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'Metabolic Relevance of cold-activated Brown Adipose Tissue in healthy normal weight and obese Men'

Dissertation
for Fulfillment of Requirements
for the Doctoral Degree of the Universität zu Lübeck
from the Department of Natural Sciences

Submitted by
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Lübeck 2017

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Date of oral examination: 10th April 2018

Approved for printing. Lübeck, 12th April 2018

Declaration of Originality

I declare that this dissertation is the result of my own original work.

This thesis has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Furthermore, I confirm that I have clearly referenced all sources (either from a printed source, internet or any other source) used in this work in accordance with departmental requirements.

Signature……………….. Date...

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Abbreviations

[^{18}F]-FDG	[^{18}F]-fluorodeoxyglucose
A	Adrenaline
ALT	Alanine-aminotransferase
AMA	American Medical Association
ANP	Atrial natriuretic peptide
AST	Aspartyl-aminotransferase
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body Mass Index
BMP4	Bone morphogenic protein 4
BMP7	Bone morphogenic protein 7
BMP8b	Bone morphogenic protein 8b
cAMP	Cyclic AMP
cond	Condition
CPT1α	Carnitine palmitoyltransferase 1 isoform alpha
CPT1β	Carnitine palmitoyltransferase 1 isoform beta
Ct	Cycle threshold
DA	Dopamine
Deiodinase type 2	DIO2
DIT	Diet-induced thermogenesis
EMG	Electromyogram
FA	Fatty acid
FFA	Free fatty acid
FFAR4	Fatty acid receptor 4
FGF21	Fibroblast growth factor 21

FFM	Fat free mass
FM	Fat mass
fT_4	Thyroxine free T_4
G0S2	G0/G1 switch gene 2
G3P	Glycerol-3-phosphate
GGT	Gamma-glutamyltransferase
GPR120	G protein-coupled receptor 120
HEC	Hyperinsulinaemic-euglycaemic clamp
HF	High-frequency
HPA-axis	Hypothalamus-pituitary-adrenal axis
HPT-axis	Hypothalamus-pituitary-thyroid axis
HRV	Heart rate variability
HSL	Hormone sensitive lipase
IL-6	Interleukin 6
ivGTT	Intravenous glucose tolerance test
kcal	Kilocalories
LF	Low-frequency
LF/HF	Ratio of LF and HF power components
LPL	Lipoprotein lipase
Mets	Metabolic syndrome
mRNA	Messenger RNA
NA	Noradrenaline
NASH	Nonalcoholic steatohepatitis
NBP	Brain-type natriuretic peptide
NEFA	Non-esterified fatty acid
NST	Nonshivering thermogenesis
NTC	No template controls

oGTT	Oral glucose tolerance test
PBMCs	Peripheral blood mononuclear cells
PCOS	Polycystic ovary syndrome
PDC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase lipoamide kinase isozyme 4
PET/CT	Positron emission tomography–computed tomography
PGC-1α	Proliferator-activated receptor γ coactivator 1α
PKA	Protein kinase A
PPARα	Proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor γ
PRDM16	PR domain zinc finger protein 16
qPCR	Quantitative polymerase chain reaction
REE	Resting energy expenditure
ROIs	Regions of interest
RQ	Respiratory quotient
RT	Reverse transcriptase
SLC25A20	Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20
SNS	Sympathetic nervous system
SOPs	Standard operation procedures
SRS	Symptom rating scale
SVB	Sympathovagal balance
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TSH	Thyroid stimulating hormone
UCP1	Uncoupling protein 1
VAS	Visual analogue scale
VCO_2	Rate of elimination of carbon dioxide

VO_2	Rate of elimination of oxygen
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization
WPS	Water-perfused suit

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RQ = VCO_2/VO_2 19

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M = Total glucose infusion mmol/L×kg body weight^{-1}×60min^{-1} 21

Abstract

Cold exposure has been shown to activate brown adipose tissue (BAT) in human adults and is thereby associated with improved insulin sensitivity and lipid metabolism. To date, molecular mechanisms underlying the metabolic benefits of cold-induced BAT activation are not fully identified and data in obese humans are even less clear. When activated, BAT utilises large amounts of circulating fatty acids (FA) released from white fat depots, suggesting pathways regulating lipolysis and FA β-oxidation to mediate metabolic effects of cold-induced BAT activation in humans. The present work aimed at addressing effects of moderate cold-induced BAT activation on human glucose and lipid metabolism, and comparing them between normal weight and obese individuals. In addition, this study sought to identify underlying systemic transcriptional mechanisms for metabolic effects of BAT activation.

Therefore, fifteen normal weight and fourteen obese metabolically healthy male volunteers participated in a cross-balanced repeated within-subject study with two experimental conditions. Using a water-perfused suit participants were exposed to thermoneutrality (22°C to 25°C) and moderate cold (16°C to 18°C, shivering excluded), respectively. Cold-induced BAT activation was quantified by [^{18}F]-FDG-PET/CT in a subset of volunteers. Botnia clamp procedure was applied to determine insulin sensitivity before and upon exposure to moderate cold, complemented by measurements of resting energy expenditure (REE), respiratory quotient (RQ), hormones, catecholamines, circulating lipids and individual food preference. Furthermore, selected target genes within lipolytic and FA β-oxidation pathways were assessed before and after exposure to moderate cold in human peripheral blood samples using quantitative polymerase chain reaction (qPCR) analysis.

Cold exposure increased plasma noradrenaline (NA) and dopamine (DA) concentrations, and induced BAT activation as proven by PET-CT imaging. Glucose tolerance was significantly improved in obese but not normal weight men, while insulin sensitivity increased exclusively in the normal weight study cohort during moderate cold. In accordance with increased expression of selected genes involved in lipolysis and FA-oxidation, circulating triglycerides (TG) markedly increased in both groups during cold exposure as compared to thermoneutrality. In addition, exposure to moderate cold lowered serum thyroxine (fT_4) exclusively in the obese study cohort.

In summary, exposure to moderate cold induced sympathetic nervous system (SNS) activity and BAT activation in humans, leading to improved glucose metabolism in normal weight and obese men. In addition, cold-induced SNS and BAT activation was accompanied by alterations in mRNA expression levels of genes regulating lipid metabolism resulting in

elevated serum TG. Altogether, these results indicate increased lipid mobilisation from white fat to cover the additional need of energy substrates during non-shivering thermogenesis (NST). These data support the concept of human BAT metabolism significantly contributing to whole-body glucose and lipid utilisation in a very coordinated manner in normal weight and obesity.

To conclude, the present work presents novel insights into underlying molecular mechanisms linking cold exposure to BAT activity and improved metabolic health in humans, thereby highlighting BAT activation as a potential therapeutic target in metabolic medicine.

Zusammenfassung

Die Prävalenzraten von Übergewicht und Adipositas sind über die letzten Jahrzehnte weltweit dramatisch gestiegen. Dementsprechend ist auch eine deutliche Zunahme von Patienten mit dem metabolischen Syndrom zu verzeichnen. Das metabolische Syndrom umfasst u.a. Übergewicht, erhöhte Blutfette und Diabetes mellitus Typ 2 (charakterisiert durch verminderte Insulinsensitivität und gestörte Insulinsekretion). Mit Nachdruck wird daran geforscht neue Erkenntnisse zur Pathogenese sowie zu effektiven Präventions- und Therapiemethoden zu finden. Aktivierung von braunem Fettgewebe wurde wiederholt als aussichtsreiche Methode zur Verminderung verschiedener Komponenten des metabolischen Syndroms, insbesondere der Glukose- und Fettstoffwechselstörung, postuliert. Das braune Fettgewebe ist eine spezielle Fettgewebsform, die bei kühlen Umgebungstemperaturen aktiv Wärme erzeugt ohne zusätzliches Muskelzittern erforderlich zu machen. Zur Initiierung und Aufrechterhaltung zitterfreier Thermogenese metabolisiert braunes Fettgewebe primär freie Fettsäuren und Glukose und reduziert damit im Blut zirkulierende Lipide und Glukose. Diese Eigenschaften machen kälteaktiviertes braunes Fettgewebe zu einem potenziellen Kandidaten für die Therapie von Übergewicht und metabolischem Syndrom. Bisher ist jedoch nicht abschließend geklärt, über welche Mechanismen braunes Fettgewebe potentiell positive Effekte auf den menschlichen Energiestoffwechsel ausüben kann.

Ziel der vorliegenden Arbeit ist es, zunächst die Effekte von kälteaktiviertem braunen Fettgewebe auf Glukose- und Lipidstoffwechsel bei schlanken und adipösen metabolisch gesunden, jungen Männern zu untersuchen und miteinander zu vergleichen. Zudem wurden mögliche regulatorische Einflüsse von kältestimuliertem braunen Fettgewebe auf Transkriptionsmuster relevanter Gene der Lipolyse und Fettsäureoxidation in zirkulierenden Blutzellen untersucht.

Insgesamt wurden fünfzehn normalgewichtige (BMI 18.5-24.9) und vierzehn adipöse (BMI 30-34.9 kg/m^2) junge Männer in einem balanciert-randomisierten Messwiederholungsdesign thermoneutralen Bedingungen (22.0-25.0°C) und moderater Kälte (16.0-18.0°C) zur zitterfreien Aktivierung von braunem Fettgewebe ausgesetzt. Dabei wurden relevante Marker des Glukose- und Lipidstoffwechsels untersucht sowie Transkriptomanalysen selektierter Zielgene durchgeführt. Überdies wurden zusätzliche Parameter des Energiestoffwechsels, Ruheumsatz und respiratorischer Quotient, sowie individuelle Präferenz für bestimmte Nahrungsmittel erfasst. Die kälteinduzierte Aktivierung brauner Fettdepots wurde in einer Subgruppe mittels [^{18}F]-FDG-PET/CT überprüft.

Die PET/CT-Bildgebung bestätigte kälteinduzierte Aktivierung von supraclaviculären braunen Fettdepots. Zudem führte die Kälteexposition zu einer starken Aktivierung des sympathischen Nervensystems, gekennzeichnet durch einen deutlichen Anstieg der Plasma-Konzentrationen von Noradrenalin und Dopamin, was maßgeblich die Signalkaskade brauner Fettzellen initiiert. Im Weiteren verbesserten sich während moderater Kälteexposition die Glukosetoleranz in den adipösen und Insulinsensitivität in den schlanken Probanden. In beiden Gruppen zeigten sich erhöhte Konzentrationen zirkulierender Triglyceride unter kälteaktiviertem braunen Fettgewebe. Analog zu diesem Befund war die mRNA von vier relevanten Fettstoffwechsel-Genen, nämlich PDK4, CPT1α, SLC25A20 und G0S2 vermehrt exprimiert. Ferner sank unter kälteinduzierter Aktivierung von braunem Fettgewebe die Serum-Konzentration von fT_4 einzig in der adipösen Studienkohorte ab. Die übrigen Hormone (TSH, Cortisol, Gesamt-Ghrelin und Leptin), Blutfette (Gesamtcholesterin, HDL und LDL) und Messgrößen (Ruheumsatz, respiratorischer Quotient, Ernährungspräferenzen) blieben in beiden Studienkohorten von kälteaktiviertem braunen Fett unbeeinflusst, ebenso die mRNA von CPT1β und PPARα.

Allgemein verdeutlichen die vorgenannten Befunde die enge Interaktion zwischen sympathischem Nervensystem und braunem Fettgewebe bei der Integration von Prozessen des Glukose- und Lipidstoffwechsels über molekulare Pfade der Lipolyse und Fettsäureoxidation. Die aus der gesteigerten Lipolyse zusätzlich bereitgestellten Energieträger werden möglichweise zur Aufrechterhaltung der zitterfreien Thermogenese im braunen Fettgewebe genutzt. Methodisch zeigt die vorliegende Arbeit überdies, dass systemische Transkriptomanalysen aus zirkulierenden Blutzellen sinnvoll zur weiteren Hypothesengenerierung genutzt werden können. Diese vereinfachte Alternative zu Gewebebiopsien eröffnet interessante neue Ansätze in der humanen Stoffwechselforschung zu BAT. Bezüglich postulierter gewichtsreduzierender Effekte von braunem Fettgewebe bleibt darauf hinzuweisen, dass im Rahmen dieser Untersuchung von Akuteffekten der BAT-Aktivierung weder bei schlanken noch übergewichtigen Männern eine Erhöhung des Ruheumsatzes festgestellt wurde.

Abschließend ist festzuhalten, dass die Ergebnisse der vorliegenden Arbeit für das Potenzial von kältestimuliertem braunen Fettgewebe als wirksame Interventionsmethode bei Störungen des Glukose- und Lipidstoffwechsels sprechen und zwar sowohl bei schlanken als auch adipösen Männern.

Chapter I. Introduction

1.1. Background

1.1.1. The complex burden of obesity

In recent years prevalence rates of overweight and obesity substantially increased worldwide [1]. In Germany, almost two thirds of men and half of all women are overweight or obese [1], and according to the World Health Organization (WHO; [2]) more than 20% of the overall German population is obese. The WHO defines overweight and obesity as 'abnormal or excessive fat accumulation that may impair health', in concrete numbers the Body Mass Index (BMI) quantifies and categorizes different nutritional states in adults (see table 1). Calculated as body weight (kg) divided by squared body height (m^2), BMIs ranging from 25 to 29.9 kg/m^2 indicate overweight or 'pre-obesity', while values >30 kg/m^2 are classified as obesity and further subdivided into 3 classes of severity, which are 'obesity class I' as mildest form (BMI 30 – 34.9), 'class II' as moderate obesity (35 – 39.9) and finally extreme obesity as 'class III' (BMI >40 kg/m^2). In contrast, normal weight is indicated by BMI between 18.5 and 24.9 kg/m^2, and underweight is considered BMI <18.5 kg/m^2 [2].

In a rather simplistic view, obesity emerges from a sustained energetic imbalance with energy intake exceeding energy expenditure. In everyday life this is reflected by consumption of high caloric food without sufficient physical activity as compensatory intervention, thus finally leading to storage of excess energy substrates mainly in white fat depots, liver and muscles [3]. In fact, overweight and obesity originate from a multifactorial interplay of individual behaviour, socio-ecological factors, but also genes and other physiological predispositions including hormones impacting on energy homeostasis [3–5]. Apart from this complexity, essential for reducing body weight is a negative energy balance with reduced calorie intake. Attempts to achieve weight loss primarily focus on changing the individual´s behaviour towards a healthy lifestyle as least invasive option, however clinical treatment strategies further comprise cognitive restructuring, pharmacological treatment and bariatric surgical interventions [4]. For compelling reasons, the individual motivation to overcome overweight and obesity should not only be driven by cosmetic concerns, as excess body weight has turned out to be more than merely an issue of aesthetical perception: Obesity is accompanied by numerous severe comorbidities, most prominent type 2 diabetes mellitus (T2DM), dyslipidaemia and hypertension, together defined as the metabolic syndrome (Mets; [6]). Furthermore, obesity is associated with higher risk for cancer, heart failure, psychiatric

disorders and many other diseases. According to clear statements of the WHO [7] and also other Associations as the American Medical Association (AMA) [8] obesity is recognized a chronic disease. Primarily driven by the increasing rates of obesity worldwide, Mets has become a major global health burden [9]. It is known that the individual components of Mets are closely interrelated and presuppose each other as e.g. dyslipidaemia impacts on insulin resistance, a hallmark of T2DM [10]. A second hallmark of T2DM is impaired pancreatic first phase insulin secretion in response to an acute increase in blood glucose, which, together with the aforementioned insulin resistance in tissues and organs is responsible for limited metabolic control and contributes to the pathogenesis of hyperglycaemia [11]. Both insulin secretion from pancreatic β-cells and insulin sensitivity are major components of physiological glucose homeostasis, as their precisely coordinated interaction determines the amount of blood glucose available in the circulation and the extent to which organs and tissues can use glucose as energy substrate for proper functioning. Insulin sensitivity refers to the cell´s capacity to utilise circulating glucose from the blood stream mediated by insulin. In insulin resistance as seen in T2DM the cells responsiveness to insulin is impaired despite normal or even compensatory elevated insulin secretion. In consequence, glucose cannot enter insulin resistant target tissues resulting in hyperglycaemia, thereby causing endothelial damage within small blood vessels and arteries [12,13]. In addition to impaired glucose metabolism, in particular visceral obesity is often accompanied by an aberrant blood lipid composition, characterised by elevated levels of circulating triglycerides (TG) and free fatty acids (FFA, also referred to as 'non-esterified fatty acids', NEFA), low HDL cholesterol and elevated LDL cholesterol [14]. Elevated FFA and TG levels, as metabolized in the liver, accumulate as lipid droplets in the cytoplasm of hepatocytes and finally cause non-alcoholic fatty liver disease (NAFLD). In obese humans NAFLD is a maladaptive process and can progress to non-alcoholic steatohepatitis (NASH), liver cirrhosis and finally cancer [15]. Free fatty acids used for hepatic TG formation are delivered by i) diet, ii) de novo lipogenesis and iii) lipolysis in adipose tissue, all of which are likely to be affected in obesity towards the promotion of increased FFA trafficking to the liver [14]. In turn, such elevated levels of circulating FFA are associated with insulin resistance of peripheral tissues, such as skeletal muscle and adipose tissue. Therefore, to the extend adipose tissue itself becomes insulin resistant, compensatory hyperinsulinaemia and a further rise in lipolysis is to be expected, since insulin principally facilitates lipogenesis and suppresses lipolysis. Thus, the obese

insulin resistant patient appears to be caught in a vicious circle of both impaired glucose and fat metabolism, which further affect each other [16].
In sum, obesity is a complex metabolic disorder raising multi-faceted issues for the single patient, social systems and health care systems [5]. This complexity stimulated a large body of multidisciplinary research with the overarching goal to identify underlying mechanisms and integrate those findings into the development of novel effective treatment strategies to combat obesity and Mets.

Table 1. BMI classification according to WHO

BMI	Nutritional status
< 18.5	Underweight
18.5 – 24.9	Normal weight
25.0 – 29.9	Overweight
30.0 – 34.9	Obesity class I
35.0 – 39.9	Obesity class II
> 40.0	Obesity class III

1.1.2. Can brown adipose tissue help to defy obesity and the metabolic syndrome?

In order to combat obesity and Mets the optimum therapy would address both glucose and lipid metabolism. In this respect, thermogenic brown adipose tissue (BAT) has attracted much attention as novel target, as this particular type of fat produces heat through combustion of fatty acids (FA), i.e. esterified fatty acids, and glucose as fuel [17]. Required FA and glucose derive mainly from the circulation as either TG-rich lipoproteins or in an albumin-bound form as NEFA, thereby lowering levels of circulating chylomicron- and very-low-density lipoprotein (VLDL)-bound TG as well as blood glucose [17,18]. As already shown in rodents, activated BAT might offer new options to lower elevated blood lipid concentrations, thereby reinstalling euglycaemia and improving insulin sensitivity [19,20].

1.2. Brown adipose tissue in humans

1.2.1. Physiology and Functions

'Body fat' comprises not only the depots of white adipose tissue (WAT) as main storage site for excess energy, but also beige and brown adipose tissue present in mammals, including humans. Giving a brief overview, in addition to storing vast amounts of lipids and providing thermal insulation WAT also acts as endocrine organ secreting numerous adipokines relevant for energy metabolism and inflammation, such as e.g. leptin, adiponectin, fibroblast growth factor 21 (FGF21) and interleukin 6 (IL-6; [21,22]). Beige adipocytes develop in subcutaneous depots of WAT from the same precursor cells as their white hosts, but have the potential to acquire thermogenic capacities in response to various activators, such as β_3-adrenergic stimulation, in the context of e.g. prolonged cold exposure, exercise as well as in presence of various hormones and metabolites [23]. This process has been termed 'browning' of WAT, therefore beige adipocytes are also referred to as inducible 'brite' or 'brown-like' adipocytes, moreover as 'recruitable brown adipose tissue' [24]. The type of adipose tissue featured in the present work is the genuine brown adipose tissue (BAT) in the context of human energy metabolism, which will be introduced and extensively discussed in the following sections.

Brown adipose tissue is a specialized compartment of fat, acting as a thermogenic organ by dissipating heat from energy substrates and thereby prevents hypothermia in both young and adult animals [17]. In human neonates and infants, BAT is located mainly around internal organs, in interscapular and supraclavicular regions as well as next to the carotid artery and jugular vein. With increasing age BAT mass declines. In human adults BAT depots are mostly found in the supraclavicular region and around the spine [25]. The characteristic macroscopic brown appearance of BAT is due to heme cofactors in the mitochondrial enzyme cytochrome oxidase [26]. Brown adipocytes are derived from a Myf5+ myogenic lineage rather than adipogenic progenitor cells, i.e. BAT shares a common precursor with skeletal muscle cells but not with white adipocytes. This, at first glance, surprising origin explains why BAT and WAT serve markedly different functions with BAT being specialized for lipid catabolism rather than storage [27,28]. Brown adipose tissue is highly vascularized, rich in mitochondria and contains many small lipid droplets. These features, together with the BAT-specific transmembrane protein uncoupling protein 1 (UCP1), provide the thermogenic capacity required for BATs primary function referred to as nonshivering thermogenesis (NST) whenever the organism is exposed to cold [17]. Nonshivering thermogenesis is a cold-induced

increase in heat production entirely provided by BAT without contribution of muscle activity in form of shivering, and serves as an adaptive mechanism for the maintenance of normothermia in response to environmental temperature [29]. Cold sensation initiates NST in BAT via activation of the sympathetic nervous system (SNS), which is centrally controlled by the hypothalamus. Since BAT is densely innervated by sympathetic signals, SNS is crucially involved in the regulation of BAT development and its thermogenic function. Upon ambient cold BAT is activated by noradrenaline (NA) as released from sympathetic fibres and bound to β_3-adrenergic receptors of brown adipocytes. Activation of β_3-adrenergic receptors triggers the cyclic AMP − phosphokinase A signalling pathway to initiate NST, which requires brown adipocytes to combust fatty acids and glucose as fuel [30]. However, within BAT the released energy is not used for re-transport of protons into the mitochondrial matrix. Located in the inner mitochondrial membrane of brown adipocytes, UCP1 uncouples mitochondrial respiration from oxidative phosphorylation, thereby allowing protons to directly re-enter the mitochondrial matrix without passing the ATP synthase complex, hence, the energy directly dissipates as heat from the tissue [17]. Figure 1 schematically illustrates BAT thermogenesis.

1.2.2. BATs metabolic relevance and potential as anti-obesity target

Increasing the cellular turnover of substrates such as FFA and glucose, thereby clearing excessive energy substrates from the circulation is associated with reduced risk to develop insulin resistance. By utilising FFA and glucose as energy substrates for NST, both lipid and glucose metabolism converge during BAT thermogenesis, thus making BAT an interesting target for treating obesity and Mets [32]. The evaluation of BATs metabolic potential is closely linked to the essential question of whether BAT is relevant –or negligible– for human energy homeostasis. Therefore, precise determination of BAT mass and its metabolic contribution was a critical starting point in this field of research. Indeed, BATs capacity to metabolise FFA and glucose upon acute cold exposure has been shown to exert notable influence on energy metabolism in animals and humans, e.g. increased energy expenditure, improved insulin sensitivity, reduced hypercholesterolaemia and accelerated fat clearance [33–37].

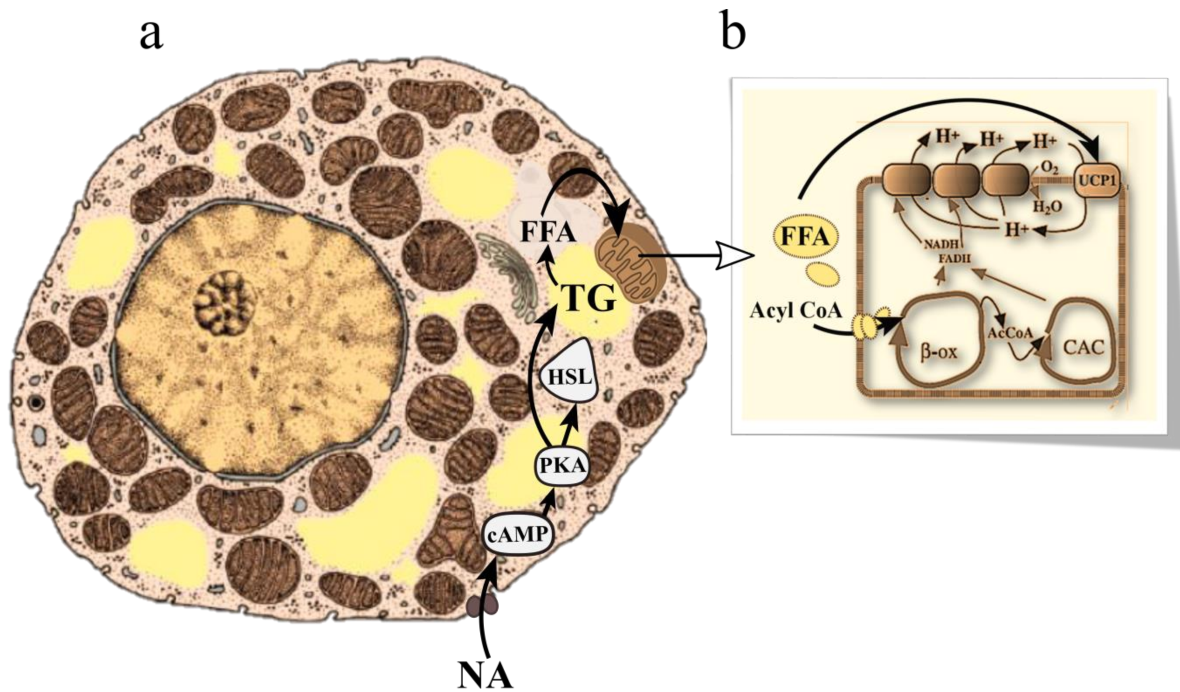


Figure 1. *Sympathetic activation of UCP1-mediated nonshivering thermogenesis in brown adipocytes and detailed schematic illustration of respiratory uncoupling in BAT mitochondria.* Upon cold stimulation the sympathetic nervous system releases noradrenaline (NA) which binds to β_3-adrenergc receptors on the plasma membrane of brown adipocytes (**a**). This triggers the signal transduction cascade: adenylate cyclase is activated and converts ATP to cyclic AMP (cAMP). Cyclic AMP activates protein kinase A (PKA), thereby initiating the catalytic subunit to detach from the regulatory subunit. This induces the break-down of TG to free FFA by hormone sensitive lipase (HSL) and adipose triglyceride lipase. The released FFA activate UCP1 (**b**) and enter the mitochondria, where they serve as substrates for β-oxidation. In turn, activated UCP1 uncouples the proton motive force from oxidative phosphorylation by generating a proton leak, resulting in dissipation of the proton motive force directly as heat. This proton motive force constitutes an electrochemical gradient built up by electron transfer, which promotes pumping of protons via complex I, III and IV from the matrix back to the intermembrane space (modified from Junqueira's basic histology: text and atlas, 12th edition by Anthony Mescher 2013 and Cannon 2004 [17,31]).

In general, both presence and volume of functional BAT are associated with a lower BMI and a favourable metabolic state, whereas in overweight and obese individuals as well as T2DM patients and elderly people relatively little amounts of functional BAT have been detected [35,38–42]. This finding raised the question of whether decreased BAT activity is a consequence of obesity or rather resembles an underlying metabolic property contributing to obesity and insulin resistance.

To study BAT activation in humans, [^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG) Positron emission tomography-computed tomography (PET/CT; see section 2.8.) imaging is considered as gold standard diagnostic tool to localise and quantify functional BAT volume, often complemented by additional methods to assess metabolic parameters such as circulating hormones, lipids and resting energy expenditure (REE, see section 2.5.). Experimental protocols to explore BAT physiology in humans generally require volunteers to be exposed to moderate cold aiming at activating BAT while avoiding shivering at the same time, as increased muscle activity will also impact metabolism. Using combined cooling-[^{18}F]-FDG PET/CT protocols, previous studies quantified BAT volume in adult humans with a range between 9 g and 170 g [33,43]. Based on PET/CT data from one selected volunteer in a study of Virtanen et al. it was estimated that 63 g of cold-activated BAT contribute to 5 % of whole-body energy expenditure. In other words, over a one-year period such amount of fully activated BAT could metabolise energy substrates equivalent to approximately 4.1 kg of WAT [44,45]. However, this calculation likely does not estimate the real contribution of BAT to human energy metabolism precisely. This is first due to interindividual differences in BAT mass and second, because the utilised glucose quantified using [^{18}F]-FDG as marker for functional BAT mass is not the primary energy source for NST. Activated BAT predominantly (~90 %) metabolises FFA, while glucose accounts for only 10 % of total BAT metabolism [44,46,47]. Nevertheless, even the conservative estimate of a 5 % contribution of BAT to human energy metabolism appears significant and reasonable to consider BAT as a potential target to treat major components of the Mets [48]. The relevance of BAT for human energy metabolism is also supported by its remarkable plasticity in terms of activation and volume. This is reflected by observed seasonal variations in BAT volume with higher activated BAT mass detected during winter as compared to summer and the potential to recruit BAT by means of prolonged cold exposure, also referred to as 'acclimation' [49,50]. Furthermore, browning of WAT occurs after prolonged cold exposure, i.e. beige adipocytes in WAT depots obtain BAT-like features and express UCP1 to provide thermogenesis like genuine brown adipocytes [22]. This induction of BAT-like properties might be another potential mechanism to treat obesity and Mets, since recruiting further thermogenic adipocytes will, in turn, increase the total of combusted energy substrates. The effectiveness of cold acclimation protocols to enhance BAT activation has been proven in normal weight and obese humans as well as in T2DM patients reflected by increased [^{18}F]-FDG uptake into supraclavicular BAT depots. However, the

extent to which obese individuals benefit from increased BAT activation following cold acclimation periods with respect to weight loss and metabolic improvements is less clear. To date, conclusive data from controlled moderate cold acclimation interventions in obese patients is sparse. A study using PET/CT demonstrated increased BAT metabolism in cold acclimated obese participants. However, the gain in BAT activity was not accompanied by elevated REE as a prerequisite for weight loss [51]. In contrast, normal weight men and overweight T2DM patients exhibited reduced fat mass and improved insulin sensitivity following cold acclimation, but also in these studies no changes in body weight were reported [32,35,50,52,53].

Together, acute exposure to moderate cold is an effective stimulus to induce BAT thermogenesis in human adults, with growing evidence supporting BAT to be functionally relevant in humans in terms of a significant contribution to energy homeostasis. This is further supported by BATs marked dynamic and plastic properties in response to changing ambient conditions as reflected by increases in BAT activity and mass in normal weight and obese individuals following cold acclimation periods. However, the potential of recruited BAT to effectively reduce body weight requires further studies.

1.3. Effects of cold-induced BAT activation on

1.3.1. Resting energy expenditure and respiratory quotient

Resting energy expenditure is the proportion of daily energy expenditure required to maintain vital physiological functions of a resting, but awake, individual in a fasted state and measured during thermoneutrality [54]. In general, REE varies substantially between individuals as main determinants of REE include fat free mass (FFM), e.g. skeletal muscle mass, body weight, sex and age [55]. In recent years also cold-activated BAT has been recognized to increase REE in humans. In their pioneering study on cold-induced BAT activation in normal weight and overweight or obese humans van Marken Lichtenbelt and colleagues reported significantly higher REE of about 1.2 MJ/day (286 kcal/day) in both normal weight and obese individuals. Additionally, they observed a shift in RQ (see section 2.5. for details) from 0.83, which indicates primarily protein oxidation, to 0.78, reflecting 74 % fat oxidation as a result of increased fat utilisation [56,57]. Furthermore, Ouellet and her colleagues stated a 1.8 fold

increase in REE, while Yoneshiro and co-workers expressed their finding in more tangible numbers, namely a plus of 410±293 kcal/day. Finally, also Chondronikola et al. reported a 15 % increase in REE in BAT-positive participants [33,34,58]. Likewise, treatment with selective β_3-adrenergic receptor agonists, which are known for their potential to activate BAT, were found to increase REE: In overweight and obese individuals administration of L-796568, a selective β_3-adrenergic receptor agonist, increased REE by up to 8 % [59]. In addition, the β_3-adrenergic receptor agonist mirabegron, an approved drug to treat overactive bladder, has recently been demonstrated to stimulate BAT depots in normal weight individuals, accompanied by higher REE of 203±40 kcal/day [60]. Nevertheless, there are also studies without any changes in REE following cold-induced BAT activation: The cold-acclimation PET/CT study by Hanssen et al. did not observe a rise in REE in cold acclimated obese participants despite a notable gain in BAT volume [51]. This suggests that acute vs. prolonged exposure to moderate cold might have different effects on REE, at least in obesity. Consistently, no measureable effects of cold-activated BAT on REE in normal weight human individuals after a 6 hours/day cold acclimation intervention were detected by van der Lans and her co-workers [50]. These studies suggest that acute BAT thermogenesis might boost REE by 200 kcal/day as conservative lower limit, while in contrast cold acclimation procedures and resulting increases in BAT activation appear ineffective to facilitate weight loss in humans.

1.3.2. Effects of cold-induced BAT activation on glucose metabolism

Glucose is mainly utilised as energy substrate for self-maintenance of all cells. Furthermore, glucose is used for glycerol-3-phosphate (G3P) synthesis, which constitutes the carbon backbone for esterification of FFA to TG, and is the basis of energy demanding steps supporting this process. In BAT significant amounts of glucose are stored as glycogen and converted to lactate by anaerobic glycolysis [17]. Several studies investigated effects of cold-induced BAT activation on insulin-mediated glucose utilisation and found significant improvement in glucose homeostasis. In more detail, Orava et al. reported increased glucose uptake into BAT - but no other tissues - of healthy volunteers following 2 hours of exposure to 17°C, thus supporting BATs capacity to clear plasma glucose from the circulation [43]. Opposing results were presented by Ouellet and her colleagues who did not observe changes

in glucose disposal after 2 hours of cold exposure to 18°C as compared to thermoneutrality, and therefore suggested a minor contribution of BAT metabolism to whole-body glucose uptake [33]. Only one but retrospective PET/CT study found BAT volume to be inversely associated with fasting plasma glucose, implying metabolically active BAT to improve glucose tolerance and prevent T2DM. However, after adjustment for age, sex and BMI statistical significance disappeared [61]. Although growing evidence implicates a contribution of cold-activated BAT to glucose homeostasis in humans, further research on BATs impact on human glucose metabolism is required to examine additional important markers of glucose metabolism, such as pancreatic β-cell capacity/activity and the glycaemic response to acute glucose challenges, e.g. during oral (oGTT) or intravenous glucose tolerance tests (ivGTT) in humans.

1.3.3. Effects of cold-induced BAT activation on lipid metabolism

During NST TG and FFA are incorporated into BAT following hydrolysation of the circulating TG to FA and monoacylglycerol provided by lipoprotein lipase (LPL; [18,62,63]). Cold-induced BAT thermogenesis has been demonstrated to significantly accelerate plasma TG clearance and reduce hypercholesterolaemia in designated mouse models of hyperlipidaemia. Further evidence suggests that cold-activated BAT might exert substantial impact on human lipid metabolism as well. In normal weight healthy volunteers acute cold-induced BAT activation was associated with elevated appearance rates of FFA from WAT lipolysis and increased uptake of dietary FFA into BAT depots [33,64]. Although prolonged cold exposure of five to eight hours has been shown to increase whole-body lipolysis, FFA cycling and FFA oxidation also in obese men [65], it is unclear whether acute cold exposure is also capable to induce BAT activation and associated increased lipid mobilisation to the same extend in obese volunteers as in normal weight. Apart from this, it is important to note that none of the available human studies were able to demonstrate increased TG clearance as shown in rodents. Therefore BATs relevance as TG-clearing organ in humans remains uncertain.

1.4. Regulation of BAT differentiation, thermogenesis and recruitment

Environmental, metabolic and nutritional as well as endocrine factors have been identified to regulate human preadipocyte differentiation into mature brown adipocytes, BAT thermogenesis and *de novo* recruitment/browning.

Apart from ambient cold, exercise has been reported to promote browning in WAT, probably mediated by the peptide irisin released from working muscles, however, in humans supporting evidence for exercise-induced browning is inconclusive [30]. The same is true for diet-induced thermogenesis (DIT). Nutrition has been found to influence BAT activity at least in rodents [66]. In turn, specific nutritional interventions as high-fat diets can induce the 'whitening' of beige adipocytes, i.e. these cells develop increased lipid storage capacities and have the potential to even counteract the thermogenic function of genuine BAT in mice [22,67]. Contrary to high-fat diets, hot and spicy food ingredients appear to not only cause a perception of 'inner heat waves' experienced by many people after consumption. Experimental evidence confirms that particular dietary supplements exert thermic effects through BAT in humans: Capsaicin, the active essence of pepper responsible for the hot sensation upon consumption, has been shown to stimulate BAT thermogenesis in humans [68]. Furthermore, capsinoids as non-pungent counterparts of capsaicin and another sort of pepper ('grain of paradise') have been reported to increase REE in humans with detectable BAT depots [69,70]. However, evidence for a general role of nutrients stimulating BAT activity in humans is yet to be established [71–73]. Moreover, endogenous catecholamines, hormones and enzymes are important regulators of BAT functioning: NA as sympathetic transmitter functions as main activator of BAT and is crucially involved in BAT recruitment, moreover dopamine (DA) has recently been shown to increase mitochondrial mass and thermogenesis in brown adipocytes [17,42,74]. Furthermore, insulin is essential for glucose uptake into BAT, but probably rather to ensure sufficient glucose supply than directly supporting BATs thermogenic activity [43]. Additional important hormonal factors for both thermoregulation in general and BAT thermogenesis are the thyroid hormones, glucocorticoids and also sex hormones estradiol as well as androgen [79–82]. On a transcriptional level peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), peroxisome proliferator-activated receptor γ (PPARγ) and PR domain zinc finger protein 16 (PRDM16) are key players of BAT thermogenesis and adipogenesis. As crucial triad for thermogenesis, PGC-1α regulates mitochondrial biogenesis, oxidative metabolism and is even capable of inducing the expression of UCP1 in adipocytes, while PPARγ is involved in the differentiation of both white and brown adipocytes. Finally,

PRDM16 is essential for the development of brown adipocytes by suppressing marker genes inducing differentiation into myoblasts [22,24,28]. Particularly involved in the regulation of BAT differentiation and browning in humans additional hormonal factors have been identified including FGF21, bone morphogenic proteins (BMPs) BMP4, BMP7 and BMP8b, atrial and brain-type natriuretic peptides (ANP and NBP, respectively) and retinaldehyde [22,23].

1.5. Objective and aims of the present work

Cold-induced BAT thermogenesis is a complex process that depends on numerous regulatory factors. Although general mechanisms of BAT activation and its effects are rather well understood, the precise mechanistic link between cold-induced BAT activation and metabolic effects in humans are not clarified. The potential capacity of BAT for glucose and lipid clearance exerts direct impact on hyperglycaemia, insulin resistance and hypertriglyceridaemia, and is therefore considered the driving mechanism for the metabolic improvements observed upon activated BAT, at least in normal weight human individuals. To date, results on health benefits upon cold-activated BAT in obese humans are still inconclusive. Metabolic improvements associated with BAT thermogenesis are subject to transcriptional regulation of genes within catabolic pathways adding another layer of complexity [65,79,80]. In this respect, current research suggests that BAT thermogenesis signals a shortage in energy supply to stimulate mobilisation of stored energy substrates from WAT [45,81]. For that reason, particularly genes within pathways regulating lipolysis and FFA β-oxidation appear as further mechanistic candidates.

In conclusion, it has not been finally clarified yet whether cold-induced BAT thermogenesis exerts beneficial metabolic influence to comparable extent in normal weight and obese humans. Furthermore, the molecular mechanisms underlying cold-induced BAT activation and associated metabolic improvements in humans are not fully established. Therefore, the objective of the present work is to investigate the effects of cold-activated BAT in normal weight and obese metabolically healthy men, moreover to reveal underlying regulatory mechanisms of BAT thermogenesis and metabolic health. In order to study also transcriptional regulation of genes involved in energy metabolism without regulatory requirements of taking biopsies from human BAT, messenger RNA (mRNA) of selected

target genes was isolated from human peripheral blood. Target genes were chosen due to their regulatory functions mainly within lipolytic and FFA β-oxidation pathways and according to previous metabolic studies both in humans and rodents, which used peripheral blood mononuclear cells (PBMCs) for transcriptional analyses [82–85]. Approximately 80 % of tissue-specific genes are also detactable in the human peripheral blood transcriptome [86]. Moreover, even specific markers for BAT activation, WAT and browning have been found in rat PBMCs during cold stimulation [87–89]. Therefore, this alternative approach might offer a simplified alternative for analysis of systemic gene transcription levels in human metabolic research.

The specific aims of the present study regarding metabolic consequences of exposure to moderate cold and subsequent BAT thermogenesis in young normal weight and obese metabolically healthy men are:

i) Proving BAT activation during individualised cooling protocols.

ii) Assessing and comparing consequences of acute cold-induced BAT activation on main parameters of human glucose and lipid metabolism, REE and RQ, as well as transcriptional changes of metabolically relevant genes in circulating blood cells.

iii) Evaluation of the hypothalamus-pituitary-thyroid (HPT) axis and hypothalamus-pituitary-adrenal (HPA) axis, respectively, as potential mediators between acute BAT thermogenesis and associated beneficial effects on human energy homeostasis.

iv) Assessing potential effects of acute cold-induced BAT activation on the homeostatic and hedonic regulation of hunger and appetite.

Therefore, the following hypotheses were tested:

Cold-induced activation of BAT

i) Is detected by increased [^{18}F]-FDG uptake into typical BAT depots after exposure to moderate cold as compared to thermoneutrality as well as marked SNS activation in both normal weight and obese individuals.

ii) Is followed by distinct alterations in key parameters of human glucose and lipid metabolism as well as REE and RQ with more pronounced effects in normal weight as compared to obese individuals.

iii) Modulates mRNA expression of genes within lipolytic and FA β-oxidation pathways in circulating blood cells.

iv) Is related to significant modulation of the HPT-axis in both cohorts.

v) Is followed by increased orexigenic signalling and preference for high caloric foods.

Chapter II. Methods and materials

This chapter presents the experimental methods and protocols applied in the present study. Specific standard operation procedures (SOPs) used for the Botnia clamp, indirect calorimetry and food preference tests are described within each corresponding section after the theoretical principles of that method.

2.1. Participants

Fifteen healthy normal weight (BMI 18.5 - 24.9 kg BW/m^2) and fourteen obese (BMI 30 - 34.9 kg BW/m^2) metabolically healthy male volunteers participated in two experimental sessions at least 2 weeks apart. Exclusion criteria were chronic or acute illness, current medication of any kind, smoking, alcohol or drug abuse, obesity and diabetes in first degree relatives. To control for early-stage impairment of glucose metabolism in the obese participants, HbA1c >5.9 % was an additional exclusion criteria. A subset of three normal weight men participated in an additional FDG-PET/CT imaging study to prove cold-induced BAT activation. All participants gave written informed consent. The study was approved by the local ethics committee of the University Lübeck according to the declaration of Helsinki.

2.2. Experimental design and cooling protocol

The study protocols are shown in figure 2a and 2b. Participants arrived at the research unit after a 12-h overnight fast at 8:00. After a short introduction the volunteers rated their actual subjective condition on a symptom rating scale (SRS) and a visual analogue scale (VAS) to control potential confounding variables such as subjective stress or discomfort (see supplemental material A.1. and A.2.). Next, two computer-based food preference tests 'liking' and 'wanting' were completed (see chapter 2.7.) to assess hedonic and homeostatic components of food choice, respectively. Thereafter participants put on standardised underwear, comprising muscle shirt and long johns, and body weight was measured on a calibrated column scale (seca, Hamburg, Germany). Next, a mobile heart rate monitor (ActiHeart, Camntech, Cambridge, UK) was adhered to the participants chest (V2 and V5, 4th intercostals) and electrodes for electromyogram (EMG) recordings to examine potential shivering were fixed to the participants upper arm, thigh and belly (Neurofax EEG-9200, Nihon Koden, Tokyio, Japan). Two 18-gauge venous catheters (Vasofix Braunüle, B. Braun,

Melsungen, Germany) were inserted into antecubital veins of both arms for subsequent blood sampling and infusions. Participants put on a water-perfused suit (WPS, ThermoFlash, Buchenberg, Germany) that was worn throughout the entire experimental procedure (supplemental figure D.1.). For the normal weight participants, the cooling protocol started with an adaptation period of 60 minutes to either thermoneutral (22.0°C) or moderate cold (18.06±0.07°C, mean±SEM) as minimal temperature without shivering, which was maintained during the entire experiment. In case of shivering, as detected by EMG in one normal weight participant, temperature of the WPS was increased by steps of 1.0°C until shivering terminated completely. After the adaptation period REE was assessed via indirect calorimetry (Vmax™ Encore metabolic cart, Carefusion, Kelberg, Germany), as described in detail in section 2.5. At 11:00 (or 12:30 in the obese, see below) the Botnia clamp (for details see section 2.6.) was initiated, which, in brief, is the combination of a frequently sampled ivGTT followed by a hyperinsulinaemic-euglycaemic clamp (HEC; [90]). For measurement of relevant metabolic parameters in the normal weight participants blood was sampled after arrival (basal, 0 minutes), after the indirect calorimetry (after 100 minutes) and during euglycaemic steady state of the HEC (after 300 minutes). In addition, core body temperature was measured at baseline, after thermal adaptation, as well as 60, 120, and 180 minutes after start of the ivGTT using an ear thermometer (Braun ThermoScan type 6014, Braun GmbH, Kronberg, Germany). A slightly amended protocol was applied in the obese cohort (figure 2b), i.e. a second adaptation period and indirect calorimetry were introduced after the first indirect calorimetry, moreover the clamp was extended by 30 minutes. After putting on the WPS, each obese participant was first adapted to thermoneutral 25°C for 45 minutes, a first indirect calorimetry was conducted to assess REE during thermoneutrality and after completion a blood sample was taken as standardised thermoneutral baseline value. Next, the participant was adapted a second time for 45 minutes to either thermoneutral 25°C again or to 16°C, depending on the experimental session. The lower minimum temperature of 16°C, relative to 18°C in the normal weight cohort, was applied, because obese individuals are known to show higher insulative responses than their normal weight counterparts during cooling protocols [91,92]. After calorimetry at the end of the second adaptation period the protocol was similar to the normal weight cohort, except for the HEC, which was performed 30 minutes longer to ensure a stable euglycaemic steady state. In consequence of the modifications, in the obese cohort main metabolic blood parameters were sampled after the

first indirect calorimetry (basal, 0 minutes), after the second indirect calorimetry (100 minutes) and during euglycaemic steady state of the clamp (after 330 minutes). After the HEC food preference tests were completed a second time in both cohorts.

2.3. Symptom rating scale (SRS) and Visual analogue scale (VAS)

Autonomic or neuroglycopenic symptoms were rated five times during the experiment on a symptom rating scale (SRS), consisting of 27 items covering different physical as well as mental symptoms, such as racing heart, fatigue and headache. Strength of the symptoms was rated on a scale ranging from 0 ('weak') to 9 ('strong'). After completion of the SRS the participants further rated their current feelings of hunger, thirst, mood and well-being on a visual analogue scale (VAS), shown as a straight black line of 100 mm length ranging from 0 mm ('not at all') to 100 mm ('extremely'). Also, three additional VAS items assessed the Participants ´s current need for food and the respective gustatory quality (sweet or savoury).

2.4. Sympathovagal balance (SVB)

Sympathovagal balance (SVB) was assessed to complement measures of plasma catecholamines. The SVB refers to the proportion of sympathetic and parasympathetic impact on autonomic regulation of the sinus-astral node [93]. It is derived from recordings of heart rate variability (HRV), i.e. variance of intervals between heartbeats, also known as 'R-R intervals'. By applying fast-Fourier transformation low-frequency (LF) and high-frequency (HF) power spectra of the recorded heart rate over time are determined, whereby LF ranges from 0.04 to 0.15 Hz and HF from 0.15 to 0.40 Hz. The LF band is considered to primarily reflect sympathetic activity, while the HF component indicates the parasympathetic tone. The ratio of the calculated LF and HF power components (LF/HF) is a commonly used HRV index to quantify SVB. In general, elevated basal values are associated with increased risk to develop coronary heart disease or ischemic heart failure and, in contrast, lower LF/HF ratios reflect a healthy cardiovascular phenotype [87–89].

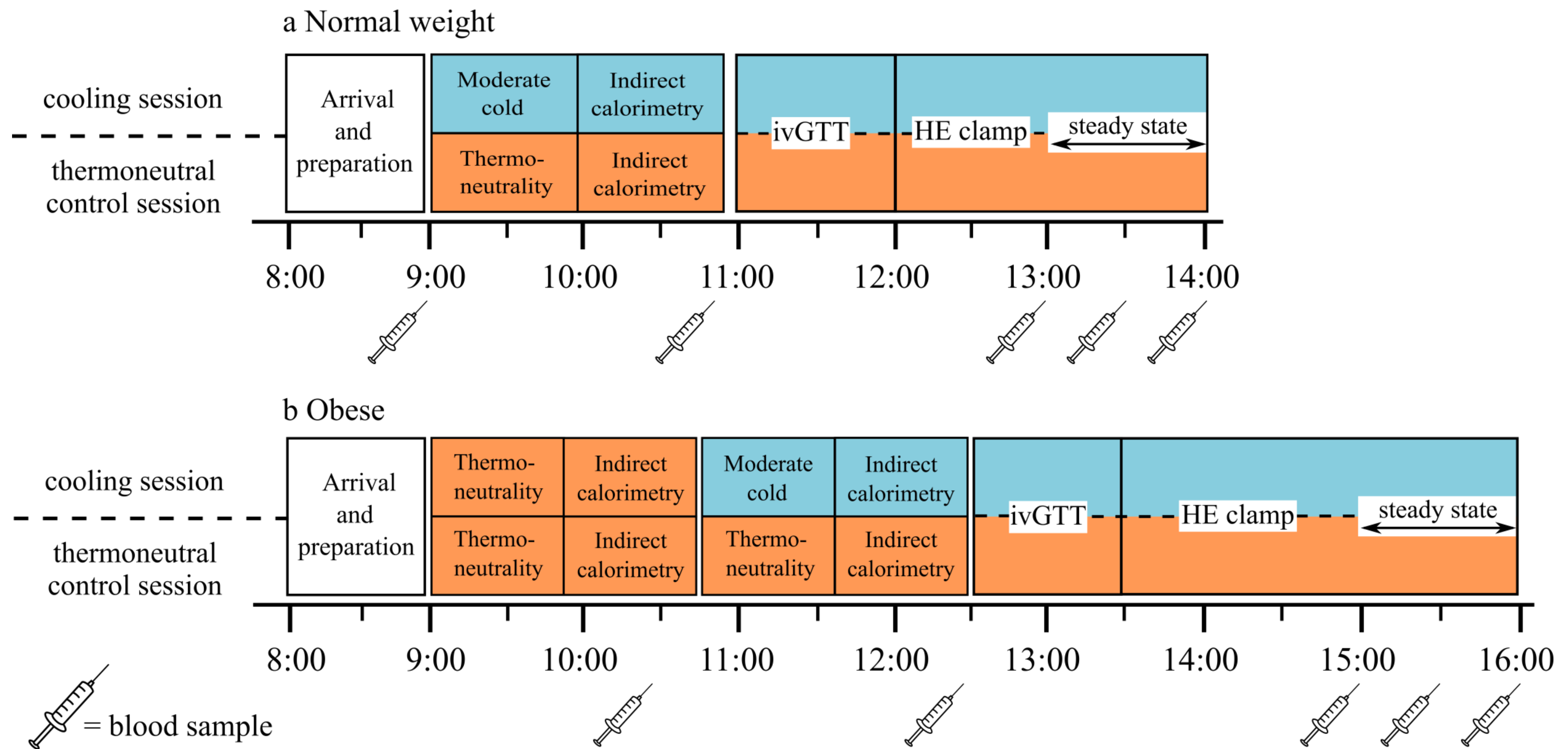


Figure 2. *Experimental design in the normal weight and obese cohort.* The study was conducted in a cross-balanced repeated within-subject design on two separate days with two experimental conditions. The normal weight participants (**a**) were kept at thermoneutrality (22.0°C) on one study day (thermoneutral control session) and exposed to moderate cold (mean temperature 18.06°C ± 0.07 SEM, shivering excluded) on the other study day (cooling session). Obese participants (**b**) were adapted twice to thermoneutral 25°C for 45 minutes (thermoneutral control session) for the thermoneutral control session, while for the cooling session they were first adapted to thermoneutral 25°C for 45 minutes and subsequently adapted to moderate cold of 16°C for another 45 minutes. Temperature conditions were realised using a water-perfused whole body suit which was worn throughout the experiment. Thermal adaptation, ivGTT and hyperinsulinaemic-euglycaemic clamp were performed on both study days.

2.5. Indirect calorimetry

Indirect calorimetry is a standard method to determine the REE by measuring produced carbon dioxide (VCO_2) and consumed oxygen (VO_2) in breathing air. Measuring both exhaled VCO_2 and inhaled VO_2 allows the indirect determination of heat produced by the body, from which, in turn, the energy expenditure can be calculated. To generate ATP as the body´s most important energy carrier VO_2 needs to be spent while heat is produced, hence each created ATP corresponds to a certain amount of heat, which can be determined from oxygen consumption.

The indirect calorimeter used in the present study ('Vmax™ Encore metabolic cart') is a canopy system characterised by a rigid plastic canopy hood placed over the volunteer's head, collecting the patient´s respiration mixed with air, from which the actual minute volume is determined in a breath-by-breath approach. The system uses an air dilution method to assess exhaled minute volume with variable flow, which allows adjustments to maintain the fraction of expired VCO_2 between 0.5 and 1.0 %. Concentrations of VCO_2 and VO_2 in expired and inspired air are measured by an infrared gas analyser and a galvanic fuel cell. Before measurements can be accomplished, both the gas sensors and mass spectrometer require initial calibrations using two gas mixtures (26% O_2 balance nitrogen, and 16% O_2, 4% CO_2 balance nitrogen) and a 3.0 L air syringe, respectively.

For the final calculation of REE from VCO_2 and VO_2 the Vmax™ system applies the modified Weir equation:

Equation 1.

$$REE\ (kcal/day) = [VO_2\ (L/min) \times 3.9] + [VCO_2\ (L/min) \times 1.1] \times 1440\ min/day$$

Another major parameter obtained from indirect calorimetry is the 'Respiratory Quotient' (RQ), i.e. the ratio of VCO_2 produced to VO_2 consumed:

Equation 2.

$$RQ = VCO_2/VO_2$$

The RQ is a dimensionless measure reflecting relative proportion of metabolised carbohydrate, fat and protein. Each of these energy substrates produces a different amount of

energy as heat, expressed in kilocalories (kcal), and CO_2, which needs to be removed from the system. In consequence the RQ varies according to the macronutrient composition of food. For macronutrients the RQs are:

i) Carbohydrates = 1.0
ii) Fat = 0.7
iii) Protein = 0.81

For optimal measurements the following SOP was used: Before calibration of the flow sensors a 30 min warm-up phase was allowed. After subsequent calibration the participant was placed in bed in a supine position and instructed not to move during the measurement, to keep the eyes open (blinking allowed) and to breathe normally. The canopy hood was carefully placed over the volunteer´s head and the measuring system started. During the calorimetry the participant was left alone and shielded from bright light as well as ambient noise, while the investigator monitored the session from the adjacent control room. Each individual trial lasted about 35 minutes (maximum 45 minutes), during which all unexpected events or potential disturbances as well as observed movements were noted by the investigators in order to exclude the corresponding value measured at that time point from subsequent analysis. In addition, the first 5 minutes of each trial were systematically discarded. Steady state REE was defined as mean REE of 5 consecutive values showing the least variation with a maximum cut-off of 10 %. Main outcome variables for statistical analysis were steady state values of REE and RQ.

2.6. Botnia clamp

The Botnia clamp is the gold standard method to assess insulin secretion from pancreatic β-cells and the insulin sensitivity as two key parameters of glucose homeostasis. The Botnia clamp was introduced by Tripathy and his co-workers in 2003 combining a frequently sampled ivGTT with a subsequent hyperinsulinaemic-euglycaemic clamp (HEC; [90]). For the ivGTT a glucose bolus of 0.3 g per kg body weight of a 20 % glucose solution is injected intravenously at time point 0, and subsequent blood samples are collected 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 minutes after the glucose load. The glycaemic response is evaluated as area under blood glucose curve (AUC) for the initial 20 minutes after administration of the glucose

bolus and over the entire duration of the ivGTT as total AUC. AUC is calculated according to the trapezoidal rule.
At minute 60 the HEC is started with continuous fixed infusion rates (1.0 IU/kg body weight) of short-acting human insulin (Insuman Rapid 40 I.E.; Sanofi-Aventis Deutschland GmbH) for 120 minutes (150 minutes in the obese study cohort). At the same time plasma glucose concentration is maintained stable at 5.5 mmol/L via adjustable infusion of 20 % glucose solution. Glucose infusion rate is calculated from dynamics of measured (2 to 3 minutes intervals throughout the clamp) plasma glucose by a clamp software (EKF diagnostic GmbH, Barleben, Germany). Approximately 60 minutes after starting the HEC hepatic glucose production is halted and blood glucose reaches a stable euglycaemic plateau, which is maintained over additional 60 minutes ('euglycaemic steady state') until the end of the clamp. From the cumulative glucose infusion rates during euglycaemic steady state whole-body glucose utilisation as measure of insulin sensitivity, termed *M*-value (Equation 3), is calculated:

Equation 3:
M = Total glucose infusion mmol/L $\times$ kg body weight$^{-1}\times$ 60min^{-1}

For the Botnia clamp the following SOP was used: Volunteers were placed in bed in a comfortable semi-upright position. The ivGTT started with administration of the glucose solution via one of the permanent intravenous catheters, while blood was sampled from the other catheter according to the timeline described above. After the first 30 minutes following the glucose bolus the volunteer was allowed to engage in sedentary pursuits such as reading between the subsequent blood collections until the end of the clamp. For blood collection and infusion of insulin and glucose during the HEC a special blood drawing system was used (supplement B.1.). Stable euglycaemia was monitored for at least 30 minutes following the end of the HEC before the participants were discharged.

2.7. Food preference tests

The potential influence of cold-induced BAT activation on hedonic appetite control was studied with computer-based test paradigms. Hedonic appetite comprises both a pleasure component and a motivational component of food related behaviour, termed 'liking' and 'wanting', respectively. While liking reflects the subjective pleasure elicited by food, wanting refers to the motivational aspect of food reward and related expression of behaviour [96]. Tests were conducted using the experimental control software 'E-prime' version 2.0 (Psychology Software Tools, Pittsburgh, PA) on a 17" laptop running Windows 10 as operating system. Both tests operate according to the same principle: Forty-two photographs of different food items are presented on the screen of a notebook, varying in terms of calorie content (low vs. high) and taste (sweet vs. savoury). Subjective liking of the different food items is assessed by rating each picture with respect to general liking ('How appetising do you find this food?') on a scale ranging from 1 ('not appetising') to 5 ('very appetising'). The same way, current wanting, i.e. motivation to eat the presented food at the moment (1 'not at all' to 5 'very much'), is assessed. During both tests, the respective ratings are entered directly into the actual image mask via the keyboard.
For 'liking' and 'wanting' the following SOP was used: The participant was seated at a desk with the notebook placed in front of him. First, the test 'liking' was completed. According to the instructions, the participant was asked to view and rate each image without a time limit, and informed that there were no 'correct' or 'false' answers. The investigators made sure that the task was clear, started the test and left the room. After 'liking' was completed, the same instructions were given for the 'wanting' test and the procedure started.

2.8. [^{18}F]-FDG PET/PET-CT protocol

To proof cold-induced BAT activation three normal weight volunteers of the study cohort were included into a BAT imaging study in Vienna, where [^{18}F]-FDG PET/CT scans were performed during thermoneutrality (23°C ambient temperature) as well as after moderate cold exposure on two study days separated by at least 14 days. To ensure an optimal course of the protocol, a cooling garment was used (CoolShirt Systems, Stockbridge, Georgia, USA; supplemental figure D). Subjects underwent PET/CT measurements in the morning after an overnight fasting period and sixty minutes after [^{18}F]-FDG application (2.5MBq/kg body weight) on each study day. PET/CT scans were performed using a Siemens Biograph 64 True

Point scanner (Siemens Healthcare, Erlangen, Germany). A static PET/CT scanning protocol was used to determine the [^{18}F]-FDG-uptake; the protocol started with a low dose CT scan (120 kV, 50mAs) immediately followed by PET acquisition in 3D mode of 3-min. [^{18}F]-FDG uptake was assessed and CT was used for attenuation correction and for anatomical localisation of brown fat. PET/CT images were acquired from the base of the skull to mid-thigh. BAT volume and activity were quantified using Hermes Hybrid 3D Viewer (Hermes Medical Solutions, London, UK). The regions of interest (ROIs) were generated in the axial fusion image composed by the static [^{18}F]-FDG PET image and the CT image at a Hounsfield unit (HU) range between -180 and -10 according to the BARCIST criteria [97]. ROIs were drawn semi-automatically using a threshold of SUV 2. Only ROIs located within areas with fat density (determined by CT) were considered for brown fat quantification. To determine the effects of cold exposure on skeletal muscle, cubes were selected as volumes of interest in three different muscles. Average [^{18}F]-FDG uptake in the thickest part of M. triceps brachii and M. gluteus maximus as well as in the lumbar (L4) part of M. erector spinae were analysed as previously reported [72].

2.9. Laboratory analyses

Plasma glucose concentration was assessed by the glucose oxidase method (EKF Biosen C-Line glucose analyzer, EKF Diagnostic, Barleben, Germany).

Serum insulin, TSH, fT_4 and cortisol were analysed using the IMMULITE 2000 Immunoassay System (Siemens Healthcare, Erlangen, Germany). Detection limits and average within-assay coefficients of variation of the assays were: Insulin, 2.0 µUI/mL and ≤5.3 %; TSH 0.04 µUI/mL and ≤12.05 %; fT_4 0.3 pmol/L and ≤7.8 %; cortisol 5.5 nmol/L and ≤7.4 %.

Blood lipids TG, HDL, and total cholesterol were measured using the ARCHITECT c Systems (Abbott Diagnostics, Wiesbaden, Germany), LDL was calculated from total cholesterol and HDL. Detection limits and average within-assay coefficients of variation of the assays were: TG 0.06 mmol/L and ≤3.6 %; total cholesterol 0.13 mmol/L and ≤4.0 %; HDL 0.06 mmol/L and ≤1.2 %.

Plasma total ghrelin and leptin concentrations were measured using radioimmunoassays (RIA; Millipore Corporation, Billerica, USA). Detection limits and average within-assay coefficients of variation of the assays were: Total ghrelin 93 pmol/L and ≤10.0 %; leptin 0.437 ng/mL ≤8.3 %.
For catecholamine measurement high-performance liquid chromatography followed by subsequent chemical detection was performed using the Recipe ClinRep™ kit (Recipe Chemicals + Instruments GmbH, Munich, Germany).

2.10. Statistical analysis

All statistics were performed using RStudio version 3.1.0. All reported values are mean±SEM. To test for systematic effects of cold-induced BAT activation vs. thermoneutrality data sets of normal weight and obese participants were analysed separately using 2-way repeated measurements ANOVAs including within-subject factors 'condition' (cond; 2 levels, 'thermoneutrality' vs. 'moderate cold') and 'time' (3 levels, 'basal' vs. ' after thermal adaptation' vs. 'euglycaemic steady state'; 2 levels for hormones that were measured at baseline and after adaptation or at baseline and during euglycaemic steady state, respectively; 5 levels for core body temperature, SRS and VAS, respectively). In addition, impact of weight status, i.e. 'normal weight' and 'obese', was assessed by pooling the data of both cohorts and calculating 2-way repeated measurements ANOVAs with the between-subject factor 'weight statuts' (2 levels, 'normal weight' vs. 'obese'). For analysis of the food preference tests 'liking' and 'wanting' 2-way repeated measurements ANOVAs with an additional within-factor factor 'food quality' (3 levels, 'high calorie savoury', 'high calorie sweet', 'neutral low calorie') and the between-factor 'weight status' were calculated. Effects of interest are significant condition×time (cond×time) interactions with differences between 'thermoneutrality' vs. 'moderate cold' at time-factor levels 'after thermal adaptation' and/or 'euglycaemic steady state', furthermore condition×time×weight status (cond×time×weight) as well as condition×weight status (cond×weight) interactions and main effects of factors 'condition' and 'weight status'. Significant main effects of factor 'time' or time×weight status (time×weight) interactions are not relevant for the addressed research question and are not reported. P-values <0.05 two-sided were considered significant. Values for insulin, TSH, fT_4,

cortisol, total cholesterol, HDL-cholesterol, LDL-cholesterol, TG, total ghrelin and leptin during euglycaemic steady state are averages of 3 blood samples taken during the last 60 minutes of the HEC. For insulin sensitivity and post-hoc comparisons paired and unpaired t-tests were performed, in case of violated normality assumption paired and unpaired Wilcoxon signed-rank tests were used. Multiple comparisons were taken into account and adjusted according to Bonferroni-Holm correction. Greenhouse-Geisser and Huynh-Field corrected values are reported in case of violated sphericity. Integrals for AUCs were calculated applying the trapezoidal rule from blood samples taken during the ivGTT for plasma glucose curves and over the euglycaemic steady state for blood lipids.

2.11. Transcriptional analysis using quantitative real-time PCR

2.11.1. Whole blood RNA extraction, reverse transcription and primers

For determination of mRNA in human peripheral whole blood the PAXgene™ Blood RNA System (PreAnalytiX, QIAGEN, Germany) was used. According to the manufacturer´s instructions blood samples were collected in PAXgene™ Blood RNA Tubes and RNA was extracted using the PAXgene™ Blood RNA Kit. The isolated RNA was reverse transcribed to cDNA with random decamers in the RETROscript™ kit (Ambion, USA). For determination of all target mRNA (PDK4, CPT1α, CPT1β, G0S2, FFAR4, SLC25A20 and PPARα) commercially available established primers were employed (TaqMan™ Gene Expression Assays).

2.11.2. Quantitative real-time PCR measurement and data processing

Real-time qPCR was conducted on a QuantStudio™ 7 Flex System (Applied Biosystems, USA). The genes 18S and ACTB were used as housekeepers. Optimal cDNA concentrations and reliable exclusion of amplifying genomic DNA was tested by running probe qPCRs on samples of different dilution rates, i.e. 1:2, 1:4, 1:10, and 1:100, eventually a dilution ratio of 1:2 proved optimal. The qPCR was run in duplicate for each sample including no template controls (NTC) and reverse transcriptase (RT) controls with a total volume of 20 µl per well containing 10 ng of cDNA. Levels of gene expression are given as cycle threshold (Ct value), which reflects the level of gene detection in terms of reactions reaching a fluorescent intensity

above background. The PCR cycle at which the sample reaches this level is called Ct. The Ct values obtained from qPCR measurements were normalized according to the $2^{-\Delta\Delta}$Ct method. In short, expression of the target gene is normalized to both expression levels of the housekeeping genes and to each corresponding control sample (-ΔΔCt). The resulting negative value is taken as exponent of 2 and represents the fold change of the treated target gene relative to the untreated control, i.e. the sample collected after adaptation to thermoneutrality. In the present study, validity of 18S as most stable reference gene was confirmed using the Normfinder algorithm [98].

2.12. Study approvals

In accordance with the Declaration of Helsinki, the study was approved by the local ethics committee of the University Lübeck (AZ 12/030). PET/CT studies in a subset of the initial cohort was approved by the Ethics Committee of the Medical University of Vienna (EK 1032/2013). Written informed consent was received from all participants before inclusion into both studies.

Chapter III: Results

3.1. Participant characteristics

Anthropometric characteristics and routine blood chemistry at the initial medical examination in fifteen normal weight and fourteen obese metabolically healthy male volunteers are summarized in Table 2. Age, blood pressure (systolic and diastolic) and plasma creatinine were comparable between both study cohorts (p=0.550, p=0.111, p=0.642 and p=0.275, respectively). As intended by study design, body weight and BMI were notably higher in the obese as compared to the normal weight participants (both p<0.001). In addition, values of the assessed liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) in the obese men exceeded those in the normal weight (ALT p=0.001, AST p=0.006 and GGT p=0.046), but were all within the normal range.

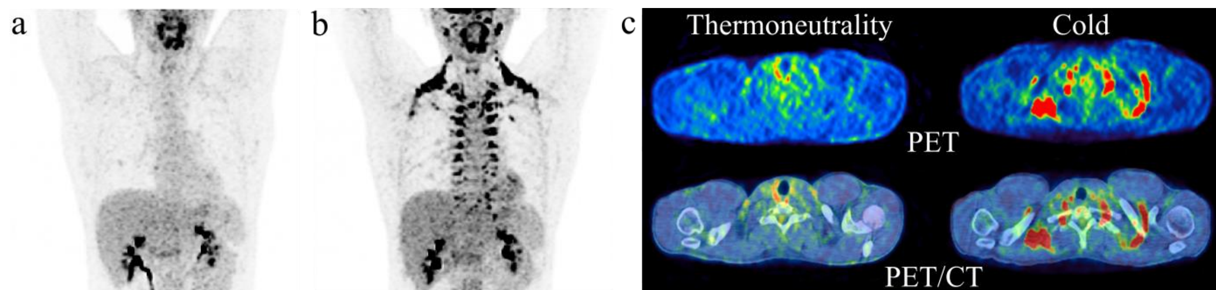
Table 2. Anthropomorphic characteristics and routine blood chemistry at the initial medical examination

	Normal weight (n=15)		Obese (n=14)	
Parameter	Mean±SEM	Range	Mean±SEM	Range
Age	27.1±0.8	24 - 26	25.3±0.8	24 - 27
Weight	79.5±1.9†††	68 - 92	106.8±6.5	94 - 125
Blood pressure (SYS) mmHg	127.4±3.1	110 - 140	133.4±2.7	118 - 160
Blood pressure (DIA) mmHg	76.1±2.1	65 - 88	77.6±3.1	65 - 92
BMI (kg/m^2)	23.3±0.3†††	22 - 25	32.6±0.9	30 - 35
GGT (U/l)	19.9±2.0†	11 - 38	31.0±4.9	22 - 45
AST (U/l)	23.1±1.2††	16 - 33	28.8±2.9	24 - 37
ALT (U/l)	19.0±1.7†††	10 - 31	47.8±6.5	40 - 67
Plasma creatinine (μmol/l)	78.5±2.3	67 - 107	85.4±8.5	67 - 104

GGT=gamma-glutamyltransferase — † p<0.05 normal weight vs obese
AST= aspartyl-aminotransferase — †† p<0.01 normal weight vs obese
ALT= alanine-aminotransferase — ††† p<0.001 normal weight vs obese

3.2. Effects of cold exposure on BAT metabolism, SNS activity and body core temperature

As proof of concept, three normal weight participants were subjected to [^{18}F]-FDG PET/CT scans at thermoneutrality and moderate cold, respectively. Cold exposure increased [^{18}F]-FDG uptake in cervical, supraclavicular and paravertebral BAT areas, confirming cold-induced BAT activation (figure 3a, b and c). At the same time, cold exposure did not alter [^{18}F]-FDG uptake in skeletal muscle (figure 3e), confirming unchanged metabolic muscle activity during cold.



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BAT	Before cold exposure				After cold exposure			
Subject	SUVmean	SUVmax	Volume cm³	TLG	SUVmean	SUVmax	Volume cm³	TLG
1	2,25	2,82	3,78	8,52	4,45	22,55	148,4	659,92
2	2,41	4,57	11,69	28,13	2,53	6,81	42,4	107,19
3	-	-	-	-	2,46	4,98	18,11	44,65

- = n.d.
SUV=standardised uptake values
TLG = total lesion glycolysis

e

Muscle	Before cold exposure	After cold exposure
Subject	SUV mean	SUV mean
1	0,64	0,46
2	0,60	0,64
3	0,52	0,64

Figure 3. *BAT-glucose uptake increases in response to moderate cold exposure.* Representative coronal PET slices showing [^{18}F]-FDG uptake in typical BAT depots (**a**) during thermoneutrality and (**b**) after exposure to moderate cold in a healthy man (subject 03). (**c**) Axial PET and PET/CT fusion slices showing [^{18}F]-FDG uptake into supraclavicular BAT depots under thermoneutrality (left) and after moderate cold (right). Summary of PET/CT parameters of all 3 subjects in (**d**) BAT and (**e**) skeletal muscle.

Table 3. Fasting glucose levels, hormone and catecholamine concentrations, insulin sensitivity, REE, RQ, SVB and core body temperature during thermoneutrality (22.0°C and 25.0°C in the normal weight and obese study cohort, respectively) and cold-induced BAT activation (18.0°C and 16.0°C in the normal weight and obese study cohort, respectively) at baseline, after adaptation (100 minutes) and during euglycaemic steady state of a HEC (300 and 330 minutes in the normal weight and obese study cohort, respectively). Data are mean±SEM. n=29.

	Basal (0’)				After temperature adaptation (100’)				During euglycaemic steady state (300 - 330’)			
	Thermoneutrality		Before cold exposure		Thermoneutrality		Moderate cold		Thermoneutrality		Moderate cold	
Parameter	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese
Plasma glucose (mmol/L)	-	-	-	-	4.23±0.11	4.47±0.07	4.33±0.18	4.35±0.06	4.96±0.03	4.90±0.06	4.92±0.06	4.92±0.05
Insulin (µIU/ml)	5.21±0.73	8.20±1.03	6.79±1.72	10.33±1.36	4.66±0.77	7.70±0.99	5.64±1.13	9.17±1.09	66.07±4.5	91.92±11.1	72.30±5.3††,**[c]	121.13±13.1*,***[a]
Insulin sensitivity (*M*-value)	-	-	-	-	-	-	-	-	9.9±0.7	9.5±0.9	11.9±1.1*	9.8±0.8
Dopamine (ng/L)	55.5±4.5	18.0±0.6	64.9±6.9	19.2±0.9	-	-	-	-	68.3±5.4†††	17.4±0.4	104.6±11.6*,*[a],†††	29.6±2.4**,***[a]
Noradrenaline (ng/L)	230.4±33.3	289.1±27.1	269.7±38.8	244.1±22.4*	-	-	-	-	338.8±36.7	388.1±33.8	604.1±48.1***,**[a]	617.6±24.8***,***[a]
Adrenaline (ng/L)	36.8±3.1	41.5±15.8	34.5±3.7	34.6±7.6	-	-	-	-	29.3±3.6	24.4±4.9	31.8±6.1	20.8±2.9
TSH (µIU/ml)	2.0±0.2(†)	1.4±0.2	2.0±0.3	1.4±0.1	1.4±0.1†††	1.3±0.2	-	1.2±0.1	1.2±0.1	1.1±0.1	1.3±0.2	1.2±1.3
fT_4 (pmol/L)	13.9±0.39	14.0±0.4	13.6±0.39	14.4±0.6	13.8±0.4	15.0±0.4	13.3±0.4	14.1±0.4*	13.3±0.4	14.8±0.4	13.0±0.4	14.1±0.5(*)
Cortisol (nmol/L)	468.1±21.5†††	285.0±29.4	464.0±27.5†††	258.0±20.0	358.1±33.8††	206.2±15.6	362.5±20.0††	214.5±20.9	283.3±31.7	214.6±22.0	267.3±23.9	193.2±10.2
Ghrelin (total; pg/mL)	1,198.5±82.4†††	472.3±46.7	1,188.9±94.5†††	475.7±42.2	1,191.8±71.3†††	500.2±45.7	1,203.6±80.4†††	559.0±62.1	-	-	-	-
Leptin (ng/mL)	4.0±0.4†††	26.4±4.7	3.8±0.4†††	29.5±4.4	3.6±0.4††	25.9±4.3	3.1±0.3††	27.5±4.3	-	-	-	-
REE	-	1,852.0±54.0	-	1,862.0±58.0	1,849.0±81.0††	1,979.0±56.0	1,834.0±69.0††	2,086.0±52.0	-	-	-	-
RQ	-	0.77±0.02	-	0.79±0.01	0.83±0.01†††	0.79±0.02	0.85±0.02†††	0.78±0.01	-	-	-	-
Sympathovagal balance	2.9±0.4	2.6±0.5	2.6±0.5	2.4±0.4	2.6±0.3	2.7±0.4	2.6±0.4	2.7±0.6	2.7±0.4	4.8±0.8	2.9±0.6	3.5±0.8
Body core temperature (°C)	36.2±0.08	36.4±0.11	36.4±0.11	36.5±0.09	36.2±0.08	36.3±0.07	36.2±0.06	36.3±0.06	36.2±0.04	36.4±0.08	36.0±0.13	36.3±0.09

* $p<0.05$ thermoneutral vs moderate cold
** $p<0.01$ thermoneutral vs moderate cold
*** $p<0.001$ thermoneutral vs moderate cold
(*) $p<0.10$ thermoneutral vs moderate cold

† $p<0.05$ normal weight vs obese
†† $p<0.01$ normal weight vs obese
††† $p<0.001$ normal weight vs obese
(†) $p<0.10$ normal weight vs obese

*[a] $p<0.05$ interaction cond×time
**[a] $p<0.01$ interaction cond×time
***[a] $p<0.001$ interaction cond×time

*[b] $p<0.05$ interaction cond×weight

**[c] $p<0.01$ interaction cond×time×weight

Results of catecholamines NA, DA and A as well as SVB are listed in table 3. Sympathetic activation substantially increased in both cohorts upon cold exposure as indicated by significantly higher concentrations of circulating plasma NA (normal weight $p<0.001$ and obese $p<0.001$; figure 4a and b) and DA (normal weight $p=0.026$ and obese $p=0.008$; figure 4c and d) while no effects were observed on levels of A neither in normal weight nor obese men (both $p=1.0$; interactions all $p>0.05$). Corresponding significant cond×time interactions in both cohorts underlined the changes in both NA and DA during euglycaemic steady state of the clamp in the BAT-activated state relative to the thermoneutral condition (normal weight NA $p=0.009$ and DA $p=0.024$; obese NA $p<0.001$ and DA $p<0.001$). Baseline NA levels differed between conditions in the obese men ($p=0.012$), but the increase in plasma NA during cold exposure remained significant after baseline correction ($p<0.001$). No other baseline differences were observed (normal weight NA $p=0.216$, DA $p=0.109$; obese DA $p=0.583$). Levels of circulating moreover DA differed between the study cohorts with generally lower circulating DA in the obese relative to the normal weight men ($p<0.001$). SVB as additional marker of sympathetic activation was neither modulated by BAT activating cold exposure nor associated with weight status throughout the experiment ($p\geq0.484$ for all comparisons). Values of body core temperature are shown in table 3. Exposure to moderate cold was not accompanied by changes in body core temperature in neither cohort (normal weight $p=0.108$ and obese $p=0.359$) and no differences were observed between normal weight vs. obese men ($p=0.144$).

3.3. Effects of cold-induced BAT activation on REE and RQ

Results of the indirect calorimetry measurements are shown in table 3. Exposure to moderate cold did not modulate REE and RQ in neither cohort (normal weight REE $p=0.751$ and RQ $p=0.694$; obese REE $p=0.925$ and RQ $p=0.589$). REE significantly differed between normal weight and obese individuals in both conditions ($p=0.007$; figure 5) with obese individuals showing higher resting metabolic rates than normal weight men. Like REE, the RQ was notably different between study cohorts with obese men showing a lower RQ than the normal weight ($p<0.001$).

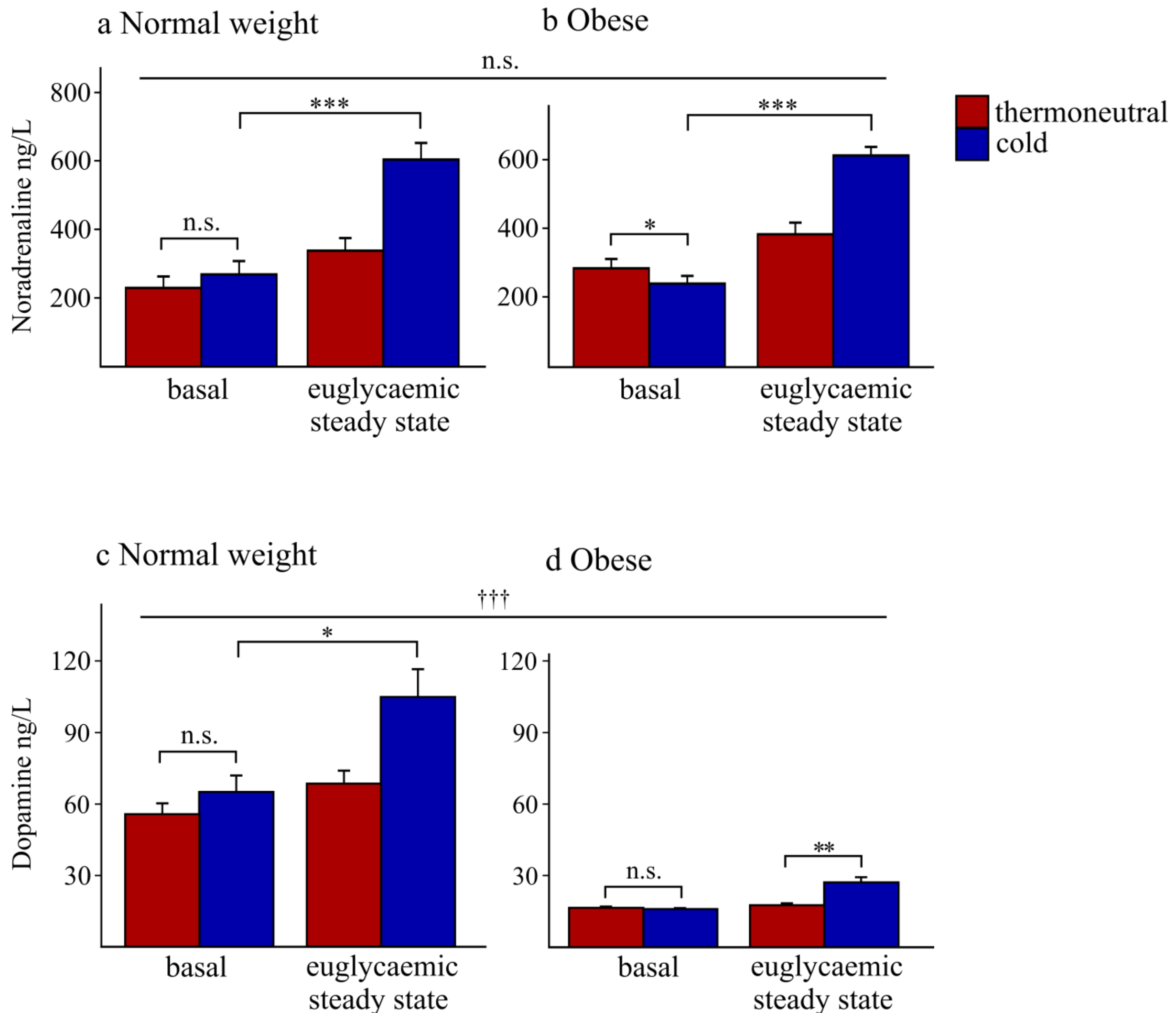

Figure 4. *Sympathetic activation during cold-induced BAT thermogenesis.*
Plasma NA and DA concentrations at baseline and during euglycaemic steady state of the HEC in normal weight and obese men during thermoneutrality (red) and upon exposure to moderate BAT-activating cold (blue). Levels of NA significantly increased during cold-induced BAT activation in both cohorts (**a** normal weight $p<0.001$ and **b** obese $p<0.001$, 2-tailed paired t-tests; n=15 and n=14, respectively). In the obese men the observed effect of cold exposure on NA remained significant after baseline correction ($p<0.001$, 2-tailed paired t-test; n=14). Levels of plasma DA were also markedly elevated in both normal weight (**c**) and obese men (**d**) during cold exposure relative to thermoneutrality (normal weight $p=0.026$, n=15; obese $p=0.008$, n=14; 2-tailed paired t-tests). In addition, plasma DA was notably lower in the obese (**d**) relative to the normal weight men (main effect weight status $p<0.001$†††, 2-way repeated-measurements ANOVA; n=29).

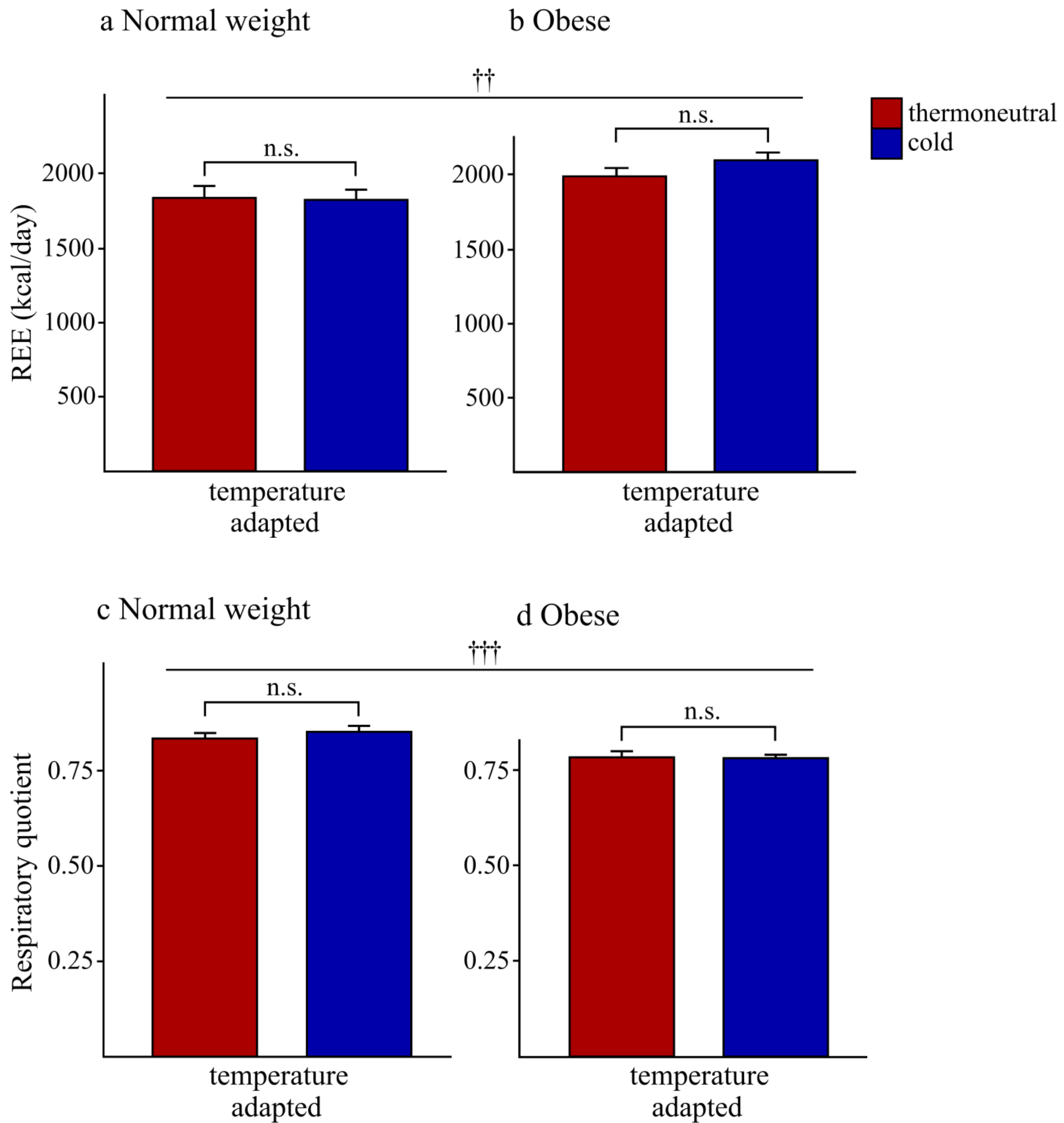

Figure 5. *REE and RQ are not affected by cold-induced BAT activation.*
REE and RQ measured in normal weight and obese men after thermal adaptation during thermoneutrality (red) and exposure to BAT-activating moderate cold (blue). Cold-induced BAT activation did not change daily REE and RQ in neither cohort (normal weight REE p=0.751 (**a**) and RQ p=0.694 (**b**); obese REE p=0.925 (**c**) and RQ p=0.589 (**d**); 2-way repeated measurements ANOVAs; n=13 [normal weight] and n=14 [obese]). REE significantly differed between study cohorts with the obese men showing higher REE than the normal weight (p=0.007††; 2-way repeated measurements ANOVA; n=27). Furthermore, the RQ significantly depended on the weight status, i.e. values of the obese men were lower than those of the normal weight (p<0.001†††; 2-way repeated measurements ANOVA; n=27) in both conditions, indicating differences in primary energy substrate utilisation between the cohorts.

3.4. Effects of cold-induced BAT activation on glucose metabolism

Levels of plasma glucose and serum insulin as well as insulin sensitivity *(M*-value*)* are given in table 3. Plasma glucose was comparable between conditions in both the normal weight and obese cohort after the adaption phase and during euglycaemic steady state of the HEC (all $p \geq 0.117$). For the glycaemic response ANOVA revealed a significant cond×time×weight interaction ($p=0.002$), i.e. an improvement in glucose tolerance in the cold condition was found exclusively in the obese cohort, indicated by a reduction in the total area under the plasma glucose curve (AUC; 338.16±9.5 during thermoneutrality vs. 310.06±11.0 mmol/L×60min^{-1} during moderate cold, $p=0.039$; figure 6b). This difference was most pronounced during the first 20 minutes after administration of the glucose bolus ($p=0.018$). At later stages, i.e. minute 30 to 60, during thermoneutrality and moderate cold both AUCs converged (68.8±3.07 during thermoneutrality vs. 61.08±3.37 mmol/L×60min^{-1} during moderate cold; $p=0.06$). In contrast, no statistical difference was observed in the normal weight participants for both the AUC over the initial 20 minutes nor the entire duration of the ivGTT (AUC initial 20 minutes following iv glucose during thermoneutrality 149.78±2.9 vs. cold exposure 148.08±3.8 mmol/L×60min^{-1}, $p=0.838$; total AUC during thermoneutrality 292.5±7.5 vs. cold exposure 282.1±11.4 mmol/L×60min^{-1}, $p=1.0$; figure 6a). Glucose excursions during the ivGTT under thermoneutral conditions were in general elevated in obese as compared to normal weight men (all $p<0.001$; figure 6c). However, the maximum in blood glucose during ivGTT in obese men was significantly lower upon cold-induced BAT activation ($p=0.022$) but still higher as compared to the normal weight cohort ($p=0.019$; figure 6c). Serum insulin levels in obese men increased significantly upon cold exposure during euglycaemic steady state of the HEC compared to the thermoneutral condition ($p=0.019$; cond×time×weight interaction $p=0.003$), but were not changed between conditions during HEC steady state in the lean cohort and $p=0.908$). Whole-body insulin sensitivity (expressed as *M*-value) was significantly improved upon cold-induced BAT activation relative to thermoneutrality in the normal weight, but not in the obese men ($p=0.035$ and $p=0.662$, respectively; figure 7), although in the pooled analysis the cond×weight interaction missed statistical significance ($p=0.117$).

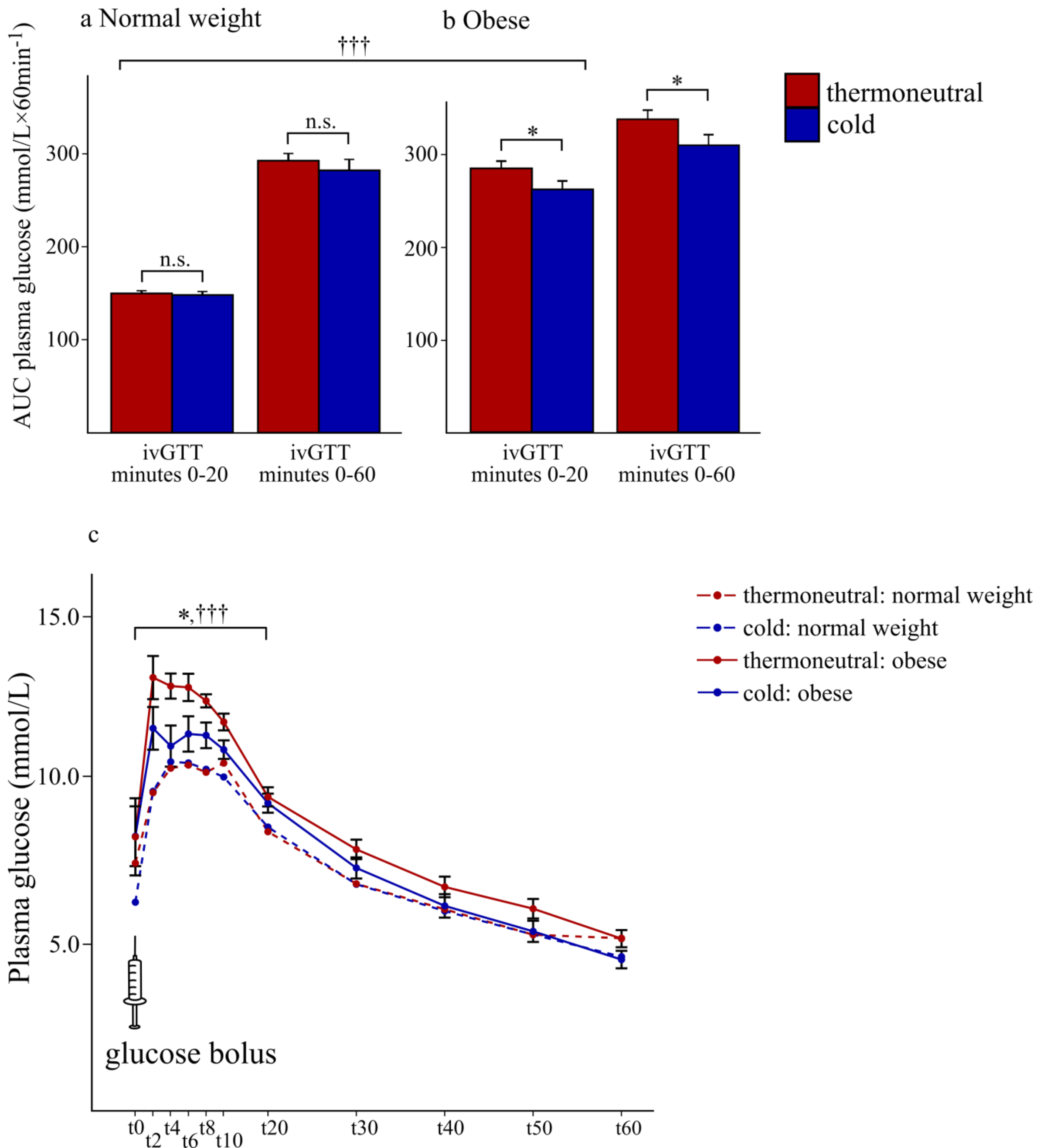

Figure 6. *Improved glucose tolerance during cold-induced BAT activation in the obese men.* AUCs of plasma glucose from the start to minute 20 of the ivGTT and over the entire 60 minutes of the ivGTT during thermoneutrality (red) and upon cold-induced BAT activation (blue) in normal weight (**a**) and obese men (**b**). Plasma glucose curves (**c**) throughout the ivGTT during thermoneutrality (red) and upon exposure to moderate cold (blue) in normal weight (dashed) and obese (solid) men. Plasma glucose was markedly lower after administration of the glucose bolus upon BAT activation than during thermoneutrality in the obese men (**b** AUC from minute t0 to t20, p=0.018, total AUC p=0.039; 2-tailed paired t-test; n=14), while glucose tolerance was not different between conditions in the normal weight (**a** p=0.838, 2-tailed paired t-tests; n=15). After the glucose bolus plasma glucose peaked notably higher in the obese as compared to the normal weight men in both conditions (**c** during thermoneutrality p<0.001, upon cold-activated BAT p=0.014; 2-tailed unpaired t-tests; n=29).

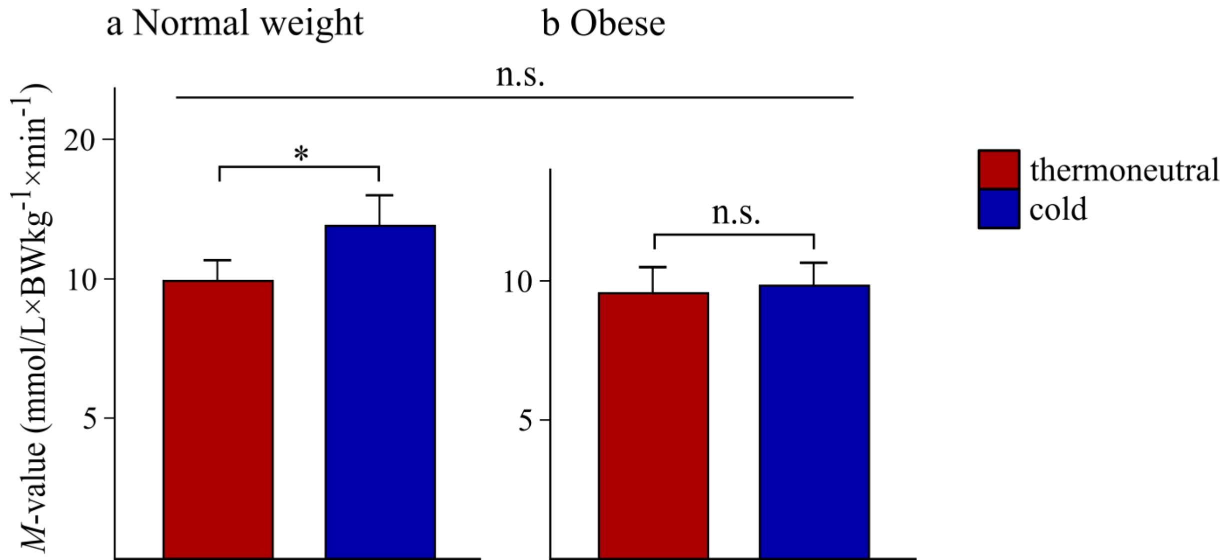

Figure 7. *Insulin sensitivity improves upon cold-activated BAT in normal weight men.*
Insulin sensitivity, expressed as *M*-value, in normal weight (**a**) and obese (**b**) men during thermoneutrality (red) and cold-induced BAT activation (blue). The *M*-value significantly increased upon cold exposure relative to thermoneutrality in the normal weight participants, but not in the obese (normal weight p=0.035 and obese p=0.662, respectively). Study cohorts were not statistically different (p=0.286, 2-way repeated measurements ANOVA; n=29).

3.5. Effects of cold-induced BAT activation on lipid metabolism

Concentrations of serum lipids and corresponding AUCs covering the euglycaemic steady state of the HEC are given in table 4. At baseline, concentrations of all measured lipids were comparable between conditions (all $p \geq 0.069$, respectively). Levels of circulating TG significantly increased in the obese participants upon exposure to BAT-stimulating cold during euglycaemic steady state of the HEC (p=0.037; figure 8b), but were not modulated by cold treatment in the normal weight cohort (p=0.748; figure 8a). The AUCs of TG significantly increased upon cold-activated BAT as compared to thermoneutrality in both the normal weight and obese participants (p=0.001; figure 9a and 9b), and this effect was more pronounced in obese (p=0.016) relative to normal weight (p=0.059) men. Circulating total cholesterol, HDL and LDL were not affected by cold-induced BAT activation in none of the study cohorts (total cholesterol normal weight p=0.522 and obese p=0.483, respectively; HDL normal weight p=0.688 and obese p=0.312, respectively; LDL normal weight p=0.629 and obese p=0.644, respectively). Likewise, the AUCs of those lipids were unchanged by cooling (total cholesterol p=0.178; HDL p=0.727; LDL p=0.303). Regarding differences between the study cohorts circulating HDL during

euglycaemic steady state of the HEC and the corresponding AUC were lower in the obese relative to the normal weight men (circulating HDL during thermoneutrality p=0.012 and moderate cold p=0.618; AUCs in both conditions p<0.001). Circulating TG, total cholesterol and LDL were comparable between the study cohorts throughout the experiment (TG p=0.476; cholesterol p=0.586; LDL p=0.261), and only AUCs of total cholesterol were notably lower in the obese men relative to normal weight in both conditions (both p<0.001; AUCs TG, HDL and LDL all p≥0.150, respectively).

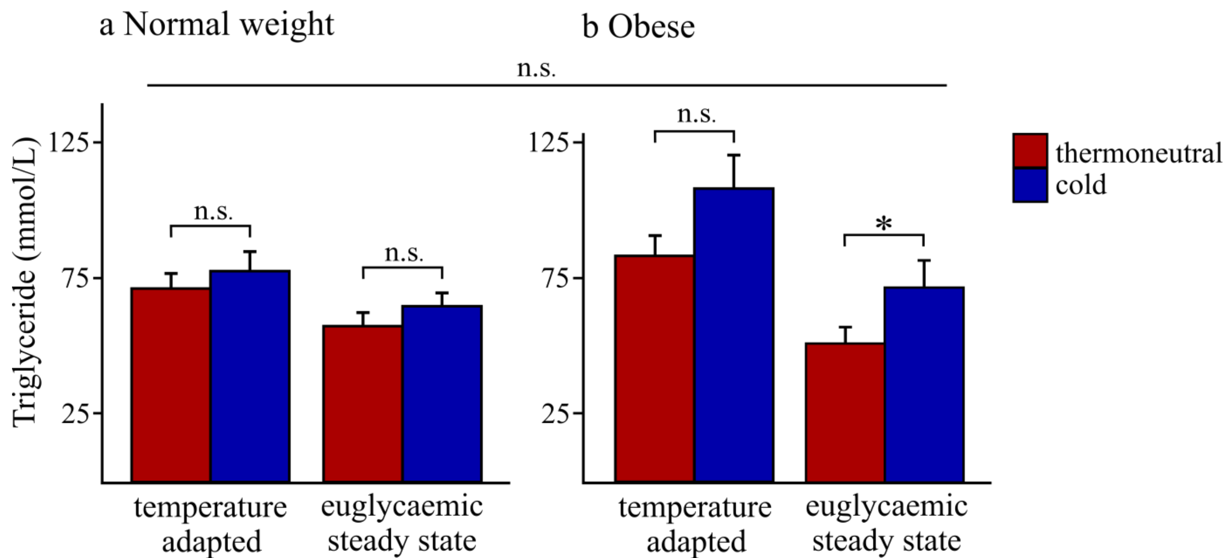

Figure 8. *Circulating TG increases during cold-induced BAT activation in obese men.* Concentrations of TG after thermal adaptation and during euglycaemic steady state of the HEC upon thermoneutrality (red) and exposure to moderate cold (blue) in normal weight (**a**) and obese men (**b**). Serum TG notably increased upon exposure to moderate cold during euglycaemic steady state of the HEC in the obese (p=0.037, 2-tailed paired Wilcoxon signed-rank test; n=14), but remained unchanged in the normal weight study cohort throughout the experiment (p=0.748, 2-tailed paired Wilcoxon signed-rank test; n=14). Serum TG was not associated with weight status (p=0.102, 2-way repeated measurements ANOVA; n=28).

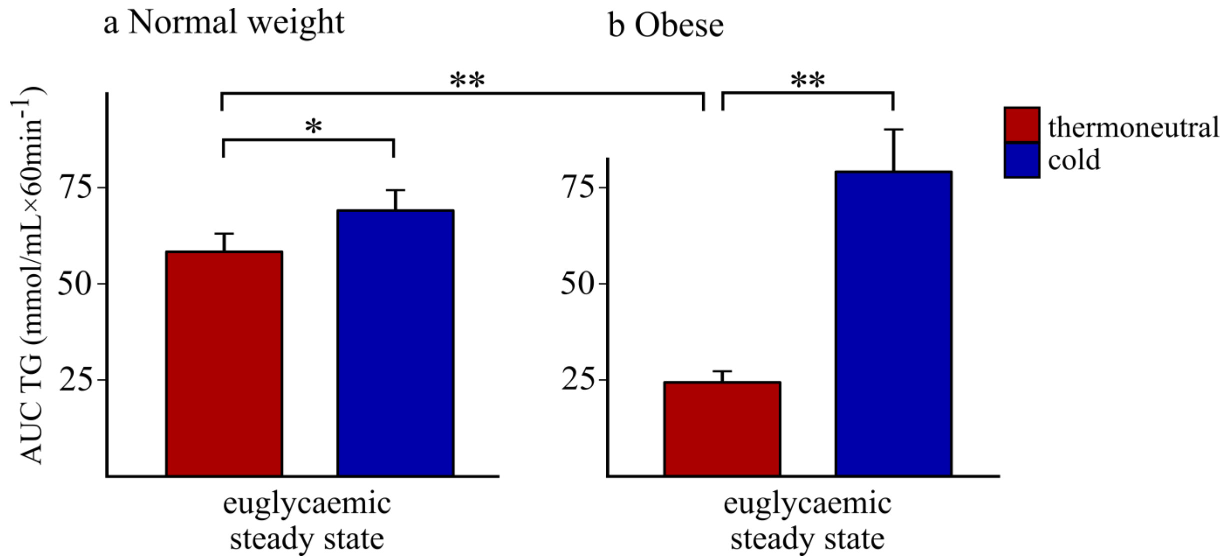

Figure 9. *Increased TG during euglycaemic steady state upon cold-induced BAT activation.* AUC of serum TG over the euglycaemic steady state of the HEC during thermoneutrality (red) and upon cold-activated BAT (blue) in normal weight (**a**) and obese men (**b**). In both study cohorts TG increased during 60 minutes of maintained euglycaemia in the cold relative to the thermoneutral session (normal weight p=0.05, n=14; obese p=0.016, n=14; 2-tailed paired Wilcoxon signed-rank tests).

Table 4. Serum concentrations and AUCs of lipids TG, total cholesterol, HDL and LDL during thermoneutrality (22.0°C and 25.0°C in the normal weight and obese study cohort, respectively) and cold-induced BAT activation (18.0°C and 16.0°C in the normal weight and obese study cohort, respectively) at baseline, after adaptation (100 minutes) and during euglycaemic steady state of HEC (300 and 330 minutes in the normal weight and obese study cohort, respectively). Data are mean±SEM. n=29.

	Basal (0')				After temperature adaption (100')				During euglycaemic steady state (300 - 330')			
	Thermoneutrality		Before cold exposure		Thermoneutrality		Moderate cold		Thermoneutrality		Moderate cold	
Parameter	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese
Cholesterol (mmol/L)	4.2±0.17	4.02±0.20	4.27±0.19	3.96±0.17	4.11±0.16	4.02±0.21	4.12±0.19	4.14±0.20	3.97±0.16	3.40±0.18	4.0±0.17	3.58±0.15
AUC (mmol/L×60min^{-1})	-	-	-	-	-		-	-	237.41±9.62†††	110.27±5.56	240.03±10.17†††	116.98±4.40
LDL (mmol/L)	2.37±0.18	2.72±0.17	2.43±0.19	2.65±0.13	2.32±0.16	2.76±0.18	2.34±0.19	2.78±0.15	2.26±0.17	2.30±0.15	2.27±0.17	2.41±0.12
AUC (mmol/L×60min^{-1})	-	-	-	-	-		-	-	72.46±5.40	74.34±4.76	72.82±5.52	78.71±3.72
HDL (mmol/L)	1.28±0.06	1.10±0.05	1.28±0.06	1.10±0.05	1.24±0.06	1.11±0.05	1.22±0.05	1.14±0.06	1.21±0.06†	0.96±0.05	1.20±0.05	1.00±0.05
AUC (mmol/L×60min^{-1})	-	-	-	-	-		-	-	72.4±3.57†††	30.88±1.49	71.82±3.17†††	32.47±1.60
Triglyceride (mmol/L)	0.88±0.05	0.97±0.08	0.93±0.08	1.17±0.15	0.80±0.06	0.94±0.08	0.85±0.07	1.22±0.14	0.65±0.06	0.75±0.09	0.73±0.06	1.06±0.15*
AUC (mmol/L×60min^{-1})	-	-	-	-	-		-	-	38.60±3.14	44.25±5.31	45.59±3.49(*)	62.99±8.74*

* $p<0.05$ thermoneutral vs moderate cold † $p<0.05$ normal weight vs obese

(*) $p<0.10$ thermoneutral vs moderate cold ††† $p<0.001$ normal weight vs obese

3.6. Effects of cold-induced BAT activation on mRNA of PDK4, CPT1α, CPT1β, SLC25A20, G0S2, FFAR4 and PPARα in the obese study cohort

Results of gene transcription analyses are given in table 5. Expression levels of all target transcripts were comparable at baseline between conditions (all $p \geq 0.296$, respectively). Cold-induced BAT activation significantly increased relative mRNA expression levels of PDK4, CPT1α, SLC25A20 and G0S2 (p=0.006, p=0.017, p=0.041 and p=0.002; figure 10). No changes between conditions were observed in CPT1β and PPARα (p=0.157 and p=0.135, respectively). FFAR4 was regarded as not expressed due to Ct values above 35 (see supplemental table C.1.). Repeated analyses using normalisation to the mean of all control samples yielded comparable results (supplemental table C.1.). As additional finding the reference gene ACTB varied systematically with the experimental conditions (p=0.034). In contrast, expression patterns of 18S remained unchanged between conditions or time points of measurements (p=0.641 and p=0.119), therefore only 18S was considered as suitable reference. Mean Ct values of target and reference genes are given in supplemental table C.1.

Table 5. Fold-changes of target gene mRNA levels normalized to expression of each corresponding control sample, and Ct values of reference genes during thermoneutral 25.0°C as baseline and after adaptation (100 minutes) to BAT-activating moderate cold (16.0 °C, shivering excluded), respectively. Data are means±SEM. n=14 (obese men only).

	Basal (0’)		After temperature adaptation (100’)	
	Thermoneutrality	Before cold exposure	Thermoneutrality	Moderate cold
Target gene (fold-change)			(control sample)	
CPT1α	1.29±0.23	1.70±0.42	1.0±0.0	2.34±0.56*
CPT1β	1.15±0.18	2.04±1.00	1.0±0.0	2.62±0.74
PDK4	1.46±0.40	1.81±0.45	1.0±0.0	3.05±0.84**
SCL25A20	1.61±0.31	1.96±0.63	1.0±0.0	2.91±0.77*
PPARα	1.19±0.20	1.91±0.71	1.0±0.0	1.94±0.39
FFAR4	1.15±0.26	2.07±0.18	1.0±0.0	3.86±1.93
G0S2	1.62±0.48	2.63±1.04	1.0±0.0	4.99±1.69**
Reference gene (Ct)				
18S	14.54±0.32	14.19±0.34	14.81±0.35	14.88±0.23
ACTB	24.14±0.30	23.57±0.27	24.57±0.46	23.68±0.35*

* p <0.05, thermoneutral vs moderate cold
** p <0.01, thermoneutral vs moderate cold

3.6. Effects of cold-induced BAT activation on mRNA of PDK4, CPT1α, CPT1β, SLC25A20, G0S2, FFAR4 and PPARα in the obese study cohort

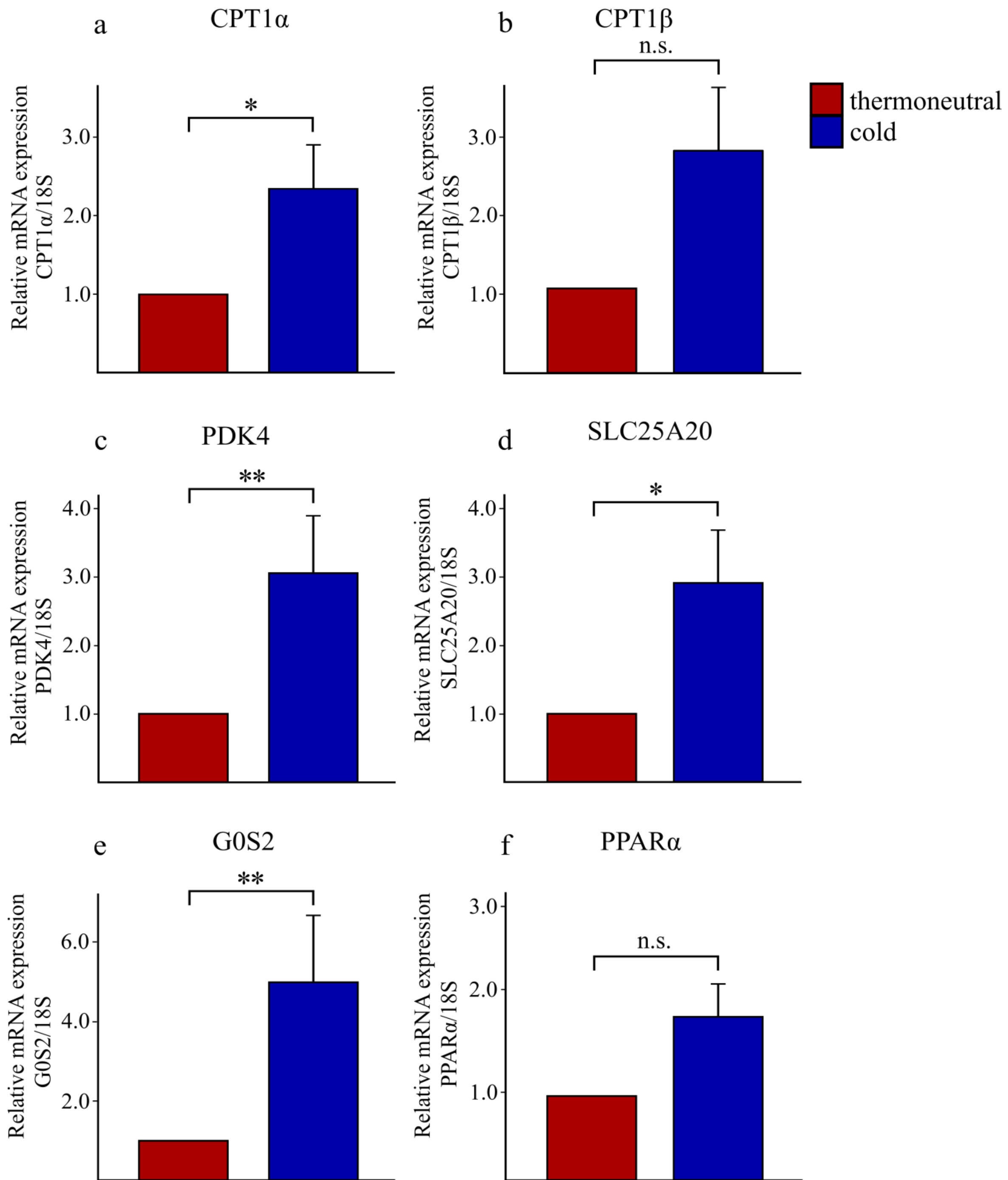

Figure 10. *Upregulated genes of lipolytic and FA oxidation pathways upon cold-induced BAT activation.* Relative mRNA expression levels using 18S as housekeeping gene measured after thermal adaptation in 14 obese, healthy men during thermoneutrality (red) and moderate cold (blue). Expression levels of CPT1α (**a**), PDK4 (**c**), SLC25A20 (**d**) and G0S2 (**e**) were significantly upregulated upon cold-induced BAT activation as compared to thermoneutrality (p=0.017, p=0.003, p=0.020 and p<0.001, respectively; 2-tailed paired Wilcoxon signed-rank tests; n=14), in contrast CPT1β (**b**) and PPARα (**f**) remained unchanged (p=0.157 and p=0.135, 2-tailed paired Wilcoxon signed-rank tests; n=14). Values were normalized to grouped control samples.

3.7. Effects of cold-induced BAT activation on hormones of the thyroidal and stress axis

Serum concentrations of TSH, fT_4 and cortisol are listed in table 3. Results for fT_4 are further depicted in figure 11. None of the measured hormones showed different concentrations at baseline between conditions (all $p \geq 0.508$, respectively). Exposure to moderate cold did not affect serum TSH throughout the experiments in neither study cohort (both $p=1.0$). In contrast, after the adaptation period circulating fT_4 was significantly reduced ($p=0.049$) and persisted to be lower during euglycaemic steady state of the clamp ($p=0.066$; figure 11) upon cold-induced BAT activation as compared to thermoneutrality in the obese participants, but not the normal weight ($p=0.134$; figure 11). Finally, BAT activation did not affect circulating cortisol in none of the study cohorts (normal weight $p=0.795$ and obese $p=0.567$).

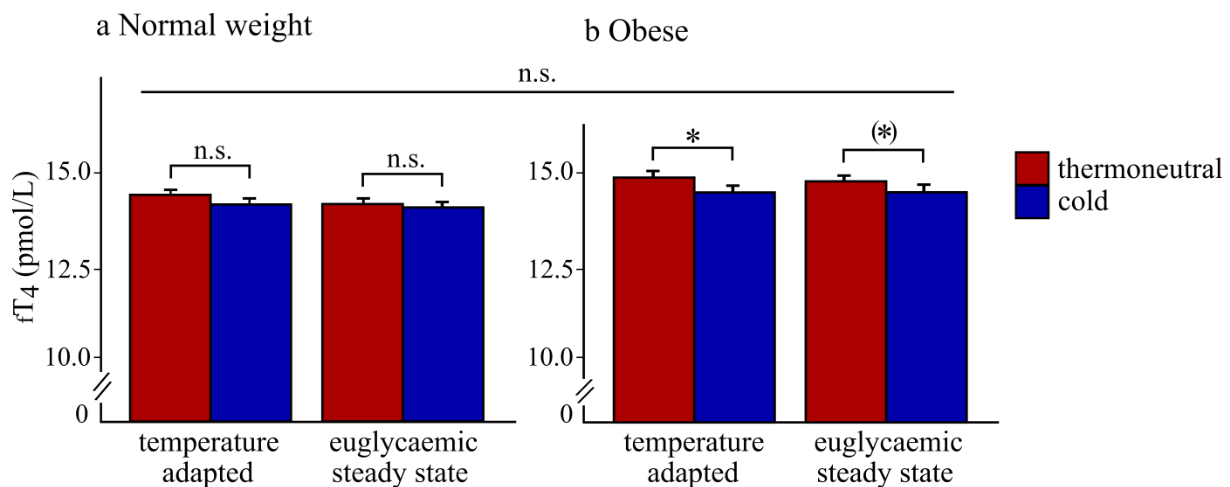

Figure 11. *Serum fT_4 decreases upon cold-induced BAT activation in obese men.*
Circulating fT_4 after thermal adaptation and during euglycaemic steady state of the HEC upon thermoneutrality (red) and cold-induced BAT activation (blue) in normal weight (**a**) and obese men (**b**). Serum fT_4 decreased in the BAT-activated state and tended to be lower during the cold euglycaemic steady state of the HEC in the obese ($p=0.049$, 2-tailed paired Wilcoxon signed-rank test; n=14), but remained unchanged in the normal weight study cohort throughout the experiment ($p=0.134$, 2-tailed paired Wilcoxon signed-rank test; n=15). Concentrations of fT_4 tended to be lower in the normal weight participants relative to the obese during euglycaemic steady state of the clamp in the cold condition ($p=0.069$, 2-tailed unpaired Wilcoxon rank sum test; n=29), but the corresponding interaction did not reach statistical significance ($p=0.798$, 2-way repeated measurements ANOVA; n=29).

3.8. Effects of cold-induced BAT activation on hormonal orexigenic/anorexogenic balance and food preferences

Levels of plasma ghrelin and leptin are given in table 3. At baseline concentrations of both hormones were comparable between conditions (total ghrelin in normal weight and obese $p=1.0$ and $p=0.855$, respectively; leptin in normal weight and obese $p=0.754$ and $p=1.0$, respectively). Cold-induced BAT activation did not affect total ghrelin and leptin in neither the obese nor the normal weight participants (total ghrelin in normal weight and obese $p=0.431$ and $p=0.192$, respectively [figure 12a and 12b]; leptin in normal weight and obese $p=0.534$ and $p=0.238$, respectively [figure 12c and 12d]). In contrast, both total ghrelin and leptin markedly differed between the study cohorts (both $p<0.001$): In the obese men total ghrelin as orexigenic hormone was notably lower as compared to the normal weight corresponding to approximately half the total plasma ghrelin levels measured in the normal weight men. Conversely, circulating leptin, a known anorexigenic hormone, in the obese participants markedly exceeded concentrations measured in the normal weight. In numbers, the difference in plasma leptin corresponded to sixfold and even more of the concentrations measured in the normal weight. Results of the food preference tests 'liking' and 'wanting' are depicted in table 6. In both tests the preferences for specific food, i.e. high calorie and savoury vs. high calorie and sweet vs. neutral and low calorie, were not modulated by cold-induced BAT activation (liking: normal weight $p\geq0.562$ and obese $p\geq0.170$, respectively; wanting: normal weight $p\geq0.780$ and obese $p\geq0.113$, respectively), and comparable between the normal weight and obese study cohorts (liking $p\geq0.111$ and wanting $p\geq0.131$, respectively).

3.8. Effects of cold-induced BAT activation on hormonal orexigenic/anorexogenic balance and food preferences

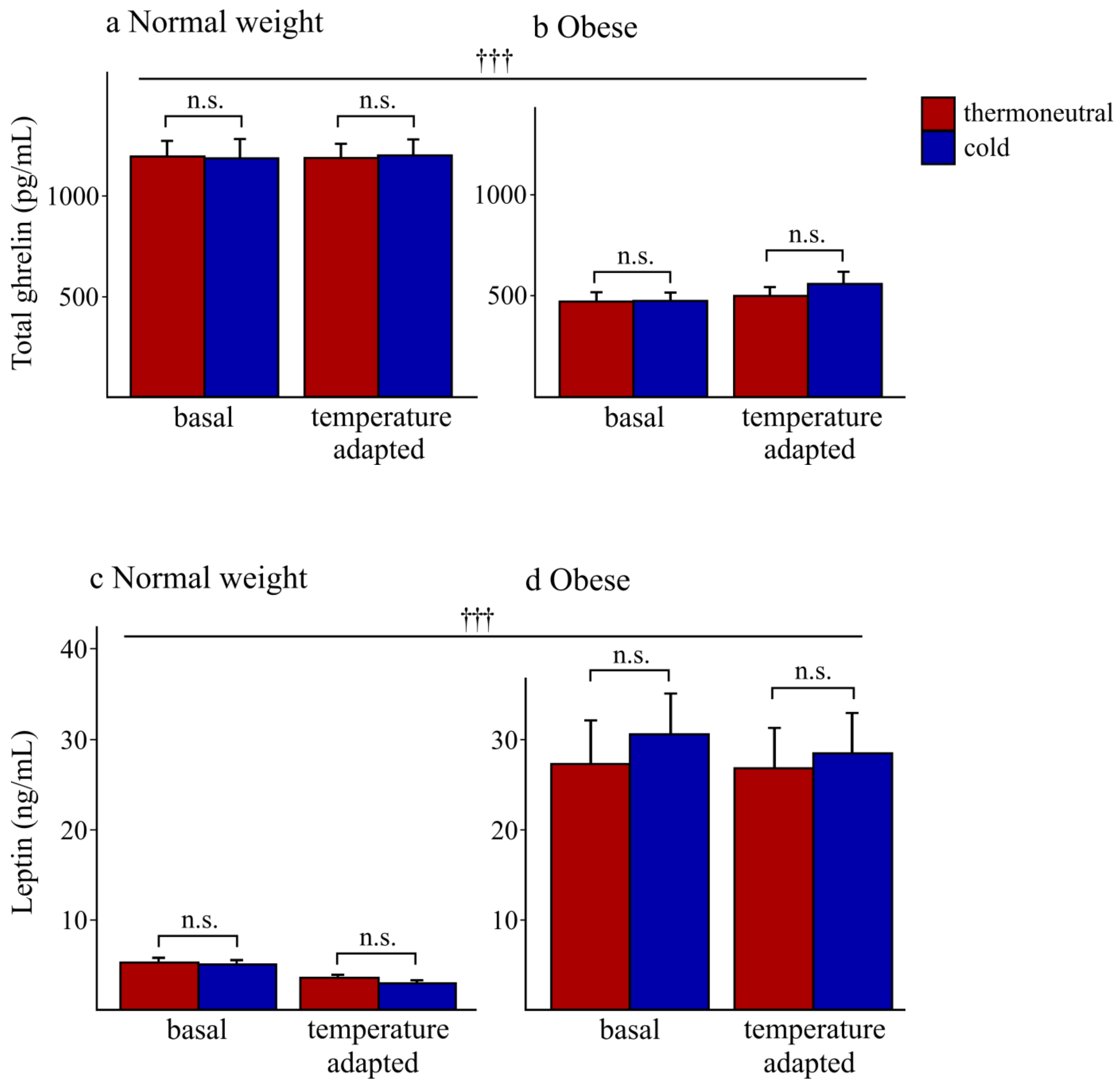

Figure 12. *Cold-activated BAT does not modulate total ghrelin, leptin and food preferences.* Circulating plasma total ghrelin and leptin measured at baseline and after thermal adaptation in normal weight and obese men during thermoneutrality (red) and exposure to moderate cold (blue). Cold-induced BAT activation did not affect total ghrelin and leptin in neither normal weight (**a** and **c;** p=0.431 and p=0.534, 2-way repeated-measurements ANOVAs; n=15 [total ghrelin]; n=14 [leptin]) nor obese (**b** and **d;** p=0.192 and p=0.238, 2-way repeated-measurements ANOVAs; both parameters n=14). Both total ghrelin and leptin were strongly associated with weight status, i.e. normal weight men showed significantly higher levels of total ghrelin (p<0.001†††; 2-way repeated-measurements ANOVA; n=29) and substantially lower plasma leptin (p<0.001†††; 2-way repeated-measurements ANOVA; n=28) as compared to the obese in both conditions.

Table 6. Mean Liking and Wanting ratings of different food during thermoneutrality (22.0°C and 25.0°C in the normal weight and obese study cohort, respectively) and after cold-induced BAT activation (18.0°C and 16.0°C in the normal weight and obese study cohort, respectively) at baseline and after the HEC (320 and 350 minutes in the normal weight and obese study cohort, respectively). Data are mean±SEM. n=29.

	Basal rating (0')				Final rating (320 - 350')			
	Thermoneutrality		Before cold exposure		Thermoneutrality		Moderate cold	
Liking	lean	obese	lean	obese	lean	obese	lean	obese
High calorie savoury	3.51±0.19	3.05±0.29	3.30±0.17	3.23±0.21	3.67±0.15	2.93±0.25	3.48±0.21	3.45±0.25
High calorie sweet	3.41±0.20	2.93±0.29	3.24±0.25	2.95±0.29	3.39±0.20	2.91±0.30	3.30±0.22	2.92±0.35
Neutral/low calorie	3.20±0.19	3.14±0.17	3.25±0.19	3.18±0.14	3.26±0.22	2.90±0.22	3.20±0.21	2.97±0.17
Wanting								
High calorie savoury	2.66±0.25	2.26±0.29	2.68±0.25	2.25±0.31	3.56±0.20	3.17±0.32	3.80±0.16	3.61±0.30
High calorie sweet	2.83±0.26	2.38±0.32	2.86±0.26	2.47±0.41	3.11±0.23	2.82±0.33	3.08±0.21	2.88±0.42
Neutral/low calorie	2.87±0.20	2.42±0.26	2.94±0.19	2.93±0.19	3.07±0.20	2.97±0.22	3.08±0.22	2.97±0.20

3.9. Effects of cold-induced BAT activation on autonomic, neuroglycopenic symptoms and subjective well-being (SRS and VAS)

An overview of the complementary subjective ratings SRS and VAS is given in table 7 and 8. Although many symptoms of the SRS differed in their rating levels between conditions and/or study cohorts none of the differences remained statistically significant after α-level adjustment. Significant interactions (cond×time and/or cond×time×weight, respectively) in the pooled analysis were found for experienced tingling (cond×time p=0.028), tremor (cond×time p=0.003), palpitation (cond×time×weight p=0.046), joy (cond×time p=0.013), vertigo (cond×time×weight p=0.044), nervousness (cond×time×weight p=0.021) and warmth (cond×time p=0.002). Concerning personal conditions assessed with the VAS no significant cond×time and/or cond×time×weight interactions were revealed. Experienced thirst in the cold condition during euglycaemic steady state of the clamp was rated significantly lower in the obese participants (p=0.027) as compared to thermoneutrality, furthermore mean ratings of being thirsty were significantly lower in the obese than the normal weight cohort during euglycaemic steady state (p<0.001), and to lesser extent at basal levels upon cold exposure (p=0.051). Finally, higher subjective ratings in the normal weight relative to the obese participants were revealed for stress at baseline during thermoneutrality (p=0.026).

Table 7. Ratings of autonomic and neuroglycopenic symptoms using SRS during thermoneutrality (22.0°C and 25.0°C in the normal weight and obese study cohort, respectively) and after cold-induced BAT activation (18.0°C and 16.0°C in the normal weight and obese study cohort, respectively) at baseline, after adaptation (100 minutes) and during euglycaemic steady state of a HEC (300 and 330 minutes in the normal weight and obese study cohort, respectively). n=29.

	Basal (0')				After temperature adaptation (100')				During euglycaemic steady state (300 - 330')			
	Thermoneutrality		Before cold exposure		Thermoneutrality		Moderate cold		Thermoneutrality		Moderate cold	
Symptom	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese
Fear	0.25±0.18	0.00±0.00	0.25±0.13	0.00±0.00	0.08±0.08	0.00±0.00	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Transpiration	0.50±0.26	0.07±0.07	0.42±0.23	0.21±0.15	0.25±0.25	0.07±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.07	0.00±0.00	0.00±0.00
Feeling unwell	0.42±0.23	0.21±0.15	0.67±0.36	0.64±0.27	0.58±0.29	0.14±0.10	0.83±0.32	0.36±0.17	0.33±0.26	0.14±0.10	0.58±0.40	0.29±0.22
Arousal	1.08±0.40	0.64±0.50	0.67±0.33	0.43±0.17	0.75±0.35	0.07±0.07	0.67±0.31	0.21±0.11	0.75±0.41	0.14±0.10	0.25±0.18	0.07±0.07
Tingling *[a]	0.17±0.17	0.00±0.00	0.17±0.17	0.21±0.15	0.08±0.08	0.00±0.00	0.42±0.29	0.29±0.16	0.08±0.08	0.14±0.10	0.42±0.29	0.07±0.07
Tremor **[a]	0.00±0.00	0.00±0.00	0.25±0.18	0.07±0.07	0.33±0.26	0.00±0.00	0.58±0.29	0.29±0.13	0.67±0.36	0.00±0.00	2.00±0.79 (†)	0.29±0.16
Hunger	3.25±0.64	2.07±0.45	0.50±0.57	2.93±0.57	4.17±0.69	3.29±0.72	4.50±0.56	3.79±0.61	4.00±0.79	2.86±0.80	3.83±0.81	4.21±0.81
Palpitation *[c]	0.50±0.23	0.00±0.00	0.25±0.18	0.21±0.15	0.25±0.18	0.00±0.00	0.17±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.25	0.00±0.00
Blurred vision	0.08±0.08	0.29±0.19	0.33±0.19	0.21±0.15	0.00±0.00	0.07±0.07	0.92±0.51	0.07±0.07	0.08±0.08	0.07±0.07	0.42±0.42	0.00±0.00
Concentration	6.08±0.50	4.79±0.70	4.75±0.75	5.14±0.76	5.08±0.62	4.07±0.63	4.50±0.65	4.71±0.71	5.00±0.59	4.07±0.74	5.17±0.73	4.57±0.74
Thirst	3.08±0.62	1.29±0.44	2.25±0.41	1.21±0.35	2.00±0.51	1.50±0.43	1.67±0.56	0.93±0.30	1.67±0.53	0.79±0.30	0.67±0.26	0.71±0.35
Anger	0.25±0.13	0.00±0.00	0.08±0.08	0.00±0.00	0.17±0.11	0.00±0.00	0.17±0.17	0.00±0.00	0.33±0.33	0.00±0.00	0.17±0.17	0.00±0.00
Headache	0.17±0.11	0.07±0.07	0.17±0.17	0.36±0.17	0.25±0.18	0.07±0.07	0.08±0.08	0.21±0.11	0.00±0.00	0.21±0.11	0.08±0.08	0.93±0.43
Satiety	2.08±0.62	2.79±0.67	1.33±0.47	2.71±0.63	1.17±0.41	2.07±0.51	1.33±0.61	1.93±0.52	1.17±0.37	1.93±0.58	1.00±0.55	1.07±0.40
Nausea	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.17±0.17	0.00±0.00	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.08	0.00±0.00
Sadness	0.00±0.00	0.00±0.00	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.08	0.00±0.00
Respiratory problems	0.08±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Joy *[a]	4.58±0.81	4.29±0.53	3.75±0.74	4.43±0.47	4.17±0.73	3.57±0.60	3.67±0.72	4.57±0.54	3.50±0.79	3.57±0.67	3.50±0.75	3.86±0.72
Tiredness	2.33±0.56	2.36±0.56	2.67±0.56	2.00±0.46	2.67±0.64	2.86±0.64	3.33±0.58	2.21±0.55	2.00±0.62	0.93±0.37	1.67±0.68	1.07±0.46
Vertigo *[c]	0.08±0.08	0.14±0.10	0.08±0.08	0.29±0.13	0.17±0.17	0.07±0.07	0.58±0.5	0.07±0.07	0.00±0.00	0.14±0.10	0.17±0.17	0.00±0.00
Nervousness *[c]	0.83±0.44	0.07±0.07	0.25±0.13	0.36±0.25	0.33±0.19	0.00±0.00	0.08±0.08	0.07±0.07	0.00±0.00	0.00±0.00	0.08±0.08	0.00±0.00
Appetite	3.58±0.84	2.36±0.61	3.50±0.56	2.86±0.58	4.17±0.77	2.64±0.7	4.58±0.62	3.79±0.70	3.33±0.95	3.00±0.81	3.50±0.92	3.57±0.83
Itch	0.00±0.00	0.00±0.00	0.08±0.08	0.07±0.07	0.00±0.00	0.14±0.1	0.00±0.00	0.07±0.07	0.00±0.00	0.07±0.07	0.00±0.00	0.00±0.00
Weakness	0.58±0.34	0.57±0.25	1.00±0.30	0.36±0.20	0.42±0.26	0.36±0.2	0.83±0.41	0.64±0.27	0.33±0.22	0.07±0.07	0.67±0.36	0.86±0.55
Warmth **[a]	2.67±0.57	2.21±0.49	2.25±0.57	3.00±0.56	1.83±0.55	1.86±0.47	0.75±0.39	1.64±0.40	1.50±0.60	1.79±0.50	0.25±0.25	1.21±0.38
Activity	3.83±0.80	2.57±0.67	3.00±0.64	2.79±0.69	3.08±0.56	2.07±0.67	2.75±0.69	2.21±0.64	2.25±0.63	2.36±0.70	2.75±0.69	1.79±0.70
Fullness	0.25±0.25	1.21±0.59	0.42±0.34	1.57±0.57	0.25±0.13	0.5±0.36	0.42±0.23	0.64±0.23	0.33±0.19	0.50±0.36	0.17±0.11	0.43±0.29

(†) p <0.10 normal weight vs obese *[a] p <0.05 interaction cond×time *[c] p <0.05 interaction cond×time×weight
**[a] p <0.01 interaction cond×time

Table 8. Ratings of current subjective well-being using an visual analogue scale (VAS) during thermoneutrality (22.0°C and 25.0°C in the normal weight and obese study cohort, respectively) and after cold-induced BAT activation (18.0°C and 16.0°C in the normal weight and obese study cohort, respectively) at baseline, after adaptation (100 minutes) and during euglycaemic steady state of a HEC (300 and 330 minutes in the normal weight and obese study cohort, respectively). n=29.

	Basal (0')				After temperature adaptation (100')				During euglycaemic steady state (300 - 330')			
	Thermoneutrality		Before cold exposure		Thermoneutrality		Moderate cold		Thermoneutrality		Moderate cold	
Personal condition	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese	lean	obese
Hungry	4.65±0.79	2.79±0.70	4.78±0.67	3.86±0.80	5.10±0.89	4.34±0.76	5.78±0.58	4.00±0.79	4.80±0.91	4.22±0.95	4.98±0.79	5.40±0.91
Satiated	1.89±0.55	3.17±0.73	1.47±0.54	2.89±0.80	1.74±0.54	2.39±0.61	1.01±0.29	1.84±0.63	1.55±0.38	2.32±0.63	2.79±0.83	1.51±0.40
Thirsty	3.74±0.64	1.54±0.45	3.62±0.53(†)	1.44±0.43	2.32±0.54	1.47±0.49	2.46±0.61	1.35±0.53	1.89±0.58	0.91±0.39	1.15±0.32†††	0.00±0.00*
Anxious	0.47±0.15	0.16±0.10	0.34±0.12	0.15±0.05	0.50±0.14	0.20±0.13	0.63±0.19	0.14±0.10	0.67±0.27	0.11±0.03	0.45±0.15	0.26±0.16
Happy	5.24±0.81	5.19±0.57	4.98±0.75	5.65±0.54	4.67±0.76	4.65±0.55	4.61±0.81	4.71±0.69	3.87±0.85	4.79±0.74	4.13±0.77	4.98±0.66
Stressed	1.47±0.34†	0.29±0.13	0.69±0.22	0.55±0.22	0.99±0.31	0.35±0.14	0.71±0.28	0.35±0.12	1.35±0.50	0.24±0.08	0.92±0.39	0.39±0.16
Sleepy	2.33±0.54	3.19±0.61	3.03±0.63	3.32±0.56	3.61±0.73	3.18±0.67	3.71±0.76	2.53±0.53	2.65±0.76	1.36±0.45	2.09±0.61	1.59±0.57
Concentrated	5.93±0.59	4.59±0.68	5.45±0.62	6.04±0.46	5.32±0.48	4.89±0.55	4.78±0.59	5.23±0.66	5.64±0.53	4.71±0.67	5.93±0.55	4.90±0.75
General craving for food	4.83±0.82	3.51±0.79	4.69±0.68	3.85±0.83	5.43±0.87	4.42±0.84	5.87±0.82	3.82±0.87	5.69±1.00	4.27±1.00	5.78±0.88	5.66±0.98
Craving for sweet food	4.09±0.81	1.71±0.68	2.92±0.57	2.30±0.90	4.31±0.79	2.19±0.66	4.14±0.66(†)	1.79±0.75	4.58±0.99	2.59±0.81	4.70±0.87	3.55±0.97
Craving for savoury food	4.28±0.81	2.85±0.63	4.23±0.58	3.39±0.82	5.12±0.89	4.54±0.80	5.72±0.78	3.34±0.84	5.53±0.99	4.36±1.04	5.85±0.91	5.08±1.00

* p <0.05 thermoneutral vs moderate cold

† p <0.05 normal weight vs obese

††† p <0.001 normal weight vs obese

(†) p <0.10 normal weight vs obese

Chapter IV. Discussion

4.1. Moderate cold activates the SNS and BAT thermogenesis in humans

Exposure to moderate cold was employed to induce BAT thermogenesis via stimulation of the SNS in the present work in young normal weight and obese healthy men.

The effectivity of the applied cooling protocol to increase metabolic BAT activity was confirmed by [^{18}F]-FDG PET scans in a subset of three normal weight participants. In line with previous reports, cold exposure did not affect [^{18}F]-FDG uptake into skeletal muscles, another metabolically important tissue potentially affecting glucose metabolism, and furthermore did not reduce body core temperature [34,50,52,65,99,100]. In sum, these observations prove BAT to be more important than skeletal muscle for cold-induced glucose uptake in fasted, non-shivering humans.

Cold-induced stimulation of the SNS as a mechanistic factor for BAT activation was further confirmed by the robust increase of NA in both normal weight and obese men. The importance of SNS activation, and in particular NA as major player in thermoregulation and SNS-mediated BAT-thermogenesis in humans is well established [45]. Besides NA, also levels of plasma DA were found notably increased in both normal weight and obese men in the present work suggesting also relevant dopaminergic impact on BAT metabolism in humans. This finding is consistent with a recent cell culture study from our group by Kohlie and Iwen et al. [74] who demonstrated direct influence of DA on mitochondrial mass and thermogenesis in murine brown adipocytes *in vitro*. In addition, increased levels of DA in BAT following cold exposure have previously been shown in rats [101]. Since ambient cold usually does not affect the adrenal medulla [102,103], A levels remained unchanged in the present work. Besides the marked increase in NA and DA the strong sympathetic activation upon cold exposure was not mirrored by changes in the SVB in neither study cohort. Although SVB has not been studied in the context of cold-induced BAT thermogenesis before, the result of unchanged SVB during ambient cold presented here is consistent with a previous study which sought to reveal effects of ambient heat and cold on heart rate control by the arterial baroreflex. During thermoneutrality as well as heat of 45°C or cold of 10°C for 40 – 65 minutes, respectively, SVB as expressed by LF/HF ratio remained unchanged [104]. The differential effect of SNS activation with increased circulating plasma catecholamine concentrations but unchanged SVB is likely due to the cardiovascular specificity of SVB. SVB is related to baroreflex function rather than whole-body sympathetic outflow in general.

Therefore, general effects of cold-induced SNS activation might be missed by this parameter [105].

Together, our findings confirm that the cooling protocol applied in the present work activated BAT thermogenesis via stimulation of the SNS and furthermore indicate NA and DA to be important regulators of SNS-induced BAT activation. Of interest, sympathetic drive on the cardiac system as reflected by SVB in not modulated by moderate cold.

4.2. REE and RQ are dependent on weight status but not influenced by cold-induced BAT activation

Using indirect calorimetry REE and RQ were determined in normal weight and obese men during thermoneutrality and in the state of cold-induced BAT activation. The cold-activated BAT was not affecting REE and RQ in none of the study cohorts in the present work, but both parameters significantly varied between the normal weight and obese men. Since van Marken Lichtenbelt et al. in 2009 impressively demonstrated increased REE and a drop in RQ towards facilitated fat oxidation following cold-induced BAT thermogenesis in both normal weight and obese participants, changes in REE associated with BAT activation have been repeatedly shown humans [33,34,56,58]. Nevertheless, these findings are countered by studies that did not detect any changes in REE upon cold-induced BAT activation, which complicates a conclusive evaluation of BATs capacity to burn sufficient extra calories to lose weight [35,50]. The fact, that no effects of active BAT on REE and RQ were detected in the present work might be due to the sample sizes of thirteen normal weight and fourteen obese participants included in the analysis of REE, which might have decreased statistical power to reveal differences between conditions. Also, most of the aforementioned studies reporting higher REE following cold-induced BAT activation were also limited by various factors, most important the lack of controlling for muscle shivering, but also subcategorisation of participants into 'high BAT activators' and 'low BAT activators' according to [^{18}F]-FDG uptake during PET/CT scanning [33,34,58].

On this background, it cannot be concluded whether or not cold-induced BAT thermogenesis may help to effectively burn sufficient calories to reduce body weight and attenuate overweight or obesity. However the present work suggests that neither normal weight nor obese men increase their daily REE by means of moderate cold treatment concomitant with

careful exclusion of increased muscle activity by shivering. With respect to the ongoing discussion of whether normal weight people do benefit from cold-induced BAT thermogenesis, while overweight and obese are supposed to do not, it is important to point out that the findings from the present study do not support the notion of differential effects of cooling on REE and RQ depending on weight status.

Although general metabolic differences between normal weight and obese individuals are not the primary focus of this work, the strong influence of weight status on REE and RQ found here shall briefly be addressed: The impact of weight status on REE and RQ is reflected by significantly higher resting metabolic rates and lower RQs in the obese men relative to normal weight. It is well established that REE increases as a function of body size and FFM, which is higher in overweight and obese humans relative to normal weight counterparts [101–104]. In line with this, a higher REE in obese as compared to non-obese individuals has already been shown in previous cross-sectional studies suggesting the increase to arise from compensatory mechanisms, such as FFM expansion in order to limit further body weight gain [110]. In fact, the comparison of REE between normal weight and overweight or obese cohorts requires appropriate control for body composition in order to reveal real differences between the groups. As no such normalisation has been implemented in both the cross-sectional studies mentioned above and the present work, it is likely that after adjustment the normal weight individuals might exhibit even higher REE than obese or both groups might show comparable resting metabolic rates as previous studies suggest [56,102,105–108]. Finally, regarding differences in RQ depending on the weight status, a lower RQ indicating higher fat oxidation in obese relative to normal weight individuals has already been reported by Blaak and colleagues, moreover data from Pima Indians, an obesity and T2DM-prone tribe, revealed an inverse relationship between RQ and fat mass (FM), consistent with the results of the present study [114,115]. As FM has been shown to contribute to fat oxidation in humans, it is proposed that the expansion of fat stores in obesity acts as an adaptive mechanism to increase the oxidative fat utilisation corresponding to high proportions of dietary fat intake [116].

In summary, findings from previous and the present study indicate varying REE and RQ depending on weight status, although its actual contribution to both parameters is probably mediated by other related factors such as FFM and FM.

4.3. *Cold-induced BAT activation differentially alters glucose metabolism and insulin sensitivity in normal weight and obese men*

The present study revealed tremendous improvements in human glucose metabolism upon BAT activation. By employing the Botnia clamp as gold standard method to assess the most relevant markers such as glucose tolerance and insulin sensitivity (*M*-value) under thermoneutral conditions and during exposure to BAT-activating moderate cold differential effects in normal weight and obese healthy men were observed. Glucose tolerance significantly improved during cold-induced BAT activation in the obese but not the normal weight cohort, while insulin sensitivity increased by up to 20 % in the normal weight men exclusively. Nevertheless, glucose tolerance of obese men must be regarded as relatively impaired when contrasted with their normal weight counterparts. Cold-induced BAT thermogenesis has been shown to improve whole-body glucose uptake and insulin sensitivity in humans before [33–35,56]. Beyond insulin sensitivity the present work provides new insights into effects of cold-activated BAT on glucose tolerance assessed by ivGTT in both normal weight and obese study cohorts. This work is the first to demonstrate that cold-induced BAT activation is capable of improving impaired glucose tolerance in obese men. Thereby, this finding underlines the metabolic relevance of cold-induced BAT thermogenesis in obesity, in particular with respect to acute glucose clearance. In addition, results suggest that in normal weight metabolically healthy humans an already prevalent normal glucose tolerance cannot be further improved, at least not by means of acute cold-induced BAT activation. Similar findings of improved glucose tolerance upon cold exposure in obesity, but not normal weight have earlier been reported in rodents. An oGTT was employed to evaluate glucose tolerance in mice with DIO and normal weight metabolically healthy control animals during ambient temperature and after cold exposure. Analogously to the present work, the glucose peak in obese mice was significantly reduced after cold exposure relative to ambient temperature, while glucose curves in normal weight control mice remained unchanged between both conditions [117]. Further support is provided by previous studies on high-intensity exercise and resveratrol supplementation, both of which improved effectively the impaired glucose tolerance in type 2 diabetic and obese patients, respectively, but failed to further boost normal glucose tolerance in healthy control participants [118,119]. Important to note, the improved glucose tolerance during cold exposure found in the present work is unlikely to result from elevated circulating insulin following the glucose bolus as various data suggest: First, in this work and also a separate study insulin secretion was assessed specifically in a normal weight study cohort to address the question of whether cold exposure

modulates the initial insulin response during an ivGTT and found it unchanged by moderate cold [120]. Apart from that, other studies in humans and rodents reported decreased, not increased, insulin levels under cold conditions relative to room temperature, with unchanged glucose tolerance in rats [100,121]. Furthermore, circulating unchallenged insulin concentrations in obese men did not change upon cold-induced BAT activation. Accordingly, insulin secretion can be considered unaffected by cold exposure in the young and healthy obese cohort included in this study as well.

The findings presented here point to differential advantageous effects of moderate cold exposure and associated BAT thermogenesis with an overall improvement of whole-body insulin sensitivity in normal weight and a significant reduction (although not normalisation) of glucose intolerance in obese men. These findings emphasise the metabolic relevance of cold-activated BAT for human glycaemic control in both populations. In this respect, the present work demonstrates the need for a differentiated consideration of the respective patient group when it comes to the evaluation of cold-activated BAT as potential treatment target in metabolic medicine. As far as this will be realised, these promising findings might be translated into novel clinical approaches tailored for specific target groups to treat diverse metabolic complications, e.g. postprandial hyperglycaemia in early-stage T2DM and obesity or the observed insulin resistance in normal weight visceral obesity and polycystic ovary syndrome (PCOS).

4.4. Cold-induced BAT activation increases circulating TG and facilitates FA oxidation

Cold-activated BAT thermogenesis requires both glucose and FA as energy substrates for NST, which are delivered to BAT via TG-rich lipoproteins or bound to albumin as NEFA. In the present study cold-induced BAT activation was accompanied by higher levels of circulating TG as compared to thermoneutrality in both normal weight and obese men indicating increased lipolysis from WAT. Other circulating lipids, i.e. total cholesterol, HDL and LDL, known to be less relevant for BAT functioning [17,18,63], remained unchanged in the BAT-activated state in the present work. As discussed above, cold exposure activates the SNS, which stimulates BAT to initiate NST and NA-dependent lipolysis from WAT. Cold-stimulated BAT thermogenesis relies on constant energy supply, therefore the rise in circulating TG during sustained cold-stimulated SNS activation observed in the present work

likely reflects facilitated lipid mobilisation to cover BATs increased demand for energy substrates [33,65,122,123]. Quantified mRNA expression of selected target genes involved in lipolysis and β-oxidation before and during cold-induced BAT activation in circulating blood cells of the obese study cohort revealed significant upregulation of PDK4, CPT1α, SLC25A20 and G0S2 upon moderate cold as compared to thermoneutrality. Together with elevated levels of circulating TG this data support the concept of increased lipid mobilisation during states of increased energy demands, like defending body core temperature by BAT thermogenesis during moderate ambient cold [45]. Thereby the presented work clearly substantiates that elevated TG-mobilisation is regulated, at least in part, by PDK4: In the activated state PDK4 pushes FA oxidation by inducing a switch from glucose catabolism to FA utilisation through inhibition of pyruvate dehydrogenase complex (PDC), which catalyses the conversion of pyruvate to acetyl CoA. This mechanism allows for metabolic flexibility during unstable conditions of nutrient availability like e.g. fasting and refeeding [124]. In human skeletal muscle PDK4 expression increases under conditions of poor carbohydrate availability as seen on diets high in fat but low in carbohydrate, fasting and exercise. This state is reflected by low insulin levels but elevated circulating FFA [84,123–125]. Increased abundance of PDK4 has also been observed in muscle of T2DM patients where it is associated with enhanced lipolysis as induced by insulin resistance [124,128]. Moreover, levels of PDK4 mRNA in muscle biopsies collected from formerly obese patients after bariatric surgery showed an inverse correlation with whole-body glucose uptake. This data propose that favouring FA oxidation over glucose utilisation mediated by increased PDK4 expression might emerge as potential compensatory mechanism to counter excessive accumulation of intracellular lipids and improve muscle insulin sensitivity [129]. The association of upregulated PDK4 and insulin resistance in T2DM stimulated interventional approaches aiming on the reduction of PDK4 via specific inhibitors. As this approach is considered a promising therapy against T2DM some drugs are already under investigation in animal models [124]. Studying effects of PDK4 is complicated by the fact that its expression is rather tissue specific. While skeletal muscle, liver and heart benefit from inhibition of PDK4, its upregulation is of advantage for WAT as major lipid storage site, since increased PDK4 promotes increased glyceroneogenesis and deposition of FA in adipocytes [124]. Taking this into account, the reviewed data together

with findings obtained from the present work do not allow generalised conclusions on metabolic benefits of upregulated PDK4 in human blood cells. Nevertheless, bearing cold-induced NST in mind it might be promising to reconsider the strategy of pharmacologically altering systemic PDK4 levels in insulin resistance, provided BATs function as sink for the circulating TG and FFA can be substantiated in humans. If this is the case, the activated BAT might prevent the insulin desensitising and lipotoxic effects of elevated lipolysis. Besides the capacity of PDK4 to switch from glucose to fat utilisation, FA oxidation is further facilitated by promoting the transport of FA through the mitochondrial membrane. This is provided, inter alia, by the CPT1 system and SLC25A20. The CPT1 isoforms α and β regulate in particular the translocation of long-chain fatty acids into mitochondria. While CPT1α is highly abundant in the liver and found all over the body, CPT1β is mainly expressed in skeletal muscles, WAT and BAT [130]. In the present work mRNA of CPT1α was found upregulated in human blood cells during exposure to BAT-activating moderate cold, consistent with findings in rat PBMCs after cold-acclimation [88]. Likewise, expression of SLC25A20, another type of mitochondrial fatty acid transporter, increased during cold-activated BAT. Considering other states of increased energetic demand beside BAT thermogenesis, these findings are in line with previous studies: As reported by Bouwens and colleagues, SLC25A20 measured in human PBMCs was upregulated after 24 hours of fasting, while transcripts of CPT1 were not affected at all by starvation [82]. In contrast to fasting, exercise as a state of acutely increased energetic needs has been demonstrated to modulate expression of both CPT1α and SLC25A20 in human muscle. In these studies increased CPT1 levels were associated with endurance training, furthermore expression of SLC25A20 increased after exercise but decreased during states of physical deconditioning [80,131]. Taking this into consideration, the observation of increasing CPT1α and SLC25A20 mRNA levels upon cold-induced BAT activation in the present study suggests that these mitochondrial carriers contribute to adaptive processes in order to facilitate FA oxidation which is required during NST. Finally, also mRNA of G0S2 was upregulated during BAT thermogenesis, a gene which actually has been demonstrated to reduce lipolysis by directly binding to and inhibiting the rate limiting lipolytic enzyme adipose triglyceride lipase (ATGL), resulting in decreased TG hydrolase activity of ATGL in adipocytes [132,133]. G0S2 expression patterns are tissue-specific and inverted in WAT and liver [134]. Furthermore, G0S2 expression depends on the current nutritional state, that is, during fasting G0S2 levels in WAT decrease, leading to elevated lipolysis and subsequent

FFA fluxes. Conversely, hepatic G0S2 expression increases upon fasting in response to elevated liver FFA levels, which suppresses the break-down of TG and preserves stored TG as energy source [135,136]. Apart from that, G0S2 is induced by states of hypoxia and acts to restore ATP production by interaction with FoF1-ATP synthase, which prevents cells from deleterious effects associated with such energy crisis [137]. Therefore, upregulation of G0S2 mRNA might serve as marker for reduced rates of cellular ATP during respiratory uncoupling in brown adipocytes. The outlined complexity of G0S2 regulation complicates the interpretation of increased systemic G0S2 mRNA in PBMCs upon cold-induced BAT activation observed in the present study. Although the majority of tissue–specific genes has been demonstrated to be also expressed in PBMCs, to date the specific functions of these systemic mRNAs are not known. As the data presented here clearly point to a cold-induced increase in lipolysis, which in general is accompanied by increased plasma FFA, it is plausible to propose that the upregulation of G0S2 in peripheral blood during moderate cold most likely mirrors the above mentioned rise in hepatic G0S2 expression observed upon fasting and increased FFA fluxes from WAT to the liver. Following this, the upregulation of G0S2 observed in this study should be considered as secondary effect of cold-induced lipolysis rather than a consequence of cold-activated BAT.

To address briefly the remaining target transcripts investigated in this work, CPT1β and PPARα were found unresponsive to moderate cold exposure in obese men, moreover FFAR4 was considered as not detected due to the high Ct values of above 35 cycles. Although CPT1β is expressed inter alia in WAT and BAT, both of which were affected in the present study by cold exposure, levels of systemic CPT1β mRNA were not changed after moderate cold treatment. Likewise, mRNA expression of PPARα was not altered by cold exposure, though it is known to crucially regulate genes involved in FA oxidation and is moreover associated with obesity and browning of WAT [138,139]. In contrast to the present work, a recent study reported upregulation of diverse transcripts involved in lipid metabolism during cold-induced BAT activation: In samples of supraclavicular BAT collected from obese volunteers before and after cold treatment mRNA of inter alia CPT1β and PPARα was found increased in the BAT-activated relative to the untreated state [65]. This finding indicates that both genes are cold-responsive in tissue samples of supraclavicular BAT. However, this study relies on molecular data from only one participant, hence the validity of the reported results is to be taken with caution. Nevertheless, the aforementioned study by Chondronikola et al. indicates

that the negative results regarding CPT1β and PPARα in the present study are probably due to low circulating levels of these transcripts in peripheral blood. Therefore, associations between cold-induced BAT thermogenesis and systemic CPT1β and PPARα require further study.

In summary, the present work found upregulation of PDK4, CPT1α, SLC25A20 and G0S2 together with increased circulating TG in obese men upon cold-induced BAT activation. This finding consistently indicates facilitation of lipid mobilisation upon sustained SNS activation during ambient moderate cold, which might arise to cover BATs elevated energy demand for maintenance of NST. Although relevant levels of FFAR4 were not detected in this study and CPT1β as well as PPARα were not changed by moderate cold, their relevance for increased FA oxidation during cold-activated BAT needs to be addressed in further research in humans.

4.5. Cold-induced BAT activation differentially alters circulating fT_4 in normal weight and obese men

Thyroidal hormones notably regulate human energy expenditure, lipid and glucose metabolism as well as adaptive thermogenesis provided by BAT. They might be therefore further mediators for the metabolic improvements associated with BAT thermogenesis [75,140]. In the present study circulating fT_4 declined after adaptation to moderate cold in the obese participants. Free T_4 is a prohormone predominantly secreted by the thyroid gland and peripherally converted to the bioactive form fT_3 by deiodinase type 2 (DIO2; [141]). Free T_3 is involved in manifold metabolic processes such as adipogenesis, lipolysis, lipogenesis, FA oxidation and thermogenesis in BAT. It has been shown in isolated brown adipocytes that NA-induced lipogenesis requires intracellular fT_4, which is subsequently converted to fT_3 as crucial prerequisite for expression of thermogenic genes in BAT [142]. Apart from metabolic processes or thyroidal diseases, reductions in fT_3 and fT_4 have been associated with physical an emotional stress in rats [143]. Both conditions were not observed in the presented study as reflected by unchanged TSH, cortisol and A concentrations as well as self-rated stress (see table 3 and 8; [143]). The decrease in circulating fT_4 during ambient cold might thus reflect accelerated serum fT_4 clearance by WAT and BAT as major sites of lipolysis and thermogenesis to provide sufficient T_4 to T_3 conversion for maintenance of stable fT_3 levels. This suggested concept is indeed supported by previous findings of catecholamine-induced increase in T_4 to T_3 conversion, but requires further examination in humans [141]. The fact

that levels of fT_4 changed exclusively in the obese men but not the normal weight might be related to the more pronounced elevation of circulating TG following cold exposure in the obese study cohort. Two recent studies consistently found levels of fT_4 negatively correlated with insulin resistance [144] and plasma TG [145] in euthyroid non-diabetic normal weight and overweight/obese volunteers. However, the strong inverse relationship between fT_4 and TG disappeared in the obese participants after linear regression analyses were performed separately for the normal weight and obese study cohorts. Apart from reduced fT_4 in the obese study cohort BAT thermogenesis was not accompanied by further effects on TSH and cortisol, respectively, in the present work, what is in line with numerous previous human studies on cold-induced BAT activation [34,50,95,142–144]. It must be mentioned here that data on TSH and its contribution to cold-induced BAT thermogenesis in humans is inconsistent, with some studies reporting positive correlations between circulating TSH and BAT activation in pathological conditions such as hyperthyroidism or hyperthyroid carcinomas, while for instance Orava and her co-workers found decreased plasma TSH following cold exposure in healthy volunteers [99,140,149,150]. Likewise, available studies on associations between cortisol and cold-induced BAT activation are conflicting. Most data obtained from rodents indicated cortisol to suppress BAT activation [151–153]. In contrast, Chen et al. found increased urinary, but not serum, cortisol upon cold-activated BAT in healthy participants [148], furthermore physiological doses of cortisol have been shown to activate primary human brown adipocytes [153].

Altogether, these findings do not permit final conclusions on the relevance of TSH and cortisol for cold-activated BAT in normal weight and obese individuals. The revealed decline in serum fT_4 following cold exposure in obese men might be indicative for increased T_4 to T_3 conversion as precondition for the expression of thermogenic genes in BAT.

4.6. Cold-induced BAT activation has no effects on the orexigenic/anorexigenic balance and food preferences

By signalling the nutritional status to the brain ghrelin and leptin are known homeostatic signals for central nervous regulation of food intake. Therefore, increased energetic demands like cold-induced BAT thermogenesis might have an impact on circulating total ghrelin and

leptin as well as food preferences to stimulate adequate behavioural responses, e.g. intake of high calorie food. In the present work levels of circulating total ghrelin and leptin as well as the rewarding value of diverse foods assessed by personal 'liking' and 'wanting' ratings were not affected during cold-activation of BAT. States of increased energy expenditure such as exercise and exposure to severe cold are accompanied by decreased leptin, thereby lowering the satiating signal and facilitating food intake, while right after having a meal leptin levels increase to terminate further food intake [154,155]. In contrast, ghrelin increases after weight loss, following sleep deprivation and after exposure to severe cold in humans, leading to increased appetite and hunger ratings, but decreases after having a meal when energy requirements are met [147,149–151]. The fact, that total ghrelin and leptin remained unchanged upon BAT activation in the present study might be due to the applied moderate cold temperatures. In the studies mentioned above participants were exposed to severe cold of 6.5°C and even 2°C, respectively, for 30 - 90 minutes, hence shorter than in the present work, but nevertheless these durations were sufficient to induce changes in ghrelin and leptin. Important to point out, the low temperatures used in the aforementioned studies are likely to stimulate massive muscle shivering, thereby further increasing energy expenditure to a significant extent. However, these studies did not control for possible contributions of muscle activity [155,158]. Although cold exposure did not affect total ghrelin and leptin in the present work, both hormones were clearly related to weight status with higher ghrelin and lower leptin in the normal weight as compared to the obese men. These results are consistent with previous studies, which proposed that antagonistic regulatory influence of long term leptin and insulin levels on ghrelin might be the crucial mediating factor rather than the weight status or BMI *per se* [154,159]. From an evolutionary perspective, both the positive and inverse, respectively, correlation between either leptin or ghrelin and BMI reflect beneficial physiological adaptations to either sustained energy deprivation or a persistent positive energy balance associated with obesity [154,160]. Although a direct link between leptin and the development of obesity has become obvious with respect to leptin resistance and congenital leptin deficiency, no such association has been proven for ghrelin. Therefore, its contribution to the pathogenesis of human obesity remains to be established. Beside hormonal regulation of appetite, food intake is also regulated by numerous other factors such

as explicit and implicit food preferences referred to as 'liking' and 'wanting'. Both have never been studied in the context of cold-activated BAT in humans before, but metabolic conditions such as fasting, exercise and primary substrate oxidation have been shown to influence liking and wanting. In particular, higher levels of wanting have been linked to an increased susceptibility for overeating, leading to overweight and obesity [156–159]. In line, the efficacy of exercise as weight loss intervention has been found to be undermined by overcompensation in terms of increased energy intake after the training due to higher subjective wanting [163]. Nevertheless, in the present work neither liking nor wanting were affected by short term cold-induced BAT thermogenesis. This finding suggests that although activated BAT increases the energetic demand to defend body core temperature it does not trigger compensatory responses like increased food craving in general or shifted preferences towards high calorie savoury or sweet food. Apart from that, the findings presented here do not support previous reports of higher subjective liking and wanting in overweight or obese individuals, although the concept of 'social desirability' might have influenced the responses in both cohorts. In brief, social desirability describes the tendency of a person to convey an image in keeping with social norms and to avoid expected criticism in a 'testing' situation, e.g. interview. For that reason social desirability is a relevant response bias in social sciences [165]. As eating behaviour is crucially influenced by social norms and contexts, and high calorie food is often considered unhealthy, thus less socially accepted, it cannot be excluded that the participants of the present study understated their preferences for high caloric meals [166].

To briefly summarise the key issues of this section, the present study demonstrates that cold-induced BAT activation has no effect on total ghrelin and leptin as hormonal regulators of appetite. In line with the hormonal data, food preferences were not altered by cold-activated BAT in neither normal weight nor obese men. Therefore at least in an acute setting, beneficial metabolic effects of moderate cooling interventions to stimulate BAT metabolism are unlikely to be compensated by deleterious feeding behaviours.

4.7. Cold-induced BAT activation does not affect self-rated autonomic, neuroglycopenic symptoms and subjective well-being

Extensive studies and treatment conditions such as cold stress might induce adverse symptoms and subjective feelings of stress in volunteers. The participants included in this study did not report high levels of adverse or negatively connoted symptoms or conditions, instead 'being concentrated' gained the highest ratings in both study cohorts. The most relevant symptoms and conditions for the present intervention, which will be discussed in closer detail, comprise subjectively experienced stress, hunger, appetite and warmth as well as craving for savoury and sweet food. None of these key symptoms and dispositions were affected by moderate cold. Baseline stress levels were rated higher by the normal weight than the obese participants during thermoneutrality and craving for sweet food after thermal adaptation tended to be higher in the normal weight as compared to the obese men only in the cold condition. In contrast to the results presented here, previous studies reported ambient cold to induce stress responses like increased cortisol and immune depression, but the respective protocols applied lower temperatures and did not control muscle shivering [147,167,168]. Moreover, in a very early work Davis extensively describes increased compensatory food intake upon ambient cold in men [169]. Important to consider in this respect is that the moderate cold stimulation employed in the present work was neither accompanied by shivering nor decreased body core temperature. This might explain why no clear cold-induced effects on symptoms like hunger and warmth sensation were detected. Other reasons that may account for the results are subjective response biases in answering surveys like social desirability introduced above. In addition, it is possible that participants included in this study dissimulated and/or underreported symptoms to avoid being perceived as a sensitive person by others, for instance the investigators [170,171]. Furthermore, methodological aspects such as reduced statistical power might account for the negative results, since the need to correct for multiple comparisons frequently increases the probability of producing false negatives. Apart from effects of cold exposure, the weight status was influencing individual warmth sensation. This can be explained by the known higher insulation properties of obese as compared to normal weight individuals [92]. In line, the latter reported significantly lower warmth sensation than the obese men. Concerning individual food cravings the normal weight participants tended to rate their desire for sweet food higher than the obese after adaptation to cold in the present work. Although many studies suggested a preference for sweet food in obese humans, other reports describe higher energy intake from salty food in obesity.

4.7. Cold-induced BAT activation does not affect self-rated autonomic, neuroglycopenic symptoms and subjective well-being

Moreover, correlations between BMI and preference for fat-rich salty food have been found [172]. Since the absolute subjective ratings of craving for sweet food in the present work should be regarded as low to moderate in general, and available data on this subject is contradictory, the relevance of higher sweet craving in normal weight as compared to obese men during moderate cold as condition of increased energetic demands presented here is difficult to interpret and requires further investigation.

To sum up, the cooling protocols applied in the present work did not evoke relevant self-rated autonomic or neuroglycopenic symptoms, moreover they were not accompanied by increased hunger or food cravings, and finally did not cause other unpleasant states. As expected, cold exposure led to lower warmth sensations in normal weight relative to obese individuals. The low to moderate ratings of adverse or negatively connoted symptoms and personal conditions throughout the experiment further indicate limited influence of confounding variables caused by the research intervention.

4.8. Limitations of the present work

This thesis has some limitations that need to be addressed. First, cold-induced BAT activation was not confirmed by [^{18}F]-FDG PET scans in the entire study cohort. As discussed above, a number of previous studies have already shown increased BAT metabolism upon cold exposure both in normal weight and obese humans, although [^{18}F]-FDG uptake in general is lower in obese as compared to normal weight individuals. It is therefore justified to propose that cold-induced BAT activation is not restricted to the subgroup of participants which underwent [^{18}F]-FDG PET imaging and was also achieved in the majority of the investigated normal weight and obese volunteers. This is especially supported by the fact that the whole group of subjects was homogenous with respect to outcome parameters. Second, the study protocols in both cohorts aimed on the investigation of acute and short term exposure to moderate cold. Metabolic adaptions of BAT and other endocrine pathways in response to longer periods of cold exposure may even be more pronounced. Hence, chronic adaptions to moderate BAT-activating cold in human volunteers are to be determined in future studies. Third, only metabolically healthy men without a family history of T2DM were examined, therefore effects of cold-induced BAT activation on insulin-mediated glucose and lipid

metabolism cannot be generalised to certain patient groups with e.g. T2DM or other components of Mets and need to be addressed in future trials. Fourth, as noted above, the extent to which systemic mRNA levels detected in blood cells reflect conditions in the target tissues of the corresponding genes, and whether these circulating mRNA serve similar functions are unknown. Transcriptional analyses conducted on other than the actual target sites should therefore rather be taken as hypothesis-generating, complementary approach and require proper validation in subsequent studies using the respective target tissue. Linked to this and given that genes of interest are tissue-specific, levels of corresponding mRNA measured in peripheral blood have to be expected significantly lower than in the target tissues. The target transcripts of the present work were consistently detected at late stages of the qPCR, that is, at amplification cycles ranging from 31 to 35. For that reason, it is of crucial importance to control for amplification of genomic DNA or other unspecific signals. Apart from that, very subtle changes, which are generally difficult to detect, might not be resolved during late expression stages. Fifth, the study protocols differed in their total duration between the normal weight and obese participants. It must be emphasised that durations of cold exposure were almost identical between groups (~ 5 vs. 5 ½ hours), hence notable differences in cooling effects *per se* are unlikely to emerge from that rather short time gap. Nevertheless, differences between the study cohorts towards the end of the experiments as observed in fT_4 might have been influenced by a prolonged fasting period and slightly longer cold exposure during hyperinsulinaemic euglycaemia in the obese relative to the normal weight participants rather than reflecting real group differences.

4.9. Perspectives

The present work demonstrates that moderate cold stimulates BAT via SNS activation, which in turn is linked to improved glucose metabolism and increased lipid mobilisation. In a recent analysis related to this work, profiles of plasma FA were measured in the normal weight study cohort before and after cold exposure and revealed distinct alterations of selected circulating FA during cold [120]. Although profiling, comparison and evaluation of plasma FA profiles from normal weight and obese participants before and upon cold-induced BAT thermogenesis was beyond the scope of this work, it is worth further focusing on plasma FA or NEFA profiles also in obese individuals upon BAT activation in future studies. In contrast to rodent

studies BATs functional relevance for lipid clearance in humans has not been conclusively determined yet. However, this is crucial to permit reliable conclusions on net metabolic improvements considering the deleterious consequences lipotoxicity might have on other tissues during conditions of prolonged lipolysis. Another starting point for future investigations is offered by the consistently shown negative correlation between BMI and BAT thermogenesis, which raised a discussion about BATs relevance as target for metabolic benefits in obesity. The present work points to differential effects of moderate cold exposure and subsequent BAT thermogenesis on metabolic health in normal weight and obese men. This underlines the metabolic relevance of cold-activated BAT in both populations, but also animates to adopt a more target group oriented view in clinic and research. Following this, it is important to focus also on respective patient groups in future studies, e.g. T2DM patients, obese, women or elderly. Furthermore, the various types of cooling interventions, i.e. acute cold exposure vs. acclimation protocols, might induce differential intra-individual responses and effects between various populations. Such extensive characterisation is adding another layer of complexity to the subject, but at the same time provides very specific insights, which might result in the development of novel and tailored treatment options. Also regarding the generalisability of the obtained insights, the here reported cold-induced alterations of gene transcription involved in lipid metabolism have been determined exclusively in obese individuals. The next crucial step would therefore be to conduct the transcriptional analyses analogously in a normal weight study cohort, and optimally in BAT samples, to confirm the cold responsive target transcripts revealed in the present work. Following on from this and to conclude, in the present work targeted gene transcription analyses were conducted using human peripheral blood cells. PBMCs were used as surrogate material to circumvent the numerous restrictions of standard genetic methods in human research that arise from the collection of BAT tissue biopsies. Despite the limitations discussed above, this approach broadens the methodological spectrum in human metabolic research and enables for identification and characterisation of key transcripts in energy metabolism under various conditions or profiling of expression patterns in specific patient groups.

Chapter V. Summary and conclusion

The present work employed individualised cooling protocols together with gold standard approaches to assess glucose and lipid metabolism in normal weight and metabolically healthy obese men during cold-induced BAT activation. Additional hormonal and transcriptional key markers of energy metabolism were determined to gain insights into underlying regulatory mechanism that might act as mediatory instances between BAT thermogenesis and the associated metabolic improvements in humans. This investigation was complemented by measuring REE, RQ and food preferences to evaluate BATs potential as anti-obesity target and treatment option against Mets in humans.

The present work shows for the first time that acute exposure to moderate cold, SNS activation and BAT stimulation are associated with improved glucose tolerance and increased insulin sensitivity in obese and normal weight men, respectively, together with elevated circulating TG as indicator for facilitated lipid mobilisation in both study cohorts. As moderate cold signals the need to release stored energy from WAT for heat production the mobilised energy substrates are likely to be used as fuel for BAT thermogenesis. Consistent with this concept, systemic mRNA of genes modulating lipid metabolism, i.e. PDK4, CPT1α, SLC25A20 and G0S2, were upregulated upon cold-induced BAT activation in the obese volunteers of the present study. Through their specific properties of facilitating lipolysis and FA oxidation this cold responsive target genes might potentially mediate between cold-stimulated BAT thermogenesis and the observed metabolic improvements in humans by facilitating the removal of released FFA from the circulation. Although in the present work cold-induced BAT activation was not accompanied by increased REE, these findings highlight the close interaction between SNS and BAT thermogenesis for the integration of metabolic pathways regulating glucose and lipid handling via pathways of lipolysis and FA oxidation. Through this, the newly gained insights may provide new approaches to exploit BAT function as therapeutic intervention in metabolic medicine.

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Supplemental material A

A.1. Symptom rating scale (SRS)

Name:	Uhrzeit:	Sitzung	Pr-Nr:

Symptom-Rating-Skala

Symptom:	0 schwach	1	2	3	4 mittel	5	6	7	8	9 stark
1. Angst	0	1	2	3	4	5	6	7	8	9
2. Schwitzen	0	1	2	3	4	5	6	7	8	9
3. Körperliches Unwohlsein	0	1	2	3	4	5	6	7	8	9
4. Innere Unruhe	0	1	2	3	4	5	6	7	8	9
5. Kribbelgefühl	0	1	2	3	4	5	6	7	8	9
6. Zittern	0	1	2	3	4	5	6	7	8	9
7. Hunger	0	1	2	3	4	5	6	7	8	9
8. Herzklopfen	0	1	2	3	4	5	6	7	8	9
9. Verschwommenes Sehen	0	1	2	3	4	5	6	7	8	9
10. Konzentrationsfähigkeit	0	1	2	3	4	5	6	7	8	9
11. Durst	0	1	2	3	4	5	6	7	8	9
12. Ärger	0	1	2	3	4	5	6	7	8	9
13. Kopfschmerzen	0	1	2	3	4	5	6	7	8	9
14. Sattheit	0	1	2	3	4	5	6	7	8	9
15. Übelkeit	0	1	2	3	4	5	6	7	8	9
16. Traurigkeit	0	1	2	3	4	5	6	7	8	9
17. Atembeschwerden	0	1	2	3	4	5	6	7	8	9
18. Freude	0	1	2	3	4	5	6	7	8	9
19. Müdigkeit	0	1	2	3	4	5	6	7	8	9
20. Schwindel	0	1	2	3	4	5	6	7	8	9
21. Nervosität	0	1	2	3	4	5	6	7	8	9
22. Appetit	0	1	2	3	4	5	6	7	8	9
23. Juckreiz	0	1	2	3	4	5	6	7	8	9
24. Schwäche	0	1	2	3	4	5	6	7	8	9
25. Wärme	0	1	2	3	4	5	6	7	8	9
26. Aktivität	0	1	2	3	4	5	6	7	8	9
27. Völlegefühl	0	1	2	3	4	5	6	7	8	9

A.2. Visual analogue scale (VAS)

Proband ID: Datum: Versuch:

In welchem Ausmaß treffen folgende Aussagen zur Beurteilung ihres subjektiven Gefühles auf Sie zu? Bitte markieren Sie bei jeder Aussage die entsprechende Stelle auf der schwarzen Linie mit einem Kreuz.
Wie XXX fühlen Sie sich im Moment?

Überhaupt nicht — hungrig — Extrem

Überhaupt nicht — satt — Extrem

Überhaupt nicht — durstig — Extrem

Überhaupt nicht — ängstlich — Extrem

Überhaupt nicht — fröhlich — Extrem

Überhaupt nicht — gestresst — Extrem

Überhaupt nicht — schläfrig — Extrem

Überhaupt nicht — konzentriert — Extrem

Wie stark ist Ihr momentanes Bedürfnis nach Essen?

Überhaupt nicht stark — …generell — Sehr stark

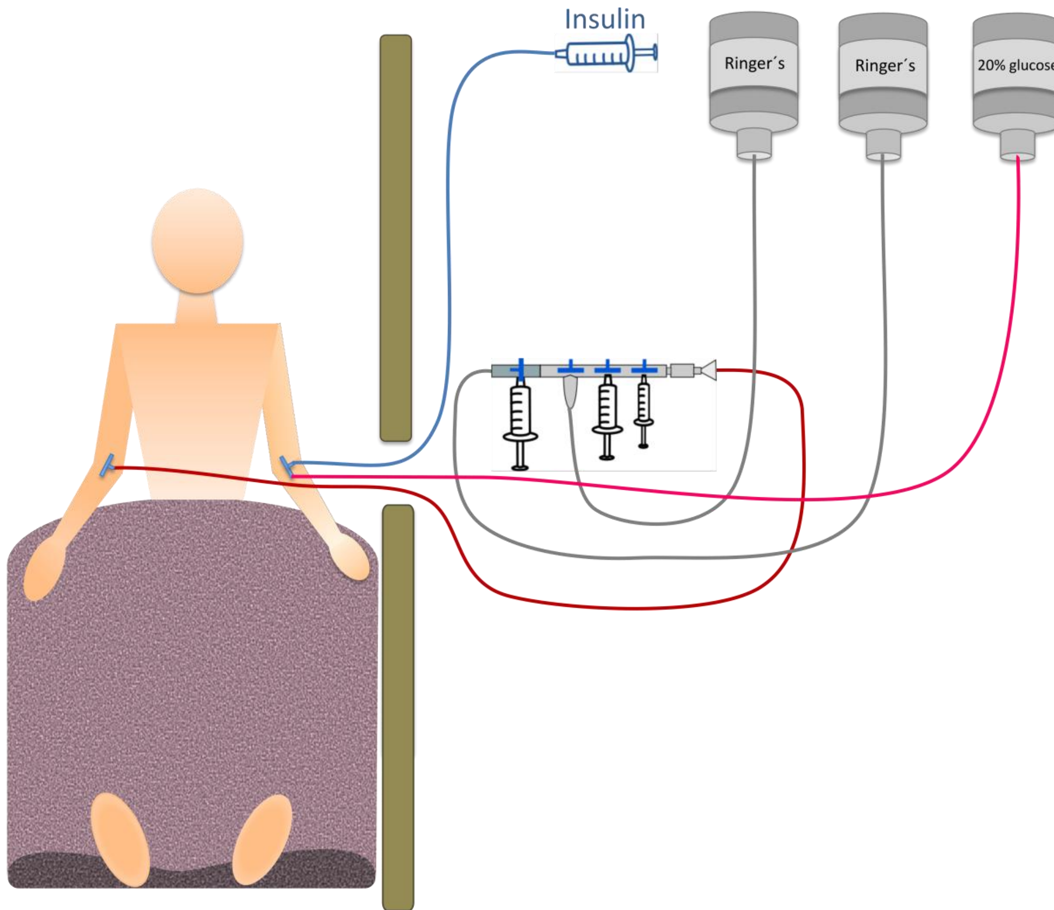
Überhaupt nicht stark — …nach Süßem — Sehr stark

Überhaupt nicht stark — …nach Herzhaftem — Sehr stark

Supplemental material B

B.1. Blood sampling system

In order to make blood collection as well as infusions of insulin and glucose during the HEC highly standardized and comfortable the blood tubing system described below was applied.
In practical terms, the tubing system allows blood glucose measurements and storage of the technical devices, i.e. clamp tower and EMG system, out of the participant´s sight in a separate control room adjacent to the experimental room, thereby keeping participants stress levels at a minimum. In brief, blood collection as well as administration of glucose and insulin infusions are accomplished via individual tubes running from the control chamber to the participant via a designated passage in the wall. Glucose and insulin are infused via separate tubes and along separate channels of a three-way stopcock connected to the permanent catheter of one arm. For blood collection a third specialised pressure tube is connected to the catheter of the other arm. The tubes for glucose infusion and blood collection are linked to a three-gang manifold, which is fixed to the edge of a table in the control chamber. Two syringes of 5 ml and 2 ml, respectively, are attached to separate ports of the manifold for blood collection, from the remaining channel a tube connected to Ringers solution provides continuous rinsing of the system and moderate volume compensation. Furthermore, the manifold is extended by an additional three-way stopcock with a 10 ml syringe attached to thoroughly flush the tubing system directly after blood collection.



Supplemental figure B.1. Schematic illustration of the system for continuous blood sampling during the HEC. Glucose (pink) and insulin (light blue) are infused via separate tubes and along separate channels of a three-way stopcock connected to the permanent catheter of one arm. Blood is sampled from the other arm (red). The tubes for glucose infusion and blood collection are linked to a three-gang manifold, which is fixed to the edge of a table in the control chamber. Two syringes of 5 ml and 2 ml (middle and right syringes, respectively) are attached to separate ports of the manifold for blood collection, from the remaining channel a tube connected to Ringers solution (grey) provides continuous rinsing of the system and moderate volume compensation. Via a 10 ml syringe (left) attached to an additional three-way stopcock the tubing system is flushed directly after blood collection.

Supplemental material C

Supplemental table C.1. Fold-changes of target gene expression levels normalized to the mean of all control samples and Ct values of target genes (in brackets) as well as reference genes after standardized adaptation to thermoneutral 25.0°C as baseline and after adaptation (100 minutes) to BAT-activating moderate cold (16.0 °C, shivering excluded), respectively. Data are means±SEM. n=14 (obese men only).

	Basal (0')		After temperature adaptation (100')	
	Thermoneutrality	Before cold exposure	Thermoneutrality	Moderate cold
Target gene (fold-change)			(control sample)	
CPT1α	1.23±0.17 (32.1)	1.55±0.41 (32.1)	1.19±0.23 (32.9)	2.01±0.26* (32.1)
CPT1β	1.27±0.40 (33.1)	1.86±0.71 (33.1)	1.63±0.56 (33.8)	2.33±0.53 (32.2)
PDK4	1.64±0.45 (33.5)	1.99±0.60 (33.5)	1.55±0.45 (34.4)	2.66±0.48* (33.3)
SCL25A20	1.66±0.39 (32.5)	1.97±0.65 (32.5)	1.41±0.33 (33.3)	2.62±0.51* (32.4)
PPARα	1.18±0.17 (31.3)	2.39±1.29 (31.3)	1.16±0.19 (32.2)	1.77±0.21 (31.6)
FFAR4	1.27±0.40 (35.3)	1.86±0.71 (35.3)	1.63±0.56 (35.7)	2.33±0.53 (35.0)
G0S2	1.29±0.23 (33.3)	3.03±1.82 (33.3)	1.52±0.42 (34.3)	4.05±1.22* (32.8)
Reference gene (Ct)				
18S	14.54±0.32	14.19±0.34	14.81±0.35	14.88±0.23
ACTB	24.14±0.30	23.57±0.27	24.57±0.46	23.68±0.35*

* $p < 0.05$, thermoneutral vs moderate cold

Supplemental material D

Supplemental figure D.1. Cooling system used in Lübeck, Germany: ThermoFlash, Buchenberg, Germany. The ThermoFlash system was initially developed to allow riding motorbikes also during cold seasons, worn underneath the everyday clothes. The soft fleece suit has flexible silicon tubes sewed in through which the cooling/warming medium (water or water glycol-mix) circulates. A star-shaped distribution system at the inlet ensures consistent flow of the medium through 5 compartments of the suit (right and left upper and lower extremities, back compartment), thereby preventing temperature differences between compartments. Temperature of the medium (water) is controlled and fixed via a recirculation cooler. Recirculation cooler used in Lübeck: Julabo FC600 S, Seelbach, Germany

Cooling system used during PET/CT cooling protocol in Vienna, Austria: CoolShirt Systems, Stockbridge, Georgia, USA. CoolShirt systems provides cooling systems mainly for surgeons to be used in the operating room, where they are exposed to e.g. hot operating room lights. Garments contain more than 45 feet of medical grade capillary tubing securely stitched on the front and back of the shirt. They are connected to a compact cooling unit via insulated hose with quick, dry disconnects. The cooling unit contains ice, water and an internal pump that supplies cool water to the COOLSHIRT as it covers up to 40% of the body.'

https://www.surgicalproductsmag.com/product-release/2012/01/surgeon-cooling-system

Supplemental material E

Participant information and written informed consent

Probandeninformation

1. Allgemeine Informationen

Bitte lesen Sie diese Probandeninformation sorgfältig durch. Der betreuende Arzt dieser Studie wird mit Ihnen noch einmal die Studie besprechen. Bitte fragen Sie ihn, wenn Sie etwas nicht verstehen oder wenn Sie zusätzlich etwas wissen möchten.

Titel der Studie: „Braunes Fettgewebe: Einfluss auf den menschlichen Fett- und Glukosestoffwechsel“

Verantwortlicher Leiter/Träger der Studie: UKSH Campus Lübeck

Zentrale Kontaktstelle: Prof. Dr. med. Sebastian M. Schmid, UK-SH Campus Lübeck, Medizinische Klinik 1, Ratzeburger Allee 160, 23538 Lübeck, Tel +49 (0) 451 – 500 0 (-> pager 2210), E-Mail: Sebastian.Schmid@uk-sh.de

Sollten Sie weitere Fragen bezüglich der Studie haben, wenden Sie sich bitte an den aufklärenden Arzt oder direkt an die oben genannte zentrale Kontaktstelle.

2. Informationen zur Studie

Hintergrund und Nutzen der Studie

Erst seit den 90er Jahren gehen Wissenschaftler davon aus, dass sogenanntes braunes Fettgewebe auch nach dem Säuglingsalter noch beim Menschen existiert. In neueren Studien konnte bei erwachsenen Probanden sogar eine Veränderung der Stoffwechselaktivität von braunem Fettgewebe in Abhängigkeit von der Umgebungstemperatur nachgewiesen werden. Der direkte Einfluss von Kälte auf das braune Fettgewebe, und somit auf Parameter des Fett- und Glukosestoffwechsels, wurde bisher jedoch fast ausschließlich nur im Tierversuch untersucht. Ob beim Menschen durch Kälteexposition der Stoffwechsel ebenso beeinflusst wird, ist noch unklar.

Mit der Teilnahme an dieser Studie helfen Sie uns, die zugrunde liegenden Mechanismen der Verknüpfung von Kälteexposition, braunem Fettgewebe und menschlichem Energiestoffwechsel besser zu verstehen.

Das Ergebnis dieser Grundlagenforschung könnte in Zukunft zur Entwicklung neuer Therapiemöglichkeiten von Diabetes und Fettstoffwechselstörungen führen. Außer einer eingehenden körperlichen Untersuchung und einer Untersuchung Ihres Blutes besteht für Sie kein Eigennutzen an dieser Studie.

Ablauf der Studie

Es handelt sich um eine balancierte, within-person-Studie im Crossover-Design. Dies bedeutet, dass Sie an zwei Versuchssitzungen, bei denen Sie unterschiedlichen Temperaturbedingungen (25°C und 16°C) ausgesetzt werden, teilnehmen. Die Verteilung erfolgt balanciert. Diese Maßnahmen sollen ausschließen, dass das Versuchsergebnis durch Verhaltensweisen und subjektive Einschätzungen der den Versuch durchführenden Personen oder der Probanden verfälscht wird. Die Teilnahme an dieser Studie ist freiwillig.

Sie werden vor Beginn der Studie körperlich untersucht. Dazu gehört eine Anamnese, eine Blutabnahme zur Kontrolle u.a. der Blutzellen- und Gerinnungswerte, des Fettstoffwechsels, der Leber- und der Nierenfunktion und des Blutzuckers. Ausschlusskriterien sind aktuelle und chronische internistische oder neurologische Erkrankungen, Bluthochdruck, Angststörungen, Alkohol- und Nikotinmissbrauch, Leistungssport (z.B. Marathonläufer) und besondere Belastungssituationen psychischer und physischer Art. Weder bei Ihnen noch bei Ihren Eltern oder Großeltern darf ein Diabetes mellitus Typ 2 bekannt sein.

Sie dürfen in den vier Wochen vor Versuchsbeginn und während der Versuche nicht an anderen Studien teilnehmen, kein Blut spenden, keine Diät beginnen. Am Vortag der Versuche dürfen Sie sich keinen ungewöhnlich hohen und niedrigen Temperaturen (z.B. Eisbaden, Saunagang) aussetzen, keinen Sport treiben und auch sonst keine körperlich anstrengende Tätigkeit verrichten. Am Abend vor dem Versuchstag sollten Sie ihre letzte Mahlzeit nicht nach 20 Uhr einnehmen und nicht später als 22 Uhr zu Bett gehen. Am Versuchstag selbst sollten Sie sich gesund fühlen und **nüchtern** zum Versuchsbeginn erscheinen.

Sie nehmen an zwei Versuchssitzungen teil (s. Abbildung 1). Jede Sitzung erstreckt sich über etwa 7 Stunden. Zu allen Versuchssitzungen erscheinen Sie **bitte pünktlich** um **7.50 Uhr** im Institut für Neuroendokrinologie, UK-SH, Campus Lübeck, Haus 50, 2. Obergeschoss.

1. Versuchssitzung
08:00 – 10:00 Vorbereitung & Anpassung an die Temperaturbedingung (25°C) 10:00 – 10:45 indirekte Kalorimetrie 10:45 – 11:30 Anpassung an die Temperaturbedingungen (25°C oder 16°C) 11:30 – 12:30 indirekte Kalorimetrie 2 12:30 – 13:30 Glukosetoleranztest 13:30 – 16:00 hyperinsulinämischer euglykämischer Clamp 16:00 – 16:30 Abschlussuntersuchung
2 Wochen Pause
2. Versuchssitzung
08:00 – 10:00 Vorbereitung & Anpassung an die Temperaturbedingung (25°C) 10:00 – 10:45 indirekte Kalorimetrie 10:45 – 11:30 Anpassung an die Temperaturbedingungen (25°C oder 16°C) 11:30 – 12:30 indirekte Kalorimetrie 2 12:30 – 13:30 Glukosetoleranztest 13:30 – 16:00 hyperinsulinämischer euglykämischer Clamp 16:00 – 16:30 Abschlussuntersuchung

Abbildung 1: Übersicht Versuchsablauf

Bei den Versuchssitzungen erfolgt nach Ihrer Ankunft im Schlaflabor die Durchführung verschiedener psychologischer (Erhebung von Wahrnehmung und Befindlichkeit) Tests. Außerdem werden zwei Venenverweilkatheter angelegt, die es uns ermöglichen, immer wieder Blut abzunehmen ohne Sie erneut stechen zu müssen

Anschließend erfolgt die Messung des Sauerstoffverbrauchs und der Kohlenstoffdioxidproduktion mithilfe einer Atemmaske, die Sie für ca. 1 Stunde tragen müssen. Die dabei gewonnenen Ergebnisse ermöglichen uns die Berechnung Ihres Ruheenergieverbrauchs (indirekte Kalorimetrie).

Danach erhalten Sie zunächst eine Zucker-(Glucose)-Infusion (Glukosetoleranztest) und im weiteren Verlauf eine gleichzeitige Infusion von Insulin und Zucker (Glucose) in einer Ihrem Körpergewicht angepassten Menge über einen Venenzugang. Über einen zweiten Zugang messen wir alle 5 Minuten Ihren Blutzucker und halten diesen auf einem normwertigen Niveau von 90 – 95 mg/dl. In diesem Verfahren (sog. „Glukose-Clamp-Verfahren") wird Ihr Blutzucker im physiologischen, d.h. normwertigen Bereich gehalten. Anschließend wird die Infusion über die Vene beendet und der Blutzuckerspiegel für weitere 30 Minuten überwacht. Während der Infusion von Zuckerlösung und Insulin ist jederzeit ein Arzt im Hintergrund, der bei Zeichen von Unverträglichkeit oder bei sonstigen Beeinträchtigungen innerhalb von kurzer Zeit einschreiten kann.

Sie tragen während der gesamten Zeit des Versuches einen Ganzkörperanzug zur Einstellung der jeweiligen Versuchstemperatur.

Es werden Blutproben zur Messung des Blutzuckerspiegels und verschiedener Hormone entnommen. Insgesamt werden Ihnen ca. 250 ml Blut im Verlauf einer Sitzung abgenommen (zum Vergleich: eine Blutspende entspricht ca. 500 ml Blut).

Darüber hinaus geben Sie jeweils drei Urinproben ab. Nach einer Wiederholung der Tests zur Wahrnehmung und Befindlichkeit ist die Versuchssitzung gegen 16:30 Uhr beendet.

Zusammenfassend besteht die Studie für Sie als Proband aus zwei Sitzungen im Abstand von mindestens zwei Wochen mit jeweils einem euglykämischen Clamp, einer Atemgasmessung und der Entnahme von jeweils 250 ml Blut, sowie der Abgabe von drei Urinproben pro Sitzung.

3. Risiken der Studie

Durch eine gleichzeitige Gabe von Insulin und Zuckerlösung werden wir Ihren Blutzuckerspiegel während des **hyperinsulinämischen euglyklämischen Clamps** im physiologischen, d.h. **normwertigen Bereich** halten. Eine Unterzuckerung ist zwar theoretisch möglich, das Auftreten derselben jedoch sehr unwahrscheinlich, da wir Ihren Blutzuckerspiegel in engem zeitlichem Abstand bestimmen und sofort auf mögliche Abweichungen aus dem Normbereich reagieren. Eine theoretisch mögliche Unterzuckerung ruft in Ihrem Körper eine Gegenregulation hervor, die Sie möglicherweise als Stress erleben (möglich ist: Zittern, Hunger, Herzklopfen, Schwitzen, Nervosität u.a.). Bei empfindlichen Personen mit einer Neigung zu Krampfleiden kann eine schwere Unterzuckerung einen Krampfanfall auslösen. Bei schweren Unterzuckerungen ist ein sehr geringes theoretisches Risiko einer Nervenzellschädigung im Gehirn verbunden, die allerdings erst bei lang andauernden und sehr tiefen Unterzuckerungen auftreten kann. Die Insulin- und Zuckerinfusion wird während des Versuchs genau geregelt, wobei die Blutzuckerwerte in Abständen von 5 Minuten und bei Bedarf noch engmaschiger kontrolliert werden.

Die Risiken der **Blutentnahme** und Infusion von Zuckerlösung in die Vene beinhalten u.a.: Fehlpunktionen mit Verletzungen von Nerven und Gefäßen mit Bildung von Blutergüssen, Venenentzündung, ggf. Armvenenthrombose, Weichteilentzündungen nach Austreten der Glukoseinfusion aus einem verletzten Gefäß. Diese Nebenwirkungen treten aber bei sachgerechter Durchführung sehr selten auf.

Die Messung des **Ruheenergieverbrauchs** über die indirekte Kalorimetrie wird von geschultem Personal durchgeführt. Sie werden dabei durchgehend überwacht, sodass Komplikationen, sehr unwahrscheinlich sind.

Die **Versuchstemperatur von 16 °C** wurde von Probanden einer vorausgegangenen Studie als moderat empfunden. Die Körpertemperatur wird zudem ständig überwacht, wodurch das Risiko einer Unterkühlung sehr gering gehalten wird.

4. Datenschutzrechtliche Informationen

Für die Datenverarbeitung verantwortlich ist Dr. med. Sebastian Schmid, Arzt am UKSH, Campus Lübeck. Die Datenerhebung erfolgt zum Zweck des oben genannten Studienziels, also der Aufklärung von Kälteexposition auf die Regulation des Fett- und Glukosestoffwechsels. Aus der Voruntersuchung erhalten wir persönliche Daten und Untersuchungsbefunde, aus den Testverfahren und Blutentnahmen während des Versuchs erhalten wir verschiedene Daten über die Reaktion Ihres Körpers auf Kälteeinwirkung, wie z.B. Hormonkonzentrationen und Energieverbrauch.

Diese Daten werden in pseudonymisierter Form, d.h. ohne direkten Bezug zu Ihrem Namen, elektronisch gespeichert und ausgewertet. Die Bestimmungen des Datenschutzgesetzes werden eingehalten. Zugriff auf Ihre Daten haben nur Mitarbeiter der Studie. Diese Personen sind zur Verschwiegenheit verpflichtet. Eine Weitergabe der persönlichen Daten an Dritte ist nicht vorgesehen. Die Daten sind vor fremdem Zugriff geschützt. Die personenbezogenen Daten werden nach Beendigung der Studie anonymisiert, soweit gesetzliche Vorgaben nicht längere Archivierungspflichten vorsehen.

5. Hinweise für den Versuchsteilnehmer

Die Teilnahme an dieser Studie ist freiwillig. Sie können jederzeit ohne Angabe von Gründen die Teilnahme an der Studie beenden, ohne dass dadurch Nachteile für Sie entstehen.

Am Tag vor den Versuchstagen sollten Sie keinen Sport treiben oder andere anstrengende körperliche Aktivitäten verrichten. Die letzte Nahrungsaufnahme vor Versuchsbeginn sollte ein reguläres Abendessen nicht nach 20:00 Uhr sein. Am Morgen des Testtages erscheinen Sie dann bitte um 8.00 Uhr zum eigentlichen Versuch. Bitte achten Sie darauf, im Anschluss an den Versuch etwas zu essen, damit sie fit und verkehrstüchtig sind.

6. Aufwandsentschädigung

Für Ihre Teilnahme an dieser Studie erhalten Sie nach Beendigung Ihrer zwei Versuchssitzungen eine Aufwandsentschädigung von insgesamt 150,- €.

Vielen Dank für Ihr Interesse.

Einverständniserklärung

zur klinisch-experimentellen Studie

„Braunes Fettgewebe: Einfluss auf den menschlichen Fett- und Glukosestoffwechsel“

Ich bestätige hiermit durch meine Unterschrift, dass ich die Informationsschrift zur Studie erhalten, gelesen und die geplanten Untersuchungen verstanden habe. Ich hatte Gelegenheit alle meine Fragen zu stellen. Diese wurden zufriedenstellend und vollständig beantwortet.

Ich wurde ausführlich – mündlich und schriftlich – über das Ziel und den Verlauf der Studie, Chancen und Risiken der Behandlung, meine Rechte und Pflichten, den mir zustehenden Versicherungsschutz und die Freiwilligkeit der Teilnahme aufgeklärt.

Ich erkläre hiermit meine Teilnahme an der oben genannten Studie und dass ich in der Zeit vier Wochen vor und während der Studie nicht an anderen Studien teilnehmen und kein Blut spenden werde.

Ich hatte ausreichend Zeit, mich gegen die Teilnahme an der Studie zu entscheiden und willige hiermit in die Teilnahme ein. Ich wurde darauf hingewiesen, dass meine Teilnahme freiwillig ist und dass ich jederzeit ohne Angabe von Gründen zurücktreten kann und/oder die Vernichtung meiner Blutproben und erhobenen Daten verlangen kann ohne dass mir dadurch Nachteile entstehen.

Meine Daten und die mir entnommenen Blutproben werden ausschließlich für die oben genannte Studie verwendet und nicht an Dritte weitergegeben. Es gelten die Richtlinien des Datenschutzes und der ärztlichen Schweigepflicht. Ich wurde über meine Datenschutzrechte informiert. Mit der Erhebung, Verarbeitung und Speicherung meiner Daten, sowie der Übermittlung im Rahmen der Studie bin ich einverstanden. Tritt im Rahmen der Studiendurchführung ein Schaden auf, der den Studienteilnehmern durch das schuldhafte Verhalten eines Beschäftigten des Universitätsklinikums Schleswig-Holstein (UKSH) zugefügt wurde, haftet die gesetzliche Haftpflicht des UKSH.

Proband:

Name, Vorname Datum Unterschrift

Betreuender Studienarzt:

Name, Vorname Datum Unterschrift

Overview of Contributions

All the experiments presented in this doctoral thesis were conducted under first supervision of Prof. Dr. Sebastian M. Schmid (Department of Internal Medicine I, Section of Endocrinology & Diabetes, University Hospital Schleswig-Holstein, Lübeck, Germany) and second supervision of Prof. Dr. Nico Bunzeck (Department of Psychology, University of Lübeck, Germany).

Comprehensive expert consultation and support was also provided by Dr. Alexander K. Iwen (Department of Internal Medicine I, Section of Endocrinology & Diabetes, University Hospital Schleswig-Holstein, Lübeck), who furthermore substantially contributed to the published manuscript 'Cold-induced brown adipose tissue activity alters plasma fatty acids and improves glucose metabolism in men'.

Data collection for the normal weight study cohort was performed by the medical students Melanie Cassens and Maren Waltl.
Data for the obese study cohort were acquired together with the medical student Leonie Rademacher.

Laboratory measurements were conducted by Martina Grohs and Susanne Behling.
Blood lipids and catecholamines were determined at the external service laboratory facility LADR (LADR GmbH, Medizinisches Versorgungszentrum, Dr. Kramer & Kollegen, Gessthacht, Germany).

Data preparation was done by Melanie Cassens, Maren Waltl, Leonie Rademacher and myself. All statistical analyses of the acquired hormonal and behavioural data were performed by myself.

The analysis of transcriptional regulation of gene expression was conducted at the Institute of Metabolic Research (IMR), University of Cambridge (UK), in cooperation with Prof. Sadaf Farooqi. Under supervision of Fleur Talbot and Dr. Edson Mendes De Oliveira I conducted the analyses (RNA extraction, cDNA reverse transcription and qPCR) in the laboratory and analysed the data.

Acknowledgements

First, I thank my principle investigator Prof. Sebastian Schmid for his supervision and support as well as all the opportunities I have received to meet and work together with other researchers, join international conferences, and spend a very interesting and inspiring research stay in Cambridge, UK.
Furthermore, I thank my second supervisor Prof. Nico Bunzeck for his support, the helpful comments and interesting exchange throughout the duration of this research project.

My thanks moreover go to Dr. Alexander Iwen for his continuous valuable advice and help with the interpretation of our hormonal data, also for the very interesting discussions we´ve had.

Also, I owe great thanks to the GRK 1957 for providing the doctoral students with many interesting and instructive events, workshops and seminars as well as for the help and support throughout the doctoral phase.

My warmest gratitude goes to my colleagues Leonie Rademacher and Anne Windjäger, who started together with me working on this exciting project and provided so much support, enthusiasm and friendship during the past 3 years!

Also I thank Maren Waltl and Melanie Noll for sharing the experimental procedures with me and for collecting the data of the lean study cohort.

Thank you, too, everyone else who helped me with advice and/or contributed by any other means to my thesis: Dr. Alaa Othman, Martina Gros, Susanne Behling and Arkan Al-Zubaidi.

Moreover, I´m very greatful to Prof. Sadaf Farooqi for the opportunity to join her research group and conduct research at her laboratory in Cambridge, also to all the team members of the 'GOOS' project, in particular Fleur Talbot, Edson Mendes De Oliveira, Elana Henning, Bas Brouwers, Lukas Stadler and Julia Keogh. I certainly enjoyed my time in your group!

Last but not least, I thank my family, in particular my parents Bärbel and Gerhard Backhaus, my partner Nick Wood together with his parents Inge and William Graham Wood, and finally my close friends for all your ongoing support, encouragement, ideas, interest, patience and love throughout this very intense times!