma was stored at -80 °C until further analyses. Blood collected in serum-tubes was stored at room temperature for at least 30 minutes to allow coagulation, followed by centrifugation and storage at -80 °C until further analyses. Glucose (Hk-CP, Axonlab, Amsterdam, The Netherlands), triglycerides (Sigma, Zwijndrecht, The Netherlands) and FFA (NEFA-HR, WAKO chemicals, Neuss, Germany) were determined enzymatically in EDTA plasma derived from the meal test samples using a Pentra 400 (Horiba, Montpellier, France).

Analysis of lipid particles via NMR spectroscopy

Blood collected in serum-tubes, stored at -80 °C were analyzed to specifically measure concentrations of lipoprotein particles using nuclear magnetic resonance (NMR) spectroscopy (Axinon Magnetic Group Analysis, Numares health, Regensburg Germany).

Statistical analyses

Subject characteristics are reported as mean ± SD. Other results are reported as mean ± SE. Data are presented for n=14, unless otherwise indicated. All data were evaluated for normal distribution. Data of three different meal test days was compared using a total (AUC) and an incremental area-under-the curve (iAUC). The AUC/iAUC were calculated for the duration of the meal test, starting at 9.00 AM and ending at 5.00 PM. Differences between meal tests were analyzed with a repeated measures ANOVA for parametric data and with a Friedman's ANOVA for non-parametric data. Statistical significance was set at $p<0.05$. Statistical analyses

were performed using IBM SPSS version 26.0 for MacOSx. Figures were made using Graphpad Prism version 6.0 for MacOSx.

Results

Subject characteristics

14 healthy young volunteers (9 men, 5 women, age: 26.1±7.2 years) participated in this study. Volunteers were non-obese (BMI: 23.3±1.6 kg/m2), were non-smokers, did not use any medication except for hormonal anticonception in women and had an overall sedentary lifestyle (Baecke score 10.4±2.1). The participant characteristics are presented in table 1. There were no significant differences between male and female volunteers in BMI (p=0.42), age (p=0.31) and triglyceride levels at screening (p=0.77).

Table 1. Participant characteristics

Parameter	Mean ± SD
Number volunteers female, male	5, 9
Age (years)	26 ± 7.2
Body weight (kg)	72.5 ± 10.1
Height (m)	1.75 ± 0.10
BMI (kg/m^2)	23.3 ± 1.6
Glucose (mmol/L)	4.9 ± 0.4
TG (mmol/L)	1.05 ± 0.60
ASAT (U/L)	23 ± 6
ALAT (U/L)	24 ± 9
GGT (U/L)	20 ± 7
eGFR ($ml/min/1.73mm^2$)	89 ± 9
Physical activity level (Baecke score)	10.4 ± 2.1

Abbreviations: BMI, body mass index; TG, triglycerides; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma-glutamyl transferase; eGFR, estimated glomerular filtration rate according CKD-EPI method.

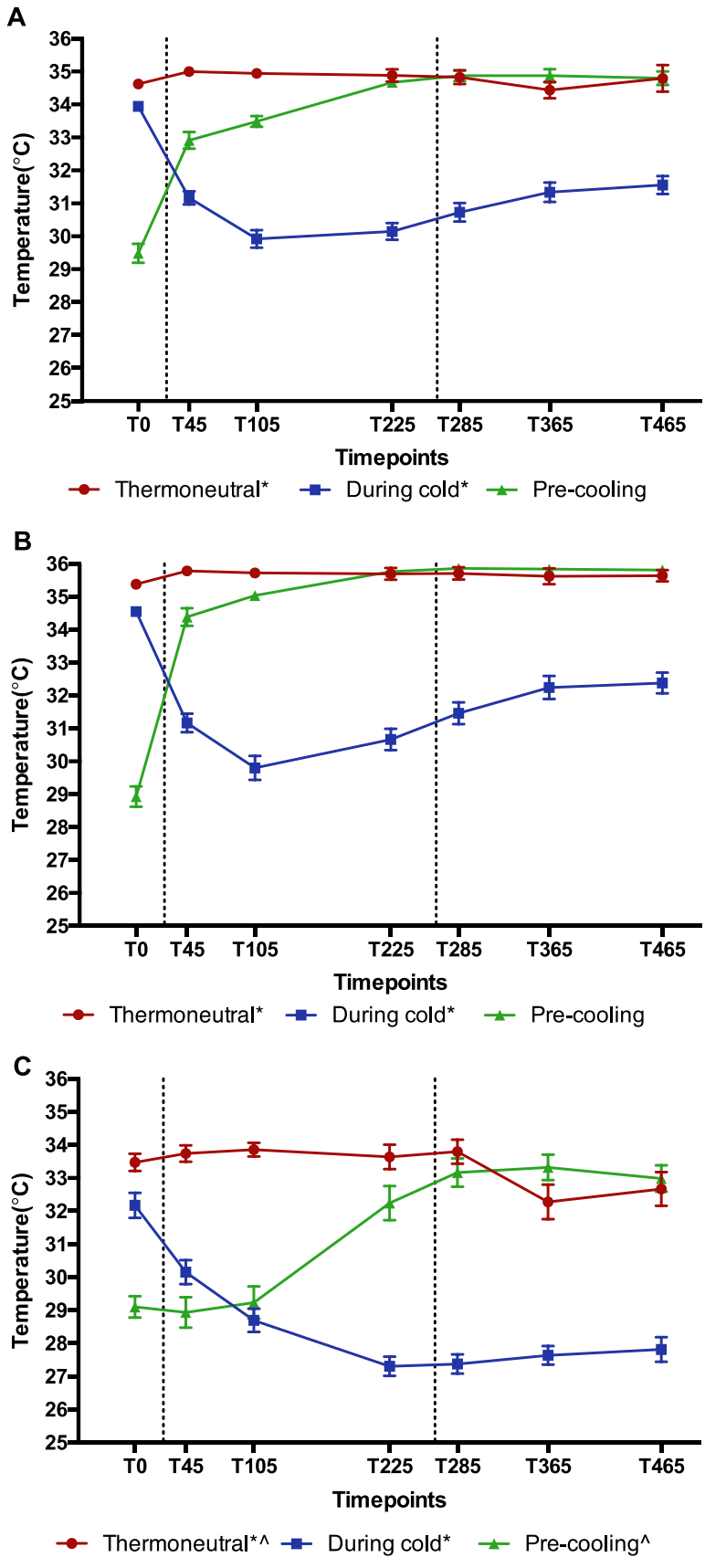
Skin and muscle temperatures

First, to investigate the body's response to the cold exposure, we measured skin and muscle temperature. During the meal test, mean skin temperature was significantly higher at thermoneutrality compared to cold (34.8 ±0.1 °C vs. 31.3 ±0.5 °C, p= 0.004), however it was not significantly different compared to the meal test with precooling (33.6±0.7 °C; p=0.1562, figure 2A). There were no significant differences between genders during cold exposure (p=0.97) or after pre-cooling (0.32). There was however a significant difference between male and female volunteers at thermoneutral conditions (34.6±0.1 °C vs. 35.0±0.1 °C; p=0.011). These results are shown in supplemental figure 2A. Mean proximal skin temperature was significantly higher during the meal test at thermoneutrality compared to cold (35.7±0.04 °C vs. 31.8±0.6 °C, p= 0.0008) however it was not significantly different compared to precooling (34.5±1.0 °C; p=0.1015, figure 2B). There was a significant difference in proximal skin temperature between male and female volunteers during the meal test at thermoneutrality (35.4±0.1 °C vs. 36.0±0.1 °C; p=0.0006). There were no gender differences in the meal test during cold (p=0.38) or with pre-cooling (p=0.26), as shown in supplemental figure 2B.

Similarly, the mean distal temperature was significantly higher during the meal test at thermoneutrality compared to cold (33.4 ±0.2 °C vs. 28.7±0.7 °C, p= 0.0004), and it showed a trend towards a significant difference compared to pre-cooling (31.3±0.8 °C; p=0.07, figure 2C). There were no gender differences in distal skin temperature during the meal tests (p=0.26 at thermoneutrality; p=0.87 during cold; p=0.38 with pre-cooling) as shown in supplemental figure 2C. The mean proximal-distal gradient did not differ significantly between the meal test at thermoneutrality compared to cold (2.4±0.2 °C vs. 3.0±0.6 °C, p= 0.17) or pre-cooling (3.2±0.8 °C, p= 0.30) as shown in figure 2D.

There were no gender differences in the meal tests at thermoneutrality, during cold or after pre-cooling (p=0.45; p=0.31; p=0.97), as shown in supplemental figure 2D. Regarding the mean underarm-finger gradient, we did observe a significantly lower gradient during the meal test at thermoneutrality compared to the meal test during cold (-0.8±0.1 °C vs. 5.7±0.9 °C, p=0.0002). The mean under-finger gradient was also higher in the meal test during cold exposure compared to the meal test with pre-cooling (1.2±0.9 °C, p=0.044) as shown in figure 2E. During the meal test at thermoneutrality there was lower gradient in the female volunteers (-1.7±0.2 °C vs. -0.2±0.1 °C; p=0.0006).

However, there were no gender differences in the meal tests during cold (p=0.38) or after pre-cooling (p=0.31), as shown in supplemental figure 2E. Muscle temperature was significantly higher during thermoneutral condition compared to cold exposure (35.1±0.2 °C vs. 31.9±0.4 °C; p<0.001).


A
Temperature(°C)
36
35
34
33
32
31
30
29
28
27
26
25
T0
T45
T105
T225
T285
T365
T465
Timepoints
Thermoneutral*
During cold*
Pre-cooling
B
Temperature(°C)
Timepoints
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Pre-cooling
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During cold*
Pre-cooling^

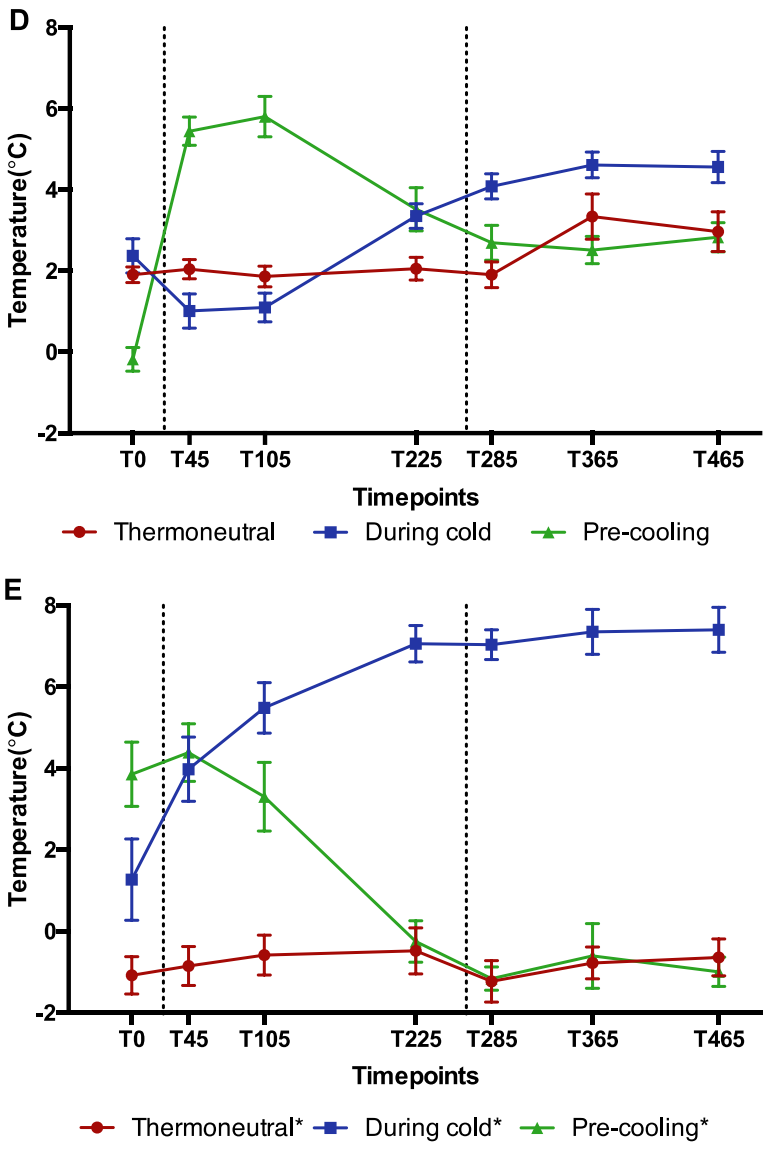


Figure 2. Skin temperatures during the meal tests

A: Mean skin temperature during the different meal tests. **B**: Mean proximal skin temperature. **C**: Mean distal skin temperature, **D**: Mean proximal-distal gradient and **E**: Mean underarm-finger gradient. The meal test at thermoneutrality is indicated as the red line, the meal test during cold exposure as the blue line, and the meal test after cold as the green line. Dashed vertical lines show the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14. Data is presented as mean ± SE. * indicates significant difference. ^ Indicates trends towards significant difference.

Postprandial energy and substrate metabolism

Next, we examined if cold exposure had an effect on energy expenditure during the meal test (9.00AM until 5.00PM. Total energy expenditure during the meal test, calculated as AUC, was significantly higher during cold exposure compared to thermoneutrality (2772±130.3 kJ vs. 2613±166.2 kJ; p=0.0203) and precooling (2772±130.3 kJ vs. 2587±110.6 kJ; p=0.0052) (figure 3A). These differences were similar in the male volunteers (p=0.015), however there were no differences in the female volunteers (p=0.54). Similar results were found when the iAUC values were calculated, with significantly higher values with the meal test during cold exposure (481.2±55.8 kJ) compared to thermoneutrality (481.2±55.8 kJ vs. 338.2±27.5 kJ; p=0.0031) and compared to precooling (481.2±55.8 kJ vs. 302.3±36.6 kJ; p=0.0494) (figure 3A). Again in male volunteers we observed the same differences (p=0.014), while in the female volunteers these were absent (p=0.21). Results for male and female volunteers are shown in supplemental figure 3A.

To calculate diet-induced thermogenesis, we subtracted average energy expenditure at rest from energy expenditure during the meal. DIT, expressed as a percentage of resting energy expenditure, was 10.5±1.2% at thermoneutrality. During cold exposure, this percentage was 17.4±3.4 %, consisting of both diet-induced thermogenesis (DIT) and cold-induced thermogenesis (CIT). These data suggest that the difference between the DIT and CIT+DIT was 159±64 kJ or 6.1±2.3 %, which can be attributed to CIT, and suggests additive effects of CIT and DIT.

For male volunteers, average DIT was 10.8±1.7%, and average DIT+CIT was 19.5±4.7%. For female volunteers, DIT was 10.1±2.0% and DIT+CIT was 13.7±4.8%. This suggests that while DIT% is similar in both male and female volunteers, there is a higher though non-significant increase in energy expenditure in cold in men compared to women (8.7±3.2% vs. 3.6±3.4%; p=0.49).

The increase in energy expenditure during cold exposure was mainly accommodated by an increase in fat oxidation. Thus, iAUC of fatty acid oxidation upon the meal test was significantly higher during cold exposure compared to precooling (8.6±1.6 g vs. 2.1±0.7 g; p=0.0023) and tended to be higher compared to thermoneutrality (8.6±1.6 g vs. 5.1±1.0 g; p=0.0785), as shown in figure 3B. For male volunteers these differences were also present, as fatty acid oxidation was higher during cold compared to at thermoneutrality (11.1±2.1 g vs. 5.4±1.3 g; p=0.05) and compared to after pre-cooling (2.2±1.0 g; p=0.001), however they were absent in female volunteers (p=0.31), as is shown in supplemental figure 3B. No significant differences in iAUC of carbohydrate oxidation were observed between the different meal tests, as shown in figure 3C. This was similar for both male (p=0.70) and female volunteers (p=0.99), as shown in supplemental figure 3C.

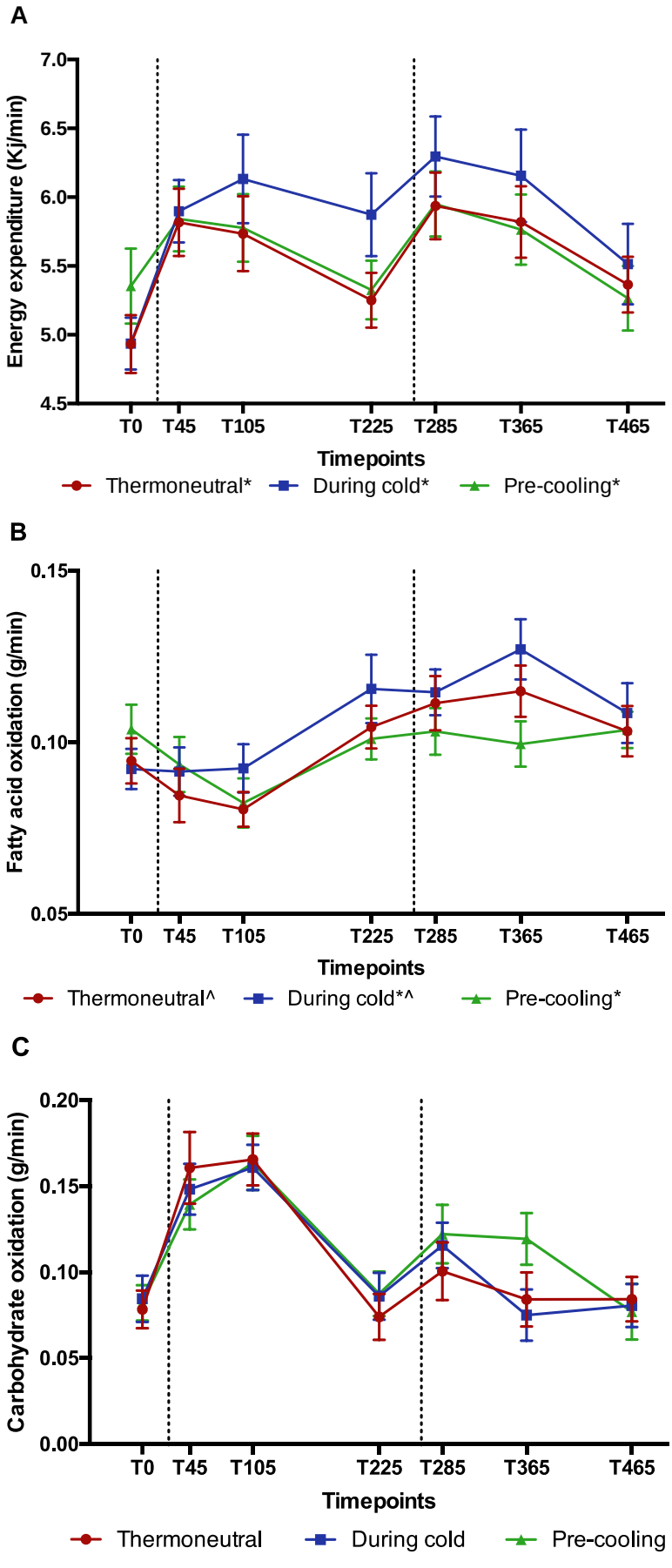

A
Energy expenditure (Kj/min)
7.0
6.5
6.0
5.5
5.0
4.5
T0
T45
T105
T225
T285
T365
T465
Timepoints
Thermoneutral*
During cold*
Pre-cooling*
B
Fatty acid oxidation (g/min)
0.15
0.10
0.05
T0
T45
T105
T225
T285
T365
T465
Timepoints
Thermoneutral^
During cold*^
Pre-cooling*
C
Carbohydrate oxidation (g/min)
0.20
0.15
0.10
0.05
0.00
T0
T45
T105
T225
T285
T365
T465
Timepoints
Thermoneutral
During cold
Pre-cooling

Figure 3. Energy expenditure and substrate oxidation during the meal tests
A: Energy expenditure, **B**: Fatty acid oxidation and **C**: Carbohydrate oxidation during the meal tests. The meal test at thermoneutrality is indicated as the red line, the meal test during cold exposure as the blue line and the meal test after cold as the green line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14. Data is presented as mean ± SE. * indicates significant difference. ^ Indicates trends towards significant difference.

Activity of brown adipose tissue via 18F-FDG PET/CT

To investigate if the interindividual differences in physiological response to cold exposure were related to the individuals BAT activity, the latter was assessed using FDG-PET/CT after four hours of acute non-shivering cold exposure. On average, BAT volume was 66.8±12.4 mL, with an average activity of 3.2±0.3 SUV and a maximal activity of 8.9±1.2 SUV. The average BAT activity showed a trend towards significant positive correlation with the (CIT; 159±64 kJ or 6.1±2.3%) (r=0.46; p=0.097). However, there was no significant correlation between DIT and average BAT activity (r=0.09, p=0.74), or between fatty acid oxidation and mean BAT activity (r=0.34, p=0.23).

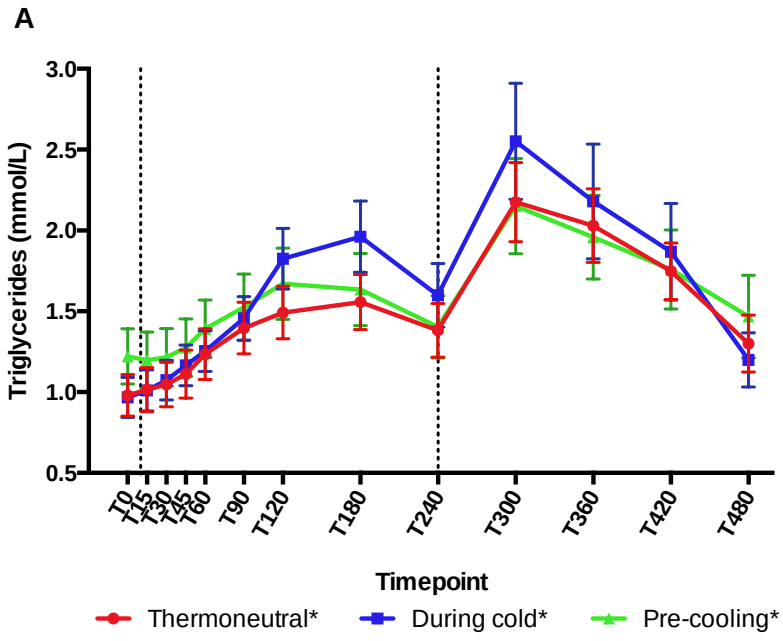
Postprandial substrate kinetics

Postprandial area-under-the-curve values (AUC) for triglycerides were not significantly different between the meal tests at thermoneutrality, during cold or with pre-cooling (respectively: 1.6±0.2 mmol/l/min vs. 1.8±0.2 mmol/l/min vs. 1.7±0.2 mmol/l/min; p=0.315), as shown in figure 4A. Remarkably, the incremental area-under-the-curve values (iAUC) for triglycerides were significantly higher during cold exposure compared to thermoneutrality (8.2±0.1 mmol/l/min vs. 6.2±0.1 mmol/l/min; p=0.0134) or to precooling (8.2±0.1 mmol/l/min vs. 4.6±0.1 mmol/l/min; p=0.0295).
In male volunteers the differences in iAUC were similar, with higher iAUC values during cold compared to at thermoneutrality (1.4±02 mmol/l/min vs. 0.8±0.1 mmol/l/min; p=0.098). For female volunteers these differences were also present (0.4±0.03 mmol/l/min vs. 0.2±0.1 mmol/l/min; p=0.063), as shown in supplemental figure 4A.

Postprandial AUC of plasma FFA was significantly higher during cold exposure compared to thermoneutrality (550.2±30.1 umol/l/min vs. 392.7±25.5 umol/l/min; p=0.0001) and compared to precooling (550.2±30.1 umol/l/min vs. 433.9±35.2 umol/l/min; p=0.0052). For male volunteers these differences were also present, with higher AUC during cold compared to at thermoneutrality (552.4±36.8 umol/l/min vs. 413.2±33.1 umol/l/min; p=0.004) or compared to pre-cooling (454.1±50.4 umol/l/min; p=0.055). This was similar in female volunteers, with higher values during cold compared to at thermoneutrality (546.3±61.9 umol/l/min vs. 355.7±38.2 umol/l/min; p=0.063), as shown in supplemental figure 4B.

Similar effects were observed for iAUC, which was significantly higher during the meal test during cold compared to the meal test at thermoneutrality (278.0±28 umol/l/min vs. 235.4±19.8 umol/l/min; p=0.0419) or the meal test with pre-cooling (278.0±28 umol/l/min vs. 172.8±17.3 umol/l/min; p=0.0017), as shown in figure 4B. For male volunteers these differences were also present, with higher AUC during cold compared to at thermoneutrality (280.9±29.0 umol/l/min vs. 233.4±21.0 umol/l/min; p=0.055) or compared to pre-cooling (151.7±16.1 umol/l/min; p=0.004). This was absent in female volunteers (p=0.40). Results for genders are shown in supplemental figure 4B.

Postprandial AUC of plasma glucose was significantly lower in the meal test during cold exposure compared to the meal test at thermoneutrality (5.3±0.1 umol/l/min vs. 5.8±0.1 umol/l/min; p=0.0067) and compared to the meal test with pre-cooling (5.8±0.1 umol/l/min vs. 5.9±0.1 umol/l/min; p=0.0052), as shown in figure 4C. For both male (p=0.072) and female volunteers (p=0.057) there was a tendency towards significant differences, as shown in supplemental figure 4C.



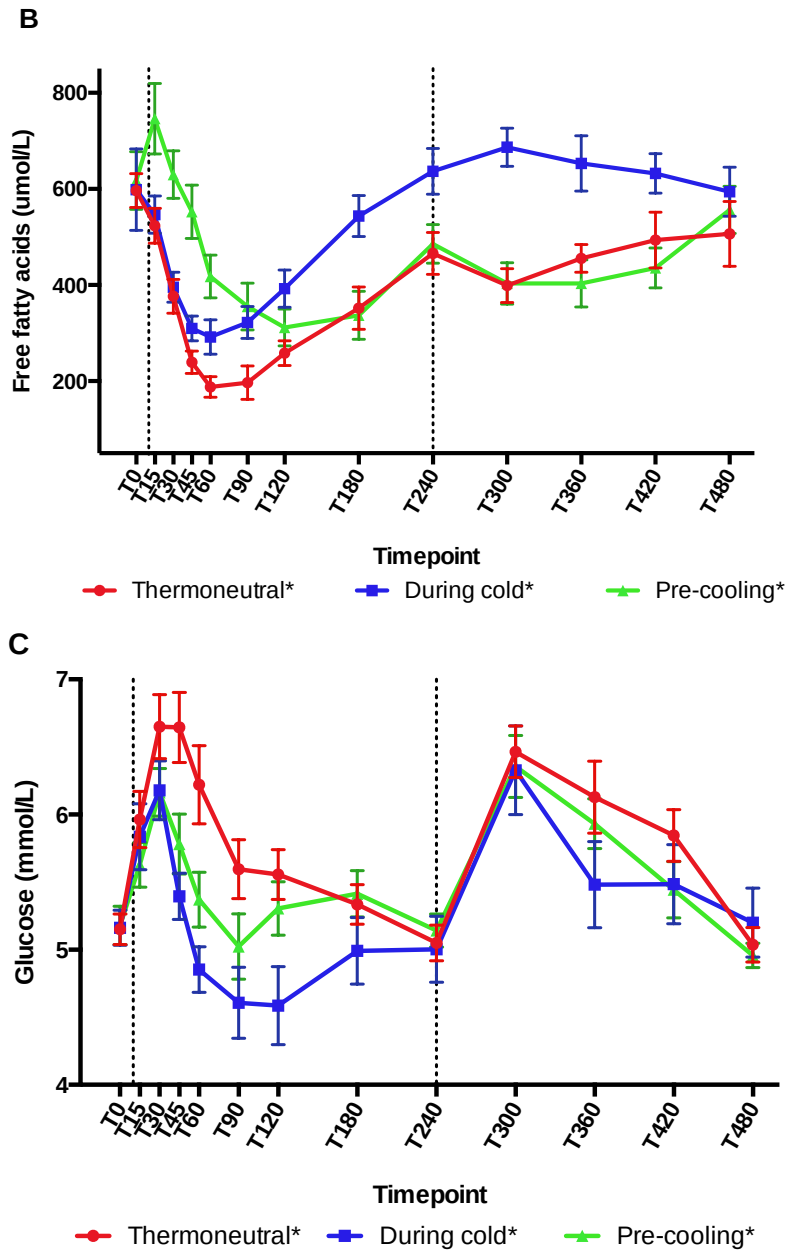
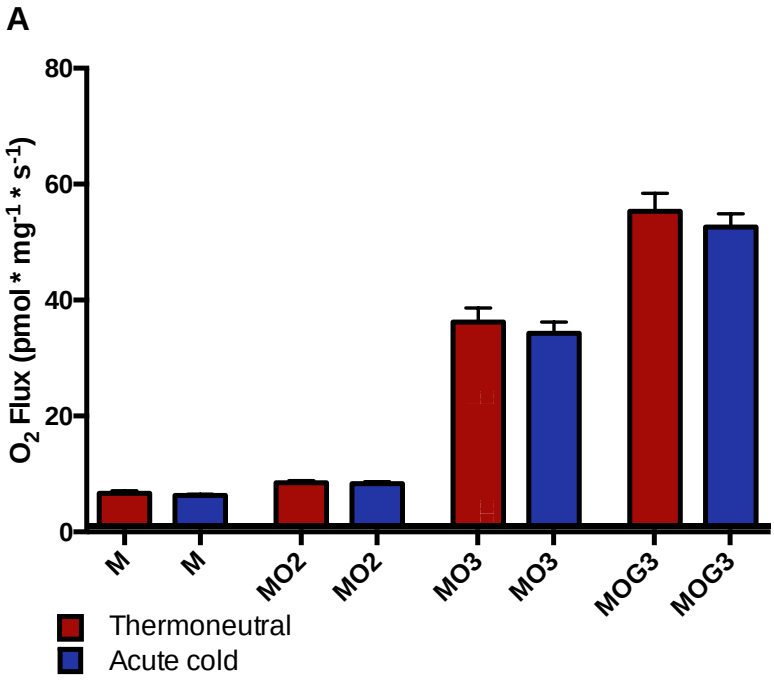


Figure 4. Plasma levels during the meal tests

Plasma levels of **A**: Triglycerides, **B**: Free fatty acids and **C**: Glucose during the meal tests. The meal test at thermoneutrality is represented as the red line, the meal test during cold as the blue line and the meal test after cold as the green line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14. Data is presented as mean ± SE. * indicates significant difference. ^ indicates trends towards significant difference.

Mitochondrial respiration

To investigate if acute cold exposure affects skeletal muscle mitochondrial function, the latter was determined in muscle biopsies taken during acute cold exposure and under thermoneutral conditions. No significant differences were observed in state 2 respiration (malate + octanoylcarnitine (MO), p=0.78) or in ADP-stimulated state 3 respiration (p=0.35). Addition of glutamate (MOG3) (p=0.94) and succinate (MOGS3) (p=0.43) did also not lead to a difference between the cold and thermoneutral conditions. Next, maximal uncoupled respiration was measured using FCCP titration, and was not significant different between the two conditions (p=0.15). Mitochondrial reserve capacity, as defined by state 3U/ state 3 upon was significantly higher thermoneutrality compared to cold exposure (1.26±0.02 vs. 1.20±0.02; p=0.006), reflecting that under cold exposure, the skeletal muscle mitochondria have less reserve respiration. To investigate leak respiration, we performed a second trace in which we determined ADP-stimulated state 3 respiration upon malate + pyruvate and leak respiration up addition of oligomycin (state 4o). No significant differences were observed between the thermoneutral and cold conditions for state 3 (p=0.94) or state 4o respiration (p=0.74) as shown in figure 5B.



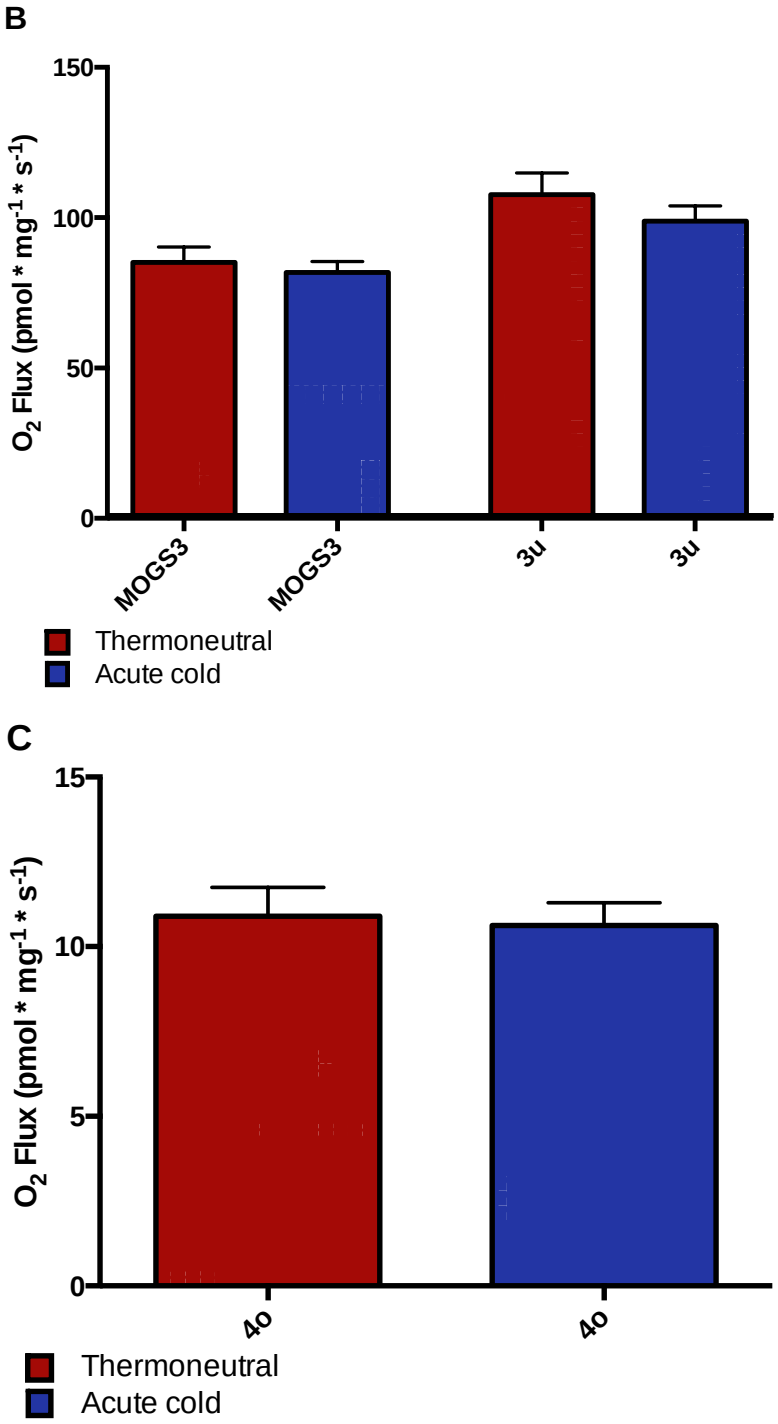


Figure. 5. Mitochondrial respiration.
A, maximally coupled respiration upon substrate only (state 2). **B,** maximally coupled respiration on substrate and ADP (MOGS3) and FCCP-induced uncoupled (3u) respiration. **C,** leak respiration upon oligomycin (state 4o). Data is presented as mean ± SE. n=14 in A, B, n=12 in C. M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; CytC, Cytochrome C; FCCP, Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone.

Discussion

Hyperlipidaemia is known to be one of the most important risk factors for developing cardiovascular disease (2). Reducing hyperlipidaemia could therefore decrease cardiovascular morbidity and mortality. There are indications that cold induced activation of brown adipose tissue leads to an increase of the clearance of blood lipids. Therefore, in this study we aimed at investigating the effects of acute non-shivering cold exposure on postprandial lipidaemia.

Previous studies indicate potential metabolic benefits of cold exposure in humans, via activation of brown adipose tissue and/or skeletal muscle. Cold exposure, both acute and chronic exposure, has been shown to increase energy expenditure (13, 25). In addition, cold acclimation has shown to improve glucose metabolism in patients with diabetes mellitus (17). In our study, we observed a mean DIT of 10.5±1.2%. Interestingly, the percentual increase in postprandial energy expenditure during cold exposure, reflecting a combination of CIT and DIT, was 17.4±3.4% percent, suggesting additive effects of cold and food intake on energy expenditure in humans, as was described before by Dauncey et al (35). Interestingly, the current study shows that cold exposure increased energy expenditure during the meal test on top of DIT. It has previously been shown that under fasting conditions, cold-induced thermogenesis (CIT) without shivering ranges from 0-30% (36). The increase in energy expenditure is accounted for 66.7% by an increase in fat oxidation, as was found previously upon cold exposure (37). Interestingly this increase in energy expenditure during cold exposure was less in the female volunteers, though not statistically significant. This could indicate that in women there is less additional energy production needed to compensate for the cold exposure. As can be seen from the temperature results there was no difference between male and female volunteers during the cold exposure.

Preclinical research in rodents has suggested that cold-induced activation of BAT leads to the clearance of circulating triglycerides (10, 11). Here, we tested the hypothesis that also in humans, cold exposure would lead to a reduction in post-prandial triglyceride levels. We hypothesized that cold exposure would acutely lower triglycerides due to the increased usage of lipids during cold exposure in muscle and BAT. In addition, we wanted to test the hypothesis that cold exposure before a meal would deplete intracellular lipid stores in BAT, leading to an increased postprandial TG uptake after cold exposure to replenish lipid stores in BAT. Therefore, we performed postprandial triglyceride kinetics during and upon cold exposure. However, contrary to our hypothesis and in contrast with findings in animals, we observed an increase in both postprandial triglycerides and free fatty acids levels during cold exposure, which may partially be explained by an increase in lipolysis that occurs with acute cold exposure (8). Furthermore, precooling also did not lower postprandial triglyceride or fatty acids levels. Together, these results are in contrast to the findings in preclinical studies and suggest that in humans cold exposure does not lead to an enhanced lipid clearance.

We previously showed that cold acclimation improved substrate metabolism and insulin sensitivity in T2D (17). Here, we found that postprandial glucose levels were lower with acute cold exposure and pre-cooling, which suggest that acute cold exposure could have a potential positive effect on glucose clearance and would be consistent with our previous finding of beneficial effects of cold exposure on insulin sensitivity (17). In the latter study, we showed that cold exposure can lead to an increase in skeletal muscle glut4 translocation, and it is tempting to speculate that enhanced GLUT4 translocation in muscle is responsible for the improved glucose clearance upon cold exposure in the current study.

Although brown adipose tissue is a potent tissue, in humans the amount of brown adipose tissue may not be enough to affect lipid consumption and therefore lower plasma levels, as was discussed previously (38). Skeletal muscle would be another tissue that could contribute to lipid consumption during cold exposure due to increased leak respiration (39, 40), however we did not observe any effects in maximal coupled or uncoupled respiration between the thermoneutral and cold conditions. Additionally we also did not observe any difference in leak respiration (state 4o) in skeletal muscle tissue, which indicates an absence of any increased proton leak during acute cold exposure. This absence is also observed in other cold exposure studies (23).

Although in rodents, the acute cold exposure was enough to lower lipid levels (10), in humans the acute cold exposure without shivering did not lead to decreased lipid levels. Additionally there is the option that cold acclimation could have a significant effect on triglyceride clearance. In cold-adapted winter swimmers it is shown that there are lower levels of markers of cardiovascular risk compared to non-adapted controls (41). Therefore future studies might focus on longer periods of cold exposure or on the effects of shivering thermogenesis, in order to investigate the potential of cold as a treatment option in the fight against cardiovascular diseases.

Reference list

1. Organization WH. Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016. Geneva; 2018.
2. Nordestgaard BG, Varbo A. Triglycerides and cardiovascular disease. Lancet. 2014;384(9943):626-35.
3. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. Jama. 2007;298(3):299-308.
4. Austin MA, McKnight B, Edwards KL, Bradley CM, McNeely MJ, Psaty BM, et al. Cardiovascular disease mortality in familial forms of hypertriglyceridemia: A 20-year prospective study. Circulation. 2000;101(24):2777-82.
5. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J Cardiovasc Risk. 1996;3(2):213-9.
6. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
7. Baba S, Jacene HA, Engles JM, Honda H, Wahl RL. CT Hounsfield units of brown adipose tissue increase with activation: preclinical and clinical studies. J Nucl Med. 2010;51(2):246-50.
8. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. J Clin Invest. 2012;122(2):545-52.
9. Khedoe PP, Hoeke G, Kooijman S, Dijk W, Buijs JT, Kersten S, et al. Brown adipose tissue takes up plasma triglycerides mostly after lipolysis. J Lipid Res. 2015;56(1):51-9.
10. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
11. Berbee JF, Boon MR, Khedoe PP, Bartelt A, Schlein C, Worthmann A, et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. Nat Commun. 2015;6:6356.
12. Dong M, Yang X, Lim S, Cao Z, Honek J, Lu H, et al. Cold exposure promotes atherosclerotic plaque growth and instability via UCP1-dependent lipolysis. Cell Metab. 2013;18(1):118-29.
13. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
14. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
15. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.

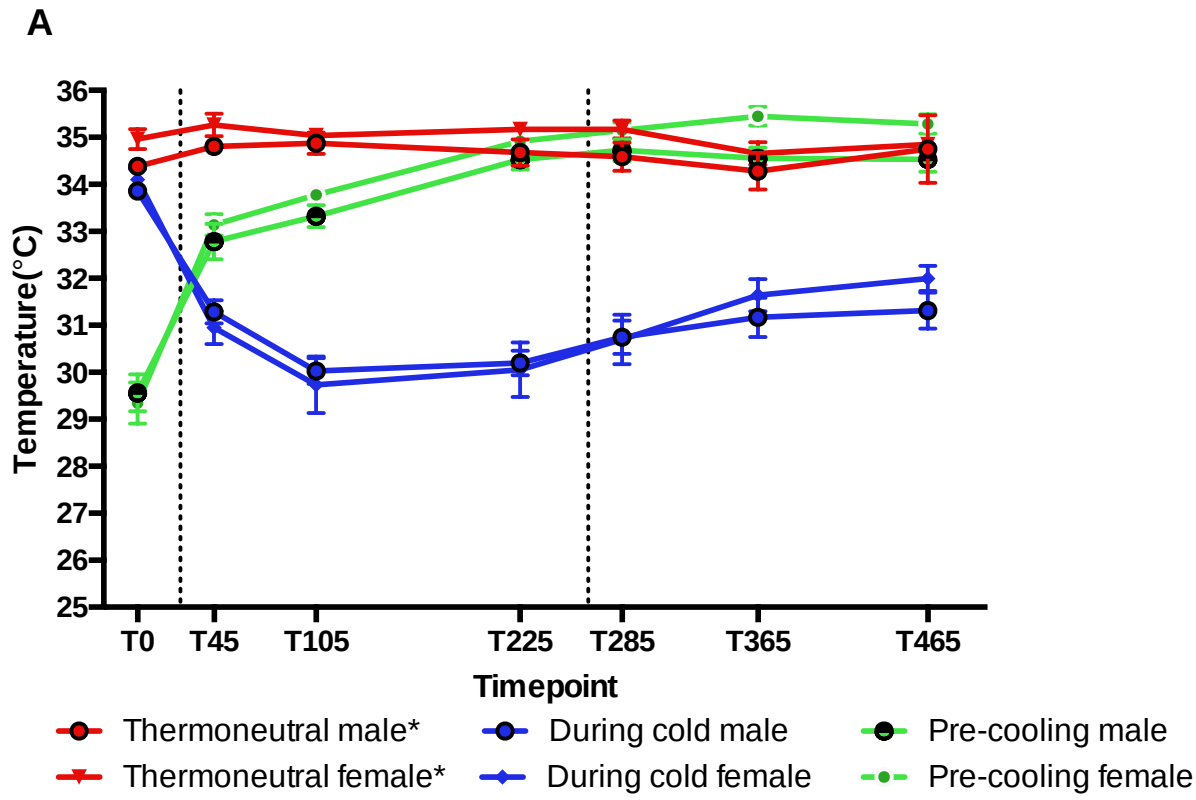
16. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, van Marken Lichtenbelt WD. Brown adipose tissue in morbidly obese subjects. PLoS One. 2011;6(2):e17247.
17. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
18. Muzik O, Mangner TJ, Leonard WR, Kumar A, Janisse J, Granneman JG. 15O PET measurement of blood flow and oxygen consumption in cold-activated human brown fat. J Nucl Med. 2013;54(4):523-31.
19. Weir G, Ramage LE, Akyol M, Rhodes JK, Kyle CJ, Fletcher AM, et al. Substantial Metabolic Activity of Human Brown Adipose Tissue during Warm Conditions and Cold-Induced Lipolysis of Local Triglycerides. Cell Metab. 2018;27(6):1348-55 e4.
20. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, et al. Brown Adipose Tissue Activation Is Linked to Distinct Systemic Effects on Lipid Metabolism in Humans. Cell Metab. 2016;23(6):1200-6.
21. Blondin DP, Tingelstad HC, Noll C, Frisch F, Phoenix S, Guerin B, et al. Dietary fatty acid metabolism of brown adipose tissue in cold-acclimated men. Nat Commun. 2017;8:14146.
22. Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta. 2001;1504(1):82-106.
23. Wijers SL, Schrauwen P, Saris WH, van Marken Lichtenbelt WD. Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. PLoS One. 2008;3(3):e1777.
24. Wijers SL, Schrauwen P, van Baak MA, Saris WH, van Marken Lichtenbelt WD. Beta-adrenergic receptor blockade does not inhibit cold-induced thermogenesis in humans: possible involvement of brown adipose tissue. J Clin Endocrinol Metab. 2011;96(4):E598-605.
25. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
26. Baumgartner S, Mensink RP, Plat J. Effects of a Plant Sterol or Stanol Enriched Mixed Meal on Postprandial Lipid Metabolism in Healthy Subjects. PLoS One. 2016;11(9):e0160396.
27. Boellaard R, O'Doherty MJ, Weber WA, Mottaghy FM, Lonsdale MN, Stroobants SG, et al. FDG PET and PET/CT: EANM procedure guidelines for tumour PET imaging: version 1.0. Eur J Nucl Med Mol Imaging. 2010;37(1):181-200.
28. Vosselman MJ, Brans B, van der Lans AA, Wierts R, van Baak MA, Mottaghy FM, et al. Brown adipose tissue activity after a high-calorie meal in humans. Am J Clin Nutr. 2013;98(1):57-64.
29. Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. Can J Sport Sci. 1991;16(1):23-9.

30. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol. 1949;109(1-2):1-9.
31. van Marken Lichtenbelt WD, Daanen HA, Wouters L, Fronczek R, Raymann RJ, Severens NM, et al. Evaluation of wireless determination of skin temperature using iButtons. Physiol Behav. 2006;88(4-5):489-97.
32. Vosselman MJ, Hoeks J, Brans B, Pallubinsky H, Nascimento EB, van der Lans AA, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. Int J Obes (Lond). 2015;39(12):1696-702.
33. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. Acta Physiol Scand. 1967;71(2):140-50.
34. Hoeks J, van Herpen NA, Mensink M, Moonen-Kornips E, van Beurden D, Hesselink MK, et al. Prolonged fasting identifies skeletal muscle mitochondrial dysfunction as consequence rather than cause of human insulin resistance. Diabetes. 2010;59(9):2117-25.
35. Dauncey MJ. Metabolic effects of altering the 24 h energy intake in man, using direct and indirect calorimetry. Br J Nutr. 1980;43(2):257-69.
36. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R285-96.
37. Vallerand AL, Jacobs I. Rates of energy substrates utilization during human cold exposure. Eur J Appl Physiol Occup Physiol. 1989;58(8):873-8.
38. Fernandez-Verdejo R, Marlatt KL, Ravussin E, Galgani JE. Contribution of brown adipose tissue to human energy metabolism. Mol Aspects Med. 2019;68:82-9.
39. Bal NC, Maurya SK, Pani S, Sethy C, Banerjee A, Das S, et al. Mild cold induced thermogenesis: are BAT and skeletal muscle synergistic partners? Biosci Rep. 2017;37(5).
40. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. J Physiol. 2017;595(6):2099-113.
41. Kralova Lesna I, Rychlikova J, Vavrova L, Vybiral S. Could human cold adaptation decrease the risk of cardiovascular disease? J Therm Biol. 2015;52:192-8.

Supplementary data

Supplementary table S1. Nutritional information of meal test shake

	Content	Energy %
Energy	755 kcal	100
Fat	50.8 gr	60.6
Saturated fat	26.1 gr	31.1
Unsaturated fat	23.8 gr	28.4
Cholesterol	0.9 gr	1.1
Carbohydrate	62.3 gr	33.0
Protein	10.9 gr	5.8



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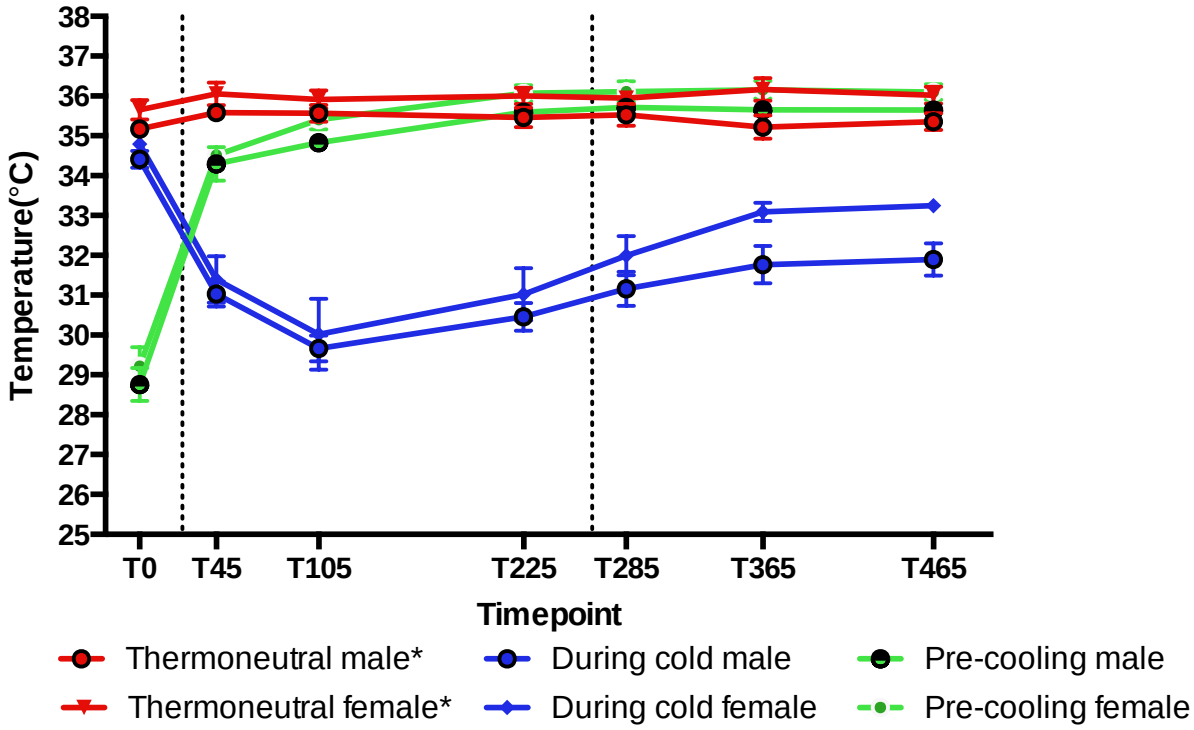
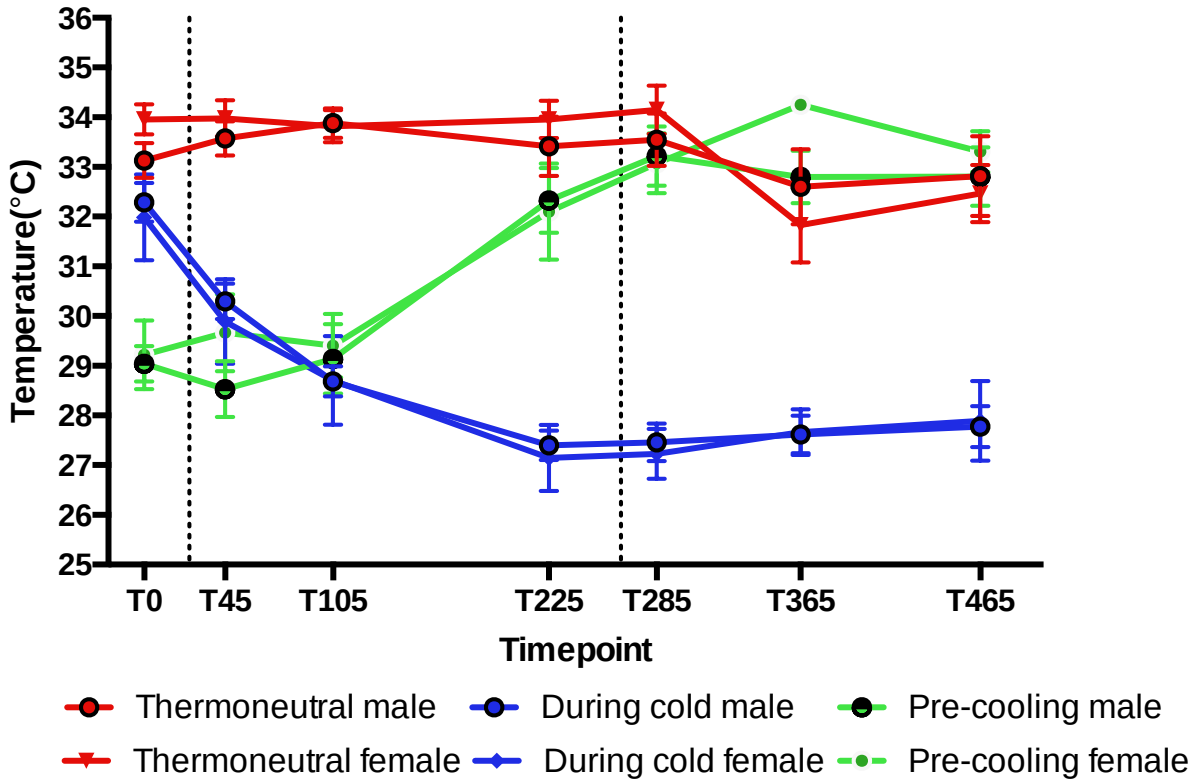
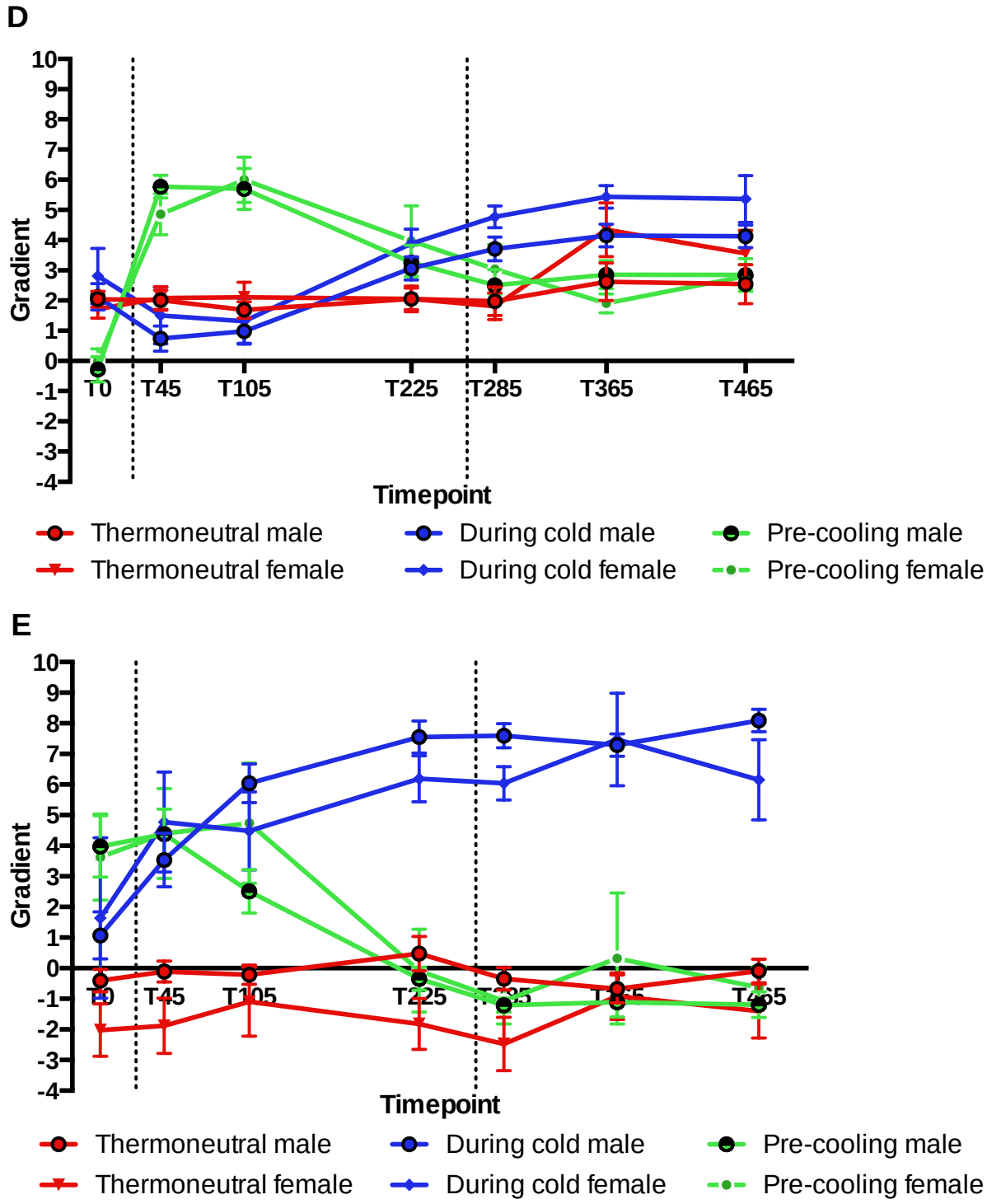

Temperature(°C)
38
37
36
35
34
33
32
31
30
29
28
27
26
25
T0
T45
T105
T225
T285
T365
T465
Timepoint
Thermoneutral male*
During cold male
Pre-cooling male
Thermoneutral female*
During cold female
Pre-cooling female

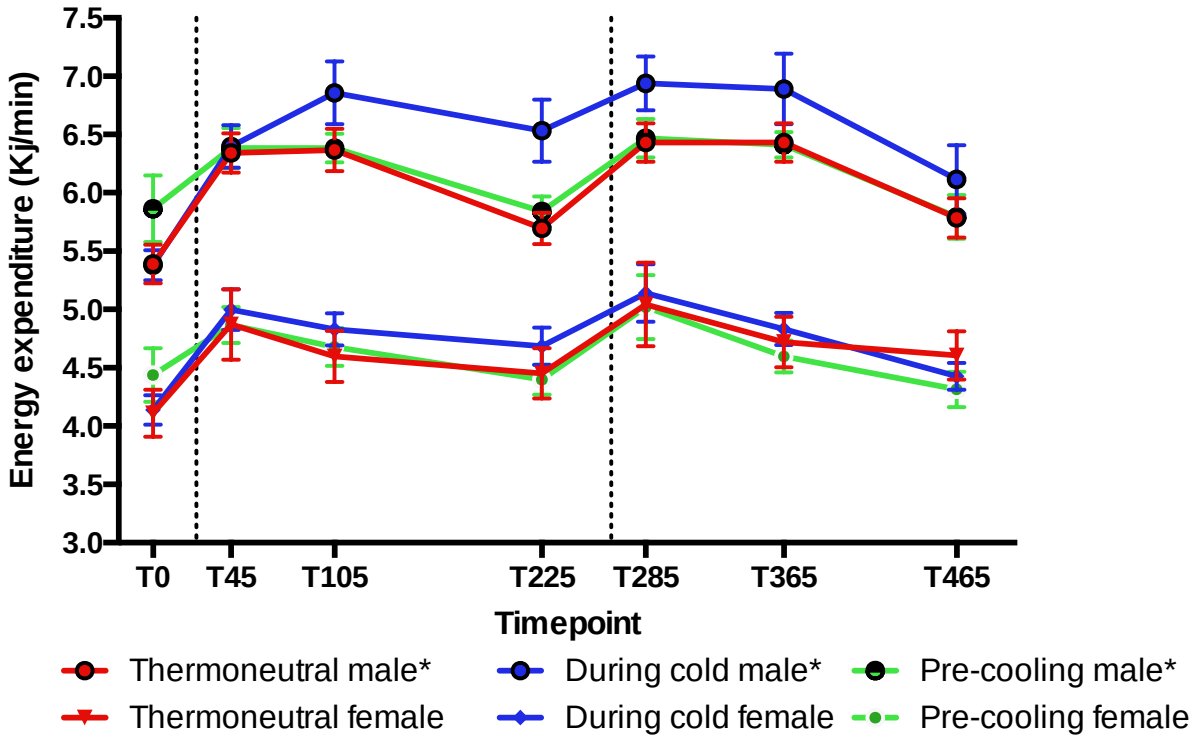
C


Temperature(°C)
36
35
34
33
32
31
30
29
28
27
26
25
T0
T45
T105
T225
T285
T365
T465
Timepoint
Thermoneutral male
During cold male
Pre-cooling male
Thermoneutral female
During cold female
Pre-cooling female

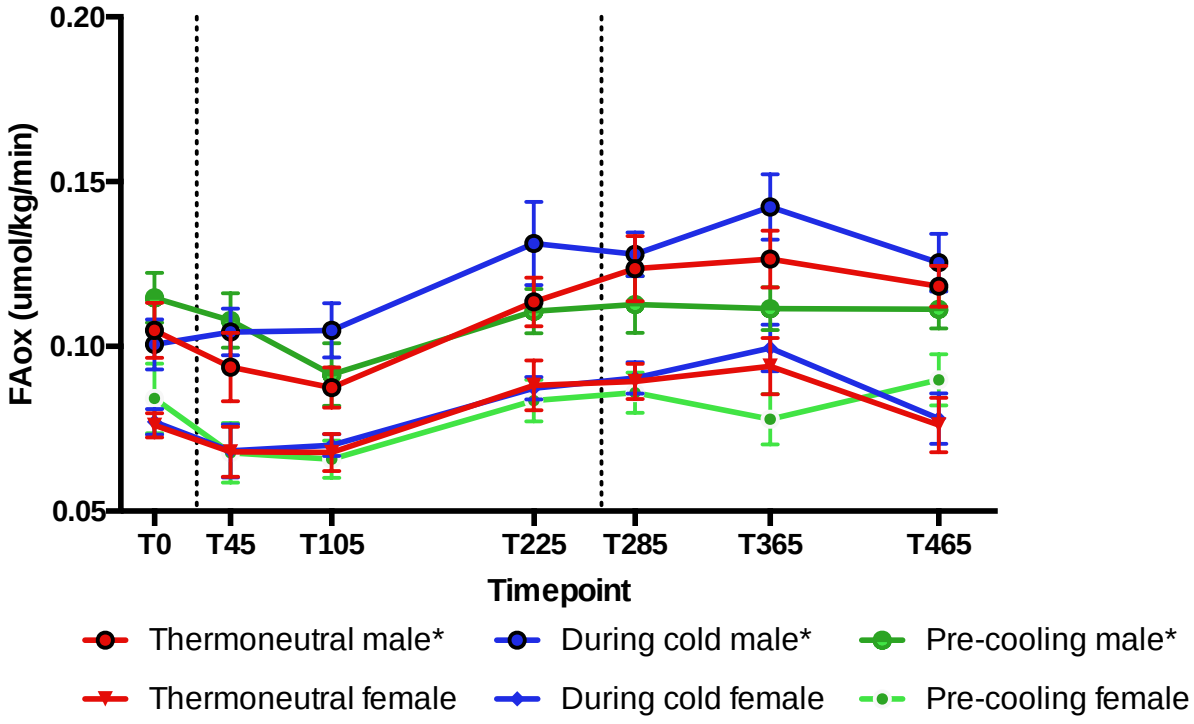


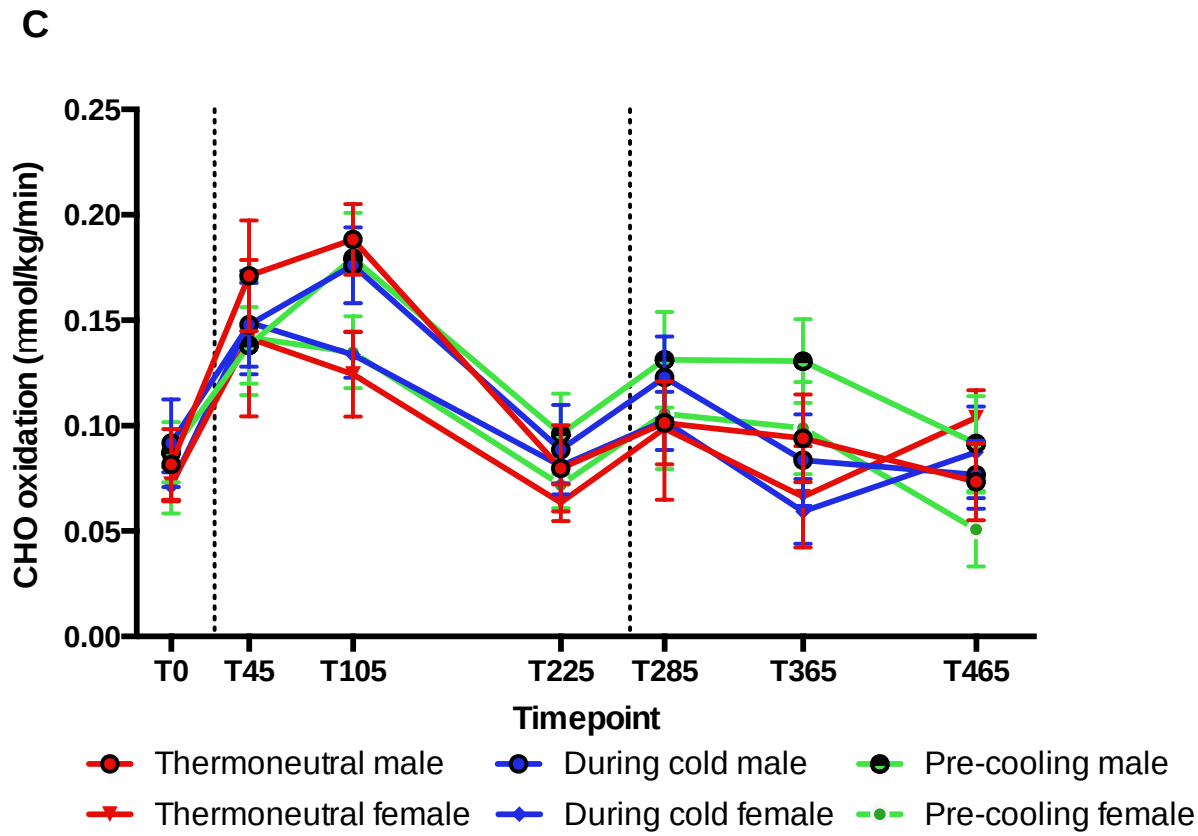
Supplemental figure 2. Skin temperatures during the meal tests for different genders
A: Mean skin temperature during the different meal tests. **B**: Mean proximal skin temperature. **C**: Mean distal skin temperature, **D**: Mean proximal-distal gradient and **E**: Mean underarm-finger gradient. The meal test at thermoneutrality is indicated as the red line, the meal test during cold exposure as the blue line, and the meal test after cold as the green line. Dashed vertical lines show the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14 (9 male, 5 female). Data is presented as mean ± SE. * indicates significant difference. ^ indicates trends towards significant difference.

A


Energy expenditure (Kj/min)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
T0
T45
T105
T225
T285
T365
T465
Timepoint
Thermoneutral male*
During cold male*
Pre-cooling male*
Thermoneutral female
During cold female
Pre-cooling female

B

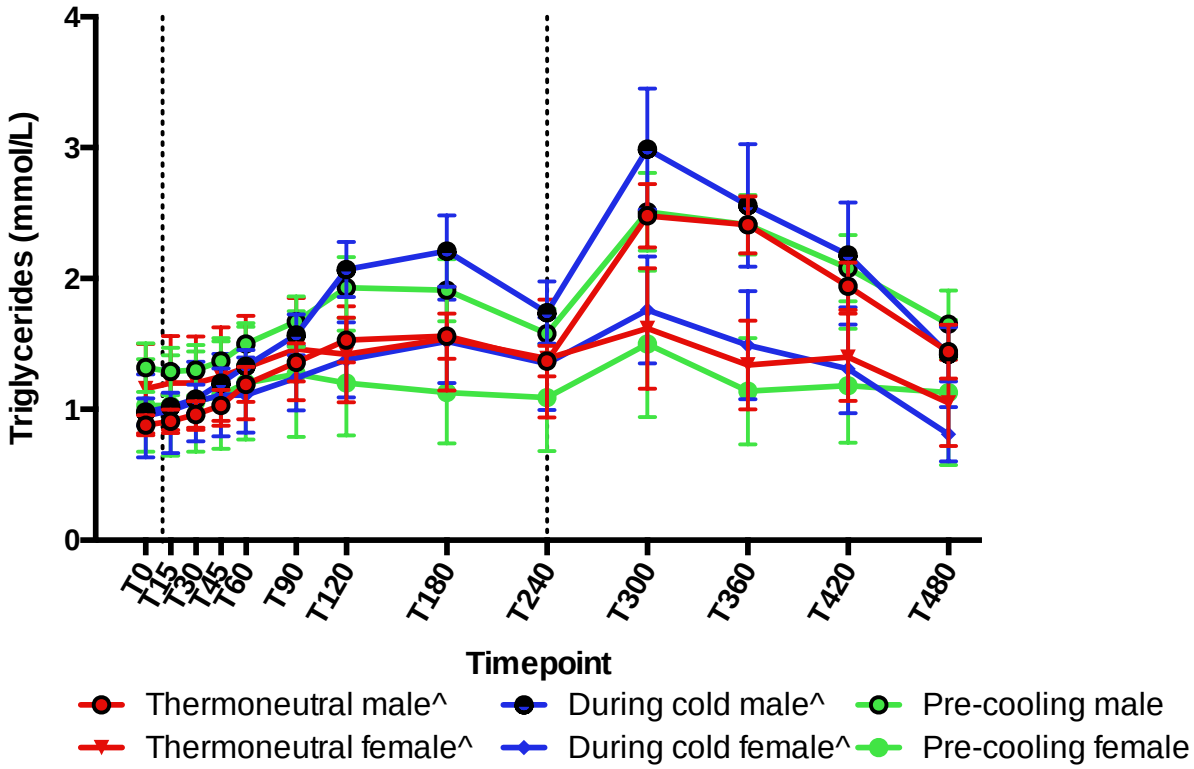

FAox (umol/kg/min)
0.20
0.15
0.10
0.05
T0
T45
T105
T225
T285
T365
T465
Timepoint
Thermoneutral male*
During cold male*
Pre-cooling male*
Thermoneutral female
During cold female
Pre-cooling female



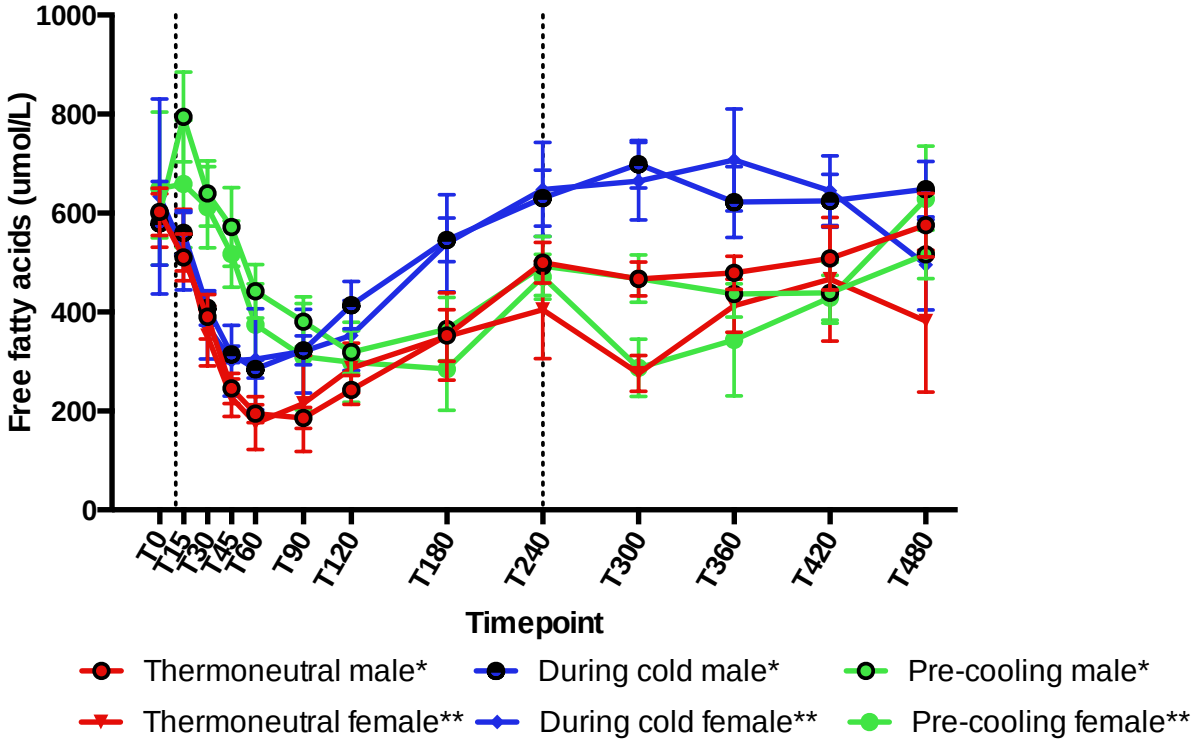
Supplemental figure 3. Energy expenditure and substrate oxidation during the meal tests for different genders

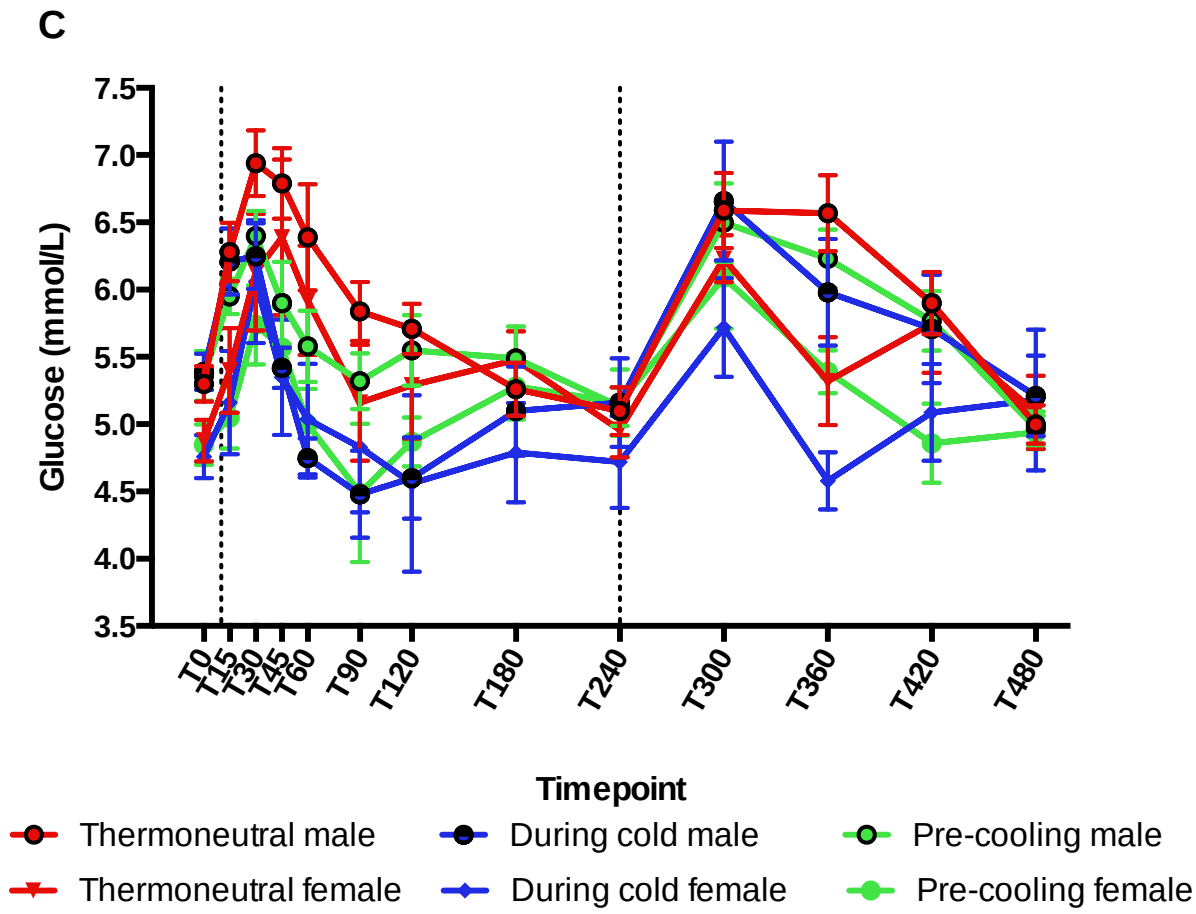
A: Energy expenditure, **B**: Fatty acid oxidation and **C**: Carbohydrate oxidation during the meal tests. The meal test at thermoneutrality is indicated as the red line, the meal test during cold exposure as the blue line and the meal test after cold as the green line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14 (9 male, 5 female). Data is presented as mean ± SE. * indicates significant difference. ^ indicates trends towards significant difference.

A


Triglycerides (mmol/L)
0
1
2
3
4
T0
T15
T30
T45
T60
T90
T120
T180
T240
T300
T360
T420
T480
Timepoint
Thermoneutral male^
During cold male^
Pre-cooling male
Thermoneutral female^
During cold female^
Pre-cooling female

B


Free fatty acids (umol/L)
0
200
400
600
800
1000
T0
T15
T30
T45
T60
T90
T120
T180
T240
T300
T360
T420
T480
Timepoint
Thermoneutral male*
During cold male*
Pre-cooling male*
Thermoneutral female**
During cold female**
Pre-cooling female**



Supplemental figure 4. Plasma levels during the meal tests for different genders
Plasma levels of **A**: Triglycerides, **B**: Free fatty acids and **C**: Glucose during the meal tests. The meal test at thermoneutrality is represented as the red line, the meal test during cold as the blue line and the meal test after cold as the green line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. n=14 (9 male, 5 female). Data is presented as mean ± SE. * indicates significant difference. ^ indicates trends towards significant difference.

Chapter 6
Absence of ^{18}F-Fluorodeoxyglucose uptake using PET/CT in Madelung's disease: a case report

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Madelung's disease

Multiple symmetric lipomatosis, also known as benign symmetric lipomatosis or Madelung's disease is characterized by symmetric development of lipomas in the cervical-thoracic region. (1) Madelung's disease mainly affects males and incidence is further increased with alcohol abuse early in life. (2-4) Clinically, patients can be divided in two sub-groups: Type 1, with symmetrical gross masses in the upper part of the body; and Type 2, with more general obesity. (5) The disease is further associated with neurological disturbances such as myopathy and cerebellar ataxia. (6)

When researching genetic and protein markers in these lipomas, several studies detected uncoupling protein 1 (UCP1) in biopsies taken from these lipomas. (7) Normally, mitochondria build up a proton gradient across the membrane by combustion of substrates such as glucose or fatty acids. This proton gradient is used by ATPase to produce ATP. UCP1, when activated by adrenergic stimulation, uncouples this process, and energy is released in the form of heat. (8) UCP1 is specific for brown adipose tissue (BAT), which could mean that BAT plays a role in Madelung's disease. Interestingly, these UCP1 positive cells derived from the lipomas did not respond to adrenergic stimulation, and may therefore be unable to produce heat. (7) In other samples taken from lipomas in patients suffering from Madelung's disease there was evidence for mitochondrial dysfunction due to mutations in the mitochondrial DNA (mtDNA) such as m.8344A > G. (6) Mitochondrial dysfunction would lead to the same disturbed function of BAT as seen with the dysfunctional adrenergic stimulation. (6)

Expression of UCP1 in lipomas associated with Madelung's disease could suggest the presence of BAT. However due to defective BAT through lack of UCP1, we hypothesize no BAT activity will be measured via uptake of ^{18}F-FDG following cold exposure. Furthermore, we expect to find the presence of UCP1 mRNA in the collected adipose tissue biopsies derived from the lipomas.

A 68-year old male patient (BMI: 36.2 kg/m^2), with the clinical diagnosis of Madelung's disease was referred to the Maastricht University Medical Centre for research to detect the presence of active brown adipose tissue. Anamnestically, our patient extensively used alcohol form from the age of 16, contributing to the clinical diagnosis. The patient suffered from symmetric lipomatosis mainly around the upper arms and abdomen, with relative sparing of the cervical region (picture 1A). Recent laboratory results showed a near-to-normal thyroid function (TSH: 4.8 mIU/L (ref 0.3-4.6mIU/L); fT4: 15.3 pmol/L (ref 10-23 pmol/L). The patient further had not used beta-blockers in the last year.

To evaluate the presence and activity of BAT, the patient underwent an individualized cooling procedure, after which an ^{18}F-FDG-PET/CT (Gemini TF PET/CT; Philips, The Netherlands) was

performed, as described before. (9) Before and after the cooling procedure energy expenditure was measured using indirect calorimetry (ventilated hood) to determine non-shivering thermogenesis (NST).

The patient arrived at the research institute at 8.30 AM, and only consumed a light breakfast at 6.00 AM. At 8.45 AM the patient ingested a telemetric pill (CorTemp HT150002; HQ Inc.) to measure core temperature. At the same time wireless temperature sensors (iButton; Maxim Integrated Products, San Jose, CA) were attached at the 15 International Organization for Standardisation (ISO) – defined sites to measure skin temperature. An intravenous cannula was placed in the antecubital vein in the right arm. The patient was wrapped in a water-perfused suit (ThermaWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne Israel). The water-perfused suit was connected to two cooling installations (Blanketrol III; Cincinnati Sub-Zero, Sharonville OH).

The water-perfused suit was heated to 36 °C to provide a thermoneutral condition. Indirect calorimetry was performed using a ventilated hood system (EZCAL; Maastricht Instruments, Maastricht, the Netherlands) to measure resting energy expenditure. After this thermoneutral period, the cooling procedure was started. The water temperature was lowered with 4 °C each 15 minutes, with concomitant measuring of the blood pressure and pulse. When the patient started to visibly shiver, the temperature was heated to 34 °C for 5 minutes to stop the shivering. A stable temperature was reached at 27 °C. After the stable temperature was reached, a second indirect calorimetry was performed. At 12.45 PM, a bolus of 77 MBq of ^{18}F-FDG was injected intravenously. After the injection the patient was instructed to lie still for 1 hour at the stable water temperature of 27 °C.

After 1 hour, at 13.45 PM, the PET/CT protocol was performed with a low-dose CT scan (120kV, 30 mAs), immediately followed by a static PET scan (6 bed positions, 4 minutes per position) covering the area from the skull to the illiac crest.

After the ^{18}F-FDG-PET/CT scan, two adipose tissue biopsies were taken under local anaesthesia. One biopsy was taken at the lateral side of the right upper arm at the location of the lipomas. The second biopsy was taken from the abdominal subcutaneous fat deposit, just right of the umbilical as a control.

The adipose tissue samples were collected on a water-permeable membrane and rinsed with demi water. Afterwards samples were sealed in paraffin or frozen in melting isopentane for further analysis. RNA from the adipose tissue was extracted using Trizol reagent followed by protocol described in the RNeasy kit from Qiagen (Hildenberg, Germany). UCP1 mRNA expression was determined using a CFX384 Touch Real-Time PCR Detection System from BioRad Laboratories (Hercules, CA) with a Taqman gene expression assay (Hs00222453_m1) as described before. (10)

Cold exposure increased resting energy expenditure from 5.2 kJ/min to 9.7 kJ/min. Though the protocol was designed to induce only non-shivering thermogenesis, the patient did shiver occasionally in the final stages of the cooling procedures. Non-shivering thermogenesis plus probably some shivering thermogenesis (as the per cent increase in energy expenditure during cold exposure above the resting energy expenditure) was 53.6%. The respiratory quotient remained 0.83. Core temperature was 37.3 °C during the thermoneutral period and dropped to 36.5 °C during cold exposure. Following cold exposure, the mean skin temperature dropped from 34.0 °C to 32.1 °C.

The ^{18}F-FDG-PET/CT scan performed after the cooling procedure showed no uptake of ^{18}F-FDG in the adipose tissue depots in the cervical-thoracic region (Figure 1B). Furthermore, no uptake of ^{18}F-FDG was observed in both upper arms.

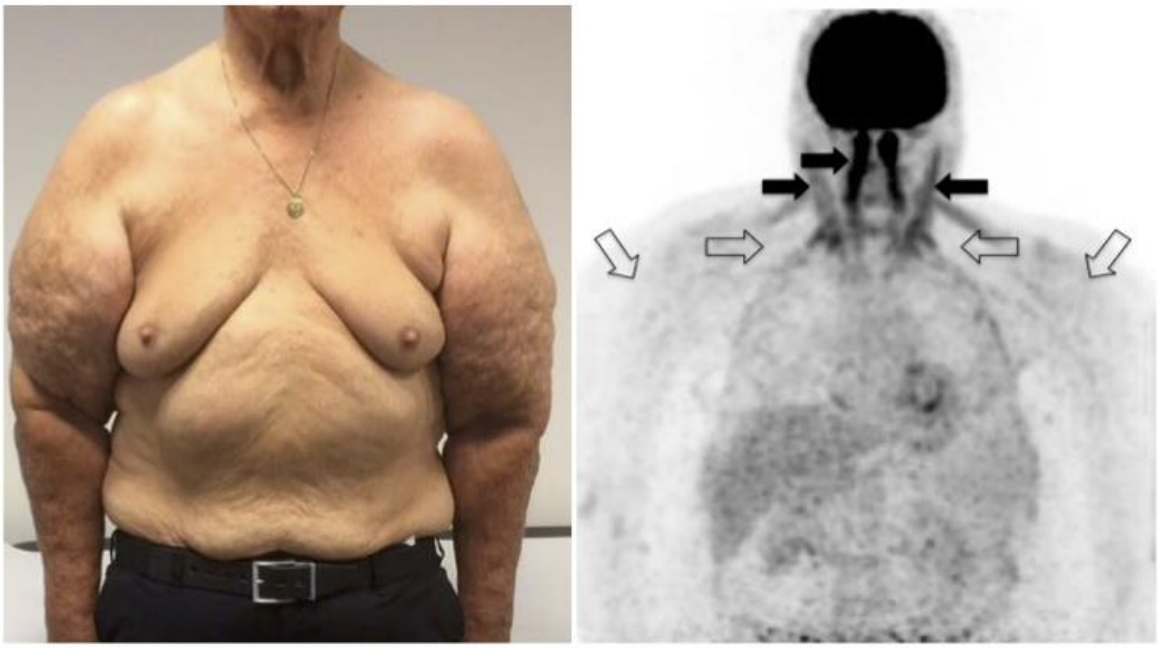

Figure 1.
A: Clinical view of the patient.
Noticeable is the subcutaneous lipomatosis in both upper arms and abdomen, with relative sparing of the cervical region. **B**: PET image using 18 F-FDG following the cooling procedure. The 18 F-FDG uptake in the cervical region is caused by active muscle tissue due to shivering (filled arrows). Note the absence of 18 F-FDG uptake in the deep cervical adipose tissue and lipomas in both upper arms (clear arrows)

An adipose tissue biopsy was derived from the lipoma and subcutaneous white adipose tissue. Biopsies from sites were sealed in paraffin and afterwards stained with haematoxylin-eosin (HE). The HE-staining showed large lipid droplets in both biopsies. The tissues from the arm (Figure 2) and the abdominal subcutaneous fat deposit (Figure 3) both showed the

microscopic structure of white adipose tissue (WAT) and not of BAT. Subsequently, we examined UCP1 mRNA expression in both biopsies. Cq values for the samples derived from the lipoma and subcutaneous white adipose tissue were 32.9 and 33.7 respectively. As a reference, we included a human hibernoma, which had a Cq value of 19.4.

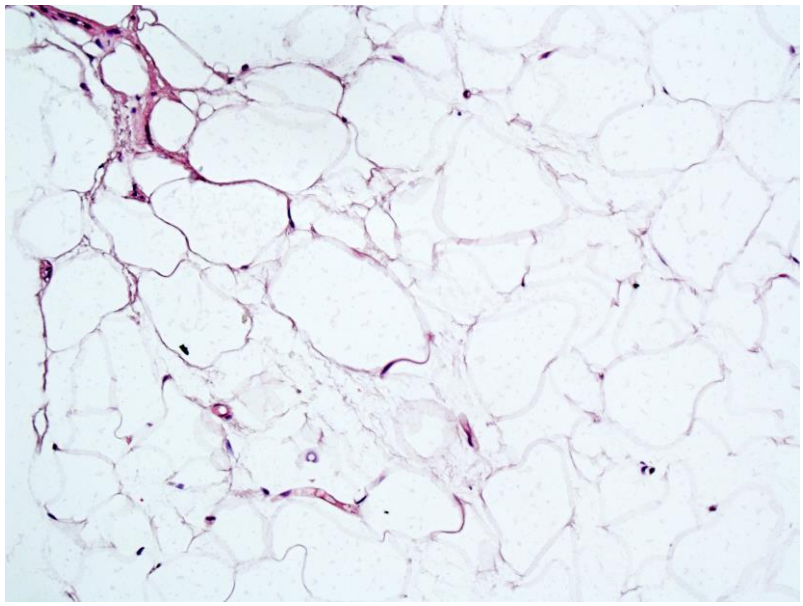

Figure 2: Adipose tissue from lipoma of right upper arm (Magnification: 200x)

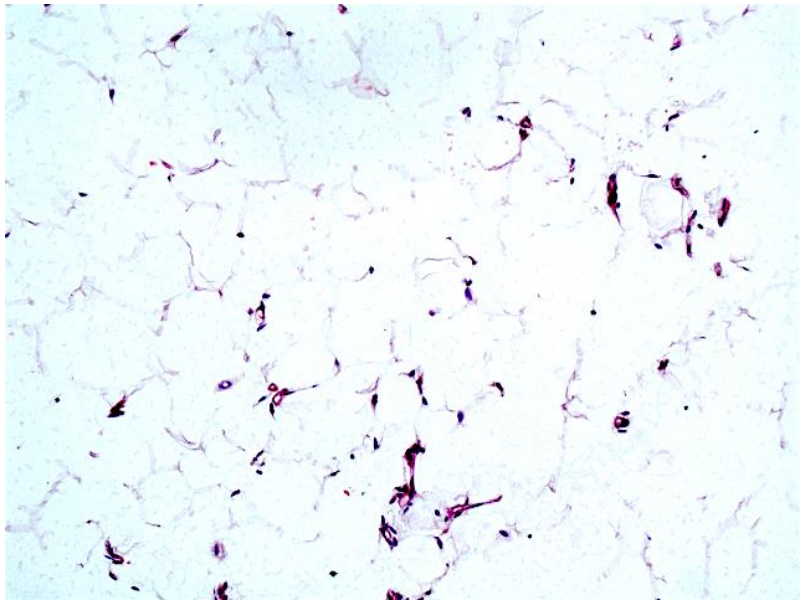

Figure 3: Subcutaneous white adipose tissue from abdomen (Magnification: 200x)

In earlier studies, the ectopic adipose tissue in Madelung's disease was UCP1 positive, although the typical cellular structure of BAT was absent, as was the response to adrenergic stimulation. (6, 7) One theory behind this is that the ectopic adipose tissue in Madelung's disease is caused by a defect in the adrenergic pathway in brown adipocytes. Although UCP1 is present, adrenergic stimulation does not increase UCP1 expression in Madelung's disease.

(7) The lack of UCP1 expression would impair mitochondrial uncoupling that could lead to BAT thermogenesis. The impaired thermogenesis would mean that the body is less able to protect the core temperature in a cold environment. This would mean that the sympathic nervous system activity will remain present. This would in turn lead to proliferation of adipocytes, specifically at the locations where BAT is normally found, such as the cervical, inter-scapular and para-renal regions. (7)

Our patient showed normal physical responses to cooling, as seen in the sustained core temperature and the temperature gradient in the lower arm. Energy expenditure increased upon cold exposure as expected under conditions of normal thermoregulation. Heart rate and blood pressure increased slightly upon cold exposure. Also these physical responses can be expected under conditions of normal thermoregulation in humans following cold exposure.

As hypothesized the ^{18}F-FDG-PET/CT scan showed no uptake of the ^{18}F-FDG tracer in neither the cervical-thoracic region nor the upper arms, where the ectopic lipomas were situated in our patient. Based on the ^{18}F-FDG-PET/CT scan, no active BAT was found. Based on the hypothesis that a dysfunctional adrenergic pathway leads to less UCP1 expression and reduced or absent thermogenesis, this could explain the lack of metabolic activity upon cold exposure and the lack of tracer uptake in the normal BAT regions in our patient.

In line with the ^{18}F-FDG-PET/CT results, the adipose tissue taken from the right upper arm showed a typical morphology of WAT, as did the control tissue taken from the abdominal adipose tissue (Figure 2 and 3). The qPCR performed on these tissues showed no presence of UCP1. Due to the clinical and genetic phenotypes described in the literature, it is possible that our patient has yet another causal mechanism. (5-7) This could potentially explain why we were unable to find any sign of BAT presence in the biopsy material.

The use of an ^{18}F-FDG-PET/CT scan combined with the cooling procedure is an established method to detect the presence of active BAT. (9) Though the prevalence in young healthy (lean) adults is high, BAT is not always detected in all lean subjects. (10) In addition in studies with elder and obese subjects, the prevalence of BAT is lower in both obese and elder subjects compared to young lean subjects. (9) This may explain the absence of active BAT in our patient, whose BMI was 36.2 kg/m^2, in the cervical-thoracic region where BAT is usually found in humans.

The supposed defect adrenergic pathway in Madelung's disease could also explain the absence of tracer uptake in both upper arms. In the adipose tissue biopsies, we only found cells resembling white adipocytes without any expression of UCP1 mRNA. An in vitro model with primary cultured adipocytes derived from patient adipose tissue biopsies could be used to examine UCP1 expression under basal or after norepinephrine stimulation. A potential culture of primary adipocytes would be another option to evaluate the dysfunctional

adrenergic pathway. However these experiments would require freshly isolated adipose tissue biopsies.

Concluding, in a patient with clinical Madelung's disease we found no active BAT, neither in the lipomas nor in the cervical-thoracic region. Adipose tissue biopsies were absent of multilocular adipocytes associates with BAT, while qPCR revealed no presence of UCP1.

Although Madelung's disease could be caused by defective stimulation of BAT, we found no proof of any BAT presence in our patient. Further research could focus on the causal mechanism for Madelung's disease and the possible relationship between the lipomatosis and BAT.

References

1. Busetto L, Strater D, Enzi G, Coin A, Sergi G, Inelmen EM, et al. Differential clinical expression of multiple symmetric lipomatosis in men and women. Int J Obes Relat Metab Disord. 2003;27(11):1419-22.
2. Enzi G, Busetto L, Ceschin E, Coin A, Digito M, Pigozzo S. Multiple symmetric lipomatosis: clinical aspects and outcome in a long-term longitudinal study. Int J Obes Relat Metab Disord. 2002;26(2):253-61.
3. Leffell DJ, Braverman IM. Familial multiple lipomatosis. Report of a case and a review of the literature. J Am Acad Dermatol. 1986;15(2 Pt 1):275-9.
4. Lee HW, Kim TH, Cho JW, Ryu BY, Kim HK, Choi CS. Multiple symmetric lipomatosis: Korean experience. Dermatol Surg. 2003;29(3):235-40.
5. Enzi G. Multiple symmetric lipomatosis: an updated clinical report. Medicine (Baltimore). 1984;63(1):56-64.
6. Plummer C, Spring PJ, Marotta R, Chin J, Taylor G, Sharpe D, et al. Multiple Symmetrical Lipomatosis--a mitochondrial disorder of brown fat. Mitochondrion. 2013;13(4):269-76.
7. Enzi G, Busetto L, Sergi G, Coin A, Inelmen EM, Vindigni V, et al. Multiple symmetric lipomatosis: a rare disease and its possible links to brown adipose tissue. Nutr Metab Cardiovasc Dis. 2015;25(4):347-53.
8. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
9. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, van Marken Lichtenbelt WD. Brown adipose tissue in morbidly obese subjects. PLoS One. 2011;6(2):e17247.
10. Vosselman MJ, Hoeks J, Brans B, Pallubinsky H, Nascimento EB, van der Lans AA, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. Int J Obes (Lond). 2015;39(12):1696-702.

Chapter 7
General discussion

Introduction

Under thermoneutral circumstances the basal metabolism provides enough heat during resting conditions to maintain the body core temperature (1). During these conditions the body temperature regulates the heat loss of the body by changing blood perfusion in the skin and extremities. The heat loss is increased in higher temperatures by vasodilation, and decreased in lower temperatures by vasoconstriction. In the cold the body can, in addition, increase the heat production (1). This can be achieved by two means: shivering thermogenesis (ST) and non-shivering thermogenesis (NST). The non-shivering thermogenesis is effected by activation of brown adipose tissue (2), at least in rodents. To which extend brown adipose tissue is involved in NST in adult humans remains a question.

Cold exposure activates the sympathic nervous system, with nerve endings present in depots of brown adipose tissue. Noradrenaline release takes place in these nerve endings after stimulation by cold, which in turn activates beta-3-adrenergic receptors in the membrane of brown adipocytes (3, 4). This leads via a cascade of reactions to the activation of the uncoupling protein 1(UCP1) found in the inner membrane of the mitochondria in the brown adipocyte. The mitochondria break down substrates such as glucose and fatty acids to build up a proton gradient across the mitochondrial inner membrane. Instead of using the potential energy from this proton gradient to produce adenosine tri-phosphate (ATP), brown adipocytes release heat through a regulated means of uncoupling. This accomplished by UCP1 (3). The potential energy of the proton gradient is then released as heat (5), which will then protect the core body temperature from lowering, at least in small mammals.

In 2009 several research groups presented independently clear proof of depots of functionally activated brown adipose tissue in adult humans (6-8). However, the amount of BAT, detected by metabolic imaging, is relatively small compared to little mammals. The reason may be that humans have a relative low surface to volume ratio linked to their body size, which means there will be less heat loss in the cold. Additionally, in our modern society, there is less need for active brown adipose tissue as we are less exposed to cold temperatures.

In the majority of these studies, radioactive tracers such as 18F-FDG are used in combination with positron emission tomography (9, 10). 18F-FDG can be used to show the uptake of glucose by active tissues, however it does not provide detailed information regarding the metabolism of brown adipocytes as seen in the review presented in Chapter 2 of this thesis. Other metabolic studies have used different radio-isotope tracers, which also show that the total contribution of BAT to whole body energy metabolism is small (11) . Despite these results, BAT can still play an important role in substrate metabolism.

The effect of non-shivering cold acclimation

Another study of this thesis focused on the effect of mild cold acclimation without shivering during the daily cold exposure. A twelve-day cold acclimation study was performed in patients with type 2 diabetes mellitus, as described in chapter 5. In this study we took special care that any shivering was prevented. In order to separate the effect of acute cold and cold acclimation we performed the insulin sensitivity measurements on the day without cold exposure, after the cold acclimation period. In this study, we did not see any improvements in insulin sensitivity. Notably, there was also no translocation of active glucose transporters, such as GLUT4, to the membranes of the skeletal muscle cells. The absence of the GLUT4 translocation is most likely responsible for the lack of improvement in insulin sensitivity.

Next to substrate utilization during cold exposure, there is an increased release of substrates to fuel the increased energy expenditure. While the increased energy expenditure and substrate oxidation might seem opportune, the results from the acute cold exposure study show that there are actually higher blood levels of lipids and fatty acids. This would mean that the increased consumption of fatty acids does not match the increased release into the blood stream. More prolonged cold exposure, as in cold acclimation could result in an decrease in plasma lipid levels , especially in view of the relatively small amount of active brown adipose tissue in humans. Research by Blondin et al. showed an increased oxidative capacity in BAT after shivering cold acclimation, which indicates that more severe cold or a prolonged exposure period could increase the contribution of BAT to human energy and substrate metabolism (15). Cold acclimation could thus improve the metabolic activity of brown adipose tissue, which could increase the bodily response to cold exposure. This could lead to more improvements in glucose and lipid metabolism, as well as maintaining these improvements for a longer time period. Additionally, these improvements may persist even when the cold stimulus has been removed.

In conclusion, despite earlier mild cold acclimation studies, that included (low) shivering levels, our study showed that actual non-shivering cold acclimation may be insufficient to induce improvements in glucose metabolism in patients with type 2 diabetes mellitus.

As indicated above non-shivering thermogenesis (NST) is attributed mostly to brown adipose tissue activation, while shivering thermogenesis (ST) is attributed to skeletal muscle activity. More prolonged cold exposure leads to adaptations by the body, which is known as acclimation. Cold acclimation leads to an increase in BAT volume and activity, as shown by Hanssen et al and by Van der Lans et al. (12, 13). Concomitantly a decrease in shivering activity is seen, which indicates that non-shivering thermogenesis is increased during acclimation (14, 15). The increased energy expenditure during cold acclimation has to be compensated by

increased consumption of substrates such as fatty acids and glucose. This leads to the question which effects cold acclimation could have on glucose- and lipid metabolism.

The role of brown adipose tissue activation on lipid- and glucose metabolism

As brown adipose tissue is activated during cold exposure substrates are used to produce the required heat. Fatty acids are the foremost substrate present in brown adipocytes. This can be observed by CT imaging, showing a decrease of lipid internal stores in brown adipocytes after acute cold exposure (16, 17), and by the increase in fatty acid uptake by BAT during cold exposure, as seen in animals (18) and with fatty acid tracers in humans (19). Additionally,after cold exposure lower lipid and cholesterol levels were observed in adult humans (19, 20). As cold exposure increases the uptake and consumption of fatty acids in BAT, we hypothesized that during cold exposure lipid levels in the blood will decrease.

Therefore in this thesis, in chapter 4, we investigated if during acute non-shivering cold exposure and after pre-cooling post-prandial serum lipid levels in humans will be reduced. Secondly, it was studied if these changes in blood lipids could be linked to brown adipose tissue presence and activity. Additionally we also investigated the role of skeletal muscle in acute non-shivering thermogenesis, as it has been shown that in response to cold mitochondrial uncoupling occurs skeletal muscle (21). Notably, we observed that acute cold exposure increased post-prandial lipid levels, in contrast to the data from mice studies. This increase can be attributed to either an enhanced intestinal uptake from dietary lipids or to increased lipolysis (22) during cold. The lipolysis during acute cold could impede any improvements seen by the increased uptake and consumption of fatty acids by BAT activation.
By using two meals during each test day, we intended to study the so-called ‘second meal’ effect, which refers to the observation that postprandial lipid levels normally are higher after the second meal. During the first meal lipids can be temporarily stored in the intestines. However, the second meal effect was present during both thermoneutral and cold conditions. This indicates that there are still lipids retained after the first meal, even during cold.

Glucose is also taken up by brown adipose tissue, which is then used as substrate for maintaining the mitochondrial proton gradient or in anaplerotic reactions, although the exact role of glucose has not yet been established (11). Therefore, BAT could serve as a glucose sink, reducing plasma glucose levels, which is favourable for type 2 diabetes mellitus. Additionally, cold acclimation has been shown to increase insulin sensitivity. By using a hyperinsulinemic euglycemic clamp technique (23) Hanssen et al observed an improvement in insulin sensitivity after 10 days of cold acclimation in both obese and diabetic volunteers (24). However, the

improvement in insulin sensitivity was not correlated with BAT activity, although it should be noted that in type 2 diabetes the use of FDG-PET/CT may underestimate the amount of BAT. The acclimation did have an effect on skeletal muscle, ie. translocation of glucose transporters (GLUT4)to the SM-membrane was increased. Some shivering during the cold acclimation occurred, and may be contraction of the skeletal muscles was responsible for the improvement in insulin sensitivity.

Limitations of study design

As mentioned in the previous paragraph and in this thesis, different types of study design were used. As mentioned, in chapter 3, a randomized double blind placebo-controlled study was performed. The use of this type of study design allows for control of potential research bias. In comparison chapters 4 and 5 describe prospective cohort studies which did not include control groups. This indicates a higher potential for bias, as there were no control groups in these studies. However this type of study design still allows for adequate research.

Finally, in chapter 6 the results from a case report are presented. As it describes a singular case, the results should not be generalized to the entire population and can lead to over-interpretation. However, this type of study design can be used to generate new ideas and hypotheses for future investigations.

The role of brown adipose tissue in human energy and substrate metabolism

In this thesis the main focus has been on the specific role of brown adipose tissue activation and non-shivering thermogenesis in the energy and substrate metabolism of adult humans. Our results from non-shivering cold exposure and cold acclimation are in clear contrast to previous studies involving mice. This can be attributed to the much higher amount of active brown adipose tissue in mice compared to adult humans. Additionally it indicates that non-shivering acute cold exposure and short-term acclimation are insufficient to improve human metabolism, and that shivering thermogenesis could be the solution. While shivering thermogenesis refers to non-voluntary contractions of skeletal muscles, evidence shows that skeletal muscle cells exhibit a form of uncoupling thermogenesis as well (21). The effects of cold acclimation with shivering could thus be similar to the effects of exercise. Evidence for this can be found in a recent publication, where similar gene expression profiles were found after exercise and cold acclimation with shivering (25). The link between BAT and skeletal muscle, in regards to their role in thermogenesis, is still a matter of debate. Overall the

contribution of BAT to energy and substrate metabolism is relatively small, as seen in metabolic imaging (11, 26, 27).

As BAT does not seem to fulfil a large role in human energy and substrate metabolism, BAT may have alternative functions. One such function could be linked to the localization of brown adipose tissue depots. Next to the depots in the neck region, BAT is also found next to the vertebral column (11). This would indicate that BAT is involved in keeping paravertebral nerves and ganglia warm. This would ensure that nerve conduction speed is maintained, in the ganglia and paravertebral nerves. Although this only a small part of the entire neural track between the central and peripheral nervous system, BAT could allow the nerves to maintain optimal functionality during cold exposure.

In addition to direct heat production, brown adipose tissue could also function as an endocrine tissue (28), and thus be involved in coordinating thermogenesis in skeletal muscle. Several studies have focused on endocrine factors produced by BAT, such as FGF21 (29, 30). FGF21 is secreted by BAT and can increase hepatic fatty acid oxidation and insulin sensitivity, which could help explain the results seen in clinical studies (31). As both tissues are active during non-shivering cold exposure, a potential interplay between these tissues seems logical (29, 30). Another example is triiodothyronine, or activated thyroid hormone T3, which is produced by active BAT (32) and is involved in catabolic processes in the entire body. This would show that BAT could influence other tissues to contribute to thermogenesis, effectively regulating the bodily heat production during cold exposure. Another potential hormone is Neuregulin 4 (NRG4) which is produced by brown adipocytes during cold exposure. NRG4 can decrease de novo lipogenesis in hepatocytes (31) making it an interesting potential hormone in the fight against dyslipidaemia. The endocrine products of brown adipocytes could thus influence whole body metabolism during cold exposure, making it an interesting topic in this field. Additionally, it would be interesting to investigate more deeply the metabolic interplay between BAT and other tissues, such as skeletal muscle tissue and liver. More severe and even longer periods of cold exposure could therefore be used to push the human body even further, with increased energy expenditure and substrate oxidation.

Table 1. **Function of BAT**	
Energy metabolism	Non-shivering thermogenesis
Substrate metabolism	Uptake and oxidation of glucose and lipids
Heating	Heating of paravertebral nerves and ganglia
Endocrine	Stimulating hepatic metabolism/ increasing thermogenesis (e.g thyroid hormones)

Table 1. An overview of the different functions of human BAT.

In conclusion the results from this thesis show that non-shivering thermogenesis, during acute cold exposure and after a 2-week period cold acclimation did not lead to metabolic improvements. This indicates that the uncoupling thermogenesis in brown adipose tissue and skeletal muscle is insufficient in adult humans to improve lipid and glucose metabolism. What remains unknown for now is the potential of BAT after more prolonged cold acclimation, and what can be expected after more severe cold exposure with shivering thermogenesis. Additionally, the interaction between BAT and other tissues, such as skeletal muscle, deserves more attention in future research.

References

1. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol. 2011;214(Pt 2):242-53.
2. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R285-96.
3. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
4. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. FASEB J. 2009;23(9):3113-20.
5. Klaus S, Casteilla L, Bouillaud F, Ricquier D. The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. Int J Biochem. 1991;23(9):791-801.
6. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.
7. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
8. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
9. Bauwens M, Wierts R, van Royen B, Bucerius J, Backes W, Mottaghy F, et al. Molecular imaging of brown adipose tissue in health and disease. Eur J Nucl Med Mol Imaging. 2014;41(4):776-91.
10. Chondronikola M, Beeman SC, Wahl RL. Non-invasive methods for the assessment of brown adipose tissue in humans. J Physiol. 2018;596(3):363-78.
11. Richard MA, Pallubinsky H, Blondin DP. Functional characterization of human brown adipose tissue metabolism. Biochem J. 2020;477(7):1261-86.
12. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.
13. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
14. Davis TR. Chamber cold acclimatization in man. Rep US Army Med Res Lab. 1961;475:1-8.
15. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. J Physiol. 2017;595(6):2099-113.

16. Baba S, Jacene HA, Engles JM, Honda H, Wahl RL. CT Hounsfield units of brown adipose tissue increase with activation: preclinical and clinical studies. J Nucl Med. 2010;51(2):246-50.
17. M UD, Raiko J, Saari T, Saunavaara V, Kudomi N, Solin O, et al. Human Brown Fat Radiodensity Indicates Underlying Tissue Composition and Systemic Metabolic Health. J Clin Endocrinol Metab. 2017;102(7):2258-67.
18. Berbee JF, Boon MR, Khedoe PP, Bartelt A, Schlein C, Worthmann A, et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. Nature communications. 2015;6:6356.
19. Blondin DP, Tingelstad HC, Noll C, Frisch F, Phoenix S, Guerin B, et al. Dietary fatty acid metabolism of brown adipose tissue in cold-acclimated men. Nat Commun. 2017;8:14146.
20. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. J Clin Endocrinol Metab. 2014;99(3):E438-46.
21. Wijers SL, Schrauwen P, Saris WH, van Marken Lichtenbelt WD. Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. PLoS One. 2008;3(3):e1777.
22. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. J Physiol. 2015;593(3):701-14.
23. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214-23.
24. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
25. Nascimento EBM, Hangelbroek RWJ, Hooiveld G, Hoeks J, Van Marken Lichtenbelt WD, Hesselink MHC, et al. Comparative transcriptome analysis of human skeletal muscle in response to cold acclimation and exercise training in human volunteers. BMC Med Genomics. 2020;13(1):124.
26. Muzik O, Mangner TJ, Granneman JG. Assessment of oxidative metabolism in brown fat using PET imaging. Front Endocrinol (Lausanne). 2012;3:15.
27. Muzik O, Mangner TJ, Leonard WR, Kumar A, Janisse J, Granneman JG. 15O PET measurement of blood flow and oxygen consumption in cold-activated human brown fat. J Nucl Med. 2013;54(4):523-31.
28. Villarroya J, Cereijo R, Villarroya F. An endocrine role for brown adipose tissue? Am J Physiol Endocrinol Metab. 2013;305(5):E567-72.
29. Bal NC, Maurya SK, Pani S, Sethy C, Banerjee A, Das S, et al. Mild cold induced thermogenesis: are BAT and skeletal muscle synergistic partners? Biosci Rep. 2017;37(5).

30. Bal NC, Singh S, Reis FCG, Maurya SK, Pani S, Rowland LA, et al. Both brown adipose tissue and skeletal muscle thermogenesis processes are activated during mild to severe cold adaptation in mice. The Journal of biological chemistry. 2017;292(40):16616-25.
31. Scheja L, Heeren J. The endocrine function of adipose tissues in health and cardiometabolic disease. Nat Rev Endocrinol. 2019;15(9):507-24.
32. Silva JE. Thyroid hormone control of thermogenesis and energy balance. Thyroid. 1995;5(6):481-92.

Valorisation

Societal relevance

Our modern Western lifestyle, hallmarked by an excess of food intake and lack of exercise, and characterized by low levels of 24h energy expenditure, presents us with an increase in patients with the metabolic syndrome. The metabolic syndrome is characterized by obesity, type 2 diabetes mellitus and cardiovascular disease. The prevalence of obesity has increased by almost 80%, from 7% in 1980 to a staggering 12.5% of the population worldwide in 2015 with approximately 650 million people being obese (1). Complications of obesity such as dyslipidaemia, type 2 diabetes mellitus and atherosclerosis are currently among the leading causes of death in the World with an estimated 19.5 million deaths for diabetes mellitus and cardiovascular diseases (2).

In the Netherlands, a similar trend is occurring with an estimated 50,2% of inhabitants who are either overweight or obese (3), indicating that the Netherlands are not immune to the worldwide trends. This is also seen in the high occurrence of patients with metabolic syndrome.

To combat metabolic syndrome, it is highly relevant from a physiological perspective to find ways to counteract this diseases and to search for treatments and preventive measures. In general, many interventions have been focused on improving glucose and lipid metabolism by using exercise and dietary restrictions. However, long-term adherence to exercise training programs and dietary regimes is generally poor, warranting the exploration of alternative strategies. One such strategy is cold exposure and the activation of brown adipose tissue (BAT). The discovery of functional active brown adipose tissue in adult humans in 2009 (4-6) boosted research for the treatment of metabolic syndrome.

In this thesis, the use of cold exposure to mitigate insulin resistance in patients with T2DM was investigated. Additionally the effects of cold exposure with potential activation of BAT on post-prandial hyperlipidaemia were studied, which is of interest to reduce the risk of developing metabolic syndrome. In turn this would lead to a reduced chance of developing cardiovascular diseases such as myocardial infarction.

Thus, cold exposure can be seen as an addition therapy to common lifestyle interventions. Cold exposure could also be an alternative for exercise for patients who have limited options for physical exercise. Finally, by applying lower temperatures in in buildings, a healthier indoor environment can be created for virtual all people, potentially reducing the chance of developing obesity or metabolic syndrome.

Though the conditions tested in the studies of this thesis did not always show positive effects on metabolic health parameters, more research in this field is warranted to further develop

metabolic therapies. Thereby, both a cold therapy and/or a healthier living environment can potentially be created, relieving the societal burden of diabetes mellitus and cardiovascular disease by reducing medication use, hospitalization rates, and the costs for society.

Economic relevance

Due to the increasing number of people with metabolic syndrome, dyslipidaemia and type 2 diabetes mellitus, there is an increasing financial burden on society. It is estimated that in 2010 about 1.62 billion euros are spent on obesity alone, and approximately 1.14 billion euros on diabetes (7). Since the health care in the Netherlands is partially financed by the entire adult population, there are a number of healthy people who are also paying for the medical costs of the patients suffering from obesity and diabetes. It is therefore reasonable to investigate potential methods of reducing the health care costs. Nowadays, a healthy diet and physical activity are established alternative therapies against metabolic syndrome. However these therapies are often difficult to maintain for a prolonged period and might not be useful for everyone. This thesis is aimed at alternative therapies in the form a healthy thermic environment or cold exposure. We expect these alternative strategies to lead to a reduction in the number of patients with metabolic syndrome, in the costs of the health care system and the amount of medical leave.

Practical Relevance

As shown in this thesis we often make use of direct cold exposure using a water-perfused suit to investigate the effects on metabolic health. This kind of instruments can potentially be used for a cold therapy. In a daily-life situation this could be impracticable, as it would mean that people would have to commit to purchasing suits and the expensive and large cooling installation. Further research should thus be aimed at practical implementations, such as lowering heating in buildings, creating more dynamic indoor conditions, or developing cheap cooling instruments in order to (temporally) reduce ambient temperatures.

A potential limitation is the expectation that participants often experience the temperatures used in this thesis as very cold and unpleasant. This could make maintaining these temperatures a challenge for the practical application. However, research points out that test subjects can adapt to the colder environments.

Therefore the application of cold exposure should be done in an easy-to-use manner, such as cold exposure in normal living situations. This could be achieved for instance in our working environments, such as offices, or in our home situation, by lowering the thermostat in winter

or using cooling during summer periods. Another option is the use of saunas or public bathing houses with cooling baths. A regular use of easily accessible and acceptable cold conditions could be used in addition to the existing lifestyle measures.

Future research and hopes

Based on the research presented in this thesis cold exposure leading to non-shivering thermogenesis can have positive effects on metabolism and health. However, the conditions studied in this thesis revealed marginal effects in obese patients with type 2 diabetes mellitus. It could be that a relatively short period of mild cold exposure of 10 days may be too short to have any significant metabolic effects. It would be interesting for future research to look into the effects of prolonged exposure, as in several weeks or even months. This could be studied in an office environment perhaps were prolonged exposure could be tested in a larger population. Additionally, the cross talk between brown adipose tissue and other tissues such as liver and skeletal muscle needs further attention, as brown adipose tissue could have endocrine functions. This would include the secretion of hormones which can have metabolic effects in other organs.

Furthermore, exposure to more extreme cold can be an interesting topic as the higher metabolic demand of shivering could have more effects when compared to only mild cold exposure. In the future this could perhaps lead to the development of new interventions to improve metabolic health in our obese society.

References

1. Chooi YC, Ding C, Magkos F. The epidemiology of obesity. Metabolism. 2019;92:6-10.
2. WHO. Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016. Geneva, World Health Organization; 2018. [ ]. Available from: https://www.who.int/healthinfo/global_burden_disease/estimates/en/.
3. Environment NIoPHat. 2019 [Available from: https://www.volksgezondheidenzorg.info/onderwerp/overgewicht/cijfers-context/huidige-situatie - node-overgewicht-volwassenen.
4. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.
5. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
6. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
7. Luijben; MitP-PG, Hoogenveen R. Zorgkosten van ongezond gedrag. In: Milieu RvVe, editor. Bilthoven2010.

Summary

Summary

Modern society is confronted by a significant increase in the incidence of metabolic syndrome, which is characterized by high body fat mass, insulin resistance and hyperlipidaemia. The increase in total body fat mass can be attributed to a shift in the human energy balance. The human body takes in energy by the food and uses it for many life functions, while the excess amount is stored in the form of fat and glycogen. A shift towards less energy use, e.g. by reduced physical activity, and an increased energy intake can thus lead to the accumulation of fat. When body fat is present in excess amounts, it is referred to as obesity. This increase in fat tissue, especially in the abdominal region, is linked to the increase in metabolic disturbances such as elevated lipid levels and insulin resistance. The disturbed metabolism in turn will lead to increased atherosclerosis, affecting both small (micro) and large (macro) blood vessels. The increased atherosclerosis explains the high incidence of both microvascular complications, such as blindness, renal failure, and neuropathy, and macrovascular complications such as myocardial infarctions and cerebrovascular incidents. The high incidence of obesity, type 2 diabetes mellitus, and vascular complications places a huge burden on our society, both in financial costs as well as on the physical care system. Therefore new research is warranted to investigate potential methods to decrease this burden on our society.

Increasing energy expenditure could be a potential solution to prevent obesity and improve metabolism. Besides using exercise to increase energy expenditure, another option is to increase the energy expenditure related to maintaining a stable body temperature. As the body is exposed to cold, energy expenditure increases to maintain a stable body temperature. Thus, in a cold environment, energy expenditure is increased by heat production, otherwise known as cold-induced thermogenesis. During mild cold without shivering, this function is performed by brown adipose tissue (non-shivering thermogenesis). This tissue is present in humans, in small depots, can utilize substrates such as lipids to produce heat. In rodents, the huge amount of substrates used by active brown adipose tissue can be used to treat dyslipidaemia and obesity. We reviewed the function of brown adipose tissue in Chapter 2, as well as the potential contribution of this tissue to human metabolism. Brown adipose tissue could provide an answer to the obesity pandemic and the related metabolic disturbances.

Increasing brown adipose tissue activity and metabolism is thus an interesting option to combat obesity. In addition to the cold, a possible option is stimulation by means of nicotinamide riboside, a vitamin B3 precursor. In Chapter 3, we present the results of nicotinamide riboside suppletion in humans, both in vitro and in vivo. The nicotinamide riboside suppletion did lead to an increase in oxidative capacity in both human brown adipose tissue cells in vitro and in living mice. However it did not affect energy expenditure or brown

adipose tissue activity in adult humans with obesity. These results show that the promising of nicotinamide riboside in vitro and in mice may not have any effect on the whole human body. The lack of results in adult humans could be due to the low dosage of nicotinamide riboside. Another explanation could that obese humans have a relatively low amount of active brown adipose tissue.

Increasing the capacity of brown adipose tissue could be the answer to obesity and type 2 diabetes mellitus. In Chapter 4, we evaluated the effects of prolonged cold exposure, or cold acclimation on insulin sensitivity in adult humans with obesity and type 2 diabetes mellitus. In contrast to other studies, special attention was given to prevent shivering. After a twelve-day period of intermittent mild cold exposure, without shivering, we observed no improvements in insulin sensitivity when measured with a hyperinsulinaemic euglycaemic clamp technique. Neither did we observe any changes in glucose uptake or gene expression in skeletal muscle cells. This is in clear contrast to results from previous experiments involving cold acclimation. As there was evidence of muscle activity (shivering) in the earlier studies, the results in this thesis point out that non-shivering cold acclimation does not lead to metabolic improvements.

Next, we examined the effect of acute non-shivering cold exposure on lipid metabolism in young adult humans, as described in Chapter 5. It was expected that when brown adipose tissue is activated, substrates such as fatty acids are utilized to maintain metabolic heat production. This effect could be revealed by lowering post-prandial lipid levels in the blood circulation, which are typically elevated after a meal. Therefore a double high-fat meal test was used to study post-prandial lipid metabolism in order to compare lipid levels between thermoneutral and cold conditions. Despite an increase in whole-body fat oxidation during cold conditions, there was no significant decrease in post-prandial lipid levels and there was no correlation between substrate utilization and the amount of active brown adipose tissue. Additionally, no effects of the non-shivering cold exposure were observed on skeletal muscle fibers in vitro. Long-term effects on, for example, atherosclerosis have yet to be studied.

Finally, we studied the role of brown adipose tissue in Madelung's disease. As described in Chapter 6, Madelung’s disease is characterized by symmetric lipomatosis, by a yet unexplained pathophysiology. One explanation is that dysfunctional brown adipose tissue, through genetic mutations, is responsible for the lipomatosis. We therefore took adipose tissue biopsies from the lipomatosis and the abdominal tissue and compared these on both genetic expression and microscopic view. We found no relationship with active BAT and were therefore unable to find an explanation for the lipomatosis in our patient.

In conclusion, we have studied the potential of non-shivering cold exposure and BAT activation during acute exposure and after cold acclimation. In both studies, non-shivering cold exposure did not lead improvements of the metabolic parameters under study. Most

likely the lack of metabolic improvements can be attributed to the relatively small amount of active BAT that adult humans possess. Since humans have significantly more muscle tissue, shivering in more extreme cold can have more systemic effects. This could mean that periodic shivering is necessary for the development of an alternative treatment of metabolic syndrome.

Samenvatting

Samenvatting

In de huidige samenleving is er een stijgende lijn te zien in de incidentie van het metabool syndroom, wat gekenmerkt wordt door een toename in vet massa, een verstoord lipide (vet) metabolisme en insuline resistentie. De toename in vetmassa is te verklaren door een positieve energie balans in het menselijk lichaam. Het lichaam neemt energie op in de vorm van voeding en gebruikt energie voor de vele levensfuncties. Een overschot aan energie wordt opgeslagen als adipeus weefsel en glycogeen. Door een afname in energie verbruik en een verhoogde energie intake, door middel van een verhoogde voedsel intake, kan dit leiden tot stapeling van vetweefsel. Deze stapeling van vetweefsel, voornamelijk in de abdominale regio, is gelinkt aan een verstoord metabolisme zoals verhoogde lipide bloedspiegels en insuline resistentie.
De insuline resistentie leidt tot de ontwikkeling van type 2 diabetes mellitus, wat zich kenmerkt door verhoogde bloed glucose (suiker) waardes.

Het ontregelde metabolisme van glucose en lipiden leidt tot een verhoogde kans op atherosclerose. Dit verklaart de hoge incidentie van micro vasculaire complicaties zoals blindheid, nierfalen en neuropathie maar ook de macro vasculaire complicaties zoals myocard infarcten en cerebrovasculaire incidenten. De hoge incidentie van obesitas, type 2 diabetes mellitus en vasculaire complicaties heeft een grote invloed op onze samenleving. Dit is vooral zichtbaar in de hoge financiële kosten aan zorg en de belasting op ons zorgsysteem.

Zoals hierboven aangegeven is het verhogen van het energiegebruik een mogelijke optie ter preventie van obesitas. Naast lichamelijke beweging kan blootstelling aan een lage omgevingstemperatuur het energiegebruik van het lichaam verhogen. Hierdoor wordt het lichaam gedwongen om het energie verbruik van het lichaam te verhogen om de lichaamstemperatuur stabiel te houden. Deze toename in energie verbruik wordt geduid als koude-geïnduceerde thermogenese, en wordt uitgevoerd door bruin vet bij milde kou (zonder te rillen, z.g. non-shivering thermogenese), en door rillen bij lagere temperaturen (shivering thermogenese). Bij mensen is bruin vet in kleine hoeveelheden aanwezig en kan het substraten zoals lipiden verwerken tot warmte. Het verwerken van deze substraten kan bijdragen aan het behandelen van obesitas en type 2 diabetes mellitus. De functie van bruin vet weefsel wordt beschreven in hoofdstuk 2, evenals de mogelijke bijdrage en rol van bruin vet in de menselijke stofwisseling.

Zoals hierboven beschreven kan het vermeerderen van de hoeveelheid bruin vetweefsel bijdragen aan het verbeteren van metabolisme. Naast kou is een mogelijke optie stimulatie door middel van nicotinamide riboside, een vitamine B3 precursor. Nicotinamide riboside stimuleert in vitro mitochondriale activiteit en zou zodoende ook de mitochondriën in bruin vet kunnen stimuleren. In hoofdstuk 3, worden de resultaten van nicotinamide riboside

suppletie gepresenteerd. De suppletie van nicotinamide riboside resulteerde in een verhoging van de oxidatieve capaciteit in zowel menselijke bruin vet cellen als in muizen. Echter was er geen effect op het energie gebruik en bruin vet activiteit in volwassenen met obesitas. Dit laat zien dat veelbelovende supplementen die werken in vitro en in muismodellen, niet per se effectief zijn op het gehele lichaam. Dit kan mogelijk worden verklaard door een te lage dosering aan nicotinamide riboside in de proefpersonen. De ten opzichte van muizen relatief kleine hoeveelheid bruin vet weefsel in mensen kan hier ook een mogelijke verklaring voor zijn.

Een andere mogelijkheid om bruin vet te vermeerderen is langdurige regelmatige blootstelling aan kou zijn, ook wel bekend als koude-acclimatisatie. In Hoofdstuk 4, worden de effecten van herhaalde blootstelling aan kou, beschreven in een populatie van volwassenen met obesitas en type 2 diabetes mellitus. Na een periode van twaalf dagen met blootstelling aan milde kou zonder te rillen, zagen we, in tegenstelling tot eerdere studies, geen effect op insuline gevoeligheid en geen effect op de glucose opname in skelet spieren. Omdat in het vorige onderzoek er nog ril-activiteit optrad, is er in deze studie specifiek gelet op de afwezigheid van rillen bij de proefpersonen. Dit laat zien dat langdurige koude blootstelling zonder te rillen niet leidt tot een verbeterde stofwisseling in volwassenen met obesitas. Mogelijk zou er een ander effect kunnen zijn op bijvoorbeeld de bloedvaten of de functie van het hart.

In het volgende hoofdstuk, hoofdstuk 5, hebben we het acute effect van koude blootstelling en bruin vet activatie onderzocht in jonge gezonde proefpersonen. De verwachting was dat behalve het energiegebruik, er ook een toename in substraat (vetten en suikers) verbruik optreedt. Dit zou terug gezien kunnen worden in het verlagen van lipide levels, die typisch hoog zijn na een vetrijke maaltijd. Daartoe werd een maaltijd test gebruikt, met twee maaltijden met een hoog vetgehalte om het postprandiale vet metabolisme te bestuderen. Ondanks een toename in vet oxidatie, was er geen reductie in lipiden levels tijdens de koude blootstelling. Dit betekent dat acute milde kou niet leidt tot verlaging van de lipide spiegels. Er werd ook geen effect van de koude blootstelling zonder het rillen de werking van de skeletspiervezels aangetoond. Tenslotte was er geen relatie met de activiteit en hoeveelheid van het bruin vetweefsel in deze proefpersonen. Lange termijn effecten op bijvoorbeeld artherosclerose dienen nog te worden bestudeerd.

In Hoofdstuk 6, hebben de mogelijke rol van bruin vetweefsel bij de ziekte van Madelung onderzocht. Dit ziektebeeld wordt gekenmerkt door symmetrische vetstapeling. Een mogelijk pathofysiologisch mechanisme is dat dysfunctioneel bruin vet, door mutaties, verantwoordelijk is voor de vetstapeling. We hebben hierbij een patiënt met de ziekte van Madelung onderzocht. We hebben daarom vetbiopten genomen en de hoeveelheid bruin vet gemeten. We zagen op microscopisch en genetisch niveau geen verschil tussen het vetweefsel uit de lipomatose en het vetweefsel uit de buikwand. We zagen ook geen relatie

met actief bruin vet in deze patiënt, en hebben dus geen verband gevonden tussen bruin vet en de ziekte van Madelung.

In dit proefschrift is de potentie van milde koude blootstelling onderzocht, in zowel de acute blootstelling als na koude acclimatie. In de studies hebben we laten zien dat blootstelling aan koude zonder rillen niet leidt tot een verbetering in de stofwisseling. Er was geen verbetering van de insuline gevoeligheid na koude-acclimatisatie, en er was geen verlaging van lipide levels na acute milde kou. Het ontbreken van positieve effecten op de stofwisseling kan mogelijk worde terug geleid op het feit dat het om milde koude betrof. Hierbij is er een relatief kleine toename in energieverbruik, waardoor er ook te weinig substraat verbruik is om te kunnen leiden tot systemische effecten. Dit gebrek aan verbetering kan zijn veroorzaakt door de relatief kleine hoeveelheid bruin vet weefsel in volwassenen. Aangezien mensen beduidend veel meer spier weefsel hebben zou rillen bij meer extreme kou meer systemische effecten kunnen bewerkstelligen. Dit zou kunnen betekenen dat geregeld rillen nodig is voor de ontwikkeling van een therapie ter behandeling of preventie van het metabool syndroom.

About the author

About the author

Michiel Moonen was born on June 23, 1992 in Geleen, the Netherlands. He finished secondary school at Trevianum Scholengroep in Sittard in 2010, after graduating gymnasium cum laude.

Hereafter, he started his Medicine study at Maastricht University in 2010. During his medical internships, he focused on internal medicine, with choice internships in nephrology, endocrinology and general internal medicine at the Maastricht University Medical Centre.

After graduation in 2016, he started working as a PhD-student at the department of Nutrition and Movement Sciences at Maastricht University, under supervision of prof. W. van Marken Lichtenbelt and prof. P. Schrauwen. During his PhD trajectory he studied the effects of cold exposure and cold acclimation on brown adipose tissue function and lipid metabolism in humans, as demonstrated in this thesis and publications in scientific journals.

List of publications

Publications

Human brown adipose tissue: Underestimated target in metabolic disease? Biochim Biophys Acta Mol Cell Biol Lipids. 2019 Jan;1864(1):104-112.
Moonen MPB, Nascimento EBM, van Marken Lichtenbelt WD.

Absence of 18 F-fluorodeoxyglucose uptake using Positron Emission Tomography/Computed Tomography in Madelung's disease: A case report. Clin Obes. 2019 Jun;9(3).
Moonen MPB, Nascimento EBM, van Kroonenburgh MJPG, Brandjes D, van Marken Lichtenbelt WD.

Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans. Am J Clin Nutr. 2020 Aug 1;112(2):413-426.
Remie CME, Roumans KHM, **Moonen MPB**, Connell NJ, Havekes B, Mevenkamp J, Lindeboom L, de Wit VHW, van de Weijer T, Aarts SABM, Lutgens E, Schomakers BV, Elfrink HL, Zapata-Pérez R, Houtkooper RH, Auwerx J, Hoeks J, Schrauwen-Hinderling VB, Phielix E, Schrauwen P.

Nicotinamide riboside enhances in vitro beta-adrenergic brown adipose tissue activity in humans. J Clin Endocrinol Metab. 2021 Feb 1.
Nascimento EBM, **Moonen MPB**, Remie CME, Gariani K, Jörgensen JA, Schaart G, Hoeks J, Auwerx J, van Marken Lichtenbelt WD, Schrauwen P.

Metabolic responses to mild cold acclimation in type 2 diabetes patients. Nat Commun 12, 1516 (2021).
Remie CME, **Moonen MPB**, Roumans KHM, Nascimento EBM, Gemmink A, Havekes B, Schaart G, Kornips E, Joris PJ, Schrauwen-Hinderling VB, Hoeks J, Kersten S, Hesselink MKC, Phielix E, Van Marken-Lichtenbelt WD, Schrauwen P.

In preparation
Acute cold exposure leads to increased levels of triglycerides during mixed-meal tests in young healthy subjects
Moonen MPB, van Polanen N, Rense P, Jörgensen JA, Kornips E, Beckers M, Hoeks J, Wierts R, van de Weijer T, Havekes B, Plat J, Schrauwen P, Van Marken Lichtenbelt WD.

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Dankwoord

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