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‘Neuroendocrine mechanisms controlling BAT-thermogenic activity in humans’

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Abbreviations

AC	Adenylate cyclase
ATP	Adenosine triphosphate
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body Mass Index
cAMP	Cyclic AMP
CE	Cold exposure
CPT1	Carnitine palmitoyltransferase 1
DIT	Diet-induced thermogenesis
ECM	Extra cellular matrix
FA	Fatty acid
FFA	Free fatty acid
FM	Fat mass
fT3	Thyroxine free T3
HF	High-frequency
HPA-axis	Hypothalamus-pituitary-adrenal axis
HPT-axis	Hypothalamus-pituitary-thyroid axis
HRV	Heart rate variability
HSL	Hormone sensitive lipase
INI	Intranasal insulin
ICBT	Indirect core body temperature
IRT	Infrared thermography
IU	International unit
LF	Low-frequency
LF/HF	Ratio of LF and HF power components
LPL	Lipoprotein lipase
MGL	Monoacylglycerol lipase
MSKT	Mean skin temperature
MRI	Magnetic resonance imagining
mRNA	Messenger RNA
NE	Norepinephrine

NEFA	Non-esterified fatty acid
NST	Non-shivering thermogenesis
NW	Normal weight
OB	Obese
OW	Overweight
OT	Outside temperature
PET/CT	Positron emission tomography–computed tomography
PA	Physical activity
PKA	Protein kinase
PLC	Placebo
PAO	Preoptic area
qPCR	Quantitative polymerase chain reaction
RP	Reference point
ROI	Regions of interest
SCVT	Supraclavicular temperature
SD	Sleep duration
SNS	Sympathetic nervous system
SOPs	Standard operating procedures
SPARC	Secreted protein acidic reach cysteine
SRS	Symptom rating scale
TCA	Cycle tricarboxylic acid cycle
TG	Triglycerides
TN	Thermoneutrality
TSH	Thyroid stimulating hormone
UCP1	Uncoupling protein 1
VAS	Visual analogue scale
WAT	White adipose tissue
WHO	World Health Organization

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Abstract

Obesity is a common, yet unresolved burden affecting our society. Obesity is associated with numerous negative health effects, such as high blood pressure, dyslipidemia, diabetes and heart disease. In this sense, new therapeutic approaches are required. Brown adipose tissue (BAT), in response to proper stimuli such as cold environment, has the unique ability to generate heat through the energy consuming process of the non-shivering thermogenesis. Cold-induced BAT is associated with improved metabolic profile, including increased insulin sensitivity, glucose tolerance, and lipid homeostasis. Some factors have been proposed to modulate the development and function of BAT. However, factors associated with active BAT and possibly modulating its basal activation still need to be fully characterized.

With this work, we aimed at investigating the potential role of intranasal insulin (INI) as pharmacological activator of BAT. INI did not enhance BAT-thermogenic metabolism, as shown by unchanged supraclavicular (SCV) temperature and hormonal parameters as compared to placebo (PLC). However, INI significantly improved homeostatic regulation of satiety since post prandial ghrelin levels were reduced and subjective feelings of satiety increased following a standardized meal challenge. Together, these findings point toward a crosstalk between increased brain insulin signaling and the gastrointestinal system, possibly suggesting a novel mechanism for the regulation of satiety mediated by INI at the brain-gut axis level, however, independently from BAT activation.

Moreover, we aimed at assessing the relation of basal BAT-thermogenic activity with potential neuroendocrine and environmental factors, modulating sympathetic nervous system activity. By employing infrared thermography as non-invasive technique, we measured changes in body skin temperature within delimitated anatomical points, i.e., SCV, as an area co-locating with BAT, compared to the parasternal region, as reference point. We found that outside temperature, fat mass, sleep duration and noradrenaline are important predictors of basal BAT-thermogenic activity, showing a modulatory effect of ~ 44%, 18%, 4% and 3%, respectively.

Additionally, we explored the potential role of the adipokine SPARC as candidate factor promoting the decreased BAT-thermogenic response associated to obesity. To this aim, we performed transcriptomic analysis of human biopsies from WAT and BAT. We found that SPARC mRNA expression was upregulated in BAT of obese humans, as compared to expression levels in BAT of normal weight controls. Moreover, SPARC expression in BAT was positively associated with BMI and negatively with UCP1, collectively supporting a negative effect of SPARC on BAT function associated with higher BMI. Also, circulating plasma levels of SPARC were quantified in obese subjects during a personalized cooling protocol. Upon steady-state non-shivering cold exposure to ~

16°C, SPARC concentrations were increased as compared to thermoneutrality (25°). In addition, SPARC during cold exposure was negatively associated with noradrenaline and positively with insulin, respectively. Taken together, our explorative data on SPARC might implicate a potential link between upregulated SPARC in obesity and impaired-thermogenic capacity of BAT.

In summary, the identification of exogenous and endogenous factors modulating BAT activity as reported here provide the rational for future mechanistic studies to elucidate their potential in the activation or suppression of BAT. Tackling obesity by implementing the current therapeutic strategies with novel approaches targeting factors able to leverage human BAT thermogenic capacity might be a promising option.

Zusammenfassung

Adipositas ist eine immense Herausforderung für unsere Gesellschaft und mit zahlreichen negativen Auswirkungen auf die Gesundheit verbunden, wie beispielsweise Bluthochdruck, Fettstoffwechselstörungen und Typ 2 Diabetes Mellitus, was neue bzw. innovative therapeutische Ansätze erfordert. Braunes Fettgewebe (BAT, Brown Adipose Tissue) ist durch die Fähigkeit zur sog. zitterfreien Thermogenese in der Lage, Wärme zu erzeugen, wenn Reize wie eine kalte Umgebung einwirken. Kälte-aktiviertes BAT wird mit einem verbesserten metabolischen Profil in Verbindung gebracht, einschließlich gesteigerter Insulinsensitivität und Glukosetoleranz sowie verbessertem Lipidstoffwechsel. Es werden mehrere Faktoren diskutiert, die die Entwicklung und Funktion von BAT modulieren.

Im ersten Teil der vorliegenden Arbeit wurden daher die Effekte von intranasal appliziertem Insulin (INI) als pharmakologischen Aktivator von BAT untersucht. Es zeigte sich, dass im Vergleich zu Placebo (PLC) INI nicht den BAT inherenten thermogenen Metabolismus aktivierte, gemessen über die supraklavikuläre (SCV) Temperatur bestimmt via Infrarothermographie. Allerdings verbesserte INI die homöostatische Regulation von Sättigung, gemessen über eine postprandiale Reduktion der Ghrelinspiegel bei gleichzeitigem Anstieg des subjektiven Sättigungsgefühls nach einer standardisierten Mahlzeit. Diese Befunde implizieren eine Interaktion zwischen erhöhter Insulinsignalwirkung im Gehirn und dem gastrointestinalen System hin, was möglicherweise auf einen neuartigen Mechanismus zur Regulation der durch INI vermittelten Sättigung auf der Ebene der Gehirn-Darm-Achse hindeutet. Dies unabhängig von einer BAT-Aktivierung.

Im zweiten Teil dieses Projektes sollte die Beziehung zwischen der basalen thermogenen Aktivität von BAT und potenziellen neuroendokrinen und Umwelt- Faktoren quantifiziert werden. Mittels nicht-invasiver Infrarot-Thermografie wurden dafür die Veränderungen der Hauttemperatur innerhalb definierter anatomischer Punkte erfasst: i) SCV als Bereich, der mit BAT co-lokalisiert ist; ii) parasternale Region als Referenzpunkt. Es zeigte sich bei normgewichtigen Probanden, dass die durchschnittliche Außentemperatur der vorangegangenen sieben Tage, die Körperfettmasse, die Schlafdauer und zirkulierende Noradrenalinkonzenentrationen signifikante Prädiktoren der basalen thermogenen Aktivität von BAT mit einer erklärten Varianz von 44%, 18%, 4% und 3% (Gesamt: 69%) sind.

Im dritten Teil der Arbeit wurde die potenzielle Rolle des Adipokins SPARC bei Aktivierbarkeit von BAT im Zusammenhang mit Adipositas untersucht. Zu diesem Zweck führten wir eine Transkriptom-Analyse in humanem weißem Fettgewebe (WAT) und BAT durch. Die SPARC-mRNA Expression in BAT war bei Probanden mit Adipositas im Vergleich zum Expressionsniveau bei

normalgewichtigen Personen signifikant hochreguliert. Zudem war die SPARC Expression in BAT positiv mit dem BMI und negativ mit UCP1 assoziiert, was insgesamt einen negativen Effekt von SPARC auf die Funktion von BAT in Verbindung mit einem erhöhten BMI suggeriert. Darüber hinaus wurden zirkulierende Plasma SPARC bei Personen mit Adipositas während eines personalisierten Kühlungsprotokoll quantifiziert. Bei einer stabilen und zitterfreien Kälte-Exposition von 16°C waren die SPARC-Konzentrationen im Vergleich zur Thermoneutralität (25°) signifikant höher. Darüber hinaus war SPARC nach Kälteexposition negativ mit Noradrenalin und positiv mit Insulin assoziiert. Zusammenfassend unterstreichen diese explorativen Daten zu SPARC einen möglichen Zusammenhang von hochreguliertem SPARC bei Adipositas und einer beeinträchtigten thermogenen Kapazität von BAT.

Insgesamt könnte die Identifizierung exogener und endogener Faktoren, welche die Aktivität von BAT beeinflussen, die Grundlage für zukünftige Studien zur funktionellen Regulation von BAT bei Adipositas bilden. Die Therapie der Adipositas durch Kombination der derzeitigen therapeutischen Strategien mit neuartigen BAT-regulierenden Ansätzen, könnte hier einen vielversprechenden Ansatz darstellen.

I. Introduction

1.1 The epidemic of obesity and the need for novel therapeutic strategies

Currently, the prevalence of obesity is estimated to be 24% in Europe, i.e., almost one in four people is obese[1].

The World Health Organization (WHO) defines obesity as abnormal or excessive fat accumulation that presents a risk to health[2]. Body mass index (BMI), calculated as body weight (kg) divided by squared body height (m^2) quantifies and categorizes different weight status in adults. As defined by the WHO[2], BMI ranging from18.5 to 24.9 kg/m^2 indicates normal weight; BMI values between 25.0 and 29.9 kg/m^2 indicates overweight and BMI $\geq$30 obesity.

In the USA, the situation is even more dramatic, registering in 2015 an extraordinary increase in the adult obesity rate of more than 70%[3]. Accordingly, a report from 2019[4] showed an all-time high record of adult obesity rates of above 35% in nine U.S. states, two states more than just the year before. In sum, obesity rates have doubled among adults and more than tripled among children since the 1980s. This increasing rates are of high relevance, since obese individuals have a higher risk of developing chronic illnesses, including diabetes, hypertension and dyslipidemia, with life expectancy reduced from three to sixteen years[5,6].

Given the fundamental concept that a chronic positive energy balance, in which energy intake exceeds energy expenditure, leads to the development of obesity[7], conservative treatment programs focusing on reduced caloric intake along with increased physical activity have been broadly proposed to tackle obesity. Bariatric surgery has been introduced as treatment for obesity, leading to significant weight-loss and remission of obesity-related diseases including type 2 diabetes[8]. However, the invasiveness of the surgery itself as well as the severe post-operative complications make it difficult to consider bariatric surgery as general recommendation to address obesity[9].

Considering the above-mentioned escalating obesity rates worldwide, it becomes obvious that the current proposed conservative options, and the more recent treatment with surgery, are - at least in part - either ineffective[7,10] or too invasive. Hence, novel comprehensive strategies to finally counteract the growing epidemic are urgently needed.

In this scenario, brown adipose tissue (BAT) has lately attracted enormous attention as novel therapeutic target for metabolic diseases in humans[11,12]. BAT is a highly specialized type of fat tissue, capable to dissipate energy. Thus, BAT holds promising metabolic functions and substrates burning potential[13,14]. However, although maximizing BAT function seems an exciting idea to tackle obesity, currently, a number of objectives within BAT biology still warrant additional research to achieve deeper insights on how BAT's intrigue metabolism works. In this context, it is important to emphasize

that BAT is a dynamic organ, with metabolic phenotype varying in response to numerous stimuli[15]. Accordingly, factors both endogenous[16,17], i.e., molecules secreted by BAT itself as well as by other metabolic organs and exogenous[18,19], i.e., environmental and lifestyle conditions have been proposed as critical - directly or indirectly - modulators of BAT recruitment and activity.
It seems therefore realistic to believe that characterizing these factors and their modulatory roles on BAT-thermogenic function will help to identify novel comprehensive strategies to counteract obesity and improve metabolic profile in humans.

1.2 The regulation of body energy homeostasis is a complex system

Te energy metabolism is very complex, regulated by homeostatic as well as hedonic systems, together with individual predisposition such as epigenetic factors. The homeostatic system is regulated by the hypothalamus. Implicit in this regulatory system is a large number of metabolically relevant molecules secreted peripherally[20]. These molecules interact with specific regions within the hypothalamus, producing sensations of appetite or satiety. The integrated activity of these peripheral signals mainly takes place between the ventromedial hypothalamic (VMH) area, identified as the satiety centre, and the lateral hypothalamic (LH) area, including the arcuate nucleus, identified as appetite-stimulating centre[21,22]. Within the arcuate nucleus, different neuronal populations interplay producing orexigenic neuropeptides, such as the neuropeptide Y and agouti-related protein, as well as anorexigenic neuropeptides, such as proopiomelanocortin. The LH receives information from the arcuate nucleus and other appetite stimulating regions and transmits signals to the cerebral cortex as well as to the nucleus of the solitary tract. Examples of neuropeptides released in the LH are the melanin-concentrating hormone and orexin[22]. Concomitantly, the VMH receives coordinate projections from the paraventricular nucleus, LH and dorsomedial hypothalamic nucleus (DMH). Moreover, VMH is characterized by a high expression of leptin receptors[23]. Accordingly, anorexigenic hormones develop their effects primarily within the DMH[24].
From the periphery, the hypothalamic centers receive orexigenic and anorexigenic signals from the gastrointestinal tract as part of a short-term regulation[25]. These transmissions are mediated by, for instance the ‘hunger hormone’ ghrelin as well as by various satiety signals, including glucagon-like-peptide 1, cholecystokinin, peptide YY, pancreatic polypeptide and oxyntomodulin, respectively. On the other hand, the ‘satiety hormone’ leptin, which is mainly secreted from the adipocytes, is involved in a longer-term control of food intake[20].
The two hormones most closely associated with energy homeostasis leading to sensations of hunger and satiety are ghrelin and leptin, respectively. Ghrelin, identified in 1999 as first appetite-stimulating

hormone[26], increases during the fasted sate to stimulate hunger and decreases with feeding. Leptin, as adipocyte-released hormone, signals the hypothalamus regarding energy storage within the body[27]. The hedonic system, or reward-associated, is the other complementary drive that regulates energy metabolism[28]. This regulation takes place within the ventral tegmen area which highly expresses for dopaminergic neurons projecting to the nucleus accumbes of the ventral striatum. The reward-associated regulation is significantly influenced by the availability and taste of the food, increasing the desire to consume highly palatable foods even during periods of relative energy abundance[29].

1.3 Adipose tissue and its shades in obesity

It is well accepted that most mammals, including humans, possess - at least - three different types of adipose tissues: Brown, white and beige (or brite, brown within white fat), which differ in origin, appearance, functions and impact on whole-body metabolic health[30,31]. To provide a comprehensive characterization of each of these fat types is behind the aim of this work, where the main investigative focus has been on the 'classical' brown fat. However, it is important to recognize that the three fat types are part of an integrated and highly plastic organ. Therefore, in order to fully understand BAT, insights and notions on the factors responsible for their interplay as well as for their differences are required and will be, consequently, briefly introduced.

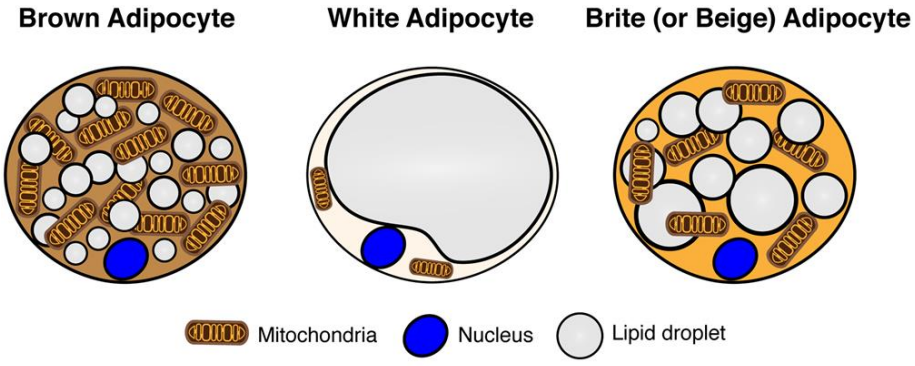
1.3.1 Not all fat cells were born equal, few were born to burn: A morphological and functional classification

Adipocytes are mesenchymal cells specialized in storing large quantities of lipids in the cytosol as lipid droplets. Adipocytes are found in subcutaneous and visceral adipose tissues, as well as in many smaller depots within or surrounding organs. Examples include the adipose tissue in the bone marrow and the one associated with the kidneys and the adrenal gland[32].

Traditionally, adipocytes have been classified as white and brown. Conventional white adipocytes are large unilocular cells, possessing few mitochondria and no uncoupling protein 1 (UCP1). Importantly, white fat cells are specialized in both, storage and release of fatty acids (FA) to continuously meet the body metabolic requirements during the rapid interchange between surplus and shortage of energy, respectively[33]. To fulfil these two main functions, white adipocytes are highly specialized in FA uptake and de novo lipogenesis, but also in FA release and mobilization via lipolysis. Additionally, white adipocytes have a remarkably endocrine capacity. WAT continuously signals by secreting molecules, named adipokines. Hence, WAT importantly contributes to the whole-body energy homeostasis[34], enabling a well-organized crosstalk between the adipose tissue and other

peripheral metabolic organs, as well as with the brain[35]. Conventional brown adipocytes, at the contrary, contain many smaller lipid droplets which confer the classical multilocular BAT-phenotype to the tissue and are abundant in mitochondria and UCP1[13]. UCP1 is highly expressed in the inner mitochondrial membrane, where it uncouples oxidative phosphorylation from the classical production of adenosine triphosphate (ATP) via regulated proton leak. As a result, these adipocytes are specialized in oxidation, rather than storage of FA. Concomitantly, brown adipocytes are responsible of converting metabolic energy into heat, which is then released from the organism. This process is known as non-shivering thermogenesis (NST) and largely under adrenergic control[13]. Accordingly, BAT is richly innervated by sympathetic nerve efferent fibers and highly vascularized. The increased mitochondrial density and vascularization give the typical brownish color to these unique adipocytes[36].

Recently, a third type of adipocyte, referred to as brown-like (or beige/brite) adipocyte, has been shown to be inducible in rodents' WAT in response to appropriate stimulations, i.e., β-3 receptor agonist treatment[37–39] or upon physiological stress, i.e., prolonged cold[39,40]. This within-adipocytes inducible process has been termed 'browning' of WAT[41]. Similar to brown adipocytes, brown-like adipocytes are rich in mitochondria and UCP1+ and, therefore, partly responsible for NST. The key morphological differences between brown, white and beige fat cells are illustrated in Figure 1[42]. Interestingly, in rodents, the inguinal fat depot (often referred to as the subcutaneous depot in mice) has been in particular demonstrated to be susceptible to browning. Visceral fat seems more resistant to undergo such morphological and functional switch, possibly owning to the decreased sympathetic innervation and β-adrenergic receptor density of this depot[40]. In light of this promising evidence, this third 'shad' of adipocyte has recently attracted considerable interest as a mean to further manipulate WAT energy metabolism. Accordingly, intensive research is currently going on in order to discover effective and feasible interventions to trigger the morphological and metabolic switch between adipocytes, possibly resulting in increased substrates utilization and whole-body energy expenditure.



	Brown	*White*	*Brite/beige*
UCP1 Expression	Positive	Negative	Positive
Mitochondrial Density	High	Low	Medium
LD Morphology	Multi-locular	Uni-locular	Multi-locular
Primary Function	Thermogenesis Endocrine	Energy storage Endocrine	Thermogenesis? Endocrine?

Figure 1. Schematic classification of morphological and general characteristics of brown, white and brite adipocytes (according to reference 42).

A quite controversial debate is still in place regarding pathways of origin and development of each type of adipocyte[43]. Initially, it was believed that the morphological variation between white and brown adipocytes would be the consequence of uncommon origin lineages. According to this theory, the latter shares a common myogenic factor 5+ (myf5+) expressing progenitor with skeletal muscle cells. This would consequently lead to two different morphological, and therefore, functional roles of the adipocytes[44]. However, this theory has lately been challenged by findings from recent studies suggesting that a subset of white fat cells could also originate from myf5+ cells[45,46]. However, the pathways that specify for brown instead of white adipocyte developmental lineage are not fully clear. Moreover, BAT has been shown to be composed of both canonical brown and specialized beige adipocytes[47,48]. Together, one can conclude that, to date, the developmental relationships among adipocytes appear more complex than initially postulated, especially pointing out the relative importance of the fat depot's anatomical location and the environmental challenges to which the adipocytes undergo.

Regarding the origin and development of brite adipocytes, the scenario is also currently unclear, but two theories have been mainly suggested. According to the first one, brite adipocytes form de novo upon stimulation from a precursor cell pool[49]. The second theory argues that brite adipocytes interconvert from existing adipocytes between a dormant and active state depending upon the presence of stimulus, i.e., reduced or increased sympathetic activation, respectively[47,50,51]. Regardless of which of these two theories may hold true, it seems commonly accepted to consider browning as a highly reversible process[41,52]. However, a still unanswered question is whether all white adipocytes can become brite under certain conditions and stimuli or not. Related to that, it makes sense to wonder whether there is a specific white adipocyte lineage that does not possibly possess browning capacity and therefore does not become brite, even under browning-inducing conditions. Importantly, in humans, browning of WAT has been demonstrated only in patients with pheochromocytoma[53], i.e., with chronically high levels of noradrenaline, and in cancer cachexia[54]. Nevertheless, it remains unclear whether browning could occur in humans upon health and physiological conditions.

Overall, the adipose tissue's highly heterogeneity, in terms of origin, develop, morphology, and functions of the adipocytes should be addressed in a context-dependent manner, considering the roles of many mediating factors. Particularly in regard to the thermogenic-capacity of brown and brite adipocytes, factors such as the anatomical proximity to the sympathetic nervous system (SNS) outflows, type of stimulation, i.e., cold exposure (CE) vs. thermoneutrality (TN), as well as its duration, i.e., acute vs chronic, seem to play a major contribution in determining their phenotype and metabolic development. It is now accepted[16,55] that understanding the functional significance of the brown adipocytes' heterogeneity, together with the neuroendocrine and environmental factors modulating their plasticity, is pivotal to advance into novel therapeutic approaches, based at targeting the thermogenic-cells potential to counteract obesity and its metabolic sequelae.

1.4 Brown adipose tissue

BAT is a highly specialized fat depot found exclusively in mammals. It holds the unique capacity of metabolizing energy substrates ultimately dissipating them as heat through the process of NST, so called as it does not involve the contribution of muscle shivering[13]. BAT was first described in hibernating mammals, initially referred to as 'hibernating gland', and has since been extensively studied in varies species, especially in rodents. The thermogenic function of BAT was first identified in 1961[56], followed by the recognition of BAT as the main site of NST across several species[13], including humans, as demonstrated in 2009[11,12,57]. From an evolutionary point of view, BAT is likely to have played a critical role in survival of mammals by safeguarding them from stress related to

nocturnal and seasonal cold. This holds true particularly in the newborn state and arousal from hibernation. Accordingly, brown depots develop prenatally and are fully functional at the time of birth, playing a key role in avoiding hypothermia in newborns[58]. In human neonates and infants, brown depots are located mainly around internal organs, in interscapular and supraclavicular (SCV) regions as well as next to the carotid artery and jugular vein. While they are fully functional at birth, BAT seems to decline with age[59]. In line, BAT depots are only found in the SCV and perirenal regions, and in less amount, in the mediastinum fossa in human adults[47,60,61]. Although present in relatively small extent in adults (from 30 to 350g is estimated to be present in healthy middle-age subjects[63,64]), BAT is considered a key player at orchestrating the whole-body energy metabolism, particularly due to its specialized burning capacity[14,65]. In fact, BAT has the potential to produce more heat per unit mass than muscle tissue through its thermogenic function[66]. It has generally been estimated that 50g of active BAT contributes to 2.5 up to 5% of the resting metabolic rate in humans[67,68].

The main stimulus for both acute activation and chronic recruitment of BAT is noradrenaline, which is essentially involved in all functional aspects of the brown adipocytes[13,69]. Noradrenaline binds to its β_3-adrenergic receptor, located on the brown adipocytes surface, activating the enzyme adenylate cyclase. This, in turn leads to increased intracellular cyclic adenosine monophosphate (cAMP) and to the activation of protein kinase A (PKA). PKA is responsible for the activation of several lipases, namely adipocytes triglycerides lipase (ATGL), hormone sensitive lipase and monoacylglycerol lipase. These enzymes break down internal triglycerides (TG) and mobilize free fatty acids (FFA) as oxidative substrate[70,71] and increases expression of a thermogenic gene expression program that includes UCP1 mRNA[72]. Carnitine palmitoyltransferase 1, located at the outer mitochondria membrane, is responsible to shuttle FFAs into mitochondria following sympathetic stimulation. FFAs serve as fuel for β-oxidation inside the mitochondria.

Notably, however, how exactly brown adipocytes choose and utilize fuel remains an important open question[42]. Nevertheless, BAT appears to have a rather high flexible substrates utilization capacity. In fact, BAT seems to be able to switch between oxidizing internally stored TGs, released upon WAT lipolysis, and circulating FFAs, released from dietary nutrients during a fasting or fed state, respectively[73]. In line, the uptake of glucose and FFA from the circulation appears to be most critical during prolonged stimulus, such as chronic cold challenge[74–76], implying that human brown adipocytes utilize internal TG as first-choice substrate to respond to acute thermogenic needs. The general notion was that endogenously mobilized FAs directly trigger UCP1 activation[41,73,77]. However, recent findings demonstrating the necessary role of ATGL in BAT thermogenesis[78] have challenged the previous theory pointing out that the explicit activator of UCP1 still remains to be

unequivocally identify. Additionally, these recent findings seem to raise the interesting possibility that thermogenesis may rely on UCP1-independent mechanisms under certain circumstances[78,79].

CE is the most potent physiological stimulus of BAT activity so far. The specific increased sympathetic outflow towards BAT upon CE is well established[80,81]. Results on acute CE in humans suggest enhanced lipid metabolism driven by cold-activated BAT through an upregulation of genes involved in the β-oxidation and lipolytic pathways[82] (also according to preliminary data from our group; paper in preparation). Improved glucose tolerance[83,84] and insulin sensitivity[84,85] have also been reported, including by our group[60], as metabolic consequences of cold-activated BAT.

Figure 2 schematically summarizes the aforementioned adrenergic metabolic pathway triggered by CE within a thermogenic brown adipocyte.

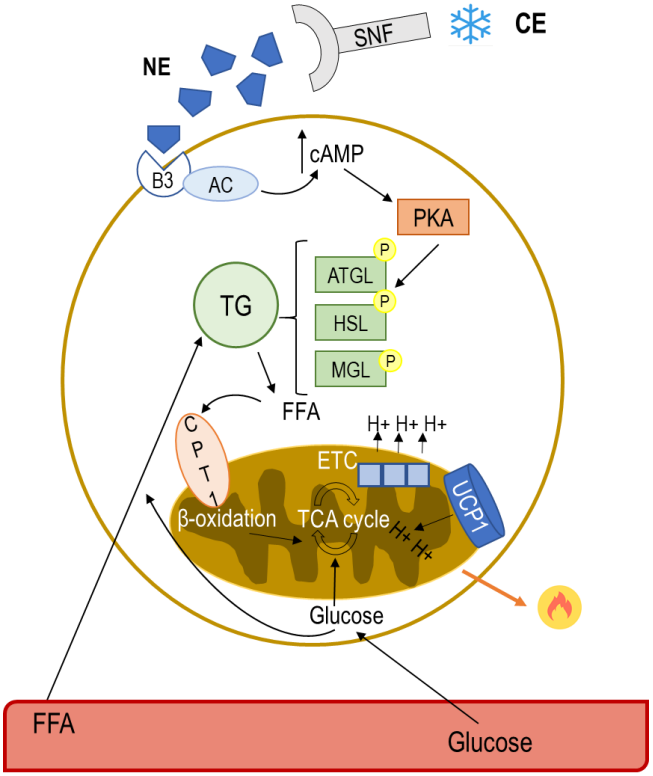


Figure 2. Schematic overview of cold-induced sympathetic regulation within a brown adipocyte. This figure was made using power point (Microsoft Office 2013) adapted from 18 . CE, cold exposure; SNF, sympathetic nerve fiber; NE, norepinephrine; AC, adenylate cyclase; PKA, protein kinase; ATGL, adipocytes triglycerides lipase; HSL, hormone sensitive lipase; MGL, monoacylglycerol lipase; CPT1, carnitine palmitoyltransferase 1; ETC, electron transport chain; TCA cycle, tricarboxylic acid cycle.

Besides its role as a defender of mammalian's core body temperature, BAT is also an important regulator of metabolic homeostasis. Remarkably, mice lacking BAT become obese[86]. Further, UCP1 targeted ablation in mice leads to increased body mass and fat mass even at TN[87]. Thus, the role of BAT and its significant contribution to energy expenditure is well reported in rodents[56,87,88].

In humans, BAT was firstly discovered in the early 1980s[58] and its thermogenic function identified in the early 1960s[89], particularly through the detection of UCP1. However, until only a decade ago, BAT was considered lost in adults and therefore addressed as an important tissue for thermoregulation in neonates only. The year 2009 marked the re-discovery of functional BAT, when three independent groups, concomitantly, published evidence for the presence of metabolically inducible brown fat in adults[11,12,57]. These studies detected active, inducible BAT by measuring the tissue's glucose uptake following cold-induced sympathetic activation by ^{18}F-flourdeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT). These aforementioned findings sparked the interest in investigating the role of this tissue in human metabolism, particularly focusing on the potential of BAT as anti-obesity treatment target. Since then, a vast body of evidence has demonstrated that both basal and cold-induced BAT activity is increased in lean compared to obese[57,90,91] and correlate negatively with BMI in adults[57,59,71,92,93] as well as in children[94]. Accordingly, total[57,59,95], visceral [59,95,96], and subcutaneous fat mass[96] as well as waist circumference[97] have been found to inversely correlate to BAT activity. Whereas, cold-activated BAT metabolism is associated positively with resting metabolic rate[57,93,98] and with cold-induced lipolysis in healthy lean subjects[99].

Of note, discerning between BAT activation versus BAT presence is not trivial in human studies. Nevertheless, several interventional studies have provided experimental evidence supporting a regulatory role of BAT activity on human's whole-body metabolism[60,84,99,100–102].

The lipid clearing effect of cold-activated BAT, already established in rodents[74], has been further characterized in humans showing[82] a cold-depended increase in whole-body FFAs oxidation and a positive association between lipolysis with BAT volume. Recent data by our group shows that acute cold-induced BAT was associated to higher levels of TG together with altered circulating concentrations of distinct FA[60]. Collectively, these results support the role of BAT in mediating lipid metabolism in humans, and lead to speculate that BAT activation induces lipid metabolization from presumably WAT stores to fulfill the increased energy demands upon activated BAT.

Diet induced thermogenesis (DIT) has also been suggested to possibly contribute to energy homeostasis through BAT activation, as indicated by pioneering studies in rodents[56,88] as well as by over- and under-feeding studies in humans[103,104]. However, the relationship between BAT activity

and DIT is still debatable, as controversial evidences do not allow to uniquely conclude whether an acute DIT-response could be attributed to BAT-activity[105,106].

It must be noted that, neither acute nor chronic interventional studies have been able to attribute weigh loss to BAT activation so far. However, Yoneshino and colleagues demonstrated that exposure to 19°C, 2h/d for six weeks led to BAT recruitment concomitantly with increased energy expenditure and decreased body fat mass which, however, was not paralleled with change in body weight[98].

Collectively, the aforementioned results point towards a metabolic health improvement mediated by BAT recruitment and activity. Despite these optimistic results, it seems fair to claim that additional interventional and mechanistic studies revealing human BAT function and regulation are warranted to further develop strategies to enhance its beneficial potentials, in terms of thermogenic and endocrine capacities.

Although so far the study of BAT has primarily focused on its thermoregulatory function and its associated regulation of glucose and lipids metabolism[13,60,84,107], there is increasing evidence that BAT may possess a secretory function[16,108]. This may be similar to that of WAT, which secretes hormones, such as leptin and adiponectin, to contribute to glucose, lipid and whole-body energy homeostasis. Accordingly, the molecules secreted from BAT, known as brown adipokines or 'batokines', could crosstalk with other tissues including WAT, bone, brain, liver and pancreas to regulate glucose and lipid homeostasis. Moreover, they might also have autocrine and paracrine functions, thus, influencing locally brown adipocytes' metabolic activities. In line with this theory, recent and growing body of evidence in some animal studies[109–111] suggests that BAT-derived autocrine and paracrine batokines act to increase - or inhibit - BAT activation and recruitment. The data point towards a regulating role of these released molecules in remodeling processes associated with the adaptive BAT recruitment in response to thermogenic stimuli. Examples of candidate batokines include proteins, peptides, hormones and mRNAs with divergent functions and interaction with different tissues[109].

Interestingly, transplantation of BAT to diet induced obese mice improved not only glucose tolerance and energy expenditure (probably exert by the transplant itself) but also increased insulin sensitivity in WAT and cardiac muscle. This suggests that the implanted BAT cross talks with these organs[85]. In two further studies in mice, BAT implantation has been shown to reverse type I diabetes, possibly by anti-inflammatory action and secretion of insulin-like growth factor 1[112,113]. Comprehensively, recent data suggest that several anti-obesogenic and anti-diabetic effects could be attributed to BAT inherent endocrine activity.

Moreover, it is important to mention that changes in the expression pattern of the white adipokines likely mirror the metabolic status of the adipose tissue. For example, an altered white adipokine

secretion pattern has been observed in compromised metabolic status, like obesity, where the adipocytes-released factors are closely related to insulin resistance, inflammation, and other obesity-induced pathogenic conditions[114]. This raises the possibility that batokines may play a similar signaling-function. Hence, elucidating the secretory and circulating patterns of the batokines may be particularly useful for the non-invasive assessment of the BAT metabolic status, upon both physiological and pathological status, like obesity. However, while several brown adipokines have been described in mice and rats[109,110], evidences of such mediators remain scarce in humans. Knowledge of batokines and its functions identified so far in animals might not be translated into human settings. The big challenge for the identification in humans is the difficult to reach anatomical location of BAT. Accordingly, the more appropriate methodologies for detecting molecules secreted exclusively by brown adipocytes in humans are still under investigation[115].

1.4.1 Brown adipose tissue in obesity and the question of being a partially dormant phenotype

Plasticity is an extremely important feature of WAT[36]. During prolonged period of positive energy balance, WAT responds by a combination of adipocytes hypertrophy and hyperplasia which progressively lead to obesity[32]. Notably, high plasticity is a remarkable signature of BAT as well[47] whereby brown adipocytes are able to continuously adapt to reorganize their morphology and metabolic function depending on environmental stimuli. Accordingly, several studies have found that BAT is highly responsive to β3-adrenerging stimulation in healthy adults, showing the tissue capacity to both expand and increase its metabolic activity upon CE[11,12,57] and β3-adrenergic agonist treatment[116]. Conversely, obesity is associated with lowered sympathetic response[117]. In line, sympathetic activity in BAT appears impaired in obese individuals upon various stimuli, such as cold[90,93], epinephrine[118], and physical activity[119], further promoting weight gain[120].

However, the molecular mechanisms that lead to reduced BAT inducible response in human obesity and its physiological implications together with its metabolic disfunctions are largely unknown.

Remarkably, rodents' studies have shown that brown and brown-like adipocytes undergo morphological and metabolic changes during conditions of chronic positive energy balance. These include accumulation of lipid droplets, reduced mitochondrial concentrations[121] and impaired glucose uptake[122], respectively. The whole of these coordinated processes has been termed 'whitening'[52,121], as it ultimately results into the conversion of brown into white-like adipocytes.

Importantly, these evidences corroborate the notion that the thermogenic phenotype of brown adipocytes is extremely plastic and requires sustained β-adrenergic stimulation to maintain its conventional thermogenic signature.

The process of whitening has also been linked to humans. This is based on initial observations showing the heterogenous morphology of the human deep-neck BAT, composed of a mixture of unilocular and multilocular adipocytes[47,123]. Recently, a study provided additional support for the 'whitening of BAT' hypothesis by unveiling, for the first time, a dormant BAT-phenotype within the perirenal adipose tissue depot of adult humans[61]. The perirenal fat is interesting due to its asymmetric access to local sympathetic activity. The upper pole of the kidneys is closer, compared to the lower pole, to the adrenal gland where norepinephrine and epinephrine are produced. Jespersen et colleagues discovered by comparing different sites of the perirenal fat that the brown cells composing the tissue have a heterogeneous signature in terms of lipid droplets accumulation and UCP1 expressions, depending on the vicinity to the local adrenergic stimulus[61]. Specifically, the conventional BAT-phenotype locates close to the adrenal gland and, therefore, to local sympathetic stimulation. Contrarily, the remaining cells of the perirenal fat are morphologically similar to WAT. The latter cells show an unilocular phenotype associated to lower – yet present - expression of UCP1 in parallel with downregulated mitochondria genes, previously established as markers of brown fat activity[61]. Thus, the authors concluded that the perirenal BAT associating with lower sympathetic local activity turns to a 'dormant' state, rather than disappear. Thus, these novel findings suggest that BAT might be equally dynamic as WAT and be able to acquire WAT-like properties upon certain stimuli such as reduced sympathetic activation. Hence, they emphasize the plasticity of BAT and seem to indicate that the morphology and thermogenic capacity of BAT closely reflect the usage or activity of the tissue in humans.

Taken together, these data additionally support the theory that conditions of altered responsiveness and sensitivity to sympathetic stimuli, as seen in obesity, could be tightly linked to the novel proposed BAT-dormant phenotype. Moreover, obesity-associated metabolites could also be related to the progressive obesity-dependent decline of BAT activity[108]. Therefore, the further characterization of the suggested BAT dormant phenotype as well as the identification of factors promoting this metabolic state could contribute at addressing BAT metabolic disfunction in obesity. In fact, this hypothesis leads to the possibility that adult humans could ultimately increase their amounts of cold-induced BAT by potentially re-activate the dormant-BAT depots. Therefore, the goal would be to identify new fat-released factors linking the 'whitened' or dormant-BAT phenotype with conditions of decreased thermogenic capacities.

While the characteristics of dysfunctional obesity-dependent BAT have been addressed in genetic and diet induced mouse models of obesity[121,122], data in humans investigating potential culprit factors are only now starting to appear[61,124], evoking further investigation. In this regard, the secreted protein acidic reach cysteine (SPARC) has recently been proposed as a secreted molecule promoting the

WAT-like phenotype of BAT[61]. SPARC is an extracellular matrix (ECM) protein expresses by several cells, including adipocytes[125–127]. SPARC has been shown to promote adipocytes structural alteration, angiogenesis, fibrosis and WAT hyperplasia as ECM protein of the adipose tissue [125,128,129]. All these mechanisms likely contribute and are associated to the role of SPARC in obesity. Therefore, the implication of SPARC in promoting obesity has been relatively well characterized. However, the potential contribution of SPARC at promoting the blunted BAT-cold response associated to obesity has not been investigated yet.

1.5 Means to activate brown adipose tissue in humans

To effectively leverage BAT's metabolic capacities, the current challenge is to identify strategies, including both lifestyle interventions and pharmacologically means, aimed at enhancing BAT adaptive thermogenesis.

1.5.1 Modulation of cold-induced brown adipose tissue activation pathways

As previously mentioned, cold is the physiological activator of BAT[13] and hence, the most frequent investigated mean to induce BAT activity in humans as well as WAT-browning in animals[11,12,41,57]. Our group was also able to prove the effectiveness of cold-induced BAT activation by employing a personalized cooling protocol and, thereby, to characterize its metabolic consequences in lean[60] and obese subjects (paper in preparation). Collectively supporting the idea that mild CE may be an effective intervention to promote the activation of BAT and the various metabolic benefits that come with it.

In parallel, pharmacological stimulation of BAT thermogenesis, aimed at increasing energy expenditure and boosting its substrates clearing capacity, is also being currently pursued as a viable anti-obesity strategy. Consistently, several pieces of evidence support that compounds acting on the SNS can modulate BAT metabolic activity[130,131]. Administration of ephedrine, a sympathomimetic molecule, increased BAT function, while, however, increasing in parallel heart rate and blood pressure[118]. Similarly, administration of the β-3 adrenergic agonist mirabegron enhanced BAT activity in healthy adults by acutely inducing BAT glucose uptake and WAT lipolysis, though, concomitantly, causing an increase in heart rate and blood pressure[116]. Although this study suggests that mirabegron administration may potentially act as an acceptable therapeutic approach to activate BAT thermogenesis, its unwanted side-effects within the cardiovascular system make the use of this compound not feasible for obese subjects. All together these data point out that, to date, there is no univocal evidence supporting the administration of sympathomimetic compounds as effective and

side-effects free activators of BAT metabolism. However, the idea of enhancing BAT thermogenic potentials via small molecules remains an exciting option, worth to be further investigated.

1.5.2 Metabolic effects of insulin: To distinguish between peripheral and central administration

Insulin receptors are vastly found in the human brain[132]. Therefore, intranasal insulin (INI) has been repeatedly used as effective and established method to selectively increase the central nervous system's insulin level. In fact, INI does not lower systemic glucose levels as it bypasses the circulation while crossing the blood brain barrier via the nasal mucosa[133]. Therefore, INI has been often used to investigate the functions of enhanced brain insulin signaling in humans[134].

Importantly, the effects on whole-body energy homeostasis mediated by enhanced brain insulin concentration oppose - almost completely - to the ones that insulin induces peripherally. As well known, insulin acts as anabolic hormone after peripheral administration (intravenous or subcutaneous). In fact, systemic insulin promotes organs substrates' uptake leading, on the long-term, to weight gain in form of muscle and fat mass. Whereas, enhanced insulin brain signaling via INI and intracerebroventricular (ICV) insulin administration, in humans and animals respectively, has been shown to induce multiple catabolic effects[135]. There is evidence from experiments conducted in various animal models, i.e., dogs[136], baboons[137] and rats[138]that ICV insulin reduces food intake and lowers body weight. Subsequently, these results have been confirmed in chicks[139] and mice[24]. In humans, similar findings support the positive regulatory role of enhanced brain insulin signaling on food behavior and energy homeostasis, particularly, by intensifying satiety[140], curbing the post prandial wanting for sweet foods[141], decreasing energy intake and (in men only) body fat[142,143]. Moreover, INI was found to improve hepatic energy metabolism[144] and even proved effective in ameliorating peripheral insulin sensitivity, both upon fasting[145] and following a meal challenge[146]. Collectively, the modulatory effects of enhanced brain insulin signaling on whole-body energy metabolism have been broadly investigated. However, none of the aforementioned studies investigated the possible metabolic role of brain insulin signaling on BAT metabolism. Thus, data in this context, especially in humans, are surprisingly scarce. Notably, data showed that ICV insulin administration in rats increased sympathetic outflow to BAT, suggesting a modulating role of central insulin in eliciting BAT thermogenic function via symphato-activation[147,148]. Accordingly, Benedict and coworkers found that INI administration increased post prandial energy expenditure with an average of ca. 17% compared to placebo in males[146]. However, although it seems tempting to speculate the involvement of BAT in modulating the reported effect of INI on DIT, no means to detect BAT activity were employed during the study. Moreover, INI was administered exclusively post

prandially, thus, whether brain insulin signaling could increase energy expenditure in non-post prandial condition remains an open question[146].

1.6 Means to detect brown adipose tissue in humans

To date, there are several experimental approaches aimed at detecting the presence and activity of BAT. However, owning to the fact that BAT is a recent 're-discovered' tissue in adult humans and is mainly found in a rather difficult to reach anatomical area i.e., SCV depot, methods to accurately assess its content and its metabolic function are currently still under investigations.

The most direct and reliable approach to detect BAT requires sampling of the tissue from human donors, i.e., BAT biopsies, usually collected within the SCV depots. Human brown adipocytes constitute a precious in-vitro model which enables to perform various range of direct methods to assess the tissue's activity. These include the quantification of genes and protein expressions level of established markers of BAT, such as UCP1, PRDM16, ZIC1 and LHX8[47,61,149]. Moreover, the determination of BAT lipid content, and measures of mitochondrial respiration[61,150] are also possible. However, invasive surgery is required to collect the biopsies. Hence, the aforementioned determinations remain limited to animal models. In humans, it could be applied only under very strict and particular conditions, e.g. oncological patients undergoing neck surgery.

1.6.1 Positron emission tomography and computed tomography

The current gold standard for imaging and measuring BAT in humans is the ^{18}F-FDG PET/CT. A functional PET-scan, by which metabolically active tissues can be visualized following the uptake of plasma ^{18}F -FDG tracer from the circulation, is combined with a CT scan, which can distinguish tissues types based on their density. ^{18}F-FDG tracer is a glucose analogue lacking the 2-hydroxyl group (-OH), which is necessary for the process of glycolysis to continue after the initial phosphorylation step. Hence, once ^{18}F-FDG enters the cells, it gets phosphorylated to ^{18}F-FDG-6-phosphate by hexokinase but cannot be further metabolized. Thus, it remains 'stuck' in the cells and can be easily visualized using PET/CT[57,95,151].

Although the ^{18}F-FDG PET/CT technique strongly renewed the engagement of many researchers in pursing the investigation of BAT, its use still holds major limitations[151].To start with, the high overall cost of the expensive radiological equipment for the PET/CT needs to be pointed out, together with the ethical and safety concerns regarding the exposure to high doses of radiation. In fact, the exposure of subjects to ionizing radiations, particularly upon repeated multiple BAT assessments or when scans would be performed in vulnerable groups, i.e., children, pregnant women or patients, makes the use

of the PET/CT scan not feasible as measuring routine technique. Notably, ethical concerns prevent the use of the PET/CT scan in clinical studies altogether in some countries, including Germany.
Additional problems have been pointed out regarding the reproducibility of the results obtained with the use of the PET/CT across labs. This limitation is mainly due to the fact that standardized conditions are required before and while performing the scans, such as temperature, diet and medications, among others, but procedure protocols still variate among studies. However, it needs to be pointed out that the lack of established standardized protocols is not limited to the PET/CT scan, but it is rather a limitation common to all the imaging techniques investigating BAT.
Moreover, due to the nature of the tracer which is taken up by all metabolically active tissues, it is necessary to avoid involuntary muscle activity, i.e., muscle shivering which could lead to increase uptake to non-BAT tissues, making the scans rather difficult to interpret and even possibly leading to bias interpretations[151]. Another issue has been brought up concerning the validity of the glucose tracer itself. As aforementioned, studies[74,75] indicate that brown adipocytes utilize internal TG stores as first substrates-choice, subsequently followed by FA and, even later, glucose uptake. Hence, depending on the nature and the duration of the stimulus, to use a glucose tracer may not be the optimal choice. Striking, in line with this observation, some studies have reported that the prevalence of active BAT quantified by ^{18}F-FDG PET/CT scan range from 1.32 to 100%, depending on the subjects population and scanning condition[152,153]. This rather big range in results could be explained by the fact that only activated BAT can be visualized. Hence, suggesting that inactive BAT, despite present and therefore possibly inducible and recruitable might be overlooked by ^{18}F-FDG PET/CT. In fact, the use of metabolic activity scans cannot distinguish whether a stimulus is insufficient for BAT activation in a given individual, or whether this individual retained no BAT at all when the scan was performed. Overall, this underscores that despite the gold standard status attributed to the ^{18}F-FDG PET/CT scan, this technique still presents some disadvantages.

1.6.2 Magnetic resonance imaging

Magnetic resonance imagining (MRI) is another quantitative technique currently under investigation in the field of brown fat biology. In fact, MRI can be safely used in longitudinal studies in human subjects of all age groups being radiation-free, enabling to understand how BAT evolves over time.
Generally, several MRI modalities can be found for biological applications, including MR-spectroscopy, chemical-shift-encoded (CSE) MRI, blood oxygenation level-dependent (BOLD) imaging, hyperpolarized MRI, and PET/MRI.

However, the CSE water–fat imaging (WFI) technique and the BOLD technique are of particular interest to quantify BAT in both active and inactive state. This constitute a big advantage of the WFI and BOLD MRI based-techniques versus the PET/CT scan, which as mentioned above, cannot be employed to detect inactive BAT.

The CSE WFI technique, by taking advantage of the small magnetic field produced by hydrogen atoms, reliably discriminates water from fat and quantifies the lipid content of the tissue[154]. In result, WFI produces images mirroring the fat/water ratio and mitochondrial density, thus enabling the identification of BAT vs WAT based on their histologic difference[155]. Specifically, it has been shown that according to the WFI, BAT shows a lower proton density fat fraction compared to WAT samples[156].

A second MRI-based technique that finds meaningful application in the quantification of BAT is the BOLD technique, typically associated with functional MRI in the brain. The intrinsic methodology of the BOLD-MRI is based on detecting changes in regional blood concentrations of oxy- and deoxyhaemoglobin[157], which are typically driven by changes in the metabolic needs of tissue. Hence, brain regions with more oxyhaemoglobin will produce higher signals which decrease after activation[157]. Interestingly, the BOLD method can be used to detect metabolic activity in any organ experiencing oxy- and deoxyhaemoglobin alterations, according to its metabolic requirements[158]. Compared with WAT, BAT has a conventional greater capillary density to sustain its thermogenic demands. Thus, changes in the BOLD MRI signal can be related to the different metabolic requirements between the two fat tissues. Studies in animals[159] and humans[158,160] have shown that activated BAT exhibits a strong BOLD signal, raising the possibility of using BOLD MRI as a valid alternative to ^{18}F-FDG-PET/CT in detecting activated BAT[161].

Overall, MRI-based techniques have two main advantages compared to PET/CT. First, they allow the quantification of BAT also in a thermoneutral state. Second, they do not rely on ionizing radiation, allowing measurements on different subjects population. Collectively it appears clear that MRI-based techniques could be of great contribution for the study of BAT in humans. Nevertheless, the current experience regarding these imaging methods in interventional studies is still limited, requiring further investigation, especially for the purpose of BAT detection in obesity.

1.6.3 Infrared thermography and thermal skin probes

Due to the heat-generating properties of brown fat, temperature assessment of the skin overlying BAT depots has been proposed as alternative technique to non-invasively detect BAT activity in humans.

An infrared thermography (IRT) camera and thermal skin probes are the two approaches most frequently used to assess skin temperature - and their changes - in relation to BAT thermogenesis.
IRT is a technique that measures emissivity, physiologically emanated by the human body, and converts this to thermal images[162]. In the last couple of years in the context of human BAT's investigation, the use of IRT as non-invasive alterative to PET/CT technique has increased exponentially[163]. That is certainly due to the fact that in 2018, continuous IRT measuring of the skin temperature within the SCV regions have been confirmed to strongly correlate with measurements of BAT activity by PET/CT[164]. Importantly, the temperature of the skin overlying the SCV regions is used as an indicator of BAT activity[164,165], due to the anatomical co-location of these depots with BAT.
The IRT technique, in contrast to PET/CT, has no radiation and is cheap and quick, allowing the possibility of detecting BAT activity on a large study cohort[166]. Additionally, IRT is highly suitable for BAT measurements in healthy individuals as well as children[167]. This was firstly demonstrated by Lee and colleagues, who used this method in 87 healthy subjects. In this study, skin temperature differed between the SCV regions and the reference point (RP), an anatomical region not co-locating with BAT, following CE[168].
However, despite being an easily accessible technique, the employment of an IRT camera requires important preparation notions to guarantee its proper use and reliable results. Accordingly, in order to capture valid thermal images, the settings of the IRT camera, such as room temperature, humidity, distance from the subject and reflective temperature, require to be assessed at each study session and properly adjusted into the camera before starting with the imaging acquisition. Additionally, before starting with the measurements, it is necessary to identify and mark correctly the anatomical points delineating the region of interest (ROI) within the SCV regions and the RP. This enables consistency when performing subsequent thermal images analyses. The pivotal importance of choosing the proper setting and performing accurate preparations before the actual thermal images are taken, makes the use of IRT, despite its great potentiality, still somewhat challenging. In fact, as for PET/CT and MRI based-techniques, a limitation concerning the use of the IRT is the variations in the SOPs across labs[166]. Generally, this issue concerning the standardization of the imaging procedures to detect BAT makes the direct comparison of studies and results among different groups problematic and needs to be urgently addressed.
Another method frequently used to non-invasively complement the assessment of BAT activity in human are thermal skin probes. These are wireless probes which are directly attached to the skin at different anatomical points. For example, iButtons have been applied over the SCV to detect changes within this region following cold challenge[84]. Alternatively, they have been applied on different

regions along the body to assess peripheral skin temperature, as a reference measure for the mean skin temperature (MSKT)[169]. Once the probes have been attached, continuous temperature recordings can be performed. Several studies have shown that skin temperature over known BAT depots is higher compared to other non-BAT related areas[170–172]. Importantly, Jange and colleagues reported comparable results between IRT and thermal probes at measuring changes in skin temperature within the SCV regions[165]. As for the IRT, the advantages of the skin probes are low costs, easy access and use, as well as the lack of radiation exposure. A major limitation of this method, however, is that the probes can provide temperature measurements exclusively for the area directly underneath. This implies that thermal probes can reliably enable the assessment of superficial BAT depots only, with the additional limitation that a thicker layer of skin, as seen in obese subjects, may lead to potential confounding in temperature records and therefore to biased results[151].

1.7 Objectives and aims of the present work

This work aimed at characterizing and modulating the sympathetic central nervous and peripheral pathways involved in mediating BAT-thermogenic activity in humans.
The specific hypotheses and aims of the studies are as follows:

Hypothesis I: Since enhanced brain insulin improves whole-body energy homeostasis in humans and BAT-thermogenesis in animals, we hypothesized that increased brain insulin signalling may trigger BAT activation by modulating the sympathetic nerve activity and the neuroendocrine stress axes in subjects tested at thermoneutrality.

Aim 1: To investigate the effect of increased brain insulin signalling as potential activator of BAT metabolism in humans.
Aim 2: To assess the relation of basal BAT-thermogenic activity with potential neuroendocrine and environmental modulating factors.

To test hypothesis I, we performed a randomized, double-blinded, placebo-controlled, cross-over design study where INI was used as method to selectively and safely reach the central nervous system, while thermography was employed to non-invasively assess basal BAT-thermogenic activity.

Hypothesis II: Increasing evidence supports the presence of BAT in subjects with obesity. Yet obesity associates with a reduced BAT-thermogenic capacity. Based on the recently proposed role of endocrine factors promoting a dormant-BAT phenotype, we hypothesized that the protein SPARC

could be a candidate adipokine mediating the dysregulated BAT-thermogenic response associated with obesity.

Aim: To explore the role of SPARC in the context of BAT-metabolism linked to obesity.

To test hypothesis II, SPARC mRNA expression was measured in human biopsies of supraclavicular BAT and subcutaneous WAT. Moreover, SPARC plasma concentrations were measured in subjects with obesity upon thermoneutrality and during a personalized non-shivering cooling protocol. Associations between SPARC concentrations and markers of metabolic health were assessed.

II. Materials and methods

2.1 Experiments related to Hypothesis I

2.1.1 Subjects and experimental design of study I

Eighteen healthy men (mean ± SEM; BMI: 21.4 ± 0.4 kg/m^2; age: 24.1 ± 0.9 years) were enrolled in this randomized, balanced, double-blind, placebo-controlled study. Exclusion criteria were chronic or acute illness, current medication of any kind, smoking, alcohol or drug abuse, and history of T2 or T1 diabetes in first degree relatives. Individuals working night shifts, and performing more than eight hours of physical exercise per week were also excluded. Subjects underwent a medical history, physical examination, and blood testing to exclude endocrine diseases, as well as sinusitis and nasal polyposis which would interfere with nasal spray administration. Subjects were asked to avoid any alcoholic or stimulant drink, strenuous exercise and exposure to extreme temperature, i.e., sauna in the preceding 24 hours before testing. All experiments were performed at Metabolic Core Unit (MCU) of the Center of Brain, Behavior and Metabolism (CBBM), University of Lübeck, on two days, at least one but not more than two weeks spaced apart. In accordance with the Declaration of Helsinki, the study was approved by the local ethics committee of the University of Lübeck (AZ 17/253) and written informed consent was received from all subjects before inclusion in the study.
Each subject participated in both experimental conditions, i.e., insulin (INI) and placebo (PLC). Each experimental session started at 8:00 and lasted till 12:10 (Figure 3). Subjects arrived at the MCU in a fasting state (from 22:00 previous experimental session) and were asked to come by any mean of transportation other than biking or walking. Upon arrival, body height was measured using a stadiometer to the nearest 0.1cm (Seca, Hamburg, Germany) while body mass and body composition were determined using air displacement plethysmography technique (BodPod, COSMED Germany GmbH, Werneck, Germany) with subjects wearing underwear. Subsequentially, standardized clothes, i.e., sleepers, light cotton long pants, and cotton shirt were given to each subject and a mobile heart rate monitor (ActiHeart, Camntech, Cambridge, UK) was placed to the subject's chest, i.e., xyphoid part of the sternum and 4th intercostal line. The device was left in place and recorded through the entire experimental session for the assessment of heart rate and heart rate variability. Concomitantly, 14 skin probes (ibuttons, Maxim Integrated, San Jose, USA) were located in specific anatomical points (Figure 4a) for constant recording of MSKT. Next, an 18-gauge venous catheter (Vasofix Braunüle, B. Braun Melsungen, Germany) was inserted into antecubital veins of the non-dominant arm for repeated blood samplings. Throughout the experiment, subjects seated in a chair located in the experiment room, kept at constant temperature and humidity, i.e., 23.07 ± 0.1 °C and 39 ± 0.01 %, respectively, as average values from the two study sessions, measured by a room thermometer

(BASE Tech Conrad Electronic SE, Hirschau Germany). A standardized acclimation period of 20 minutes (i.e., 08:40-09:00) took place in the experimental room on each study session before starting the baseline imaging acquisition with the IRT camera (FLIR T530, FLIR Systems, Wilsonville, USA). Subjects were instructed to seat in an upright position, with their back at 90°C, and to avoid movements during imaging acquisition. From each subject, neck, shoulders and upper sternum, which was used as RP, were included in the images but never the face. Double sided tapes and aluminum foil were used to delineate the two regions of interest, by marking the following anatomical areas: right and left sides of the neck in line with the chin, and on top of the right and left acromioclavicular joint. A tripod (Manfrotto stativ, Vitec Imaging Distribution GmbH, Köln, Germany) was used to sustain the IRT camera and to avoid movements during the measurements, enabling consistency between the IRT-imaging acquisition interval during the experiment (further details on the IRT method are provided in paragraph 2.1.3).

At 09:05 the first blood samples, i.e., baseline values, were collected. Moreover, subjects rated their subjective feelings on a symptom rating scale (SRS) and a visual analogue scale (VAS) to control potential confounding variables, such as subjective stress or discomfort. SRS and VAS were also used to assess the subject well-being as well as subject perceived feelings of hunger and satiety across each study session (each hour). At 9:10, the 5-minute baseline IRT-imaging acquisition started, followed by the first administration of INI or PLC, respectively. During each experimental condition, INI or PLC were administered by four puffs every 20 minutes over a period of 120 minutes. Total dose of INI was 240 IU. A 5-minutes IRT interval followed each administration of INI or PLC, for a total of four IRT intervals on each experimental condition, respectively. In addition, a 20-minutes IRT interval started at 10:50 following the meal challenge, to capture potential changes in the supraclavicular temperature (SCVT) due to DIT. To perform the meal challenge, subjects consumed 600 ml of a standard liquid meal (Fresubin energy drink, Fresenius Kabi, Bad Homburg, Germany) at a dose of 200 ml/min, (total: 60 ml, 900 kcal, 33.6 g protein, 34.8 g fat, and 112.8 g carbohydrate). In order to avoid potential bias due to change in the skin temperature induced by the temperature of the meal, we choose to serve the meal at the body-adapted temperature. Therefore, the liquid meal was pre heated and consumed at 35 ± 0.5°C.

For measurements of relevant metabolic parameters, besides the baseline blood drawn (at 09:05), additional blood samplings were collected at 09:45, 10:25, 11:10 and 12:10.

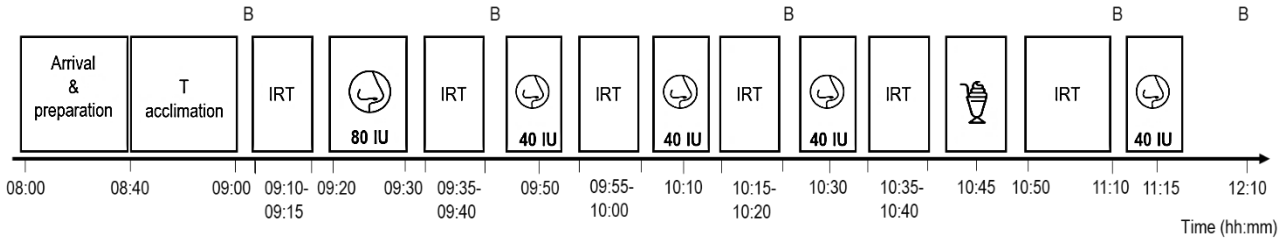


Figure 3. Experimental design of study I

The study was conducted in a cross-balanced repeated within subjects design.

T, temperature; B, blood drawn; IRT, infrared thermography; IU, international unit.

Icons: Nose, nasal spray; drink, standardized liquid meal challenge.

2.1.2 Intranasal insulin application

In total, 240 IU of human insulin (100 IU/mL; Huminsulin Normal 100, Lilly Deutschland, Bad Homburg, Germany) was administered intranasally five times during the experimental session using precision air pumps (Aero Pump, Hochheim, Germany) which fill the nasal cavity with aerosol enabling the solution to effectively target the olfactory epithelium. Each puff consisted of 0.1 mL (i.e., 10 IU of insulin or insulin dilution buffer). Insulin dilution buffer (Lilly USA, Indianapolis, USA), which exactly matches the carrier solution of human insulin was used as PLC. According to the randomization list, the study solution was bottled on the morning of each experimental session, before arrival of the subject, by laboratory technicians. Once ready, the filled bottle was handed to the investigators who - blinded regarding the experimental condition - instructed the subject on the intranasal auto administration. Subjects, as well as the investigators remained unaware of the randomization throughout the whole duration of the study. Investigators carefully monitored clinical signs of hypoglycemia. A first bolus of 80 IU (0.8 mL, four 0.1-mL puffs per nostril) of INI or PLC, was intranasally administered. Successively, the administration of 40 IU (0.4mL, two 0.1-mL puffs per nostril) of INI or PLC, was repeated every 20 minutes for four times, amounting to a total dose of 240 IU (2.4 mL; Figure 3).

2.1.3 Assessment of body temperature: Core, mean and supraclavicular skin temperature

Core body temperature (CBT) was assessed indirectly at baseline (i.e., 09:00), and repeatedly during the experiment (i.e., 10:25, 11:15, 11:35) using an ear thermometer (Braun ThermoScan type 6014, Braun GmbH, Kronberg, Germany). Body MSKT was evaluated using measurements of skin temperature obtained from the ibuttons set with a resolution 0.125˚C placed at 14 anatomical points

(i.e., forehead, neck, scapula, upper chest, abdomen, lower back, dorsal wrist, trunk, deltoid, arm, hand, lower leg, thigh and foot), while no ibuttons were placed on the SCV regions to avoid conflict with thermography measurements of the ROI co-locating with BAT (Figure 4a). Measurements were taken at 20-second intervals throughout the experiment and in parallel to the thermal imaging acquisition during the IRT intervals. The data were averaged into 1-minute intervals (i.e., three recorded values for each interval). Subsequentially, values were averaged into 5-minute intervals and analyzed. Such intervals were chosen in order to maintain consistency in the analysis of data between ibuttons and IRT methods.

SCVT was measured using an IRT camera following the standardized lab protocol. In brief, the IRT camera was positioned so that the lens was perpendicular to the larynx and the field of view included the full width of the shoulders laterally, the manubriosternal joint inferiorly, and angle of the mandible superiorly, respectively. The IRT camera was positioned 1-meter distance from the subject (Figure 4b). Room temperature and humidity, as well as the reflective temperature were measured at the beginning of the experiment and entered into the camera software settings. The human skin emissivity was also required to be entered in the camera setting and was set constant at 0.98. Before starting with the thermal imaging acquisition, it was checked that the SCV regions were free from obstructions (not covered by jewellery, hair or cloths). During the acquisition of the thermal imaging, the subject sat on a stool, in upright position but not leaning forward, directly facing the camera. To help subjects to maintain the correct position, a target marker was placed on the wall behind the camera; when images were acquired subjects were asked to look at that point. The shoulders and the upper sternum were also in the frame to provide a RP during analysis. The five selected points were previously marked during the preparation phase by applying double sided tapes and aluminium foil on the subject's skin at the following anatomical position: right and left sides of the neck in line with the chin, on top of the right and left acromioclavicular joint and sternal notch, used as RP. During the experiment, images were taken every 20-second during 5-minute intervals which occurred at baseline, after intranasal administration and after the meal challenge (Figure 3). For the analysis of the thermal images, the marked anatomical points were used to draw the ROI, which was defined as the region bounded by the left sternocleidomastoid muscle, clavicle and lateral contour of the neck as described previously[164]. The 95th percentile temperature values for the selected ROI and RP were exported into Excel (Microsoft Office 2013) and averaged using the 5-minute intervals for subsequent statistical analysis.

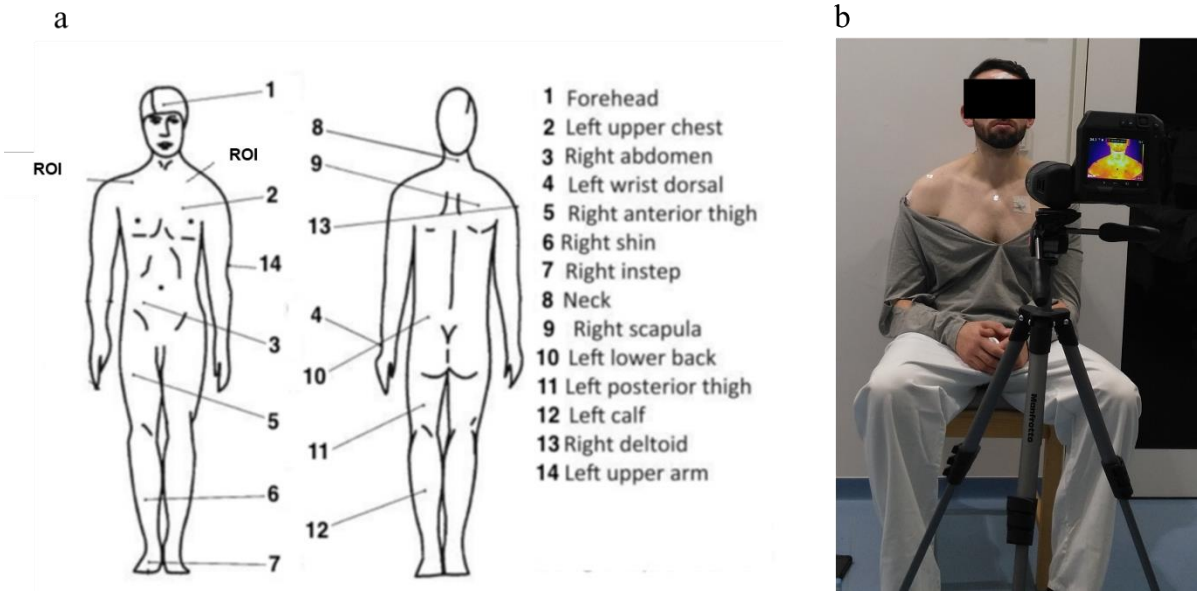


Figure 4. Illustrations of the established methodologies used to assess body temperature.
a) Anatomical location of the ibuttons; Figure adapted from[169]. b) Example of IRT imaging acquisition in one participant. ROI, region of interest.

2.1.4 Assessment of subjective feelings of well-being, satiety and hunger

Assessment of subjective feelings of well-being were performed four times during the experiment using 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 self-rated on a visual analogue scale (VAS) of 10 cm length, ranging from 0 cm (not at all) to 10 cm (extremely). Together with the SRS, subjects further rated their current feelings of hunger, satiety, thirst, mood and well-being likewise on a VAS of 10 cm length anchored from 0 cm (not at all) to 10 cm (extremely).

2.1.5 Assessment of environmental and lifestyle factors: Outside temperature, sleep duration and physical activity

To calculate the outside temperature (OT), data from the climate station of Deutsche Wetter Dienst in Lübeck (DWD, https://cdc.dwd.de/portal/) were used as source reference. These data consist of daily station observations of mean, minimum and maximal outside air temperature at two meters above ground**.** The DWD station of reference is located five kilometers away from the CBBM and the temperature was recorded hourly. For the purpose of dividing our study samples in two sub-groups to perform subsequent statistical analysis, data concerning the maximal outside temperature of the

seven days (168 hours) prior to each study visit were considered. Therefore, according to the maximal seven day average OT prior to each experimental visit, we divided our study sample in two sub-groups, i.e., 'warm' and 'cold', for values of OT >13,5 °C and ≤13,5°C, respectively.

Sleep duration (SD) and physical activity (PA) were assessed using the Motion Watch system (MotionWatch 8, CamNtech, Cambridge, UK). A MotionWatch system consists of the MotionWatch device to record data and the MotionWare software for the download and analysis of the data. The Motion Watch is a compact, lightweight, body-worn actigraph with a built-in ambient light sensor and accelerometer. The device is meant to assess activity, in terms of body acceleration with a sampling frequency of 50Hz, during daily living as well as sleeping time. At least one week before the initiation of the study, subjects were instructed on the proper use of the Motion Watch and its features (i.e., the light sensor, event marker button, and status indicator) and asked to wear it on their non-dominant wrist for seven consecutive days and nights prior to each study visit. Subjects were allowed to remove it only when showering or swimming, being the device not absolutely water resistant. During the measurement period, SD and PA data were constantly sampled and automatically stored into an internal non-volatile memory. On each experimental day, upon arrival of the subject to the CBBM, data were downloaded and stored for their subsequent analysis, i.e., sorting them into daytime activity data and sleep data to perform PA level and SD statistical analysis, respectively. Specifically, SD was measured as hours of actual sleep time averaged over the seven night prior to each study visit. Subsequentially, the seven night averaged sleep duration data were graded into categories, i.e., 'low' (≤ 7 hours of actual sleep) and 'regular' (> 7 hours of actual sleep, with no subject recording an averaged sleep duration value greater than 9.5 hour). To conduct subsequent statistical analysis, subjects were divided into one of the two sub-groups.

PA data were collected as activity counts (AC) in 1-minute intervals over the seven day prior to each experimental visit. Subsequentially, AC data were averaged into 5-minute intervals[173]. Then, intensity of PA was calculated by grading the AC intervals into percentile, i.e., 'low' intensity (AC below 33th percentile), 'moderate' intensity (AC within 33th and 66th percentile), and 'high' intensity (AC above 66th percentile). To conduct subsequent statistical analysis, these percentiles were used as cut-off to divide subjects into three sub-groups according to the seven day average level of PA intensity prior to each experimental visit.

2.1.6 Laboratory analyses

Serum fT3, cortisol and adiponectin were analysed using the IMMULITE 2000 ELISA System (Siemens Healthcare, Erlangen, Germany). The mean detection limits and intra-assay coefficients of

variation of the assays were as follows: insulin, 2.0 µUI/mL and ≤5.3 %; fT3 0.3 pmol/L; ≤7.8%; cortisol 5.5 nmol/L and ≤7.4%; adiponectin 0.49 µg/mL and ≤4.3% respectively.
Plasma leptin and active ghrelin concentrations were measured using radioimmunoassays (RIA; Millipore Corporation, Billerica, USA). The mean detection limits and intra-assay coefficients of variation of the assays were: leptin 0.437 ng/mL and ≤8.3 %; active ghrelin 4.1 pmol/L and ≤5.1%.
For the sampling of the blood used for catecholamines determination, special tubes (Kabevette Katecholamine 792 N7,5, KABE GmbH, Nümbrecht-Elsenroth, Germany) were used pre-cooled (4-5°C), as for manufacture instruction. Catecholamines concentrations were determined at the external service laboratory facility LADR (LADR GmbH, Lubeck, Germany) by performing high-performance liquid chromatography followed by subsequent chemical detection using the ClinRep kit (Recipe Chemicals + Instruments GmbH, Munich, Germany).

2.1.7 Statistical analyses

Analyses were performed using SPSS 22 (IBM, Illinois, USA) and Prism software (GraphPad, California, USA) was used to prepare the figures. R version 4.0.2 (R core team 2020) was used to calculate repeated measures correlation (Rmcorr) and to plot the corresponding graphs. Values are expressed as mean ± SEM, unless otherwise noted. To test for effects of INI vs PLC on body temperature, blood parameters and feelings of hunger and satiety, two-way repeated measures Analysis of Variance (ANOVA) was used with factors ‘condition’ (‘INI’ vs ‘PLC’) and ‘time’ (referring to baseline, post intranasal administration and post meal challenge periods). Bonferroni correction as well as paired and unpaired t-test for single time point comparisons were used as post hoc test. Ghrelin concentration was expressed as the area under the curve (AUC) calculated applying the trapezoidal rule. To test for the effects of environmental and lifestyle factors on body temperature, repeated measures ANOVA was used with the additional within-factors ‘OT (‘warm’ vs ‘cold), ‘SD’ (‘low’ vs ‘regular’), and ‘PA intensity level’ (‘low’ vs ‘regular’ vs ‘high’), respectively. Rmcorr coefficients were used to examine associations between basal SCVT and variables among neuroendocrine and environmental factors. Rmcorr analysis has been defined as appropriate statistical method for determining associations within paired repeated measures assessed with a within-subjects study design[174]. A multiple linear regression analysis was performed to identify independent factors associated with basal BAT-thermogenic activity. A p value <0.05 was considered to indicate a statistically significant difference.

2.2 Experiments related to Hypothesis II

2.2.1 Study II: Subcutaneous and supraclavicular fat biopsies for mRNA expression analysis

The mRNA analysis of human BAT and WAT has been carrying out as part of my research stay abroad at the Center for Basic Metabolic Research at the University of Copenhagen. Therefore, the fat biopsies transcriptomic data analysed originated from subjects enrolled in the study at the Center for Inflammation and Metabolism at Rigshospitalet, Copenhagen, Denmark, between 2014 and 2016. Ethical approval was received in March 2009 by the scientific ethics committee of the capital region of Copenaghen (HD-2009-020) and the study was performed according to the Declaration of Helsinki. For ethical reasons, it was not possible to perform invasive surgery in the deep neck area on healthy subjects to collect brown fat specimens. Therefore, fat biopsies were collected from 27 patients (20 women and 7 men; mean ± SEM age: 53 ± 8.04 years) scheduled for surgery due to enlarged thyroid gland with thyroid malignancy as exclusion criteria. Subjects were distributed between the BMI categories, as defined by the WHO[2], BMI: 18.5-24.9 kg/m^2, normal weight (NW, n=7); BMI: 25.0-29.9 kg/m^{2,} overweight (OW, n=10); BMI ≥30, obese (OB, n=10). Biopsy procedure was conducted following a standard protocol by an experienced surgeon at Rigshospitalitet (Denmark) and collected by Dr. med. Naja Zenius Jespersen, directly involved in the study. Specifically, the subcutaneous abdominal biopsies were obtained according to the Bergstrom needle biopsy procedure[175]. Briefly, after general anesthesia and immediately prior to initiation of the thyroid surgery, an incision of 0.5-1.0 cm was made a few centimeters below and lateral to the umbilicus and the needle was inserted to collect the subcutaneous fat biopsy. The supraclavicular biopsy was collected from the fat localized in the deep neck, either at the lymph node level 4 or level 6, depending on the specific type of surgery scheduled to remove the tumor, since the biopsy was obtained from the same surgical incision without recurring in additional surgery.

For determination of SPARC and UCP1 mRNA expression in WAT and BAT quantitative real-time polymerase chain reaction (qPCR) was used. RNA isolation from adipose tissue biopsies was performed using TRizol reagent according to the manufacture's protocol. RNA was dissolved in nuclease-free water and quantified using a Nanodrop ND 1000. Total RNA (0.25 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). cDNA samples were loaded in triplicate and the qPCR was performed. Relative quantification was conducted by TaqMan Gene Expression Assays (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). All procedures were performed according to the manufacturer's protocol. Target gene mRNA expression was normalized to the reference gene peptidyl prolyl isomerase A (PPIA) and calculated based on the delta-delta method

relative to subcutaneous fat. PPIA was chosen as preferred reference gene, as it has been specifically validated as an appropriate housekeeping gene in BAT/ WAT mRNA expression analysis studies[176].

2.2.2 Study III: Plasma analysis for quantification of SPARC after cold exposure

As next step, we aimed to quantify SPARC concentration levels in obese men following cold-activating BAT by CE vs TN. To this end, samples from a previous study conducted by our group at the University of Lübeck (AZ 12/030), in which subjects with obesity were exposed to individualized cooling protocol, were analyzed (Study III).

Fourteen males with obesity (mean ± SEM; age: 26.4 ± 0.8 years; BMI: 31.9 ± 0.5 kg/m^2) participated in this cross-balanced within-subject study, with two experimental conditions. Using a water-perfused suit (ThermoFlash, Buchenberg, Germany), subjects were exposed to TN (25°C) and to CE (16.0°C, shivering excluded). On each experimental day, subjects arrived at the research unit at 8:00, after a 12-h night-time fasting. Upon arrival, participants were asked to wear standardized underwear, i.e., sleeveless cotton shirt and long cotton pants. Thereafter, they put on a water-perfused suit which was worn throughout the entire experimental procedure. Surface electromyograms electrodes (Neurofax EEG-9200, Nihon Koden, Tokyio, Japan) were fixed to subjects' upper arm, thigh and belly to detect potential shivering. Subjects were first adapted to TN for 90 minutes after which blood samples were taken as standardized baseline values. Next, subjects were exposed to either TN or CE for 120 minutes, depending on the experimental session and blood was collected repeatedly as temperature adapted values to 25°C or 16°C, respectively (Figure 5).

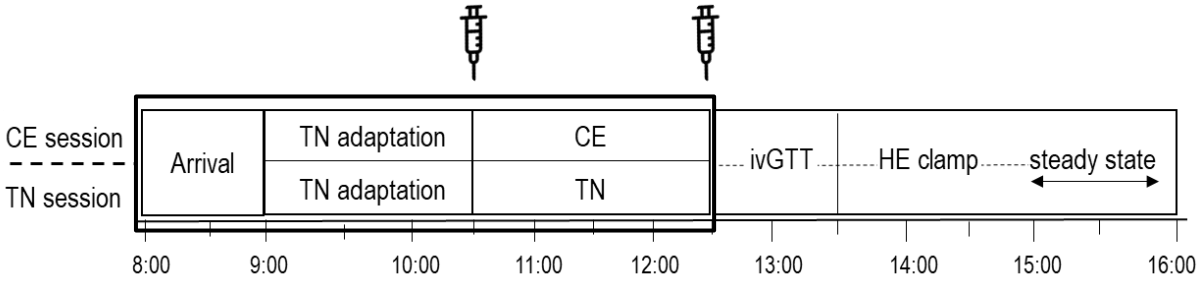


Figure 5. Experimental design of study III.

Plasma samples analyzed for the purpose of SPARC quantification are highlighted within the box. TN, thermoneutrality; CE, cold exposure; ivGTT, intravenous glucose tolerance test; HE clamp, hyperinsulinaemic-euglycaemic clamp.

Materials and methods

SPARC concentration in plasma was assessed using commercially available enzyme immunoassay kits (EIA, R&D System, Minneapolis, USA). SPARC is present in platelet granules and is released upon platelet activation. In order to quantify an accurate circulating levels of SPARC, is important to conduct the measures on platelet-free plasma. Therefore, an additional centrifugation step of the plasma samples at 10,000xg for 10 minutes at 5°C was performed to obtain platelet-free samples. After that, SPARC concentrations in human plasma were determined in duplicates using the R&D quantitative sandwich EIA kit (EIA, R&D System, Minneapolis, USA). Briefly, a monoclonal antibody specific for human SPARC was pre-coated onto a microplate, provided by the supplier. Standards and samples were pipetted into the wells and any SPARC present was bound by the immobilized antibody. After a wash step to remove any unbound substances, an enzyme-linked polyclonal antibody specific for human SPARC was added to the wells. Following one more wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. Bright yellow colour developed in proportion to the amount of SPARC bound in the initial step. A standard curve of known concentration of SPARC was created and its concentration in the samples was calculated accordingly to the colour intensity. Of note, since SPARC is detectable in saliva, precautionary measures were taken to prevent contamination of the kit while measuring, namely, a face mask was used. The assay mean detectable limit was 0.099 ng/mL with a mean intra-assay coefficient of variation of 2.1%.

2.2.3 Statistical analyses

Analyses were performed using SPSS 22 (IBM, Illinois, USA), Prism software (GraphPad, California, USA) was used to prepare the figures, and values are expressed as mean ±SEM, unless otherwise noted. To test for the effect of cold-induced BAT activation vs thermoneutrality on SPARC circulating levels, two-way repeated measures ANOVA with factors ‘condition’ (‘TN’ vs ‘CE’) and ‘time’ (‘baseline’ vs ‘after thermal acclimation’) were considered. Post hoc paired t-test was used for pairwise comparison of single time points. To assess differences in SPARC mRNA expression between fat depots (WAT vs BAT), unpaired t-test was performed. One-way ANOVA was used for the comparison of SPARC mRNA expression in BAT between BMI groups. Pearson correlation coefficients were performed to examine associations between the circulating levels of SPARC and levels of noradrenaline and insulin as well as to investigate correlation between SPARC and UCP1 mRNA expression levels. SPARC and UCP1 gene expressions were log10 transformed to obtain normal distribution of variables. A p value <0.05 was considered to indicate a statistically significant difference.

III. Results

Hypothesis I

3.1 Subjects characteristics of study I

The anthropometric and clinical characteristics of the 18 normal-weight healthy male subjects of study I at the initial medical examination are summarized in Table 1. The age of the subjects ranged from 19 to 33 years. Neither body weight nor fat mass changed significantly between visits (all $p \geq 0.293$; data no shown).

Table 1. Anthropometric and clinical characteristics of subjects of study I at the initial medical examination.

Parameter	Mean ± SEM
Age, years	24.1 ± 0.9
Height, m	180.4 ± 0.9
Weight, kg	69.7 ± 1.1
BMI, kg/m^2	21.4 ± 0.4
Fat mass, %	18.2 ± 1.3
Heart rate, bmp	65.4 ± 3.2
Systolic blood pressure, mmHg	116.1 ± 1.9
Diastolic blood pressure, mmHg	75.3 ± 2.0

N=18; BMI, body mass index.

3.2 Effect of intranasal insulin on body temperature

The overall change in SCVT over time (p=0.003) was not different between conditions (p=0.144; Figure 6). A slight increase in SCVT by 0.04°C and 0.02°C was measured 5 minutes following the meal challenge during PLC and INI, respectively, with no difference between conditions (p=0.701). 20 minutes after the meal challenge, which corresponded with the end of the IRT measures, the SCVT decreased (p=0.002) with no difference between conditions (p=0.563) with an average of 0.04°C and 0.03°C during PLC and INI, respectively.

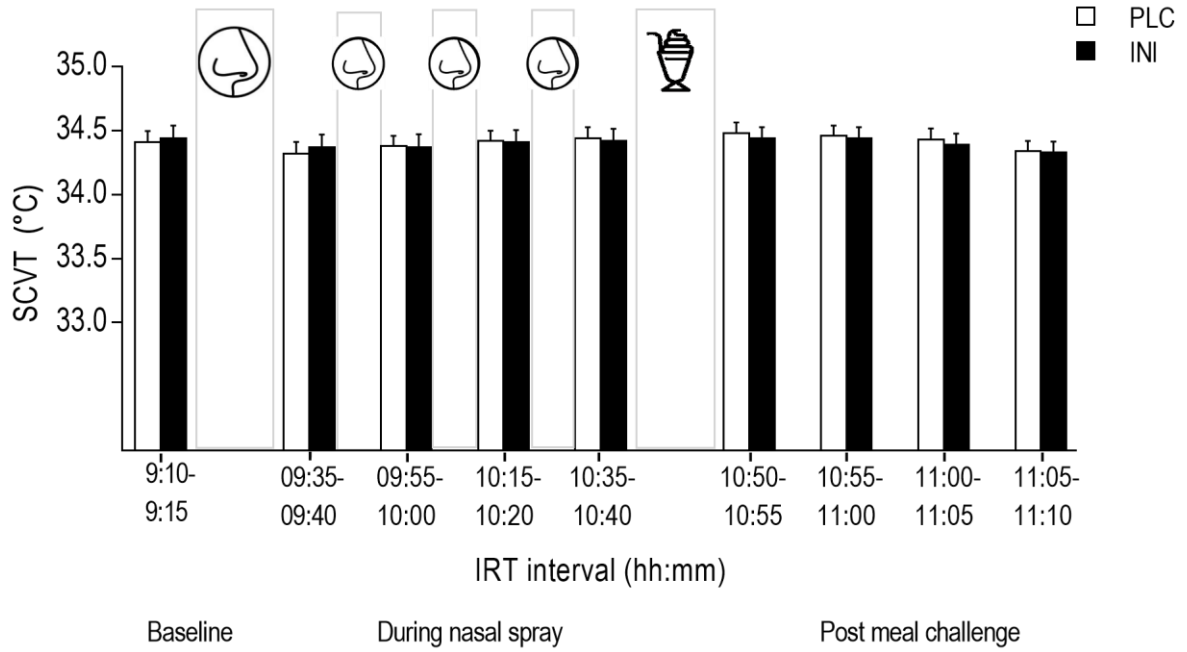


Figure 6. SCVT during experimental sessions.

INI and PLC, black and white bars, respectively. Data are mean ± SEM; n=18.

SCVT, supraclavicular temperature; IRT, Infrared Thermography; INI, intranasal insulin; PLC, placebo. Icons: Nose, nasal spray; drink, standardized liquid meal challenge.

Data of MSKT and ICBT during the experiments are reported in Table 2. MSKT and ICBT did not change during INI as compared to PLC over time (both p≥840).

Table 2. Mean skin temperature and indirect core body temperature measured at baseline, post INI and post meal challenge.

Parameter	PLC	INI	ANOVA condition*time
MSKT (°C)			
Baseline	32.25 ± 0.11	32.27 ± 0.09	0.851
Post 160 IU	31.93 ± 0.11	31.92 ± 0.08	
Post meal challenge	31.87 ± 0.12	31.85 ± 0.08	
Post 240 IU	31.99 ± 0.11	31.97 ± 0.09	
ICBT (°C)			
Baseline	36.24 ± 0.89	36.23 ± 0.86	0.840
Post 160 IU	36.24 ± 0.82	36.18 ± 0.89	
Post meal challenge	36.17 ± 0.77	36.17 ± 0.99	
Post 240 IU	36.20 ± 0.82	36.20 ± 0.86	

Values are means ± SEM; n=18; INI, intranasal insulin; PLC, placebo; IU, international unit

3.3 Effect of intranasal insulin on sympathetic nervous activity

Levels of noradrenaline and dopamine expressed as percentage change from baseline increased over time (both p<0.001) by 80% and 56%, and 48% and 39%, upon PLC and INI, respectively, while there were no differences between conditions (both p≥0.240; Figure 7a & b, respectively). Levels of adrenaline decreased over time (p<0.001) by 44% and 43% upon PLC and INI respectively, with no difference between conditions (p=0.340; Figure 7c).

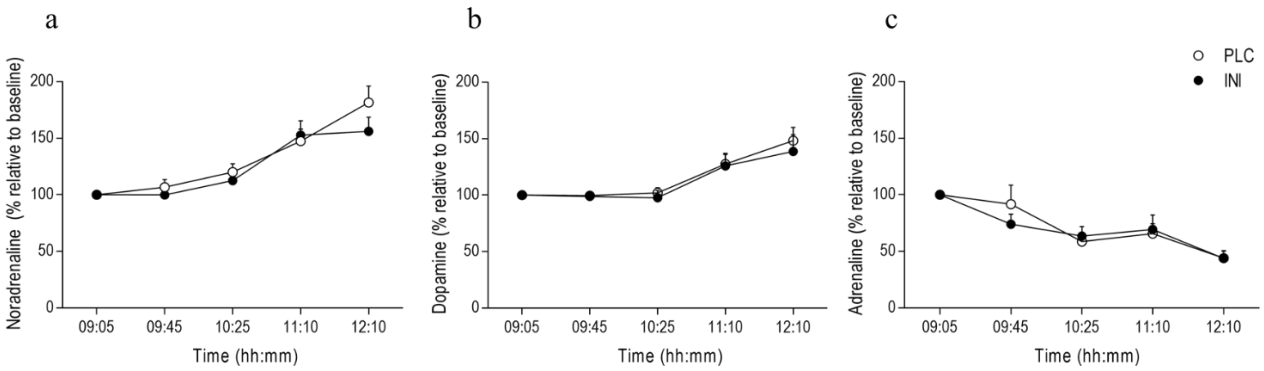


Figure 7. Catecholamines concentrations during experimental sessions.
Concentration of a) noradrenaline, b) dopamine, c) adrenaline upon INI and PLC expressed in percentage change from baseline, black and white circles, respectively. Data are mean ± SEM; n=18.

Values of HRV given as LF/HF ratio are reported in Table 3. The LF/HF ratio did not change between conditions throughout the experiment (p=0.364).

Table 3. HRV as given by LF/HF ratio at baseline, post INI and post meal challenge.

Parameter	PLC	INI	ANOVA condition*time
LF/HF ratio			
Baseline	4.99 ± 0.68	6.40 ± 1.09	0.364
Post 160 IU	5.01 ± 0.70	4.96 ± 0.76	
Post meal challenge	5.78 ± 1.02	5.53 ± 0.80	
Post 240 IU	4.33 ± 0.78	4.76 ± 0.68	

Values are mean ± SEM; n=18; HRV, heart rate variability; LF, low frequency; HF, high frequency; INI, intranasal insulin; PLC, placebo; IU, international unit.

3.4. Effect of intranasal insulin on the HPA and HPT axis activity

Levels of cortisol increased following the meal challenge ($p<0.001$) by 2.64 µg/dL and 2.36 µg/dL during PLC and INI, respectively, with no differences between conditions ($p=0.948$; Figure 8a). Likewise, although levels of fT_3 decreased over time ($p<0.001$), no difference was measured between INI and PLC ($p=0.804$; Figure 8b).

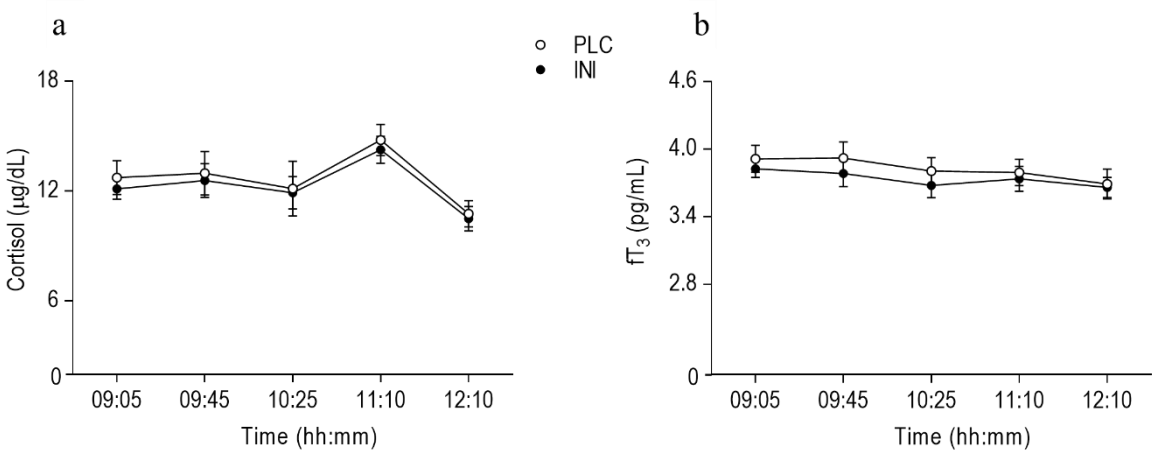


Figure 8. Cortisol and fT_3 concentrations during experimental sessions.
Concentration of a) cortisol and b) fT_3 upon INI and PLC, black and white circles, respectively. Data are mean ± SEM; n=18.

3.5 Effect of intranasal insulin on levels of leptin and adiponectin

Levels of plasma leptin and adiponectin throughout the experiment are given in Table 4. Adipokine concentrations did not change during INI as compared to PLC (both $p \geq 0.190$; Table 4). Moreover, neither levels of leptin nor adiponectin changed over time (both $p \geq 0.989$).

Table 4. Adipokine concentrations at baseline, post INI and post meal challenge

Parameter	INI	PLC	ANOVA condition*time
Leptin (ng/mL)			
Baseline	5.42 ± 0.84	5.92 ± 1.26	0.190
Post 160 IU	5.05 ±0.81	5.35 ± 1.01	
Post meal challenge	5.51 ± 0.65	5.54 ± 0.89	
Adiponectin (µg/mL)			
Baseline	10.22 ± 0.79	11.18 ±1.10	0.399
Post 160 IU	10.73 ± 0.76	11.38 ±1.36	
Post meal challenge	10.19 ± 0.76	10.56 ±1.13	

Values are mean ± SEM; n=18; INI, intranasal insulin; PLC, placebo; IU, international unit.

3.6 Effect of intranasal insulin on levels of ghrelin and subjective feeling of satiety and hunger

The levels of active ghrelin shortly failed to reach statistical difference between conditions over time (p=0.085; Figure 9a). However, a lower concentration of active ghrelin by 17.61 pg/mL was found following the meal challenge (p=0.030; Figure 9a) during INI as compared to PLC. Further, AUC of post prandial active ghrelin during INI was lower by 364.10 pg/mL (p=0.029; Figure 9b).

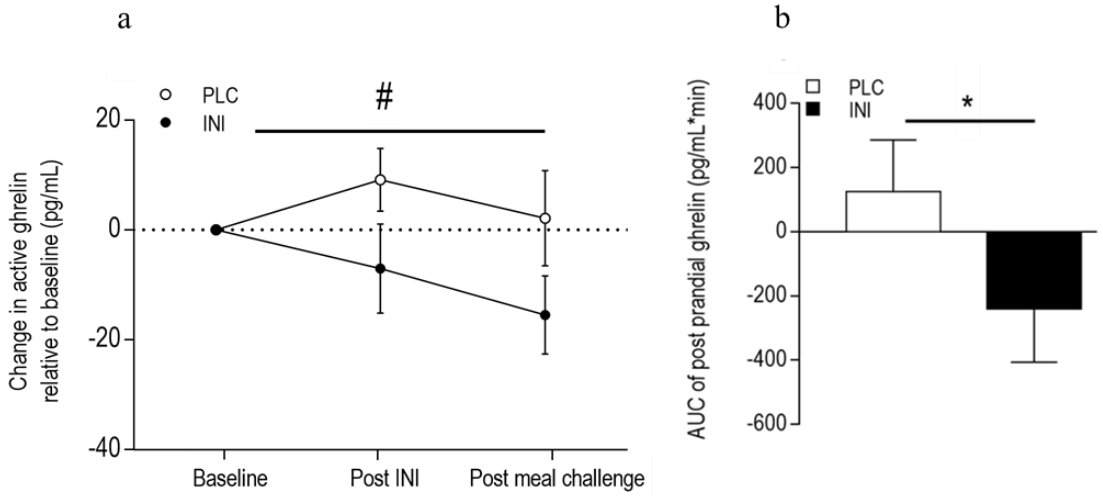


Figure 9. Active ghrelin concentrations during experimental sessions.
a) Change from baseline of active ghrelin concentrations after 160 IU of INI or PLC and after the standardized meal challenge, black and white circles, respectively. b) AUC of post prandial active ghrelin upon INI and PLC, black and white bars, respectively. Data are mean ± SEM, n=18.

Subjective feeling of satiety increased in both conditions after the standardized meal challenge ($p<0.001$) by 1.90 and 2.55 points upon PLC and INI, respectively, whereas upon INI, the postprandial reported feeling of satiety was 0.65 point greater as compared to PLC ($p=0.046$; Figure 10a). Subjective feeling of hunger decreased after the meal challenge ($p<0.001$) by 1.53 and 1.52 points upon PLC and INI, respectively, and was not different between conditions ($p=0.967$; Figure 10b).

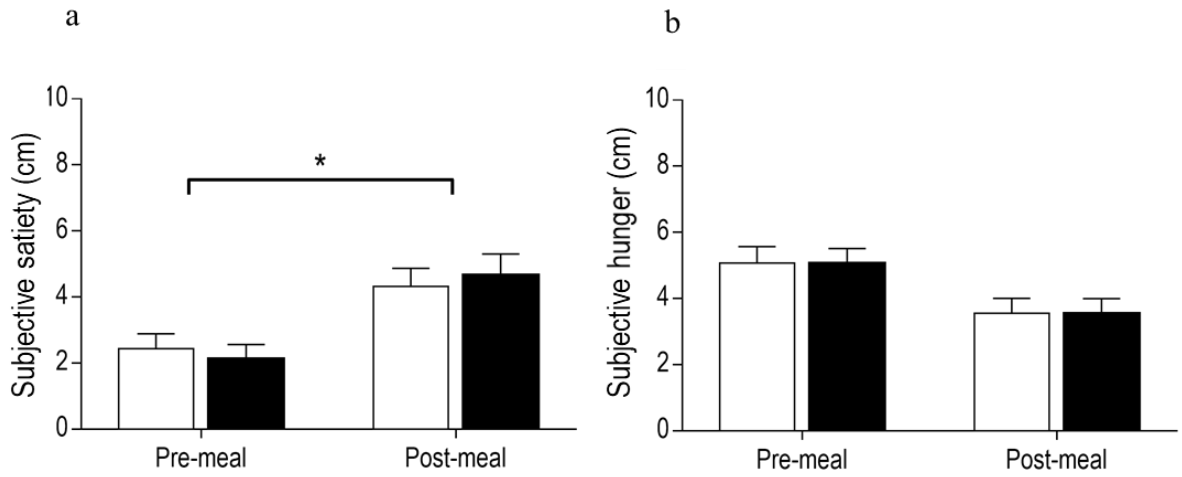


Figure 10. Subjective feelings of satiety and hunger assessed before and after a meal challenge. Subject feelings of a) satiety and b) hunger upon INI and PLC, black and white bars, respectively. Data are mean ± SEM, n=18.

3.7 Effects of neuroendocrine and environmental factors on body temperature

No association was found between basal SCVT and age (p=0.254, r_{rm}=0.274; Figure 11a), whereas basal SCVT inversely correlated with BMI (p=0.004, r_{rm}=-0.631; Figure 11b) and fat mass (p=0.046, r_{rm}=-0.462; Figure 11c), respectively.

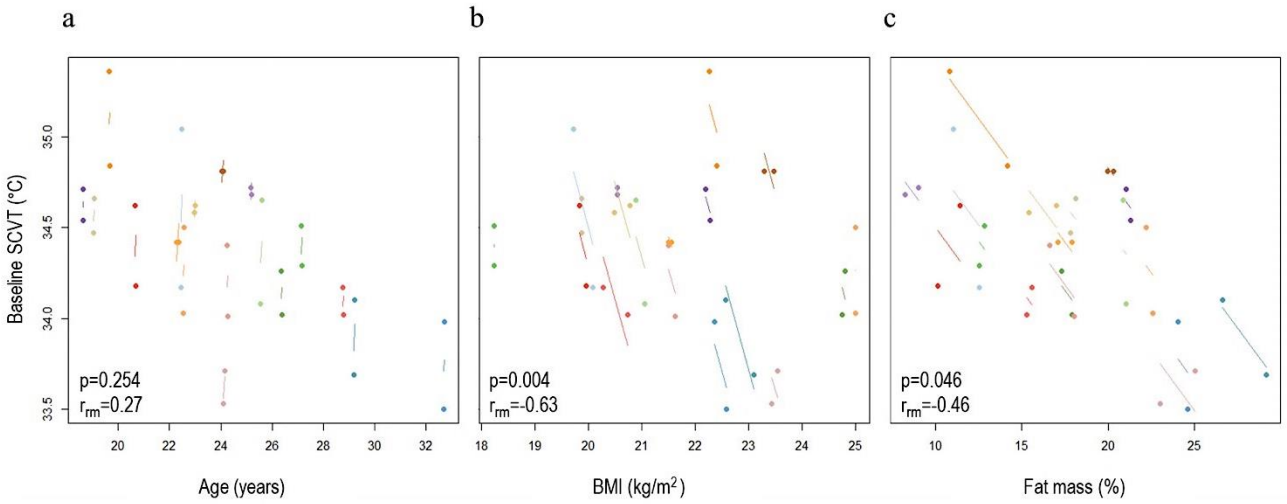


Figure 11. Associations between age, BMI, fat mass and basal SCVT.
Repeated measures correlations between SCVT and a) age, b) BMI and c) fat mass.
For the Rmcorr analysis, measurements from the same subject are given in the same colour, with corresponding lines to show the Rmcorr fit for each subject. SCVT, supraclavicular temperature; BMI, body mass index; Rmcorr, repeated measures correlational; r_{rm}, repeated measure coefficient.

To further define the relationship between body temperature and OT, subjects were divided into two sub-groups, i.e., ‘warm’ or ‘cold’, according to the 7 day average max outside temperature (7d-OT) previous to each study visit. Among the measurements of basal body skin temperatures, SCVT was higher in cold as compared to the warm group (both p<0.05; Figure 12a) for both conditions, while no differences were found in the RPT between warm and cold group (both p≥0.137; Figure 12b) for both conditions. MSKT and ICBT were also not different between groups for both conditions (all p≥0.359; data not shown). Moreover, basal SCVT inversely associated with 7d-OT (p=0.008, r_{rm}=-0.586; Figure 12c), while no association was found between basal SCVT and RPT (p=0.338, r_{rm}=-0.232; Figure 12d).
To investigate the relationship between body temperature and SD, subjects were divided into two sub-groups, i.e., ‘low’ or ‘regular’, according to the average sleep duration of the 7 night (7n-SD) previous to each study visit. Baseline SCVT was higher in the low compared to the regular sleep

group (both $p<0.05$; Figure 13a) in both conditions, while no differences were found at the RPT (both $p\geq0.332$; Figure 13b) in both conditions. MSKT and as ICBT was also not different between groups for both conditions (all $p\geq0.339$; data not shown). Moreover, inverse association was found between basal SCVT and 7n-SD ($p=0.050$, $r_{rm}=-0.454$; Figure 13c), while basal RPT was not associated with 7n-SD ($p=0.371$, $r_{rm}=0.217$; Figure 13d).

To investigate the role of PA on body temperature, subjects were divided into three sub-groups, i.e., 'low', 'medium' and 'high', according to the 7 day average physical activity (7d-PA) prior to the study visit. SCVT was not different among PA level groups for both conditions (both $p\geq0.387$; Figure 14a). Likewise, no differences were found in the RPT, MSKT and ICBT, respectively, between groups for both conditions (all $p\geq0.473$; data not shown). Additionally, no association was found between the 7d-PA high intensity level and basal SCVT ($p=0.259$, $r_{rm=}-0.272$; Figure 14b).

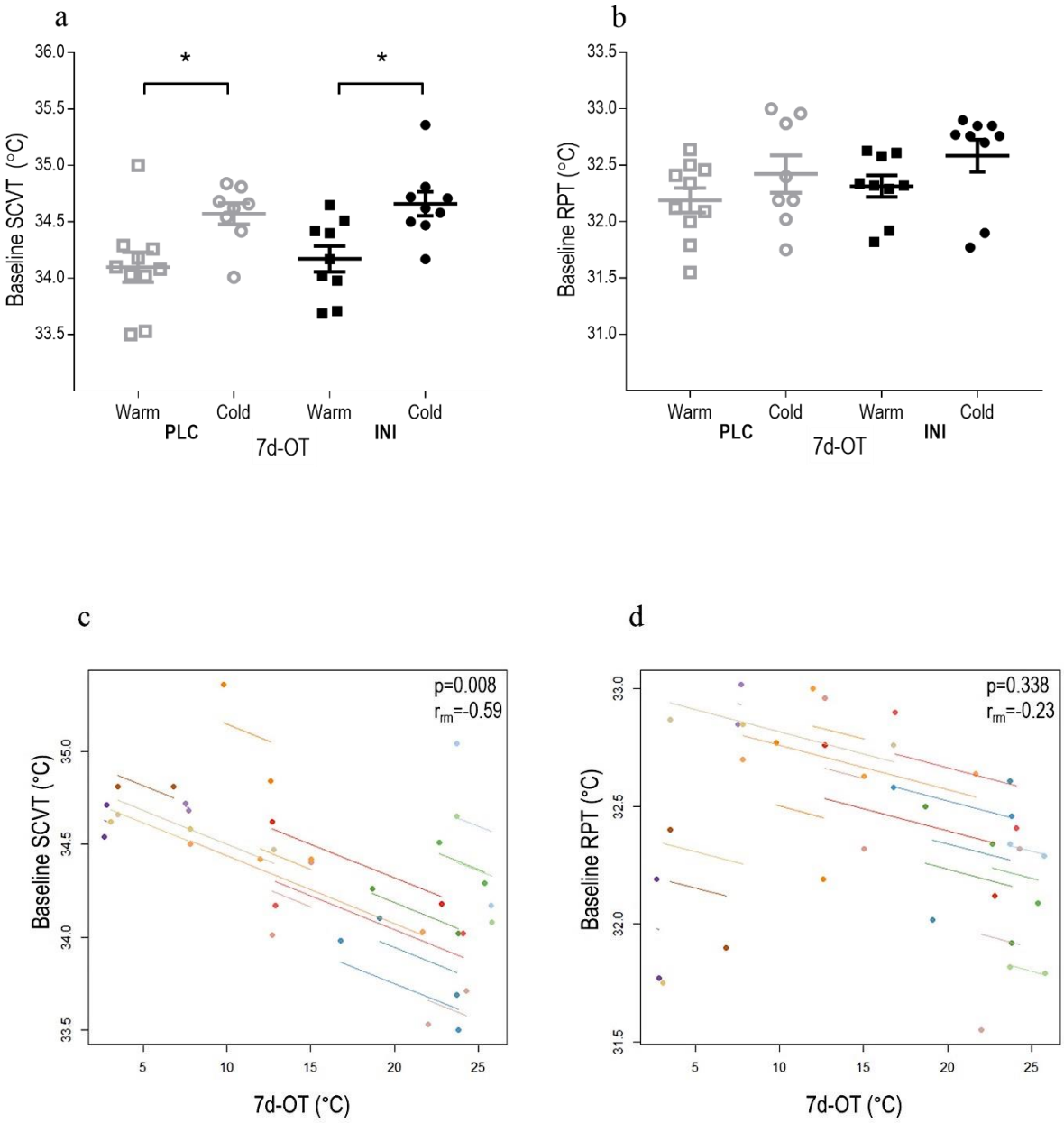


Figure 12. Associations between OT, SCVT and RPT.

Baseline a) SCVT and b) RPT in warm and cold group according to the previous 7d-OT. Bars are mean ± SEM, n=18. Rmcorr of 7d-OT with c) basal SCVT and e) basal RPT. For the Rmcorr analysis, measurements from the same subject are given in the same colour, with corresponding lines to show the Rmcorr fit for each subject. SCVT, supraclavicular temperature; RPT, reference point temperature; 7d-OT, 7 day average outside temperature; Rmcorr, repeated measures correlation; r_{rm}, repeated measure coefficient.

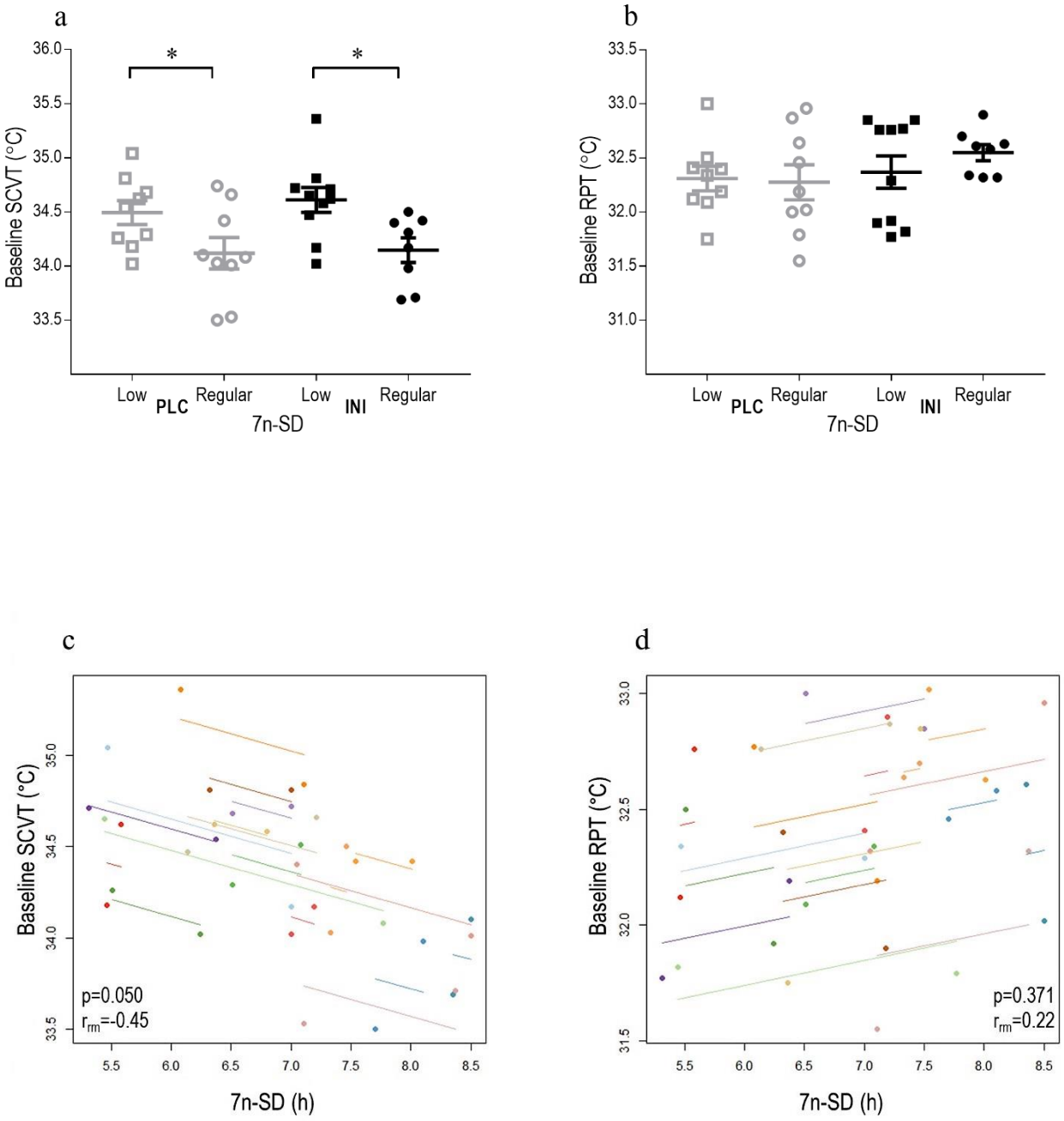


Figure 13. Associations between 7n-SD, SCVT and RPT.

Baseline a) SCVT and b) RPT in low and regular sleep group according to the previous 7n-SD. Bars are mean ± SEM, n=18. Rmcorr of 7n-SD with c) basal SCVT and d) basal RPT. For the Rmcorr analysis, measurements from the same subject are given in the same colour, with corresponding lines to show the Rmcorr fit for each subject. SCVT, supraclavicular temperature; RPT, reference point; 7n-SD; 7 night average sleep duration; Rmcorr, repeated measures correlation; r_{rm}, repeated measure coefficient.

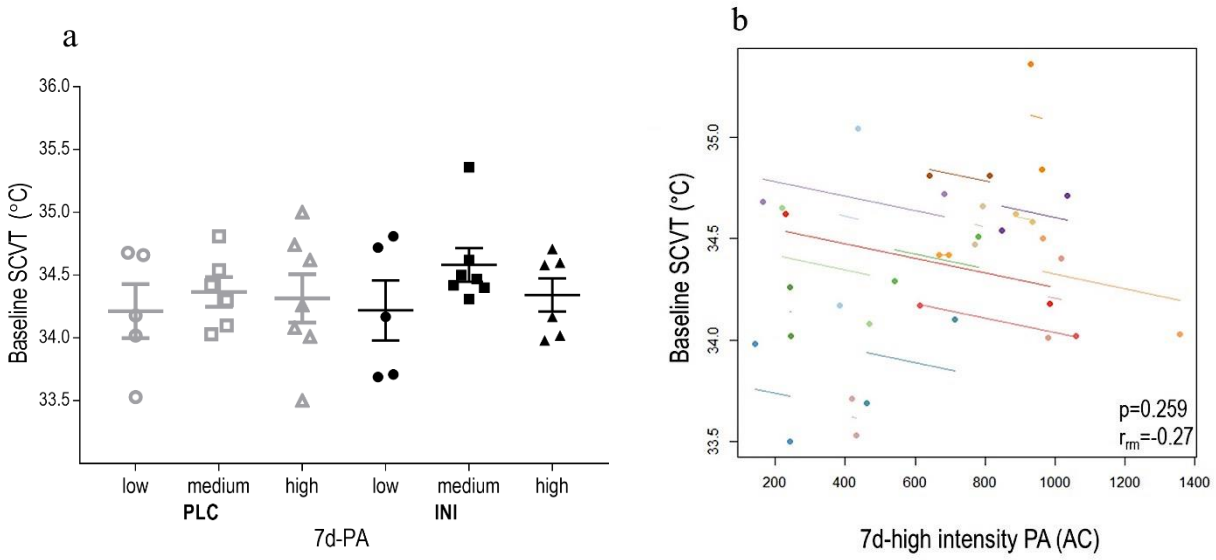


Figure 14. Association between PA and SCVT.

SCVT in a) low, regular and high level PA group according to the previous 7d-PA. Bars are mean ± SEM, n=18. b) Rmcorr between high PA and basal SCVT. According to Rmcorr analysis, measurements from the same participant are given the same colour, with corresponding lines to show the Rmcorr fit for each participant. SCVT, supraclavicular temperature; 7d-PA, 7 day average physical activity; Rmcorr, repeated measures correlation; r_{rm}, repeated measure coefficient.

Among the metabolic factors, basal SCVT positively correlated with noradrenaline at baseline (p=0.029, r_{rm}=0.500; Figure 15a), whereas no association was found with basal adrenaline (p=0.340, r_{rm}=0.231; Figure 15b). Baseline levels of neither glucose nor insulin associated with basal SCVT (p=0.588, r_{rm}=-0.132 p=0.622 and r_{rm}=-0.120; Figure 15c & d, respectively).

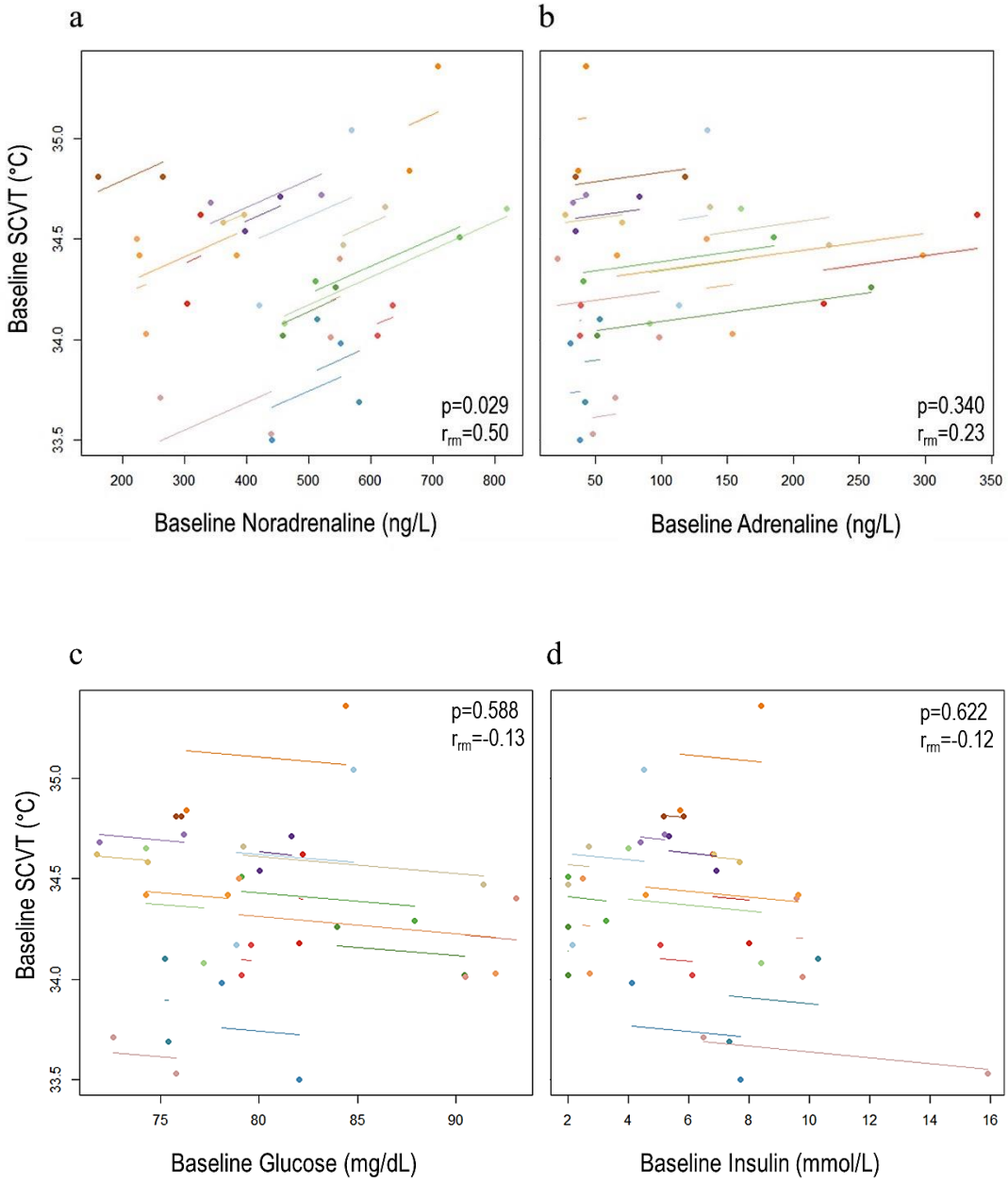


Figure 15. Associations between metabolic parameters and basal SCVT.

Rmcorr between basal SCVT and a) noradrenaline, b) adrenaline, c) glucose and d) insulin.
According to Rmcorr analysis, observations from the same participant are given the same colour, with corresponding lines to show the rmcorr fit for each participant. SCVT, supraclavicular temperature; Rmcorr, repeated measures correlational; r_{rm}, repeated measure coefficient.

Further, we sought to identify by multiple stepwise linear regression analysis which of the the neuroendocrine, environmental, lifestyle and metabolic parameters are independently associated with basal-BAT activity and to which extend they could explain its variance. Basal SCVT was inserted as dependent variable, while BMI, fat mass, 7d-OT, 7n-SD and noradrenaline were used as predictive variables. The multiple linear regression model shows a predictable variance in the basal BAT-activity among our study cohort of approximately 69% ($p<0.001$, adjusted $r^2=0.687$). In detail, 7d-OT, fat mass, 7n-SD and noradrenaline levels are significant determinants of SCVT, explaining 44% ($p<0.001$, $\beta=-0.621$), 18% ($p=0.011$, $\beta=-0.295$), 4% ($p=0.036$, $\beta=-0.235$) and 3% ($p=0.048$, $\beta=0.204$), respectively, of its variance.

Hypothesis II

3.8 SPARC mRNA expression in biopsies from human WAT and BAT of study II

In subjects with normal weight, SPARC mRNA expression in BAT was lower as compared to mRNA expression in WAT (p<0.001; Figure 16a). Whereas, SPARC mRNA expression in BAT increased with higher BMI groups (p<0.001; Figure 16b). In line, SPARC expression in BAT positively correlated with BMI (p<0.01, r=0.538; Figure 16c), while negatively with UCP1 (p<0.01, r=-0.519; Figure 16d).

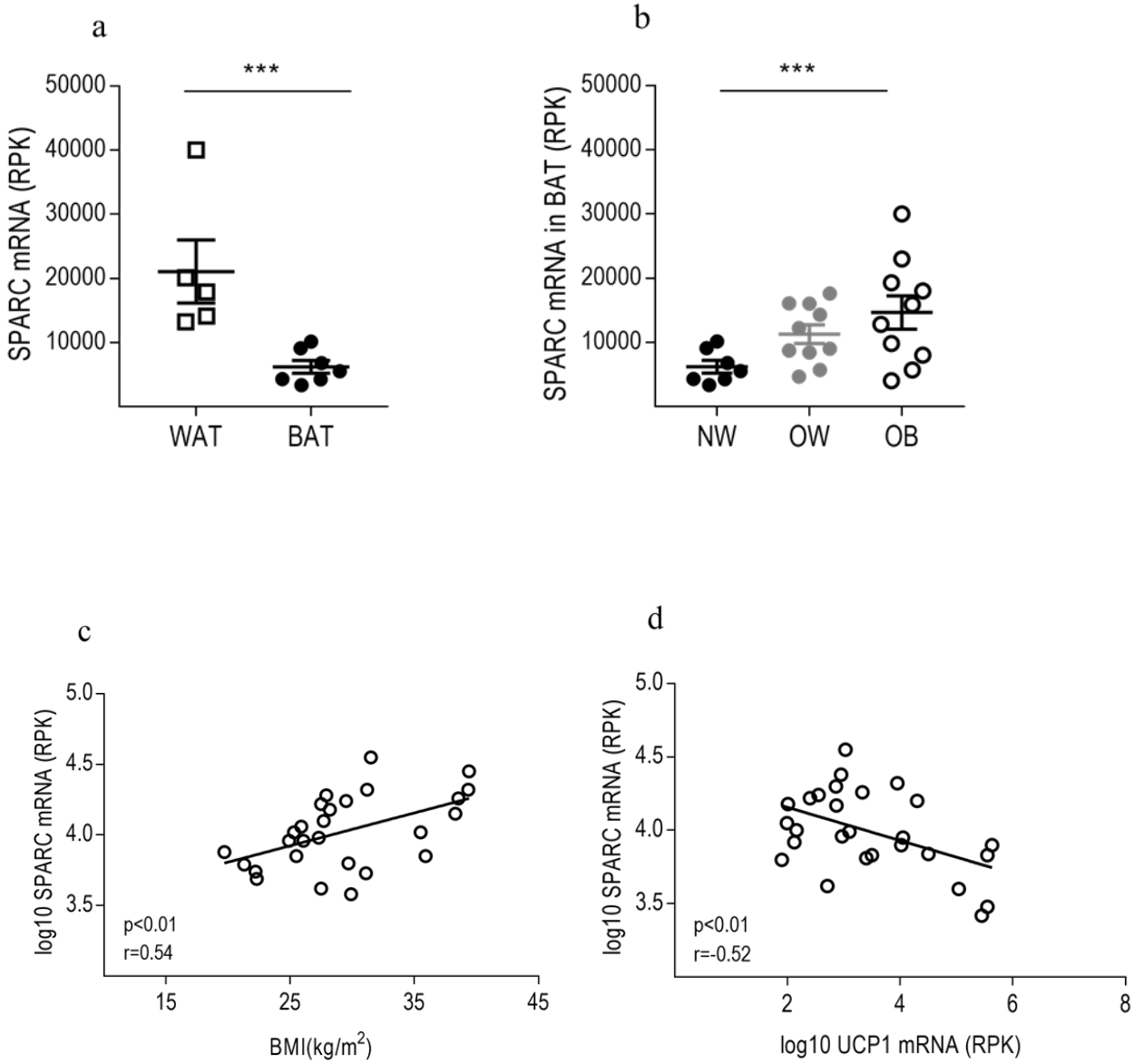


Figure 16. SPARC mRNA expression in human WAT and BAT.

a) SPARC mRNA expression in subcutanuous WAT (n=5) and supraclavicular BAT (n=7) in normal weight subjects. b) SPARC mRNA expression in BAT divided by body weight categories, normal weight (NW; n=7), overweight (OW; n=10) and obese (OB; n=10). Association between SPARC expression in BAT and c) BMI and d) UCP1. RPK, read per kilobase.

3.9 Plasma analysis of study III

In subjects with obesity, SPARC concentrations increased by 8% following 2h of CE as compared to 2h of thermal adaptation to TN (p=0.022; Figure 17a). Although SPARC levels differed between conditions at baseline (p=0.016), the increase in SPARC upon CE remained significant after baseline correction (p=0.004, Figure 17b).

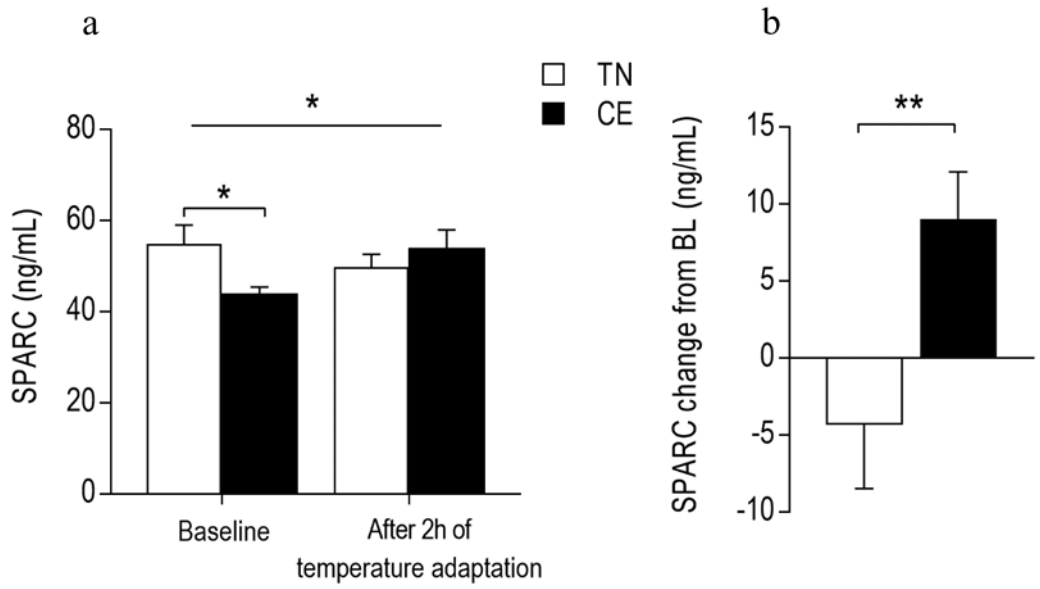
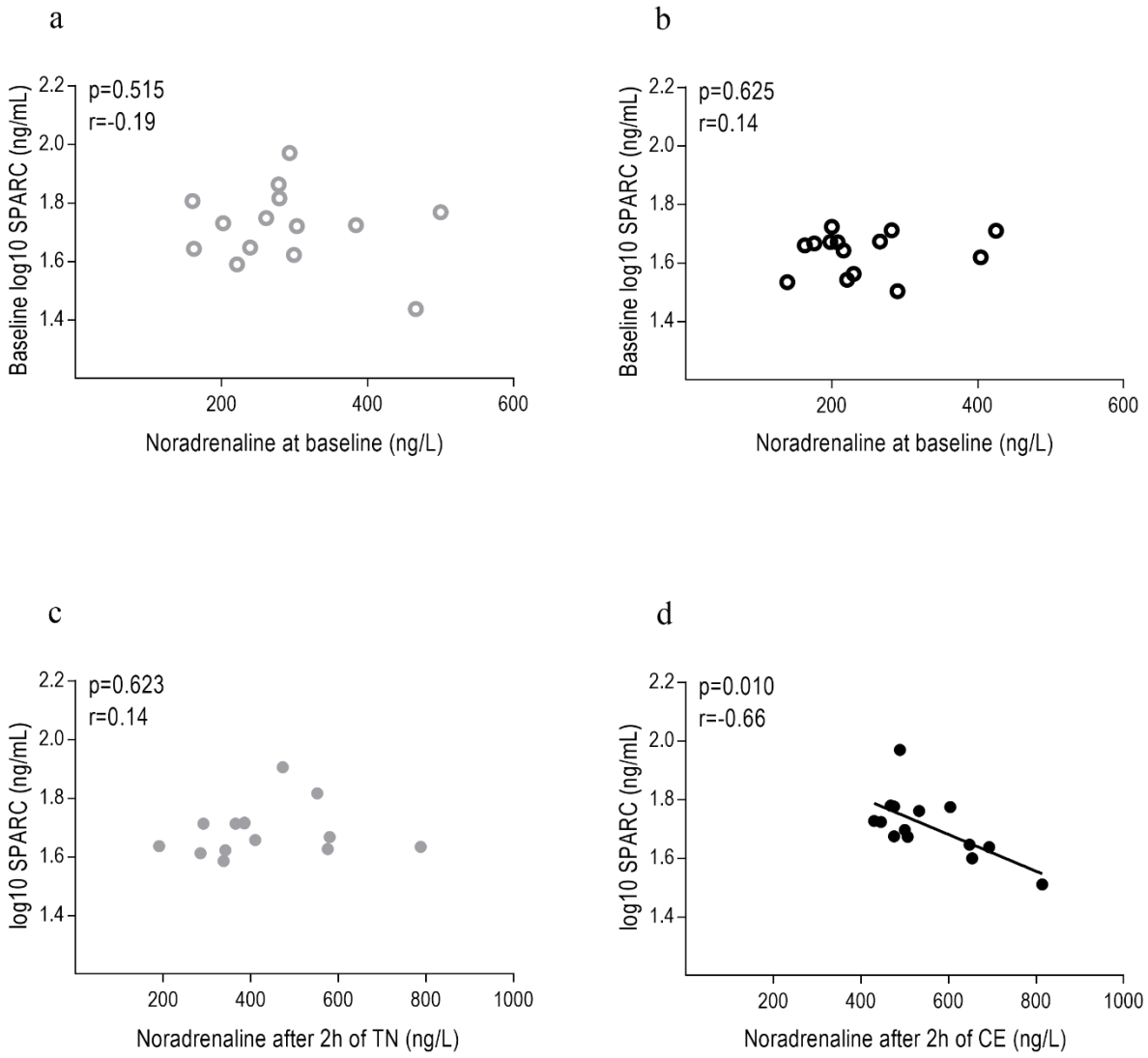


Figure 17. SPARC concentrations following cold exposure in subjects with obesity.
a) SPARC concentrations at baseline and following 2h of thermal adaptation to TN (25°C, white) and to CE (16°C, black). b) Change of SPARC concentrations from baseline upon TN (25°C, white) and CE (16°C, black). TN, thermoneutrality; CE, cold exposure. Data are mean ± SEM, n=14.

No associations were found between SPARC and noradrenaline at baseline for both conditions (p=0.515, r=-0.190 and p=0.625, r=0.143, during TN and CE, respectively; Figure 18a & b). Likewise, following 2h of thermal adaptation to TN, SPARC did not correlate with noradrenaline (p=0.623, r=0.144; Figure 18c), whereas a negative correlation was found between SPARC and noradrenaline following 2h of CE (p=0.010, r=-0.660; Figure 18d). Concomitantly, while there were no associations between SPARC and insulin at baseline for both conditions (p=0.398, r=0.245 and p=0.907, r=-0.035, during TN and CE, respectively; Figure 18e & f), nor following 2h of temperature adaptation to TN (p=0.868, r=-0.049; Figure 18g), SPARC positively correlated with insulin levels following 2h of CE (p=0.029, r=0.583; Figure 18h).



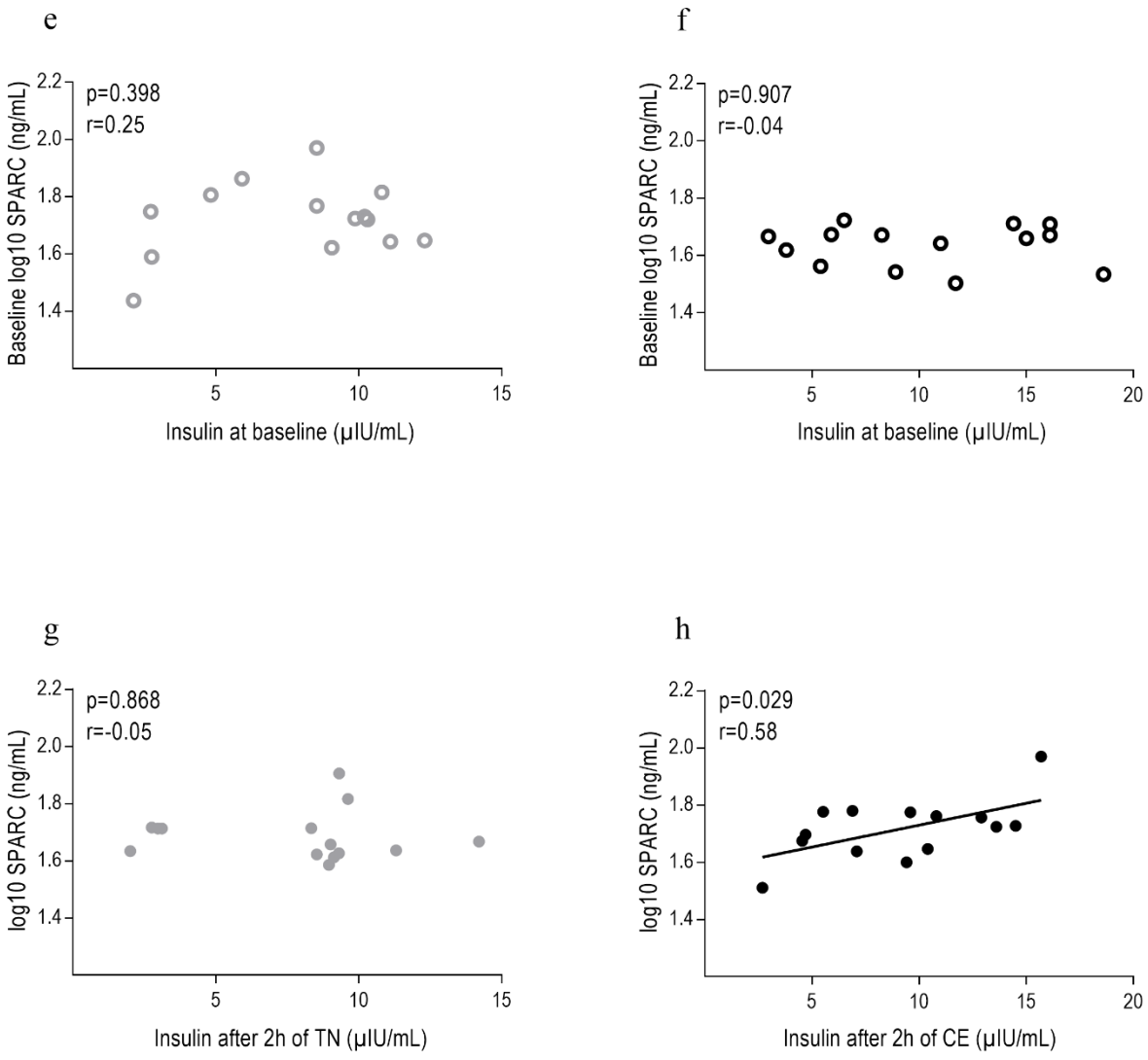


Figure 18. Associations between markers of cold-activated BAT and SPARC.

Associations between SPARC and a) noradrenaline at baseline during TN and b) during CE, grey and black circles, respectively; c) following 2h of temperature adaptation to TN and d) to CE, full grey and full black circles, respectively. Associations between SPARC and e) insulin at baseline during TN and f) during CE, grey and black circles, respectively; g) following 2h of temperature adaptation to TN and h) to CE, full grey and full back circles, respectively.

IV. Discussion

4.1 INI administration does not increase SCVT and the SNS response associated to BAT-thermogenic activity

Based on the knowledge that warm-sensitive neurons respond to central insulin causing a hyperthermic response in mice, we hypothesized that INI could represent an activator potent enough to trigger BAT function, and thereby improving metabolic profile in humans. Therefore, we determined the effects of enhanced brain-insulin signaling as activator of BAT metabolism by investigating changes on body skin temperature particularly co-locating with BAT in humans, i.e., SCV regions, during INI vs PLC.

Against our hypothesis, repeated INI administrations did not stimulate BAT-thermogenic activity indicated by an absence of increased SCVT as assessed by IRT.

Interestingly, our results are in contrast with rodents data reporting increased thermogenesis in mice following ICV[148]. In particular, a local injection of insulin into the POA of mice elevated core body temperature by directly acting on the insulin receptor expressing warm-sensitive neurons[148]. Additional retrograde tracing analysis led the authors to conclude that the promoting effect of increased brain insulin-signaling on thermogenesis was mediated through projections from the POA to BAT via the raphe pallidus[148]. The POA is a specific hypothalamic region which is known to abundantly express temperature-sensitive neurons, both warm and cold[177]. These neurons are essential for eliciting rapid responses in BAT thermogenesis, thus, making the POA a key control unit to sense and respond to skin-temperature feedbacks and thermal challenges[81,178].

To address the disagreement between our observations with the ones made in rodents showing ICV insulin promoting BAT-thermogenesis[148], several points need to be discussed. First, the control of body temperature by the SNS involves several pathways and neuronal circuits which, due to the physiologic intra-species variations, may not necessarily overlap between mammals[147,177]. Second, the mild cold stress associated to the standard housing of mice makes it often difficult to translate results of animal studies into human studies, as also recently pointed out by others[179,180]. This problem could be likely addressed by adopting a thermoneutral housing. In this way, mice's living conditions would better reflect the physiology and pathophysiology of humans' thermal conditions, thereby enabling a better metabolic comparison. Third, contrary to rodents, it is not feasible in humans to assess the exact area of the brain where insulin acts by linking to its receptors. Therefore, in this sense tentative speculations can be made only.

To assess the induction of BAT-thermogenesis potentially mediated by INI, we considered additional markers as secondary outcomes for our hypothesis and to complement the IRT measures of SCVT.

The importance of SNS activation, in particular with respect to the role of noradrenaline as major player in the SNS-mediated BAT-thermogenesis response in humans is well established[13,62]. However, levels of noradrenaline were not affected by INI in our study. Likewise, no changes were observed neither in heart rate variability nor in HPA axis activity, as shown by unchanged LF/HF ratios and circulating levels of cortisol, respectively. Together with noradrenaline, these two parameters are considered as indirect mediators of SNS response associated with induced BAT-thermogenic activity.

The LF/HF ratio refers to the proportion of sympathetic and parasympathetic impact on autonomic regulation of the sinus-astral node[181]. Accordingly, lower LF/HF ratios reflect a higher portion of parasympathetic dominance, occurring when the body conserves energy and engages in 'tend-and-befriend' behaviours, while higher LF/HF ratios indicate sympathetic dominance, occurring when the 'fight-or-flight' behaviour is engaged[182].

With regard to cortisol, it is known to play an important role in promoting maximal BAT function around the time of birth. Of note, remains to be fully established whether this is a direct effect or whether this is mediated by changes in concentrations of thyroid hormones[183]. Recently, the relation between cortisol and BAT function has been addressed in humans. In this study, a mild psychological stress was induced in healthy, lean women using a short mental arithmetic test. An IRT-camera was employed to detect changes in SCVT as an index of induced BAT activity related to the mild stress[184]. Anticipation of the mental arithmetic test led to an increase in cortisol, indicative of an anticipatory stress response associated with increased SCVT[184]. Since a parallel increase in systemic circulating catecholamines was also reported in association with higher SCVT, the increase in cortisol most probably represents an overall neuroendocrine response to acute stress via the activation of the HPA axis. Notably, central nervous insulin can induce HPA axis activity[185,186]. Its effects on the HPA axis have been proposed to be mediated by enhanced corticosteroid feedback processing in the hypothalamus[187]. Therefore, a potential role of INI at mediating cortisol levels and thereby central activation of BAT has been hypothesized. However, the results of unchanged cortisol levels between conditions underpin that INI did not induce neuroendocrine effects linked to the SNS-mediated BAT activation in our study.

The potential effect of INI at mediating the HPT axis activity was also investigated. Thyroidal hormones regulate human energy expenditure, lipid and glucose metabolism and adaptive thermogenesis provided by BAT[188]. Collectively, thyroid hormones have been suggested as further mediators for metabolic improvements associated with BAT activation[188–190]. Following this reasoning, we investigated fT_3 during INI administration as an additional indirect indicator for BAT stimulation. However, levels of fT_3 remained unchanged upon INI compared to PLC, additionally

suggesting that BAT activity was not induced by INI. However, it is important to point out that, to our knowledge, the effect of INI on thyroid hormones has not been reported in the literature so far, making it difficult to draw final conclusions. Therefore, the question whether INI could mediate the HPT axis activity and its potential subsequent effect on BAT-associated energy metabolism is still open.

For our study, we choose to administer small but repeated doses of INI, i.e., 40 IU every 20 minutes for a total dose of 240 IU, after a first bolus of 80 IU. In doing so, we aimed to reach a plateau in the insulin concentration and to maintain it throughout the entire experiment where SCVT and blood parameters have been measured. Although we could not perform analyses to selectively detect changes in the brain-insulin level upon INI administration, it seems justified to assume the effectiveness of the applied study protocol at increasing central insulin levels for at least two reasons. First, our protocol was designed accordingly to several previous studies using comparable doses of insulin applied in a similar way[133,142–144,146,191]. Especially, among these studies we used as reference, there is the pivotal experiment[133] in which - for the first time - it was shown that an administration of 40 IU of INI rapidly increases the insulin concentration in the cerebrospinal fluid, without concomitant effects on the peptide's circulating levels[133]. Of note, also the solution for the PLC administration as well as the air pumps employed in our study have been used following previously established experimental studies aimed at increasing central insulin levels in healthy humans[139–141,143,190]. Second, and much in line with the aforementioned point, we did not find any differences in the serum insulin and c-peptide levels upon INI as compared to PLC. Values of plasma glucose remained within the euglycemic range throughout the study, too. Nevertheless, our study data show an improved post prandial glucose homeostasis during INI (data not shown as the findings concerning the effect of INI on glucose metabolism are part of a MD thesis). Moreover, levels of the counterregulatory hormone cortisol were similar between conditions. These results indicate that we did not incur in insulin spillover and therefore did not trigger an anabolic effect of higher peripheric insulin concentration, supporting the effectiveness of our intervention protocol.

Taken together, the results of unchanged SNS-response's markers, concomitantly with unchanged SCVT, lead us to conclude that INI did not prove to be a pharmacological BAT-activator in our study.

4.2 INI improves homeostatic regulation of subjective feeling of satiety

INI administration to the brain has anorexigenic and catabolic properties in male subjects[142,143]. However, the underlying mechanisms of insulin as acute satiety signal in humans have not yet been fully characterized. By demonstrating that small but repeated administrations of INI decreased post

prandial ghrelin in parallel with an increase in subjective feeling of satiety, we provide further evidence supporting the notion that brain insulin acts as a satiety signal in humans.

The homeostatic regulation of satiety and hunger involves a complex cross-talk between hormone signaling from the adipose tissue, e.g. adipokines, and from the gastrointestinal tract, e.g. gut-peptides, to the hypothalamus to contribute to the whole-body energy homeostasis.

With our study, we determined whether INI could act as satiety signal by affecting concentrations of ghrelin, leptin, and adiponectin. Concomitantly, we assessed the effect of INI on perceived feelings of hunger and satiety using a standardized rating scale. The subjects were asked to associate quantitative values to the question, '*how hungry are you?'* and '*how full are you?'*, respectively, before and after the standardized meal challenge.

Interestingly, we found a greater reduction in the levels of active ghrelin upon INI, although it shortly failed to reach statistic significant over time. However, the effect of INI at decreasing ghrelin concentration became significant when analysing the difference in the AUCs of post prandial ghrelin between conditions. Notably, in line with the greater decrease in ghrelin upon INI following the meal challenge, the post prandial feeling of satiety was markedly improved upon INI, since subjects scored an average 9% higher feelings of satiety as compared to PLC.

To our knowledge, our study is the first to report an association between INI and ghrelin at improving homeostatic control of satiety in humans.

Recent evidence[192] highlighted the coordinated role of leptin and ghrelin at adjusting homeostatic regulation of hunger and satiety, thereby regulating food intake. In our study, however, leptin levels remained unchanged after the meal challenge upon INI as compared to PLC. Thus, a major effect of INI in mediating ghrelin rather than leptin concentration to acutely regulate postprandial satiety may be speculated. Supporting this hypothesis, our result is in line with a previous study[140] reporting that a single post prandial dose of 160 IU of INI did not decrease leptin concentrations over time, yet reduced the intake of palatable food in healthy volunteers. Conversely, another study[143] has shown a drop in leptin of approximately 27% with concomitant food intake reduction in men upon INI. It is important to point out, however, that in the latter study INI was chronically administered (8 weeks, 4x 40 IU/d), and the drop in leptin levels was parallel to a decrease in fat mass[143]. When this workgroup changed the modality of INI administration in a more recent study[185], they could not reproduce the aforementioned results. In particular, by administering the same daily total amount of INI sub chronically (8 weeks, 1x 160 IU/d), neither differences on leptin levels nor on body weight between conditions were found[185]. Comprehensively, these findings emphasise that metabolic effects mediated by INI change whether the drug is employed sub chronically vs chronically vs acutely, despite keeping its total dose unchanged. Additionally, these contradictory results seem to point out

that the effects of INI are dependent on the feeding state upon which the peptide is administrated, i.e., pre vs post prandial state.

In addition to the aforementioned observations regarding the controversial outcomes of INI on leptin levels[140,143,185], comparing the time of action of the two signals could be the key to explain our results. Leptin is primarily secreted by adipocytes and its circulating levels mirror the body energy reserved, consequently exerting a relative slow-action and long-term inhibitory effects on food intake[193]. Ghrelin levels more promptly respond to ingestion of food, being mainly responsible for the regulation of the timing of meal initiation and termination via the vagus nerve afferents from the gastrointestinal tract[192]. This raises the possibility that INI effectively interacts with afferents from the gut reaching the hypothalamus via the vagus nerve. Thereby, INI could mediate the short-term post prandial signal of satiety regulated by ghrelin shortly after been administered, without, however, affecting the long-term signaling of leptin.

Adiponectin has also been reported to have a role in regulating feeding behavior by sending signals to the hypothalamus[194]. Therefore, the potential change in adiponectin levels during INI was also investigated in our study, aiming to further characterize the role of INI as satiety signal.

We found no effect of acute INI administration on plasma adiponectin. This result is in line with findings from previous studies. In fact, modulatory effects of enhanced brain insulin on this specific adipokine have, to date, never been reported, despite several studies have been conducted to investigate the potential link[140,143,146]. Accordingly, it might be speculated that changes in adiponectin could be ruled out as explicatory mechanism of the anorexigenic role of INI in humans.

To conclude, we have here reported on the effect of INI at decreasing ghrelin concentrations following a standardized meal challenge, concomitantly increasing the post prandial feeling of satiety. This effect of INI unveils a potential novel mechanism mediated by INI at the brain-gut axis level to regulate satiety in humans. Encouragingly, these findings might suggest a possible interventional use of INI in patients with obesity, in whom a blunted postprandial lowering in systemic ghrelin concentration is typically seen[25,195] and thought to contribute to dysregulated energy intake.

4.3 Basal BAT-thermogenic activity is modulated by neuroendocrine and lifestyle factors

Enhanced BAT-thermogenic activity has been proposed as a potential target to tackle obesity. However, its definite role in the complex pathophysiology of obesity and associated metabolic comorbidities still need to be better elucidate[59,108,153]. It appears therefore relevant to know which factors are associated with BAT and possibly influence its basal activation.

The negative association of basal BAT activity with BMI as well as with fat mass is well known[11,63,96,196], and was confirmed by our study. Notably, our study cohort (study I) consisted of

normal weight subjects, with BMI and fat mass within the normal range. Nevertheless, meaningful inverse associations were found between these two anthropometric parameters and basal BAT activity, further emphasizing their strong relation. Interestingly, however, BMI did not remain an independent predictive factor after performing the multiple linear regression analysis. Whereas fat mass resulted as a significant predictor independently associated with SCVT, showing a modulatory effect of approximately 18%. These findings support the idea expressed in the literature[96] that variables which more accurately estimate body composition, i.e., body fat percentage rather than BMI - considered as rough estimation of the real body composition - can better predict variability within BAT activity.

We did not find any correlation between age and basal BAT in our study cohort. The association between lower incidence of cold-activated BAT and older age seems to be particularly detectable above the age of 50[59]. Thus, the missing effect of age is likely due to the relatively young age of our subjects, with the range between 19 and 33 years. Moreover, an inverse association has mainly been shown in retrospective studies examining larger study cohorts and with a larger age-range than ours[59,92,96,196]. In this regard, two studies are especially worth mentioning. The one from Yoneshino and colleagues from 2011[59] and, the more recent one by Brendle and colleagues from 2018[96]. Both studies have shown the inverse association between age and BAT-activity assessed by PET/CT scan by analyzing 162 subjects aging 20 to 80, and 260 aging 11 to 85, respectively. Thus, we assume that if we would have included also elderly subjects in our study, we would have find an association between basal BAT activity and age.

We analyzed whether environmental factors, to which one is regularly exposed upon daily living conditions, could modulate basal BAT activity and if so – to which extend. For this, we focused on OT, i.e., warmer vs colder, SD, i.e., shorter vs regular, PA, i.e., low vs medium vs high.

We were able to show that SCVT, as indicator of BAT activity[165,166], was elevated by 7 day average OT colder than 13.5°C and by 7 night SD lower than 7h. Whereas, parasternal skin temperature, considered as RPT, as well as the MSKT and ICBT were affected by neither OT nor SD. Moreover, 7 day average OT and 7 night SD inversely correlated with basal SCVT, whereas, no correlations where found between these parameters and the RP, as expected.

These data suggest that colder OT and shorter SD are modulating factors increasing skin temperature exclusively in the anatomical regions co-locating with BAT, without other concomitant changes in the body skin temperature, as indicated by unchanged RPT and MSKT.

The effect of OT on BAT recruitment is known in the literature. In particular, the incidence of metabolically active BAT has been reported to be higher during seasons with lower outdoor temperature[92,95,197]. However, it was only recently that a clear definition of the OT influencing cold-

induced thermogenesis in healthy human has been firstly described[197]. In this pivotal study, Senn and colleagues have shown that colder maximum OT averaged over the 7 days preceding an experiment had largest effects of inducing thermogenesis response to cold challenge[197]. Likewise, the daily maximum temperature averaged over 7 days best correlated with increased thermogenic activity at basal condition, as shown by increased resting energy expenditure[197]. In our study, we choose to perform our analyses in accordance to this latter evidence. Thus, in order to investigate the modulatory effect of OT on SCVT, we considered the average daily maximum OT for the 7 days preceding the experimental visit, thereby supporting the standardization of our protocol. In this respect, our results confirming the influx of colder 7 day average OT at modulating BAT activity in humans hold important new evidence to support the role of the IRT technique to non-invasively assess BAT in clinical trials.

To our knowledge, the relation between sleep duration and basal BAT activity in the context of habitual sleep behavior in humans has not been revealed yet. In our study, slightly short 7 night average SD prior to the experimental visit, i.e., ≤ of 7 h but not less than 5 h, inversely correlated with SCVT. Interestingly, we here reported a modulatory effect of SD on SCVT, despite having values of SD within the normal range in our study cohort, i.e., between 5 and 9 h/night. Accordingly, our subjects were carefully screened for absence of sleep disorders before being enrolled in the study. Moreover, shift and night working were also exclusion criteria. Thus, our data underpin a modulatory effect of SD on SCVT upon normal habitual sleeping behaviour.

A possible explanation for the inverse relation between SD and SCVT may be found in regard to the humans sleep behaviour. In fact, it has been shown that sleep behaviour tightly coincides with a conserved circadian temperature rhythm[198]. As sleep approaches, core temperature drops with the lowest pick being observed about two hours after sleep onset in humans[199]. To counteract the physiological decrease in core body temperature, humans show thermoregulatory behaviours in preparation for sleep, including curling up and using warm bedding[200]. These behaviours aimed at maximizing the covered skin surface while sleeping generate a microclimate of warmth (between 31 and 35°C[200]) around the skin. This induced-comfortable warm microclimate has been shown to facilitate sleep onset by triggering vasodilation in the torso[201] as well as in the distal part of the body, mainly at hands and feets[202]. Of note, deviation from the above mentioned warm range has a negative impact on the duration and quality of sleep[202,203]. In line, it has been shown[198,204] that centrally regulated hypothalamic pathways promoting the release of heat via vasodilatation are activated by the warm microclimate generated during sleep. Interestingly, the centrally regulated mechanisms inducing both sleep onset and vasodilatation involve the activation of warm-sensitive neurons expressed in the POA[200]. Thus, the POA is a hypothalamic region serving as a key site for both sleep

initiation and body thermoregulation. In particular, the warm sensitive neurons expressed in the POA are activated by the skin warmth stimulus. In turn, the activated warm-sensitive neurons project to the dorsal medial hypothalamus as well as to the raphe pallidus to initiate sleep and vasodilation but in parallel initiate BAT downregulation[200,205]. Thus, it could be reasoned that the bed-warmth environment per se does not promote BAT activity. Following this reasoning, we believe that the modulatory effect of lower SD at increasing basal SCVT here reported is not due to the metabolic consequence of short sleep itself. In fact, the importance of habitual sleep as contributor for the regulation of energy homeostasis and overall metabolic health is known[206–208], and has been addressed recently by our group as well[209–211]. Thus, our data should rather be interpreted in relation to the environmental temperature associated with our sleep behaviour. Accordingly, it seems fair to reason that we have detect an indirect effect of SD on SCVT, emphasising the fact that the ambient temperature at which we are exposed is the main drive for BAT-thermogenic induction. Related to our study cohort, this could mean that subjects showing a higher basal BAT-activity associated with lower SD may had spent more time in a colder environment, namely outside the bed-induced warmth climate, as compared to the regular SD group. This is further supported by the multiple linear regression analysis' result, which shows an effect of OT of 44% on SCVT, thus making it the strongest predictor among all modulating factors analyzed.

More research is certainly warranted in order to establish the role of SD on BAT function in the context of both habitual and insufficient sleep. In fact, given the association between shortened sleep and altered metabolic traits, in particular in obesity and type 2 diabetes, it would be interesting to see whether impaired sleep behaviour could alter BAT metabolism as well. In this sense, future experimental studies conducted under well-controlled and standardized laboratory conditions will contribute at sheding the light on the interaction between sleep regulation and BAT function.

PA level was also analysed as a potential predictor of basal-BAT activity in our study. However, we did not find any differences in the basal SCVT between subjects divided according to their 7 day average PA intensity levels previous to the experimental visit. Likewise, when considering the relation between the time spent at the highest level of PA and SCVT no association was found. Collectively these results indicate that PA is not a predictor factor of BAT-basal activity, even despite its intensity.

With regard to the link between PA and BAT in humans, evidences so far accumulated are still too scarce and contradictory to argue about a certain positive relation. For example, two weeks of high and moderate intensity cycling have shown to decrease insulin-stimulated BAT activity in healthy middle-aged subjects[212]. In a further study, no association was found of PA performance with BAT volume or activity following an individualized cooling challenge in young healthy men[213]. In contrast,

Dinas and colleagues reported a positive association between habitual PA and basal BAT activity, detected by PET/CT scan[214].

Data are also contradictory regarding browning of WAT possibly induced by PA. Promising results have been proposed by a study showing a positive association between PA and higher expression of browning markers in abdominal subcutaneous WAT in healthy adults[215]. However, a previous study found no differences in expression levels of markers of browning from abdominal subcutaneous WAT of endurance-trained athletes as compared to the healthy control group[216]. In sum, conclusions on the role of PA on BAT metabolism cannot be drawn. In this sense, interventional controlled trials analysing the effect of PA on human BAT recruitment as well as on WAT browning are certainly required.

More evidence from animal studies seems to agree on the existence of an exercise-induced WAT browning, although the direct effect of exercise on classic BAT remains likewise controversial[217–219]. Rodents' studies have shown that BAT holds some common developmental traits with skeletal muscle[44]. Adipocytes in mice undergo thermogenic differentiation upon acute exercise[217], suggesting that physical exercise is a stressor potent enough to promote browning of WAT as an adaptive mechanism-response. This effect has been firstly linked to the acute involvement of the SNS-stress response via catecholamine release[220]. However, it has also been suggested that acute exercise may exert its thermogenic effect on fat tissue via myokines functions, thereby promoting a cross-talk between muscle cells and adipocytes[221,222]. Investigations in this sense have been particularly focused at dissecting the role of the 2012-identified molecule irisin. It has been shown that it is released by skeletal muscle upon contraction, with the ability to promote browning in mouse inguinal adipocytes and to positively affect energy metabolic functions[218]. Interestingly, the same study showed that circulating irisin levels were higher compared to baseline after 10 weeks of regular exercise program in humans, concomitantly promoting UCP1 expression and thermogenic differentiation of white fat precursor cells, both in vitro and in vivo[218]. However, after the initial optimism of having discovered a potential novel browning-agent associated to increased exercise, following studies failed to reproduce the aforementioned results[223,224]. Therefore, the suggested role of irisin in linking PA to browning of WAT has been lately challenged.

Recently, other myokines including interleukine-6, b-aminoisobutyric acid and meteorin-like protein, have been postulated to simultaneously act on white adipocytes to promote browning in mice[222]. However, it still needs to be proved whether exercise leads to same results in humans. Therefore, future research is warranted to further elucidate if exercise-induced browning actually occurs in humans and, in this sense, which players as well as which mechanisms may be involved.

Of note, the motion watch 8 is a device commonly used in clinical and epidemiological research to assess SD and PA[225,226]. Accordingly, the motion watch 8 has been shown to be a comfortable wearable tracker device[227]. Thus, its use enables a good compliance among study participants and a comprehensive picture of both total daily PA and SD, tackling the limitations of self-report methods[225]. Nevertheless, despite the late methodological improvements in the PA assessment, it is important to point out that PA is a complex behaviour which remains not easy to assess[228]. This is especially the case with regard to the classification of its two components, leisure activity and physical exercise in subjects upon free living conditions[229].

Lastly, among the parameters categorized as 'metabolic factors' analyzed in our study, noradrenaline positively associated with basal SCVT. This finding corroborates the strong involvement of the SNS at sustaining basal BAT-activity. On the other hand, epinephrine seems not to be responsible of triggering the SNS-mediated BAT-activity at a basal level in our study and as previously shown in the literature[230].

Of note, it must be kept in mind that our data provide information about associations of various parameters with basal-BAT activity, without, however, the possibility to prove causality. In this respect, more studies are needed to establish causal relationships. Likewise, additional interventional studies can be certainly useful to confirm our results in different populations such as women, subjects with overweight and obesity, elderlies, and ethnicities other than Caucasian.

In conclusion, the modulatory effects of several environmental as well as neuroendocrine factors on basal-BAT thermogenic activity were found. In particular, OT, SD, fat mass and noradrenaline are important predictors of BAT activity under thermoneutral conditions. In light of our findings, further investigations are needed to elucidate the consequences of chronic basal-BAT induction. In fact, it seems tempting to speculate about positive metabolic consequences of chronic induced BAT. However, the long-term benefits of chronically activated BAT are yet to be comprehensively proven. Therefore, although it seems optimistic to theorize that chronic enhancement of basal-BAT thermogenic activity - e. g. by decreasing the temperature of our houses and work places, thereby increasing time spent outside our 'thermoneutral comfort zone' - will provide one more weapon in the battle against obesity, it is yet too early for final recommendations.

4.4 SPARC expression in BAT associates with increased BMI and with reduced thermogenic capacity

With this work we aimed at exploring the proposed inhibiting role of SPARC on BAT thermogenic capacity in the context of BAT-function associated to obesity. Therefore, we measured SPARC

mRNA levels in biopsies of human WAT and BAT sampled from subcutaneous and supraclavicular fat depots, respectively.

SPARC mRNA expression levels were compared in WAT vs BAT of normal weigh subjects. This analysis showed that SPARC expression in BAT was downregulated compared to its expression in WAT upon normal weight status. Contrarely, SPARC mRNA expression was upregulated in BAT of subjects with obesity, as compared to levels of SPARC expression measured in BAT of normal weight subjects. Besides, SPARC expression in BAT positively associated with BMI but negatively with UCP1. Collectively, our results seem to support a negative effect of SPARC on BAT function associated with higher BMI.

Canonically, WAT accumulates excess energy while BAT oxides substrates in response to metabolic stimuli such as over-feeding and cold, respectively. Thus, both are vital for the regulation of body metabolism and energy homeostasis. Obesity is characterized by excessive lipid storage in the adipose tissue and is due to metabolic dysregulation of energy homeostasis[10]. While the phenomena of hypertrophy and hyperplasia have been defined as the main WAT response to condition of chronic energy surplus[231], a dysfunctional activity of BAT has been also observed in obesity[95,153]. Thus, raising the possibility that the canonical role of brown adipocytes in sustaining thermogenesis could be compromised consequently to a morphological and functional reorganization of the tissue upon conditions favouring the onset of obesity. Thus, the knowledge that the plasticity of adipose tissue is not limited to WAT, being indeed a feature of rodents[36,121] as well as of humans[47,232] brown adipocytes has been achieved. However, despite this acquired knowledge, currently, the focus of nearly all research in the field of obesity prevention is at unveiling the *browning* of WAT, rather than the *whitening* of BAT. In this sense, it seems worth to raise the question whether the reduced BAT activity observed in obesity may be reconducted to the tissue entering a thermogenic dormant-state which is no longer acutely responsive to sympathetic activation, rather than to its loss. Related to this, it seems fair to hypothesized that specific molecules, e.g. fat-released factors, could be responsible at promoting such metabolic switch of the tissue by possibly cross-talking between WAT and BAT.

In this context we questioned whether SPARC could be considered as a candidate fat-released factor involved in the dysfunctional BAT-thermogenic response associated to obesity.

SPARC, also known as osteonectin and firstly identified in 1981 in bone[233], is a 32-kDa glycoprotein of the ECM that binds calcium and collagen and it is expressed by several cells, including adipocytes[125–127]. Due to its ubiquitously expression, SPARC has been investigated in the context of various pathological conditions, ranging from liver and kidney diseases and including obesity and diabetes, being WAT one of the main site of SPARC expression[125–127]. Increased levels of SPARC are found in WAT of rodents following diet-induced obesity[234]. In humans, increased expression of

SPARC was found in both visceral and subcutaneous adipose tissue of individuals with obesity and insulin resistance[235]. Furthermore, serum SPARC levels decreased following weight-loss induced by bariatric surgery in subjects with obesity[236]. Functionally, SPARC has been demonstrated to be involved in ECM remodelling, angiogenesis, fibrosis, and adipose tissue hyperplasia, respectively[125,126]. Mechanisms which are all likely to contribute to the role of SPARC in obesity. Thus, SPARC is a relatively well-characterized factor related to obesity and obesity-associated complications[126,237]. However, none of the aforementioned studies has so far reported an inhibitory effect of SPARC on BAT function in the context of obesity. Notably, a link between upregulated SPARC and decreased BAT activity has been previously shown in an animal study, demonstrating an inverse correlation of SPARC and UCP1 in artic ground squirrel during hibernation[238]. Moreover, in a recent human study investigating the perirenal adipose tissue as a novel BAT depot[61], SPARC has been associated to a whitening-looking phenotype of the tissue. In fact, a different morphological pattern of the brown adipocytes constituting this adipose tissue based on histological data was found. Particularly, adipocytes more in the proximity of the adrenergic stimulation appeared as 'canonical' brown cells, in terms of multilocular aspect and UCP1+ expression. Conversely, the adipocytes located in the lower pole of the kidney and therefore under a less local sympathetic stimulation, presented bigger lipid droplets, almost unilocular, and markedly lower UCP1 expression[61]. Moreover, the mRNA expressions of several candidate genes were compared between these two adipocytes in order to characterize the novel discovered 'partial-whitened' BAT phenotype. Strikingly, SPARC was identified as the gene having the major different expression between these adipocytes[61]. Namely, while SPARC levels were downregulated in canonical brown adipocytes, SPARC was substantially upregulated in brown cells showing the white-looking signature. Taken together, this evidence could suggest a role of SPARC in suppressing active brown fat, thereby promoting the dormant state found in the perirenal BAT.

To our knowledge, these are the only available studies directly investigating SPARC in the context of BAT metabolism, thus, making our findings here reported on the explorative association between upregulated SPARC, higher BMI and decreased thermogenic capacity novel and compelling. Nevertheless, further investigation is needed to corroborate the supressing role of SPARC on functional BAT in obesity. Undoubtedly, the identification of SPARC as a potential common characteristic of WAT, impaired BAT and BAT in obesity is highly interesting.

4.5 SPARC circulating levels increase following cold-activating BAT in obese subjects

Next, we aimed at quantifying the levels of SPARC in subjects with obesity following CE, expecting to find SPARC circulating levels changed as a consequence of cold induced BAT activity compared to TN.

SPARC levels increased following 2h of personalized CE in subjects with obesity and our observation appeared contradictory at first glance. However, this finding could be better comprehended when interpreted in the context of the impaired cold-induced BAT-thermogenic response associated to obesity[97,239]. In this prospective, our data seem to suggest that the increased circulating levels of SPARC could be responsible, or at least, a hallmark of reduced BAT-thermogenic function associated with obesity. In line with this reasoning, SPARC negatively correlated with noradrenaline, whereas positively with insulin, following CE as compared to TN. Thus, these findings further link SPARC to the tissue's blunted sympathetic responsiveness and concomitant dysregulation in markers of glucose metabolism upon obesity.

Notably, data investigating the role of SPARC in relation to cold-induced BAT activity are scarce. Thus, our study is the first - to our knowledge - to explore the role of SPARC upon cold-activated BAT in human obesity and therefore our quantitative analysis should be considered under an experimental point of view. However, we were not able to investigate the underlying mechanisms of SPARC potentially promoting the BAT-impaired phenotype as no cell materials could be collected from BAT during our cooling study. In this context, additional studies are required.

However, a possible mechanism of SPARC action would be by inducing changes in the cytoskeleton organization, such as adipocytes shapes reorganization as well as increased lipid accumulation. These are both hallmarks of obesity and previously associated to SPARC upregulation[121,122]. Accordingly, rodents' studies have shown that during conditions of chronic positive energy balance, brown and brown-like adipocytes undergo morphological and metabolic changes including accumulation of lipid droplets, reduced mitochondrial concentrations[121] and impaired glucose uptake[122], respectively. The whole of these coordinated processes has been termed 'whitening'[52,121], as it ultimately results into the conversion of brown into white-like adipocytes. Moreover, cells within other organs, i.e., skeletal muscle, liver, and pancreas are capable to take up excess fat ectopically to cope with prolonged condition of energy surplus upon obesity. This raises the question whether brown adipocytes, too, upon obesity, can reorganize their shape and function in order to adapt to the metabolic condition in which the prevailing need is to store excess energy as TG rather than leaving them in the circulation. Accordingly, the proposed idea is that brown adipocytes could be newly recruited to cope with the event of increased demand for energy storage during a constant positive energy overplus. Hence, they

would exceptionally take up excess energy in form of TG rather than conventionally release it in form of heat.

Considering our findings on SPARC together, they seem to agree with the proposed scenario in which BAT in obesity is not absent but rather found in a less-active state. Thus, SPARC may act as a fat-released factor further promoting the link between blunted BAT-thermogenic response and obesity.

Finally, it seems worth to continue studying SPARC in order to expand our knowledge on its metabolic roles possibly mediated by crosstalk between WAT and BAT. It seems also promising to envision the further study of SPARC in the context of exploring potential novel candidates for therapeutic targets, with the final goal of possibly re-activating the dormant state of BAT associated with obesity. It could be reasoned that novel agents might be successfully developed with the scope of manipulating the action of specific fat-released factors associated with BAT metabolic dysregulation. However, future studies are needed with the focus of directly testing this hypothesis in animal models and to elucidate molecular mechanisms linking SPARC to the progressive decline of BAT-thermogenic function. For example, an important first step would be to investigate the thermogenic capacities conserved in primary brown adipocytes, in terms of UCP1 expression, induction and respiratory capacity after SPARC known down as well as after its over-expression. Studies on SPARC KO mice would constitute a meaningful subsequent step. Likewise, studies employing cells in situ-staining would positively advance our current knowledge on the role of SPARC on brown adipocytes. In fact, such studies would allow to discern at a single-cell level which sub-type of cells within BAT express SPARC, both physiologically and upon adrenergic stimulation. Findings in this sense would importantly allow to better understand whether the SPARC expressing cells possibly co-express with UCP1 and other metabolic markers upon noradrenaline stimulation. In addition, in-situ imaging studies would also allow to morphologically visualize the adipocytes sub-types. This would be a pivotal step, as it would ultimately lead to corroborate the - for now only theorized - change in morphology of the brown adipocytes upon obesity, potentially partially promoted by SPARC. We theorized that this morphological change could drive a functional key-turning point resulting into a re-organization of the tissue-thermogenic capacity, rather than its disappearance upon obesity.

In conclusion, the thorough characterization of the factors modulating cold-induced BAT response at the brown adipocytes level might open up innovative strategies required to comprehensively tackle obesity and its metabolic dysregulations.

4.6 Limitation of the present work

This thesis has some limitations that need to be addressed. First, subjects of study I did not underwent a ^{18}F-FDG-PET/CT scan analysis associated with cold challenge as pre-screening test, with the purpose of confirming the actual presence of inducible BAT. Although such pre-analysis is recommended[151], it was not possible to include such procedure within our study protocol given the ethic restriction regarding the safety of PET/CT in healthy population. However, the current consensus over a number of studies[12,57,90,92,95] is that functional BAT is broadly common in the adult human population, with a prevalence getting very close to 100% in lean and young people[55]. Since with study I, we only tested young, metabolically healthy subjects it seems justified to assume that functional BAT would be present among this category.

An additional point needs to be made with respect to study III, where subjects with obesisy were tested following a personalized cooling protocol. For this study, obese but otherwise metabolically healthy and young subjects were included. Although cold-induced BAT activation was not confirmed by ^{18}F-FDG PET in the subjects of study III, we recently applied a very similar personalized cooling protocol in lean males and proved increased [^{18}F]-FDG uptake into SCV BAT depots[60]. Accordingly, we believe that the CE performed in study III activated BAT in subjects with obesity, too.

Second, the association between basal BAT and different modulating factors has been assessed indirectly by IRT technique. Although this methodology still requires further research especially to achieve a standardization among research groups, it has been repeatedly shown[151,164,168] to be a valid alternative technique to the gold standard PET/CT scan. In these terms, our findings importantly support IRT as a promising technique to investigate BAT activity in humans. This becomes particular important when other more invasive techniques, e.g., PET/CT and collection of BAT biopsies, respectively, cannot be employed.

Third, as noted above, association between basal BAT and the different modulating factors has been assessed only in young and metabolically healthy subjects (study I). However, the effects of factors, positively or negatively, influencing thermogenic BAT cannot be generalised to certain subjects' groups, such as subjects with diabetes and/or obesity, children, elderly and women and thus, need to be addressed in future trials.

Forth, for study I, subjects were tested throughout the year. Thus, data were collected at various temperature and weather conditions. However, we made sure that the two experimental visits were by maximum two weeks apart in order to minimize variation in environmental temperature. Moreover, it needs to be kept in mind that our study was designed to test subjects upon free living conditions. Thus, we standardized laboratory parameters such as room temperature and humidity as well as clothing. Moreover, we tried to maximize the standardization of subjects' condition, for

example by asking to refrain from exposure to extreme temperature, i.e., sauna and winter bath, one week before the experimental visits. It was also asked to avoid caffeinated beverages and shower 24h before and in the morning of the visit, respectively. However, we could not possibly control for environmental parameters, neither could we keep our subjects 'in captivities' for the time prior the experiments.

Finally, for the SPARC mRNA expression analysis in study II, BAT and WAT biopsies were collected from a small sample of participants and rather heterogeneous in terms of BMI. However, due to the invasivity of the technique and given the exploratory nature of the study, our analysis on human adipose tissue biopsies should still be considered under an experimental point of view. Nevertheless, further mechanistic studies are needed to establish the way of action of SPARC in the context of BAT in obesity.

V. Summary and conclusion

This work investigated the sympathetic central nervous and peripheral pathways involved in mediating BAT-thermogenic activity in humans.

The IRT technique has been employed to non-invasively investigate the role of INI as pharmacological activator of BAT. However, INI did not enhance BAT-thermogenic metabolism, as shown by unchanged SCVT and hormonal parameters, compared to PLC. Nevertheless, INI improved homeostatic regulation of satiety, by reducing ghrelin concentration following a standardized meal challenge concomitantly increasing the postprandial subjective feeling of satiety by 9%, as compared to PLC. Our study is the first to report a link between INI and the gut hormone ghrelin at improving homeostatic regulation of satiety in humans. Together, these findings point towards a crosstalk between increased brain insulin signaling and gastrointestinal system, possibly suggesting a novel mechanism for the regulation of satiety played by INI at the brain-gut axis level, however, independently from BAT activation.

Moreover, the IRT technique has also been employed to assess the modulatory effect of neuroendocrine and environmental factors on basal-BAT activity. We found that factors such as OT, fat mass, SD and noradrenaline are important predictors of basal BAT-thermogenic activity, showing a modulatory effect of 44%, 18%, 4% and 3%, respectively.

Additionally, we explored the potential role of the adipokine SPARC as candidate factor promoting the decreased BAT-thermogenic response associated to obesity. SPARC mRNA expression was upregulated in BAT of subjects with obesity, as compared to normal-weight BAT. Besides, SPARC expression in BAT positively associated with BMI but negatively with UCP1, collectively supporting a negative effect of SPARC on BAT function associated with higher BMI. Moreover, we quantified - for the first time - levels of SPARC in plasma of subjects with obesity during a personalized non-shivering cooling protocol, showing elevated concentrations of SPARC as compared to TN. In addition, during CE, SPARC negatively associated with noradrenaline and positive with insulin, two important markers of active BAT and metabolic health, respectively. Taken together our explorative data on SPARC further emphasize the plausible link between upregulated SPARC in obesity and the tissue's impaired-thermogenic capacity associated with it.

Conclusively, the identification of exogenous and endogenous factors modulating BAT activity here reported provide the rational for future mechanistic studies to elucidate the specific functional roles of such factors, and potentially to even discover others, in the suppression or activation of BAT as a mean to counteract obesity and its metabolic sequelae.

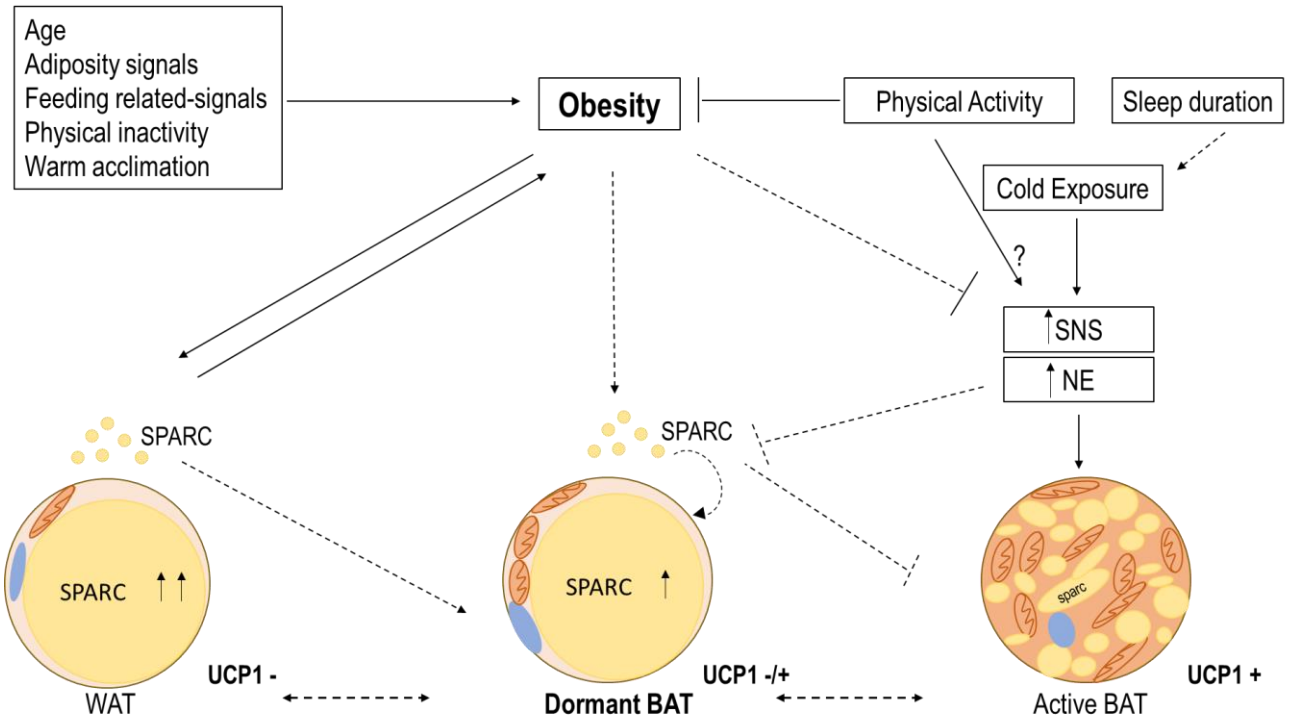


Figure 19. Schematic summary of factors modulating BAT metabolism based on the thesis' main results together with published data.

Solid arrows and lines illustrate associations established in the literature. Dashed arrows and lines illustrate associations suggested based on our findings. Factors promoting or supressing the sympathetic activity can theoretically trigger the adipose tissue plasticity, leading to the active vs dormant BAT phenotype, respectively. In obesity, several factors such as age, dysregulated feeding and adiposity-released signals, physical inactivity and warm acclimation could all be possible underlying causes of impaired sympathetic responsiveness and sensitivity progressively leading to the conversion of active BAT into dormant BAT. Moreover, we propose that the already partially impaired function of obese-BAT may be further suppressed by increased levels of SPARC associated with obesity, thereby creating a vicious cycle.

BAT, brown adipose tissue; WAT, white adipose tissue; SNS, sympathetic nervous system; NE, noradrenaline; UCP1, uncoupling protein 1; SPARC, secreted protein acidic reach cysteine.

VI. References

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Curriculum Vitae

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WORK EXPERIENCE

05.2017- Present — PhD candidate
University of Lübeck, Institute for Endocrinology and Diabetes, Lübeck, Germany

02.2014 - 04.2017 — Clinical Research Assistant
Washington University in St. Louis, Department of Nutritional Science, USA

TRAINING EXPERIENCE

11.2019 – 02.2020 — Visiting PhD Researcher
Center for basic metabolic research (CBMR), University of Copenhagen, Copenhagen, Denmark

05.2018 – 06.2018 — Visiting PhD Researcher
University of Nottingham, Division of Endocrinology, Nottingham, UK

02.2013 – 07.2013 — Internship in Human Nutrition
Universidad de Navarra, Departamento de Alimentation y Fisiologia, Pamplona, Spain

EDUCATION

10.2011 – 10.2013 — Master's degree, Food Science and Human Nutrition
University Campus Bio-Medico di Roma, Roma, Italy

09.2008 – 09.2011 — Bachelor's degree, Biological Science
University Campus Bio-Medico di Roma, Roma, Italy

ADDITIONAL INFORMATION

01.2020 – Present — Board Member of the Young Active Research in Endocrinology society (YARE)

Scientific contribution

- Invited speaker at the 63th German annual meeting for endocrinology, talk with title "Effects of small but repeated doses of intranasal insulin on human metabolism" (Giessen, **4-6.03.2020**)
- Selected abstract for oral presentation at the 21st YARE meeting, with title "small but repeated dose of intranasal insulin improve postprandial levels of glucose and ghrelin without changing peripheral insulin in healthy males" (Essen, **4-6.10.2019**)
- Selected abstract for oral presentation at the Young Investigator in Endocrinology and Diabetes Symposium, with title "Reason to chill your life: metabolic consequences of cold-activated BAT in humans "; University of Heidelberg (Heidelberg, **12.09.2019**)
- Poster "Acute BAT Activation Improves Glucose Tolerance and Beta-Cell Secretion and Facilities Lipid over Glucose Utilization in Obese Metabolically Healthy Males", presented at 2019 ENDO meeting (New Orleans, **23-24.03.2019**)
- Poster "Metabolic effects of cold-induced BAT thermogenesis in humans", presented during the 2nd ABC Symposium at Media dock (Lübeck, **14-15.03.2019**)
- Poster "Infrared Thermography as a non-invasive method to assess BAT in humans", presented during Tag der Deutschen Zentren für Gesundheitsforschung (Lübeck, **30.11.2018**)
- Invited speaker at the Impact Session during the Florida Food & Nutrition Educational Symposium & Exhibition, talk with title "Benefit of Mediterranean Diet: the connection between the territory and the quality of the products" (Tampa, **26.07.2016**)

Awards

2019 Best poster price at the 23rd annual meeting of Neuroendocrine German Society

2019 Grant awarded by Bioscientifica Trust supporting inter-lab experiences for young researches

2019 Best speaker price at 21st YARE annual meeting

2019 Travel grant awarded by DGE (Deutsche Gesellschaft für Endokrinologie)

2014 Fellowship awarded by Campus Bio-Medico di Roma for young graduates interested in gaining research experience abroad

2013 Scholarship granted by European Union to support Erasmus Placement Program

Certifications

2019 Sprachenzentrum, German exam certification, level B2.2

2018 Course "Soft skills for researches"

2017 Good Clinical Practice (GCP)

2015 TOEFL iBT, English exam certification, level C2

2013 Italian national Board Certification for Clinical Nutritionist

2007 Instituto Cervantes, Spanish exam certification, DELE, level B2

Publications

1. Treviño-Villarreal JH, Reynolds JS, Bartelt A, Langston PK, MacArthur MR, Arduini A, Tosti V, Veronese N, Bertozzi B, Brace LE, Mejia P, Trocha K, Kajitani GS, Longchamp A, Harputlugil E, Gathungu R, Bird SS, Bullock AD, Figenshau RS, Andriole GL, Thompson A, Heeren J, Ozaki CK, Kristal BS, Fontana L, Mitchell JR. *Dietary protein restriction reduces circulating VLDL triglyceride levels via CREBH-APOA5-dependent and independent mechanisms*. JCI Insight. 2018 Nov 2;3(21). pii: 99470. doi: 10.1172/jci.insight.99470.

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3. Tosti V, Bertozzi B, Fontana L. *Health Benefits of the Mediterranean Diet: Metabolic and Molecular Mechanisms.* J Gerontol A Biol Sci Med Sci. 2018 Mar 2;73(3):318-326. doi: 10.1093/gerona/glx227. Review.

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Beatrice Bertozzi