Physiology & pathophysiology of human brown adipose tissue : implications for the treatment of obesity

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Physiology & pathophysiology of human brown adipose tissue

Implications for the treatment of obesity

Guy H.E.J. Vijgen

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Physiology & pathophysiology of human brown adipose tissue

Implications for the treatment of obesity

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
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in het openbaar te verdedigen
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Chapter 1

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General Introduction

Brown adipose tissue

Brown adipose tissue (BAT) has been known since 1551 when the Swiss biologist Conrad Gesner described the observation of a body part in marmots he defined as “neither fat, nor flesh; *nec pinguitudo, nec caro*” (Figure 1).^{1,2} This description then already assumed that BAT was different from white adipose tissue (WAT) based on its macroscopic appearance. Throughout the centuries more knowledge has been obtained on BAT and in 1972 a detailed anatomical description of BAT in man was published.³ The brown adipocyte (fat cell) contains multiple fat vacuoles and a granular cytoplasm consisting of a large number of mitochondria which gives it its brown appearance.³ Except from colour (which varies from caramel-coloured - to brown - to dark red), BAT is highly vascularized, sympathetically innervated and most importantly functionally different from WAT; animal studies have confirmed that the primary function of BAT is heat production upon cold exposure and diet, in contrast to WAT that mainly serves energy storage.^{4,5}

Function of brown adipose tissue

When exposed to a cold environment, the heat production in BAT serves an important function in small animals and human newborns by preventing hypothermia.⁵ This heat production during cold exposure is defined as non-shivering thermogenesis (NST), in contrast to shivering thermogenesis (ST) resulting from skeletal muscle contractions.⁶ NST in BAT is suggested to be responsible for an increase in energy expenditure of ~50% in mice.^{5,7} In addition to NST, BAT has been shown to become active in rodents after overeating (facultative diet-induced thermogenesis; fDIT). The function of fDIT is to decrease metabolic efficiency and prevent obesity, although this is disputed by others.^{8,9} Genetically modified mice without functional BAT become obese and develop obesity-related comorbidities, suggesting BAT is important in weight management.⁷ This indicates that BAT is an important organ with a vital role in maintaining the energy balance, at least in mice. BAT produces heat by the activity of uncoupling protein 1 (UCP1), first described in 1978.¹⁰ UCP1 is located on the inner mitochondrial membrane of the brown adipocyte and is capable of producing heat.^{5,11} In the majority of the cells in the human body, the mitochondrial proton gradient is designed to produce adenosine-triphosphate (ATP), the key energy carrier in all of the human body's metabolic processes. However, in BAT the unique presence of UCP1 facilitates proton leakage over the mitochondrial inner-membrane and thus heat production instead of ATP generation.

Rediscovery of functional brown adipose tissue in adult humans

In human neonates, BAT is found in the neck along the greater vessels and muscles, supraclavicular and interscapular area, mediastinum and around the aorta, pancreas, spleen, kidneys and suprarenal glands.³ The total weight of BAT (~150-250 g) in newborns consists of 2-5% of the body's total amount of adipose tissue.⁴ BAT in adults was known for several decades, but it has only recently been shown that adult humans possess significant amounts of physiological active BAT. In 1985 Astrup et al. performed dedicated studies on human BAT and measured temperatures in interscapular and perirenal BAT before and after stimulation with the sympathomimetic ephedrine.^{12,13} Although thermogenesis increased significantly, unfortunately no contribution of BAT could be found.^{12,13} In the last decade, the evolvement of new imaging techniques made it possible to determine the functionality of human BAT. The development of positron-emission-tomography-and-computed-tomography (PET-CT), where the radioactive glucose tracer 2-deoxy-2-¹⁸F-fluoro-D-glucose (FDG) shows cellular glucose uptake, gives the opportunity to depict metabolically active tissues. FDG-PET-CT studies are clinically widely used in oncological imaging. Interestingly, it was frequently observed that FDG uptake occurred in adipose tissue (Figure 2),¹⁴ located in anatomical areas that were described in anatomical studies earlier to contain BAT.³ This FDG-PET-CT-positive adipose tissue was mostly observed in patients feeling cold and repeated scanning under warm conditions prevented the uptake in those areas (Figure 3).^{15,16} This raised scientific interest from autopsies.¹⁷ Moreover, there was a significant negative correlation between the amount of active BAT and the

Figure 2. FDG-PET-CT-image of a female patient with non-small-cell lung cancer (NSCLC) showing increased FDG-uptake in periclavicular and paravertebral adipose tissue during uncontrolled conditions (A) and absence of FDG-uptake in adipose tissue during warm conditions (B; room temperature 22-25° Celsius). Image obtained from Christensen et al.¹⁵

percentage of body fat and BMI, i.e. a low BAT activity in obesity (Figure 4).¹⁷ and a prospective study from our research group in young, healthy subjects under controlled mild cold exposure showed active BAT in the majority of the study group (96%) in anatomical regions similar to descriptive observations. Adipose tissue biopsies taken from the regions that showed FDG-uptake on PET-CT during cold exposure confirmed the histological presence of brown adipocytes (Figure 5).^{17,18} Retrospective analyses of cohorts of clinically performed FDG-PET-CT studies (mostly performed in oncological settings) throughout development in childhood and adolescence showed that BAT peaks up to observed amounts of ~1000g.¹⁹

Figure 3. PET-CT-image of a healthy, young male subject under thermoneutral conditions (thermoneutral) and after mild cold exposure (mild cold). After mild cold exposure, the black areas indicate active uptake of FDG in supraclavicular and paravertebral adipose tissue. Images obtained from van Marken Lichtenbelt et al., NEJM, 2009.¹⁷

Figure 4. Relation between Body Mass Index (BMI) and Brown Adipose Tissue (BAT) activity. Image obtained from van Marken Lichtenbelt et al, NEJM, 2009.¹⁷

Figure 5. Formalin-fixed histological slide of supraclavicular human brown adipose tissue. The multivacuolar aspect and dark-stained mitochondria are evident in the brown adipocyte (Vijgen et al., unpublished work).

However, prospective, controlled studies in healthy adolescents using metabolic imaging techniques show average volumes of BAT of $\sim 100\text{g}$ or $\sim 100\text{cm}^3$ (range $0\text{--}296\text{ cm}^3$).¹⁷ Both the amount of BAT and the number of anatomical sites show that BAT decreases with age, resulting in a decline in BAT presence being restricted to the supraclavicular/neck area or even a total absence in the last decades of life.^{3,17,20} The mechanism underlying this change in volume and distribution of BAT through life is unknown. Interestingly, recent cell studies suggest that the amount and function of brown adipocytes can change. During the development of adipocytes several factors have been identified that can influence the final differentiation into white and brown phenotypes.^{21,22} Recent reports show that stimulation of WAT depots triggers the growth of a subset of thermogenically active BRITE (brown-in-white) or 'beige' cells.^{23–25} This suggests that within human WAT a subset of preadipocytes have a certain 'plasticity', which allows differentiation to thermogenic cells depending on environmental conditions or specific stimuli.

Interestingly, recently biopsies from the supraclavicular adipose tissue area known to contain BAT in man have shown the presence of beige adipocytes and preadipocytes that could become thermogenically active *in vitro*.^{26,27} This 'plasticity' of BAT could

explain the previously observed high amounts of BAT in Finnish outdoor workers, who were exposed for longer periods of cold compared to their colleague indoor workers.²⁸ Therefore, possibly BAT in adult man could increase when stimulated to a similar extent as long-term cold exposure. Theoretically, BAT grafts developed ex-vivo could be implanted in subjects with low or absent BAT. However, this is not possible yet and at this moment most studies focus on determining the physiological and pathophysiological importance of BAT in man. In summary, BAT could have a significant role in human physiology and possibly be important in the regulation of the energy balance and weight maintenance, similar to the situation in small mammals. Therefore, this raised renewed interest in BAT in adult humans and its metabolic role in relation to obesity.

Role of brown adipose tissue in obesity

Obesity and its comorbidities were already stated as diseases by Hippocrates in his *De Priscina Medicina* (400 BC): "corpulence is not only a disease itself, but the harbinger of other diseases".^{29,30} In the past under more natural living conditions, the excess amount of adipose tissue in obesity served as an energy source during periods of food scarcity. In modern times food is always available and more than 10% of the world's adult population is obese, making obesity one of the biggest health threats in the Western world.^{31,32} Obesity is strongly associated with other diseases, such as type II diabetes, cardiovascular disease, dyslipidemia and cancer.³³ The rapidly increasing incidence of obesity and its associated diseases demand for both a preventive and curative strategy. Most obesity therapies aim at restoring the disturbed energy balance by either decreasing energy intake or increasing energy expenditure. Caloric restriction may be successful on short term,³⁴ but unfortunately it is extremely difficult to maintain weight loss, as shown by long-term follow-up studies.³⁵ One of the first promising drugs targeting obesity was 2,4-dinitrophenol (DNP), developed in the 1930s. DNP caused rapid weight loss by increasing energy expenditure through uncoupling of mitochondrial oxidative phosphorylation.³⁶ However, severe adverse effects such as hyperthermia made this drug unsuitable for large-scale use. During the last decades, several drugs were released that aimed at decreasing energy intake.³⁷ These substances targeted the central nervous system (sibutramine, rimonabant, phentermine, diethylpropion), cellular (rosiglitazone) and digestive components (orlistat) of obesity and initially showed significant long-term weight losses of 2.9-4.7 kg up to 5-10 kg after 2 years.^{34,37,38} However, increased cardiovascular risks (rosiglitazone), mood disorders (rimonabant) and abdominal

complaints (orlistat) made that these drugs are currently not recommended anymore.³⁷ Therefore, more rigorous treatments are sometimes inescapable, for example in morbid obesity. In these patients invasive bariatric surgery is an effective long-term weight loss therapy.^{39,40} In search for less invasive treatments, the quest for the 'holy grail' in obesity treatment continues and in the past years BAT has gained increasing scientific interest as a possible therapeutic target tissue for obesity in man.

In man, most studies using imaging techniques first expose study subjects to mild cold to induce BAT activity. The mild cold exposure sensitizes skin neurons and is then believed to stimulate an increase in sympathetic nervous system (SNS) activity. This increased SNS activity stimulates synapse norepinephrine (NE) release which induces β -adrenergically-mediated BAT activity.⁵ Interestingly, although β -receptors are present in human BAT,¹⁸ the exact β -adrenergic stimulus is not known yet. Recent imaging studies targeting β -adrenergic BAT stimulation with isoprenaline and ephedrine (1.0mg/kg) were unsuccessful and only high doses of ephedrine (2.5mg/kg) were able to induce some BAT activity in lean but not obese humans.⁴¹⁻⁴³ Therefore, a more detailed, cellular investigation of BAT is necessary to identify the molecular mechanisms that eventually induce UCP1-mediated mitochondrial uncoupling in the brown adipocyte to target BAT activation.

In summary, most therapeutic strategies for obesity aiming at a long-term reduction of energy intake are unsuccessful and to date no treatment has been able to sustainably increase energy expenditure. Possibly, BAT can be of great value in future obesity treatment. This thesis deals with the physiology of BAT in obesity and therapeutic options to induce BAT activity in man are explored.

Outline of the thesis

This thesis describes studies on the physiology and pathophysiology of BAT in man, in relation to the treatment of obesity. In Chapter 2, the clinical relevance of BAT in man is discussed. In Chapter 3, we observed BAT activity in morbidly obese subjects in comparison to previously studied lean subjects. Next, the effect of major weight loss by bariatric surgery in morbidly obese subjects on presence and activity of BAT was studied (Chapter 4). Since recent studies have shown that heat production in BAT and skeletal muscle could be closely related, we studied the effect of weight loss on skeletal muscle mitochondrial function (Chapter 5). Chapter 6 describes a study on the cellular physiology and the metabolic capacity of human brown and white adipose tissue. Chapter 7 describes a study on the effect of central nervous

system stimulation on BAT activity in patients with refractory epilepsy who are treated with vagus nerve stimulation. Finally, in Chapter 8, the results obtained in this thesis are discussed and implications and applications for further research are presented.

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Chapter 2

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Brown adipose tissue; clinical impact of a re-discovered thermogenic organ

The contents of this chapter are accepted for publication in Frontiers in Bioscience.

Abstract

Brown adipose tissue (BAT) is physiologically present and active in adult humans. The stimulation of BAT in man can potentially increase total daily energy expenditure and is seen as a possible target to treat obesity. Altered BAT activity is also related to other diseases and therefore the therapeutic potential of BAT could reach beyond obesity. This is supported by both in vitro and in vivo reports from animal studies, describing the possible role of BAT in both physiology and pathophysiology. In addition, since the discovery of functional BAT several clinically relevant studies have been conducted in adult man. Clinical observations report BAT activity under multiple conditions, suggesting BAT could be important in the onset and/or treatment of diseases related to the metabolic syndrome, thyroid disorders, cancer and immune system dysfunction. This review highlights those diseases or syndromes in which BAT may play a role in relation to prevention, diagnosis or therapy, by translating basic research into clinical relevance.

Introduction

Recently, active brown adipose tissue (BAT) was discovered in healthy adults.¹⁻³ In rodents, BAT is known for its high heat production capacity that can significantly increase total energy expenditure.⁴ Since the discovery of functional BAT in man, increasing energy expenditure by the stimulation of BAT is seen as a new anti-obesity target.^{5,6} Interestingly, in man BAT activity is low or absent in obese subjects. Possibly, increasing energy expenditure by stimulation of BAT could be important in weight maintenance or could create a negative energy balance leading to weight loss.

Studies in both man and animals also show active BAT under several clinical conditions. These studies suggest involvement of BAT in conditions that are associated with the metabolic syndrome (obesity, type 2 diabetes, dyslipidemia, fatty liver disease), endocrine disorders (pheochromocytoma, thyroid gland dysfunction), neoplasms (cancer cachexia, hibernoma), general inflammation and the immune response. Here, we will discuss the relevance of BAT in relation to these conditions, and its possible therapeutic impact.

Physiology of BAT

Classically, BAT is associated with cold exposure. To prevent hypothermia, increasing insulation (clothing) and change of environment are effective behavioral strategies. However, if this is not possible or insufficient, generating body heat is necessary to prevent hypothermia. Shivering in skeletal muscle is very effective to increase heat production, but this cannot be sustained for longer periods of time.⁷ Interestingly, when a subject is exposed to cold, heat production already increases prior to the onset of shivering.⁸ This process is defined as nonshivering thermogenesis (NST). In rodents BAT is the main site for NST.⁴ In man, there are indications that both BAT and skeletal muscle are involved.⁹

Mitochondrial uncoupling induces thermogenesis in BAT

NST in the brown adipocyte is facilitated by 'uncoupling' of the mitochondrial respiration from adenosine-tri-phosphate (ATP) production. The mitochondrial respiration chain generates a proton gradient across the inner mitochondrial membrane to produce ATP. However, during BAT stimulation the cross-membrane proton gradient is used to produce heat instead of ATP. An essential protein for this process, that also typifies BAT, is the uncoupling protein 1 (UCP1, Figure 1).⁴ In order

Figure 1. Uncoupling protein 1 (UCP1) mediates mitochondrial uncoupling in brown adipose tissue (BAT). Schematic overview of BAT (top left), BAT mitochondrion (top right) and inner mitochondrial membrane (A and B). The proton gradient across the inner mitochondrial membrane can be used to generate ATP (coupled respiration, depicted in A). When BAT is stimulated to become active, UCP1 facilitates uncoupling of ATP-generation to induce thermogenesis (uncoupled respiration, depicted in B).

to facilitate uncoupling by UCP1 in the brown adipocyte, free fatty acids (FFAs) are necessary.⁴ The exact contribution of FFAs to UCP1 activation is still unknown, but most likely they facilitate a membrane entrance for protons to generate thermogenesis.⁴ Besides FFAs, BAT also uses glucose during thermogenesis.⁴ The purpose of glucose-uptake in active BAT is largely unknown. Possibly, glucose is necessary to maintain the citric cycle, ATP production and the proton gradient⁴. Alternatively, glucose is converted to FFAs.⁴ The function of glucose uptake in BAT would then be to increase the amount of FFAs, necessary for mitochondrial uncoupling.

Involvement of sympathetic nervous system in BAT activation

The sympathetic nervous system (SNS) stimulates BAT to produce heat during NST. Sympathetic innervation is essential for BAT to become active, as disruption of the sympathetic fibers to BAT completely ceased BAT activity.¹⁰ In man, most

likely responses from warm- and cold sensitive neurons regulate the activation of thermogenesis.¹¹ In the hypothalamus and medulla input from these neurons is mediated into sympathetic stimuli that control BAT thermogenesis by the release of norepinephrine on the adrenergic receptors of BAT cells.¹¹ In rodents, BAT activity is associated with both adrenergic α - and β -receptor subtypes. Although the α_{1A} -receptor is abundant in BAT, stimulation could only account for maximally 10% of total BAT thermogenesis in rodents.⁴ The α_2 -receptor is even suggested to inhibit thermogenesis. The β_3 -receptor seems to be the most significant mediator of thermogenesis, as suggested from studies that have compared BAT stimulation with β_3 -agonists versus norepinephrine.⁴ Most likely β_3 -activation, and to a much lower extent α_{1A} -activation, mutually mediate norepinephrine-induced thermogenesis in mammals. Although present in BAT, the β_1 -receptor is only suggested to influence BAT activity when β_3 -receptor signaling is dysfunctional (e.g. β_3 -receptor-ablation).⁴

Thyroid hormone induces BAT activation

In addition to sympathetic stimulation of BAT, thyroid hormone plays a significant role in BAT thermogenesis. The thyroid gland mainly secretes the inactive pro-hormone thyroxine (T4), which needs to be deiodinised (lose an iodine atom) by deiodinases to triiodothyronine (T3) to become active. In rodents, T3 can stimulate BAT.¹² After conversion of T4 to T3 by the BAT-specific type-II-iodothyronine-5-deiodinase (D2), thyroid hormone enhances BAT thermogenesis.^{12,13} In addition, thyroid hormone supplementation in the brain increased BAT activity and induced weight loss in rodents.¹³ This activation of BAT could then be reversed by the β_3 -adrenergic receptor antagonist SR59230A, indicating this central thyroid activation was mediated via the SNS.¹³ These results indicate interplay at the central nervous system level between the endocrine (thyroid axis) and sympathetic regulation of BAT.

Presence of BAT in man

In humans, BAT is present in significant amounts during childhood. Compared to adults, newborns have several disadvantages regarding their maintenance of body temperature. The higher body surface-to-volume ratio may cause a more rapid heat loss. Due to low amount of muscle, the ability to induce thermogenesis by shivering is limited. Therefore NST is important in newborns.⁴ During infancy growth changes the relative surface area and the amount of BAT gradually decreases. In adulthood BAT has been reported in autopsies,¹⁴ but it was always believed to be dysfunctional.¹⁵

Strikingly, BAT has recently shown to be present in significant amounts in adults and becomes functionally active upon cold exposure.^{1,2} 2-deoxy-2-¹⁸F-fluoro-D-glucose (¹⁸F-FDG), a radioactive labeled glucose tracer, is used in nuclear imaging (positron-emission-tomography-and-computed-tomography (PET-CT)) to depict glucose uptake for tumor diagnosis. Frequently non-malignant uptake in adipose tissue was seen, which later appeared to be related to mild cold conditions during the tracer administration. Prospective, controlled mild cold exposure consistently showed this activity in supraclavicular, cervical and paravertebral adipose tissue regions. Tissue biopsies from PET-active supraclavicular adipose tissue regions confirmed BAT presence.^{1,2}

Retrospective ¹⁸F-FDG PET-CT series in infants show high amounts of BAT that even increase during adolescence.¹⁶ In adolescence, muscle mass also still increases under influence of metabolic and hormonal factors.¹⁶ Seale et al. showed that skeletal muscle and BAT share a similar developmental origin¹⁷ and therefore it is suggested the factors that induce muscle growth during adolescence could similarly influence BAT.¹⁶ After the volume of BAT peaks up to amounts of 1200 cm³ in the second decade of life,¹⁶ ¹⁸F-FDG PET-CT studies³ and histological analyses¹⁸ indicate the amount of BAT decreases with age. BAT expresses sex hormone receptors and the age-related involution of BAT could be influenced by the lowering levels of sex hormones.¹⁹ If low BAT is related to disease, the age-related BAT loss could be important in the onset of the metabolic syndrome and other pathologic conditions.

BAT in disease

Metabolic syndrome

Obesity

In mice, absence of active BAT through ablation of UCP1 causes a weight gain of >50% within 30 days after birth.²⁰ In man, dedicated studies show a strong negative correlation with BAT activity and body fat percentage and body mass index.^{1,3,21} Retrospective analyses of patient ¹⁸F-FDG PET-CT-scans reveal comparable relations.²²⁻²⁵ Possibly, due to the insulation by subcutaneous adipose tissue in obese subjects there is less need to activate BAT in daily living situations. Subjects without BAT activity show significantly lower NST,^{21,26} suggesting BAT is involved in the human energy balance. A classic overfeeding study in man showed

large differences in weight gain on a high-calorie diet.²⁷ If low or absent BAT activity would be responsible for the differences in weight gain after overfeeding, increasing BAT activity could possibly prevent or treat obesity. An explanation for the individual variation in BAT could be associated with polymorphisms in the UCP1-gene. Resting energy expenditure, sympathetic nervous system activity and the thermal effect of a meal show a relation with the UCP1 G/G genotype.²⁸ Moreover, if the UCP1 G/G genotype is accompanied by a Trp64Arg polymorphism of the β_3 -receptor gene this is associated with a lower basal metabolic rate, lower sympathetic nervous system activity and a higher amount of visceral adipose tissue.^{29,30} However, there are no studies that have directly related BAT activity and UCP1-polymorphisms yet.

In summary, active BAT is low or absent in obesity. Body composition (body fat percentage) and environmental factors (lifestyle) may cause variations in amount and activity of BAT. Increasing BAT activity could be a new target to both prevent and treat overweight.

Type 2 diabetes

The significant uptake of glucose in active BAT is shown by ^{18}F -FDG PET-CT. Stimulating glucose uptake in BAT can be beneficial in situations of impaired glucose clearance, as in type 2 diabetes. Indeed, retrospective cohort studies suggest an association with high blood glucose levels and absence of active BAT on ^{18}F -FDG PET-CT.^{24,31,32} Interesting is the case of a patient with a resected thyroid gland because of thyroid cancer and an extreme insulin resistance due to an insulin receptor mutation.³³ The therapeutic treatment of this patient with high doses of thyroid hormone was accompanied with an increase of active BAT. The suppletion with thyroid hormone also resolved the patient's hyperglycaemia, hinting towards an association between BAT activity and glucose homeostasis.

To estimate the glucose clearing capacity of BAT in man, the uptake of glucose in cold-stimulated BAT can be used. Calculations made from dynamic ^{18}F -FDG PET-CT scans during cold exposure show a mean glucose clearance in BAT of $9.1 \pm 5.1 \mu\text{mol}/100 \text{ g}/\text{minute}$ ($n=27$)³⁴ to $22.67 \pm 18.28 \mu\text{mol}/100 \text{ g}/\text{minute}$ ($n=5$) (unpublished results). To assess the possible contribution of cold stimulated BAT on whole body glucose clearance, glucose uptake in BAT (0.7 mMol glucose for 100 g

BAT per hour, based on reports above) can be compared to the glucose infusion rate in a hyperinsulinemic-euglycemic clamp test (166mMol glucose per hour).³⁵ In this comparison, BAT accounts for 0.4% of the body's maximal glucose uptake. In summary, active BAT takes up glucose and BAT in man can be important in glucose clearance. Therefore, BAT is a new potential target tissue for the treatment of type 2 diabetes.

Dyslipidemia

FFAs are essential for BAT thermogenesis (see Physiology of BAT). They are delivered to BAT by plasma circulating triglyceride-rich lipoproteins (TRLs) and cold-activated BAT in mice cleared a large amount of TRLs from the blood after a meal.³⁶ On a cellular level, BAT-released lipoprotein lipase (LPL) converted the TRLs to free fatty acids (FFA), then used for thermogenesis.³⁶ In conclusion, after BAT activation by cold exposure experimentally induced hyperlipidemia could be corrected. Although the condition of cold exposure was extreme (4°C), the reported high clearance of plasma triglycerides (>50%) provides hope for the treatment of dyslipidemia in man. In man, current treatment for dyslipidemia with statins generates a 40-60% reduction in plasma low density lipoprotein (LDL) cholesterol, a direct lipolysis product of TRLs.³⁷ Increasing BAT activity theoretically requests more lipolysis and could positively affect dyslipidemia.

Hepatosteatosi

Obesity, type 2 diabetes and dyslipidemia are all associated with an increased accumulation of fat in the liver, defined as hepatosteatosi (or non-alcoholic fatty liver disease (NAFLD)). Hepatosteatosi is associated with a decrease in liver functionality and a high risk of developing steatohepatitis and liver cirrhosis.³⁸ A retrospective analysis of ¹⁸F-FDG PET-CT-scans suggests an inverse relation between BAT activity and hepatosteatosi.³⁹ Subjects without BAT activity had a significantly higher risk of hepatosteatosi and BAT activity was inversely correlated with the

level of liver steatosis and body mass index (BMI). In addition to the association with energy balance, genetic polymorphisms of UCP1 are also associated with hepatosteatosis.⁴⁰

In conclusion, hepatosteatosis is associated with low BAT activity and prospective studies are needed to define the role of low BAT in the development of hepatosteatosis.

Thyroid disease

Patients with thyrotoxicosis (high levels of plasma T4 and T3 due to a toxic goiter) have an increase in resting energy expenditure (REE) up to 30% that returns to normal values after treatment with thiamazole.⁴¹ Upon T3-suppletion (resulting in T3 values twice as high compared to normal) in healthy subjects basal metabolic rate (BMR) increased.⁴² During hypothyroidism (low plasma levels of thyroid hormone) REE decreases 20-25%.⁴³ Even the induction of subclinical variations in thyroid function by suppletion of thyroid hormone already caused changes in REE of 5-10%.⁴³ This indicates thyroid function is relevant for at least REE in man, but the contribution to thermogenesis and BAT has not been clearly defined yet. Support for thyroid-induced BAT activity in man is given by the above-mentioned case report of a patient with a resected thyroid gland because of thyroid cancer.³³ Treatment with thyroid hormone was accompanied with active BAT on ¹⁸F-FDG PET-CT, without cold exposure.

Similar to the influence of thyroid hormone on REE, variations in thyroid function within normal ranges could also impair or stimulate BAT activity. Therefore, dedicated studies on thyroid function and BAT in man are necessary.

Pheochromocytoma

The pheochromocytoma is a tumor of chromaffin-cells. In man, chromaffin cells are present in the medulla of the adrenal gland and secrete the catecholamines norepinephrine and epinephrine. During cold exposure, BAT is activated by sympathetic stimuli from the brain that release norepinephrine to stimulate the adrenergic receptors on BAT cells.¹¹ Catecholamines released in the blood plasma induce adrenergic responses similar to activation of the sympathetic nervous system. In rodents, suppletion of norepinephrine is known to be able to increase the activity of BAT up to a fourfold.⁴ In patients with pheochromocytoma, retrospective ¹⁸F-FDG PET-CT studies often show high BAT activity even under thermoneutral conditions.^{44,45} Interestingly, the high levels of circulating plasma catecholamines

in pheochromocytoma were significantly related to the level of BAT activity.²⁵ This suggests that the principle of catecholamine-activated BAT (by suppletion of norepinephrine into the circulation) as observed in rodents also applies to man.

Cancer cachexia

During cancer cachexia high BAT activity is reported. In autopsy studies in man high BAT presence in cachectic cancer patients was already described years ago.⁴⁶ Active BAT is also often reported in ¹⁸F-FDG PET-CT-imaging of oncologic patients.⁴⁷ It is hypothesized that the high production of cytokines in cancer stimulates thermogenesis in BAT, both via the hypothalamus and directly on BAT cells. This is supported by studies in mice that show tumor implantation or stimulation with carcinogenic factors increases BAT activity.⁴⁸

Experimental denervation of BAT and β_3 -receptor antagonism reduces the effect of a cytokine-induced increase in energy expenditure.⁴⁹ Therefore, the tumor-induced high BAT activity is held responsible for the increased thermogenesis that causes body mass loss up to anorectic levels in cancer cachexia.

Hibernoma

In addition to the high BAT activity in physiological depots in patients with malignant tumors, BAT can be present within a tumor itself.⁵⁰ The rare hibernoma, a benign lipomatous tumor of brown adipocytes, was visible on ¹⁸F-FDG PET-CT-imaging.²³ Although the hibernoma shows activity on ¹⁸F-FDG PET-CT, its activity apparently seems not high enough to significantly induce weight loss.²³ In spite of the fact large amounts of BAT are present in the hibernoma, it is not activated in daily life.

Immune response

In rodents BAT thermogenesis increases during fever.⁵¹ In the inflammatory state BAT serves as the main heat-producing tissue in rodents.⁵¹ After the injection of lipopolysaccharide (LPS), an endotoxin present in bacteria, BAT is triggered to increase thermogenesis which causes fever.⁵¹ The increase in BAT activity also occurs in response to sympathetic stimuli from the hypothalamus, which itself is stimulated by pyrogenic cytokines.¹¹ Besides the sympathetic stimulus from the brain, macrophages in the blood of mice are able to release catecholamines that activate BAT.⁵² Interestingly, during fever BAT starts producing its own endogenous pyrogens.⁵¹ BAT is reported to produce interleukin-6 (IL-6), a pro-inflammatory

cytokine normally secreted by cells of the immune system, e.g. macrophages. IL-6 is not only present during fever, but is also important during inflammation and infection and is seen in other immune reactions.⁵³ In fever, IL-6 initiates the pyrogenic mediator prostaglandine E2 (PGE2) which in the hypothalamus increases the temperature setpoint.¹¹ In this perspective BAT thus could be capable of both controlling the level of fever and increasing heat production. This is interesting, because it makes BAT not only a tissue that responds when stimulated, but also a controller of thermogenesis itself. Cyclooxygenase 2 (COX-2), an important controller of PGE2 production, recruits BRITE (brown-in-white) cells in white adipose tissue (WAT) of mice.⁵⁴ These cells are non-thermogenic preadipocytes present in WAT that differ from white adipocytes because they can become thermogenic to similar levels as BAT.⁵⁵ Others factors for BRITE recruitment are a currently further explored treatment target (see Stimulation of BAT). Since IL-6, COX-2 and PGE2 are important in inflammation, increasing the amount of BAT or BRITE could be an interesting target to enhance the immune response during immunodeficiency.

Besides cold induced thermogenesis, BAT may be involved in immune competence of neonates.⁵⁶ Sudden infant death syndrome (SIDS) is associated with a depletion of BAT, as observed afterwards during autopsy.⁵⁶ It is suggested this BAT depletion could follow from maternal conditions during pregnancy. Indeed, animal studies show that maternal cold exposure increased BAT activity in newborn lambs and lactation conditions were important for BAT development in mice.^{57,58}

Results from autopsies,¹⁴ ¹⁸F-FDG PET-CT-imaging studies³ and histological analyses¹⁸ indicate the amount of BAT decreases with age. If BAT is important in the immune response, a lower amount of BAT in the elderly could possibly account to more disease susceptibility.

In conclusion, BAT can be important for our immune system. However, more prospective studies on BAT activity or recruitment during fever or immune activation are needed.

Stimulating BAT

Cold exposure

Functional BAT in man was discovered by exposing subjects to mild cold.^{1,2} Under thermoneutral conditions, healthy subjects did not show active BAT.¹ Indeed, active BAT is related to thermogenesis under mild cold exposure.^{21,26,59} During cold acclimatization (days to weeks) nonshivering thermogenesis (NST) increases.⁶⁰

In mice, the increased NST after cold acclimatization originates from increased BAT activity.⁴ Cold acclimatization also causes recruitment of BAT in rodents and primates.⁴

In man, high volumes of BAT were observed in autopsy studies on Finnish outdoor workers, who were acclimatized to cold.⁶¹ Hence, cold acclimatization could be an interesting treatment modality to increase energy expenditure by activating NST in BAT.⁷

Insulin stimulation

In mice, injection of insulin in the hypothalamus increases thermogenesis by activating BAT.⁶² In man, insulin infusion increased glucose uptake in BAT five-fold compared to baseline, from 0.9 to 4.7 $\mu\text{mol}/100\text{ g}/\text{minute}$.³⁴ This insulin stimulated glucose uptake in BAT amounted to half of the cold stimulated BAT activity. In contrast to glucose uptake during cold exposure, insulin-stimulated glucose uptake in BAT is suggested to be more active under non-thermogenic conditions (no cold exposure).⁴ Under such conditions glucose would mainly be taken up in BAT to be converted into FFAs, that later can be used for mitochondrial uncoupling during BAT activation.⁴

In summary, BAT can be stimulated by insulin to take up glucose under thermoneutral conditions. Possibly, insulin-stimulated BAT could decrease blood glucose levels and serve as a therapeutic target for type 2 diabetes.

Sympathetic stimulation

Sympathetic β -receptor agonists increase SNS activity, which could possibly stimulate BAT thermogenesis. Interestingly, cold-induced BAT activity in man can be fully blocked by the β -antagonist propranolol.⁶³ Propranolol is highly specific for β_1 - and β_2 -receptors and rodent studies indicate BAT mainly contains β_3 -receptors.⁴ This suggests BAT activity in man is mediated differently from rodents. The β -agonist ephedrine was used to stimulate BAT and increase energy expenditure many years ago, but activation of BAT was not reported possibly due to the limited methods then available.¹⁵ Since ephedrine mainly stimulates β_1 - and β_2 -receptors and propranolol shows blockage of these receptors inhibits BAT, ephedrine (or other β -receptor agonists) could be effective in activating BAT in man after all. Controversially, propranolol supplementation did not reduce cold-induced NST in man.⁶⁴ Since NST in man upon cold exposure is associated with BAT, this again supports

the β_3 -receptor specificity of BAT seen in rodents. These contrasting results could possibly become more clear after an intervention study with β_3 -receptor specific agonists.⁵

BAT stimulation via the gut

In rodents, the feeding of bile acids increases fat combustion in BAT.⁶⁵ This signal is mediated via the G protein-coupled bile acid-receptor TGR5 (TGR5), present on BAT.⁶⁶ TGR5-agonists increase energy expenditure, decrease non-alcoholic fatty liver disease (NAFLD) and improve glucose tolerance in rodents.⁶⁵ Interestingly, this process is dependent of the thyroid hormone converter D2 (for thyroid function and BAT, see BAT physiology).⁶⁷ If D2 is disrupted in mice, bile acids do not increase BAT activity anymore, indicating the importance of thyroid-induced thermogenesis in bile acid-stimulated BAT activity.⁶⁷

Gut hormones are involved in food intake regulation via the brain.⁶⁸ Rodent studies indicate the central responses involved in ingestion are most likely located in the paraventricular hypothalamus, which is also known to induce the sympathetic nervous system (SNS) and thyroid axis.⁶⁸ Increased SNS activity stimulates BAT activity, and therefore the response that mediates a decrease in food intake could stimulate energy expenditure in BAT. In man, this is illustrated by the observation that after suppletion of the appetite hormone oxyntomodulin energy expenditure was also stimulated.⁶⁹

In summary, the suppletion of bile acids and gut hormones could induce an increase in BAT activity via the gut.⁷⁰

Transforming WAT to BAT

All options described above consider the stimulation of BAT present at birth, often referred to as 'native' BAT depots. BAT in these depots was shown the share a developmental origin that is very similar to skeletal muscle.¹⁷ The presence or absence of the signaling molecule PRDM16 stimulates dermomyotomal precursors to end up as a skeletal muscle cell (absence of PRDM16) or as a brown adipocyte (PRDM16+).¹⁷ WAT seems to derive from a different developmental origin without the thermogenic capacity of BAT.⁷¹ However, within WAT depots a subpopulation of cells is indeed capable of developing into thermogenic 'beige' or BRITE (brown-in-white) cells.^{55,71} These cells possess UCP1 and are thus capable of thermogenesis. Chaffee et al. already reported 'darker yellow adipose tissue' in rhesus monkeys after 12-24 months cold-acclimatization at 5°C in comparison to controls housed

at 35°C⁴. Several studies have now reported the development of BRITE cells upon diverse stimuli under cell culture conditions and in rodents.^{55,72,73} Here, three studies are of special clinical interest because of the use of human samples.⁷³⁻⁷⁵ Irisin, a novel hormone that induced 'browning' and UCP1-expression in subcutaneous adipose tissue from mice, was shown to increase in human plasma after 10 weeks of exercise.⁷⁵ However, this study did not study the effects of irisin on human adipocytes and therefore further human studies are needed. Lee et al. show in vitro development of thermogenic adipocytes that derive from preadipocytes isolated from supraclavicular adipose tissue biopsies in both FDG-PET-CT-positive (n=2) and FDG-PET-CT-negative (n=4) subjects.⁷⁴ The stimulants used in this study were both β -agonists and proliferator-activated receptor gamma (PPAR-gamma), an agent present in several antidiabetic agents (rosiglitazone, pioglitazone). In another study, Schulz et al. treated preadipocytes isolated from human subcutaneous WAT with a mix of several agonists and showed the induction of UCP1 presence as a marker of BAT.⁷³ Although no evidence exists of BRITE recruitment in vivo in man, the cell studies suggest that transformation of non-thermogenic to thermogenic fat cells is possible in man.

Perspective

Increasing evidence appears on the important role of BAT in adult physiology in both health and disease. Several recent studies illustrate possible therapeutic potentials of BAT (Table 1, Figure 2). This overview shows that in addition to nonshivering thermogenesis, BAT could be important in the metabolic syndrome (obesity, type 2 diabetes, dyslipidemia, hepatosteatosis), neoplasms (cancer cachexia, hibernoma) and the immune system. However, to define the clinical impact of BAT on disease more basic and clinical controlled studies are needed. Recent studies clearly show metabolic activity of BAT in adult humans, although there are indications of decreasing BAT activity with ageing. Therefore, the factors that account for this decrease should be targets for future investigations. Since it is considered unethical to use PET-CT-techniques to prospectively determine BAT activity in healthy infants and for repeated measurements in healthy adults, less invasive techniques need to be developed (like magnetic resonance imaging (MRI)). On the other hand it is feasible to perform clinical studies on BAT activity and recruitment during existing therapies. Thyroid hormone suppletion, insulin therapy, weight loss treatment and sympathetic stimulation can provide much insight in the dynamics of BAT and possible clinical targets.

Table 1. Diseases associated with brown adipose tissue (BAT) activity and possible implications for therapy. Abbreviations: ¹Brown adipose tissue

Disease	Effect on BAT ¹	Evidence	Potential implication
Obesity	Decrease of BAT activity with increased BMI and body fat %	Retrospective (human) ^{22-25,31,32} Prospective (human) ^{1,3,21}	Increase BAT activity to prevent and/or treat obesity
Type 2 diabetes	Association with high blood glucose and absence of BAT activity	Retrospective (human) ^{24,31,32} Prospective evidence of insulin-mediated BAT activity ³⁴	Increase BAT activity to decrease hyperglycemia
Dyslipidemia	BAT activation decreases hyperlipidemia	Mice ³⁶	Increase BAT activity to improve hyperlipidemia
Hepatosteatorsis	Association with absence of BAT activity and hepatosteatorsis	Retrospective (human) ³⁹	Absence of BAT activity could be a risk to develop hepatosteatorsis
Hyperthyroidism	Increase in BAT activity during hyperthyroidism	Rats ¹³	Target thyroid function to correct impaired BAT activity
Hypothyroidism	Absence of BAT activity during hypothyroidism	Case-report after thyroid resection (human) ³³	
Pheochromocytoma	Association with active BAT and high plasma catecholamine levels	Pheochromocytoma patients ^{44,45}	Stimulation of sympathetic nervous system to induce BAT activity
Cancer cachexia	Active BAT during cancer cachexia	Tumor-bearing mice ⁴⁸ Autopsy (human) ⁴⁶ Cancer cachexia (human) ⁴⁷	Cancer-induced BAT activity increases cachexia
Hibernoma	High amount of active BAT in hibernoma	Human ^{23,50}	Activated BAT in hibernoma affects energy balance
Immune-activation	Immune response influences BAT activity	Cell studies ⁵² Mice ⁵⁴	BAT activity is relevant in immune activation

Figure 2. Potential therapeutic targets to stimulate BAT based on clinical observations. Schematic representation of brown adipose tissue (BAT) with beta-receptors (β_3 , β_1) and the bile-acid receptor TGR5. SNS indicates sympathetic nervous system, PPAR-gamma indicates peroxisome proliferator-activated receptor gamma, BMP7 indicates bone morphogenic protein 7, COX2 indicates cyclo-oxygenase-2, WAT indicates white adipose tissue.

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Chapter 3

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Brown adipose tissue in morbidly obese subjects

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Abstract

Background

Cold-stimulated adaptive thermogenesis in brown adipose tissue (BAT) to increase energy expenditure is suggested as a possible therapeutic target for the treatment of obesity. We have recently shown high prevalence of BAT in adult humans, which was inversely related to body mass index (BMI) and body fat percentage (BF%), suggesting that obesity is associated with lower BAT activity. Here, we examined BAT activity in morbidly obese subjects and its role in cold-induced thermogenesis (CIT) after applying a personalized cooling protocol. We hypothesize that morbidly obese subjects show reduced BAT activity upon cold exposure.

Methods and Results

After applying a personalized cooling protocol for maximal non-shivering conditions, BAT activity was determined using positron-emission tomography and computed tomography (PET-CT). Cold-induced BAT activity was detected in three out of 15 morbidly obese subjects. Combined with results from lean to morbidly obese subjects ($n=39$) from previous study, the collective data show a highly significant correlation between BAT activity and body composition ($P<0.001$), respectively explaining 64% and 60% of the variance in BMI ($r=0.8$; $P<0.001$) and BF% ($r=0.75$; $P<0.001$). Obese individuals demonstrate a blunted CIT combined with low BAT activity. Only in BAT-positive subjects ($n=26$) mean energy expenditure was increased significantly upon cold exposure (51.5 ± 6.7 J/s versus 44.0 ± 5.1 J/s, $P=0.001$), and the increase was significantly higher compared to BAT-negative subjects ($+15.5\pm8.9\%$ versus $+3.6\pm8.9\%$, $P=0.001$), indicating a role for BAT in CIT in humans.

Conclusions

This study shows that in an extremely large range of body compositions, BAT activity is highly correlated with BMI and BF%. BAT-positive subjects showed higher CIT, indicating that BAT is also in humans involved in adaptive thermogenesis. Increasing BAT activity could be a therapeutic target in (morbid) obesity.

Introduction

Brown adipose tissue (BAT) is known for its capacity to generate heat in response to cold or diet to maintain thermal balance. The regulated production of heat is called adaptive thermogenesis. BAT is the main tissue for this adaptive thermogenesis in rodents and most likely in human infants.^{1,2} Although several early anatomical studies suggested that brown adipose tissue is present in adult humans³⁻⁵, its physiologic relevance was believed to be marginal for most.^{5,6} However, recent prospective, controlled studies showed that functional BAT is detectable in lean and obese adult humans after exposure to mild cold.⁷⁻⁹ We showed a high incidence of cold induced BAT activity that was inversely related to body mass index (BMI) and body fat percentage (BF%).⁷ Other studies had similar findings.⁸⁻¹⁰

Cold-stimulated adaptive thermogenesis in BAT to increase energy expenditure is suggested as a possible therapeutic target for the treatment of obesity.^{11,12} Cold can indeed stimulate adaptive thermogenesis, but differences in body composition may be correlated with the magnitude and type of response. We showed that lean subjects increase energy expenditure significantly in response to mild cold, whereas obese subjects have a blunted cold-induced thermogenesis (CIT) and show a larger insulative response.¹³ If BAT is directly responsible for CIT, the absence of CIT in obesity suggests that BAT is reduced or absent. In addition, if the presence of BAT is structurally lower in obesity, this could be a risk factor for the development of obesity. Not being able to burn off excess calories leads to a positive energy balance and predisposes to develop obesity. This could explain the differences in weight gain in classic overfeeding studies.¹⁴

In our previous report BAT abundance in obese subjects was relatively low, though detectable in most individuals. In the current study we address BAT activity in morbid obesity, a severe form of obesity characterized by a BMI ≥ 35 kg/m² with concomitant disease or ≥ 40 kg/m² without.

To ensure maximal non-shivering thermogenesis (NST), as occurs before the onset of shivering,¹⁵ every subject was exposed to an individualized cooling protocol. We hypothesized that morbidly obese in comparison to lean subjects show reduced BAT activity upon cold exposure. Furthermore, we examined the role of BAT activity in cold-induced thermogenesis in these morbidly obese subjects.

Methods

The study was reviewed and approved by the medical ethics committee of the Maastricht University Medical Centre. Written informed consent was obtained from fifteen morbidly obese subjects, two male and thirteen female. The mean BMI was 42.1 ± 3.8 kg/m² (range 34.8-48.3 kg/m²), mean body fat percentage $48.5 \pm 4.5\%$ (range 37.9-54.7%), mean weight 123.9 ± 16.7 kg (range 98.2-155.0 kg) and mean age 39.2 ± 8.1 years (range 24-51 years). Subjects were not included when diagnosed with diabetes or use of beta-blockers. One female subject used levothyroxin for hypothyroidism and was euthyroid for several years. One female subject used azathioprine and mesalazine for Crohn's disease. They were studied in the morning from 8 a.m. to 1 p.m. in a fasted state; only water consumption was allowed after 10 p.m. the night before measurements. During the measurements the subjects wore light standardized clothing (socks 0.02 clo, shirt 0.09 clo, sweatpants 0.28 clo, underwear 0.04 clo, total clo factor 0.43 clo). Core temperature was measured by a telemetric pill (CoreTemp, USA). This measurement failed in two subjects. Skin temperature was measured by wireless iButtons at the 14 ISO-defined skin sites.¹⁶ Subjects were placed in a specially equipped air permeable tent (Colorado altitude training, USA), which functioned as a transportable climate room at the department of nuclear medicine. The tent was cooled by an air-conditioning, which can be controlled to maintain the temperature inside the tent with an accuracy of 1°C [Kingma et al., submitted]. Subjects were placed in a semi-supine position in a nephrodialysis chair to lay comfortable during the personalized cooling protocol. Previous studies in our group showed high NST in lean subjects at the fixed temperature of 16°C, without shivering.¹⁷ However, in the current study group pilot experiments showed a lower onset temperature of shivering, with high interindividual variation. Therefore we used a personal cooling protocol to ensure maximal NST in the morbidly obese state. The level of cooling (temperature) was attuned to each individual, i.e. close to the cold level that induces shivering. This protocol was performed with subsequent FDG-PET-CT-imaging, because the intraindividual response to cold exposure shows high variation.¹⁷ In order to cool the dorsal site of the body, a water perfused cooling mattress was used (cooling device; Blankett roll, Cincinatti sub zero 2000, USA). Energy expenditure was measured for three hours by indirect calorimetry (Oxycon, Jaeger, Germany). One hour in thermoneutral conditions was followed by two hours of personalized cooling. In the first hour the room temperature was $22.3 \pm 0.4^\circ\text{C}$ (baseline), followed by the second hour where the temperature of the room and cooling mattress were

decreased until subjects subjectively reported shivering. This was confirmed by continuous measurement of muscle activity (pectoralis major) by use of on-skin electrodes attached to an electromyograph (Nicolet Viking, Nicolet Biomedical Inc, USA). When shivering occurred, the air and water temperatures were increased by steps of 1°C until shivering just stopped. In this manner NST was maximized for each individual without shivering. Stable cooling conditions were reached within 28.6 ± 11.8 minutes. After one hour of cold exposure 74 MBq of 18 -Fluoro-Deoxy-Glucose (FDG) was injected. Cold exposure was maintained for another hour. To exclude the artifact of muscle activity, subjects were instructed to lay still. After this hour subjects were transferred to the positron emission tomography and computed tomography scanner (PET-CT-scanner) (Gemini TF PET-CT, Philips, the Netherlands). The scanning protocol and data interpretation were identical to our previous study protocol.⁷ Energy expenditure, room temperatures and body temperatures were sampled on a one minute basis. On a separate occasion body composition was measured with a dual x-ray absorptiometry (DXA, Hologic, type Discovery A, USA). Statistical analysis was performed with PASW Statistics 18.0 for Mac OS X 10.6.4. Reported data is expressed as means $\pm$ SD. Total BAT activity is expressed in kiloBequerel (kBq). To compare findings before and after cold exposure paired t-tests were used; unpaired t-tests were used to compare BAT-positive to BAT-negative subjects. To identify correlations between variables linear regression analyses were conducted. For the relation between brown-adipose-tissue activity and body composition, exponential curve fitting was applied.

Results

Individual cooling conditions

Temperature conditions before the onset of shivering were variable. Stable cooling temperatures were established in fourteen subjects (room temperature $14.7 \pm 2.4^\circ\text{C}$, inlet temperature of cooling mattress $13.3 \pm 4.7^\circ\text{C}$). One female subject (BMI 38.7 kg/m^2 , body fat 49.9%, age 44 years, BAT 0 kBq) did not report shivering subjectively nor electromyographically at the lowest possible room (12°C) and cooling mattress (inlet temperature of water mattress 4°C) conditions.

BAT activity

In the current morbidly obese group mean BAT activity was low (5.5 ± 14.8 kBq, range 0.0–54.5 kBq). Only three female subjects showed a cold induced increased FDG uptake in the neck and supraclavicular area on FDG-PET-CT-imaging (Figure 1). In these three subjects, the BAT activity was 4.48 kBq (BMI 42.5 kg/m², body fat 51.2%, age 47 years), 23.61 kBq (BMI 36.9 kg/m², body fat 50.8%, age 47 years) and 54.45 kBq (BMI 46.3 kg/m², body fat 53.7%, age 32 years). No BAT activity was detected in the other 12 subjects.

Energy expenditure

On average, energy expenditure during cooling compared to baseline did not increase significantly (baseline 41.9 ± 3.3 J/s, mild cold 43.7 ± 4.8 J/s, $P=0.100$) (Table 1). However, a large interindividual variation was evident. The calorimetry measurement in one female subject failed and was excluded from analysis.

Core and skin temperature

During cooling we observed a significant rise in core temperature ($37.2 \pm 0.4^\circ\text{C}$ in thermoneutral conditions (TN) versus $37.5 \pm 0.3^\circ\text{C}$ during cold exposure (T_{Cold}), $P=0.048$) and a drop in mean skintemperature (TN $31.7 \pm 0.6^\circ\text{C}$ versus T_{Cold} $27.7 \pm 1.5^\circ\text{C}$, $P<0.001$) (Table 1). Compared to thermoneutral conditions, cooling significantly increased the core-mean skin temperature gradient (TN $5.6 \pm 0.6^\circ\text{C}$ versus T_{Cold} $9.9 \pm 1.6^\circ\text{C}$, $P<0.001$) (Table 1).

Relationship of BAT - BMI

When combining the data from the current and our previous study,⁷ we found strong correlations between BAT activity and BMI ($r=0.80$, $P<0.001$), BAT activity and body fat mass ($r=0.80$, $P<0.001$), and BAT activity and BF% ($r=0.75$, $P<0.001$) (Figure 2).

In the combined group there were 26 subjects that showed active BAT on cold exposure, whereas 13 subjects did not. We therefore reanalysed our energy expenditure data in BAT-positive and BAT-negative subjects (age: 26.1 ± 7.1 yrs, range 18–47 yrs versus 37.1 ± 9.4 yrs, range 20–51 yrs, $P=0.001$, and body fat %: $25.9 \pm 11.9\%$ versus $47.2 \pm 4.7\%$, $P<0.001$ in BAT+ vs BAT- resp.). There was no difference in fat free mass, an important contributor to resting metabolic rate (RMR), between both groups (68.6 ± 9.9 kg versus 64.2 ± 9.8 kg, $P=0.195$).

RMR under thermoneutral conditions did not differ between BAT-positive and BAT-negative subjects (44.6 ± 5.2 J/s versus 42.4 ± 3.3 J/s, $P=0.123$), but after cold exposure BAT-positive subjects showed a higher energy expenditure (51.5 ± 6.7 J/s versus 44.0 ± 5.1 J/s, $P=0.001$). Very interestingly, mean energy expenditure after cold exposure increased significantly within the BAT-positive group, but not within the BAT-negative group ($+15.5 \pm 8.9\%$ versus $+3.6 \pm 8.9\%$, $P=0.001$, Figure 2C).

In the BAT-negative group the core temperature was higher, both in thermoneutral conditions ($37.1 \pm 0.4^\circ\text{C}$ versus $36.7 \pm 0.4^\circ\text{C}$, $P=0.018$) and after cold exposure ($37.4 \pm 0.4^\circ\text{C}$ versus $36.9 \pm 0.3^\circ\text{C}$, $P=0.001$). However, mean skin temperatures did not show a significant difference in thermoneutral conditions ($31.9 \pm 0.6^\circ\text{C}$ versus $32.3 \pm 0.7^\circ\text{C}$, $P=0.076$), nor during cold exposure ($28.0 \pm 1.2^\circ\text{C}$ versus $28.6 \pm 1.2^\circ\text{C}$, $P=0.135$). The gradient between core temperature and mean skin temperature was larger in the BAT-negative group in thermoneutral conditions ($5.3 \pm 0.5^\circ\text{C}$ versus $4.5 \pm 0.8^\circ\text{C}$, $P=0.004$) and during cold exposure ($9.6 \pm 1.5^\circ\text{C}$ versus $8.3 \pm 1.4^\circ\text{C}$, $P=0.023$). The gradient between core temperature and mean skin temperature was larger in the BAT-negative group in thermoneutral conditions ($5.3 \pm 0.5^\circ\text{C}$ versus $4.5 \pm 0.8^\circ\text{C}$, $P=0.004$) and during cold exposure ($9.6 \pm 1.5^\circ\text{C}$ versus $8.3 \pm 1.4^\circ\text{C}$, $P=0.023$).

Table 1. Energy expenditure, body core temperature, mean skin temperature and core-skin temperature gradient in thermoneutral conditions (TN) and during mild cold (Cold) in morbidly obese subjects.

	TN	Min	Max	Cold	Min	Max	P value
Energy expenditure (J/s)	41.9±3.3	37.9	49.6	43.7±4.8	35.7	51.1	0.100
Body core temperature (°C)	37.2±0.4	36.5	37.8	37.5±0.3	36.9	37.9	0.048
Mean skin temperature (°C)	31.7±0.6	30.4	32.9	27.7±1.5	25.2	29.6	<0.001
Core-mean skin gradient (°C)	5.6±0.6	4.9	6.7	9.9±1.6	7.3	12.2	<0.001

Figure 1. PET-images of fifteen morbidly obese subjects. Three female subjects showed BAT activity in the supraclavicular area, highlighted by a red rectangle (A). Combined PET- and CT-imaging shows FDG-uptake in supraclavicular adipose tissue (this was the morbidly obese subject that showed the most BAT activity) (B).

Figure 2. Brown adipose tissue activity in relation to body mass index (A) and body fat percentage (B). The black dots indicate the current study group, the open dots indicate previously performed measurements. Cold-induced thermogenesis (CIT), denoted in percentages, is significantly increased in 26 BAT-positive (BAT+) compared to 13 BAT-negative (BAT-) subjects (C). *: $P < 0.05$

Very interestingly, mean energy expenditure after cold exposure increased significantly within the BAT-positive group, but not within the BAT-negative group ($+15.5 \pm 8.9\%$ versus $+3.6 \pm 8.9\%$, $P=0.001$, Figure 2C).

In the BAT-negative group the core temperature was higher, both in thermoneutral conditions ($37.1 \pm 0.4^\circ\text{C}$ versus $36.7 \pm 0.4^\circ\text{C}$, $P=0.018$) and after cold exposure ($37.4 \pm 0.4^\circ\text{C}$ versus $36.9 \pm 0.3^\circ\text{C}$, $P=0.001$). However, mean skin temperatures did not show a significant difference in thermoneutral conditions ($31.9 \pm 0.6^\circ\text{C}$ versus $32.3 \pm 0.7^\circ\text{C}$, $P=0.076$), nor during cold exposure ($28.0 \pm 1.2^\circ\text{C}$ versus $28.6 \pm 1.2^\circ\text{C}$, $P=0.135$). The gradient between core temperature and mean skin temperature was larger in the BAT-negative group in thermoneutral conditions ($5.3 \pm 0.5^\circ\text{C}$ versus $4.5 \pm 0.8^\circ\text{C}$, $P=0.004$) and during cold exposure ($9.6 \pm 1.5^\circ\text{C}$ versus $8.3 \pm 1.4^\circ\text{C}$, $P=0.023$).

Discussion

In this study active BAT was detected in only three out of fifteen morbidly obese patients, suggesting that morbid obesity is associated with low BAT activity. On the other hand, the data also demonstrate that even in this group BAT is present in some subjects and can be activated by cold exposure. Nevertheless, the combined results of morbidly obese subjects with those of previously reported lean and obese subjects, clearly show that body composition is highly related to BAT activity. This is in line with lower UCP-1-presence in the intraperitoneal adipose tissue of morbidly obese compared to lean subjects.¹⁸

A limitation in our analysis is fact that our previous study was composed of male subjects, whereas the morbidly obese subjects were mostly females. However, retrospective studies have actually shown higher BAT activity in women,^{19,20} which would only further strengthen the conclusion that BAT activity is reduced in obesity. The obese subjects generally show a high insulative response. Therefore, it is important to note that we used an individualized cold exposure protocol that achieves maximal non-shivering conditions. Despite this approach, the major part of obese and morbidly obese subjects did not show BAT activation. In the three BAT-positive morbidly obese subjects, two subjects showed pronounced CIT (BAT 23.61 kBq; CIT 18.76%, BAT 4.48 kBq; CIT 6.04%). Unfortunately, in the third subject (BAT 54.45 kBq) measurement of energy expenditure failed for technical reasons. However, CIT in the two BAT positive morbidly obese subjects was high compared to the average of the morbidly obese group (mean CIT $4.45 \pm 9.33\%$). This suggests a possible relation between active BAT after cold exposure and CIT.

Since approximately two-thirds of the total group of subjects showed cold-induced BAT activity whereas one-third did not, we further tested the hypothesis that BAT-positive subjects would have higher (cold-induced) thermogenesis. Interestingly, subjects that have active BAT also have a significant increase in energy expenditure during cold exposure, whereas cold exposure did not increase energy expenditure in BAT-negative subjects. Although age is significantly different in BAT-positive and BAT-negative subjects, the range between both groups (18-47 yrs versus 20-51 yrs) strongly overlaps, with active and inactive BAT spread over all age quartiles.

After we had previously shown that BAT activity was related to RMR, the additional analysis in this study shows that BAT activity may in fact be involved in adaptive thermogenesis. The role of FFM in this process can be excluded, since there is no significant difference in FFM between the analyzed groups. Therefore, the data suggest that - like in rodents - BAT is indeed involved in cold-induced thermogenesis in humans. This is in line with earlier findings in small study groups.¹⁰

The main native BAT depot is located supraclavicular.³ That depot shows FDG-uptake after cold exposure in all subjects with active BAT. Biopsies taken from this region show typical BAT cells, mostly surrounded by white fat cells.^{8,21} A pure BAT depot, as seen in rodents, is rare in adult man. Next to native BAT, recently 'brite' or 'beige' cells derived from white adipose tissue were reported.²²⁻²⁵ These cells stem from a different cell lineage than native BAT, but they possess the ability to generate heat by mitochondrial uncoupling similar to native BAT cells.

Most morbidly obese subjects in this study had no detectable BAT activity after cold exposure. From our study, it cannot be concluded that the lack of BAT is cause or consequence of severe obesity in our subjects. However, when the amount of native BAT is minimal or absent, this could lead to severe overweight. For example, mice that lack UCP-1, essential for mitochondrial uncoupling in BAT, become obese when studied under thermoneutral conditions.²⁶ With a congenital shortage of native BAT, brite cells could therefore be of great therapeutic interest.

On the other hand, in adult man a high amount of thermogenic potential can be present throughout the body, but may remain dormant when insulation makes a metabolic response unnecessary, as may be the case in the morbidly obese state. Indeed, prolonged cold exposure in rodents and rhesus monkeys increases the amount of (brite) BAT, supporting the idea of recruitable BAT.¹ It would therefore be interesting to test if BAT can be recruited in morbidly obese subjects. Next to prolonged cold exposure, other therapeutic targets could recruit BAT: a) weight reduction decreases the insulative capacity that blunts CIT, b) pharmacological intervention, such as sympathicomimetics has a high potential and c) implantation

of cultured brown adipocytes could all counteract severe overweight.^{12,27}

In conclusion, this study shows that in an extremely large range of body compositions, BAT activity is highly correlated with BMI and BF%. Active BAT was seen in three out of 15 morbidly obese subjects. BAT-positive subjects showed higher CIT, indicating that BAT is also in humans involved in adaptive thermogenesis.

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Chapter 4

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Increase in brown adipose tissue activity after weight loss in morbidly obese subjects

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Abstract

Background

Stimulation of thermogenesis in brown adipose tissue (BAT) is a potential target to treat obesity. We earlier demonstrated that BAT activity is relatively low in obese subjects. It is unknown whether BAT can be recruited in adult humans.

Methods

Ten morbidly obese subjects eligible for laparoscopic adjustable gastric banding (LAGB) surgery were studied before and one year after bariatric surgery. The main outcome measure was BAT activity, as determined after acute cold stimulation using ^{18}F -fluoro-deoxyglucose positron-emission-tomography and computed-tomography (FDG-PET-CT).

Results

Before surgery, only two out of 10 subjects showed active BAT. One year after surgery the number of subjects with active BAT was increased to five. After weight loss, BAT-positive subjects had significantly higher non-shivering thermogenesis (NST) compared to BAT-negative subjects ($P < 0.05$).

Conclusions

The results show that in humans BAT can be recruited in the regions where it was also reported in lean subjects before. These results for the first time show recruitment of BAT in humans and may open the door for BAT targeted treatments of obesity.

Introduction

Until very recently, the role of brown adipose tissue (BAT) in human physiology was only considered to be of importance during the first years of life. However, metabolic imaging techniques (^{18}F -Fluoro-Deoxy-Glucose-Positron-Emission-Tomography-and-Computed-Tomography (^{18}F -FDG-PET-CT)) showed functionally active BAT upon cold exposure in adult man.¹⁻⁴ We previously found that BAT activity is inversely related to body mass index (BMI) and body fat percentage (BF%).² This could suggest that a low level of BAT activity may predispose to obesity, when assumed that BAT activity is an intrinsic characteristic of an individual. Alternatively, low BAT activity may be an adaptive trait of obesity – for example due to improved insulation – which would predict that functional BAT could be re-recruited when body composition would change, for example after significant weight loss. Post-obese subjects indeed report an increased cold sensation during weight loss by bariatric surgery.^{5, personal observations} It is still unknown if BAT can be recruited in man. However, the principle of recruitable BAT has been observed after prolonged cold exposure in rodents and primates.⁶ In man, seasonal variation influences BAT activity within subjects.^{3,6} *In vitro* stimulation of white adipose tissue (WAT) with peroxisome proliferator-activated-receptor-gamma (PPAR-gamma) induced the development of thermogenic 'brite' adipocytes.⁷ Moreover, treating preadipocytes isolated from human subcutaneous adipose tissue with bone-morphogenic-protein-7 (BMP7) showed brown adipogenesis.⁸ Based on these observations, BAT recruitment in man seems feasible but has not yet been shown. Therefore, we investigated BAT activity in ten morbidly obese patients before and one year after laparoscopic adjustable gastric banding (LAGB) surgery as a model for caloric restriction.

The increase in energy expenditure that is observed upon mild cold exposure in absence of shivering in skeletal muscle is defined as non-shivering thermogenesis (NST).⁹ Active BAT in adult man is suggested to contribute to NST. Indeed, subjects with active BAT show significantly higher NST than subjects without BAT activity.^{10,11} Therefore we also studied NST in our subjects.

Methods

Approval was obtained from the local ethical commission. Written informed consent was received from two male and eight female morbidly obese subjects, with a mean BMI of 41.7 ± 4.4 kg/m and a body mass of 127.0 ± 17.7 kg. One female subject used levothyroxin for hypothyroidism and was euthyroid for several years. Diabetes mellitus and use of beta-blockers were strict exclusion criteria.

Study protocol

All measurements took place several weeks before surgery between 8 a.m. to 1 p.m. under overnight fasted conditions. In the morning, subjects put on light standardized clothing (socks 0.02 clo, shirt 0.09 clo, sweatpants 0.28 clo, underwear 0.04 clo, total clo factor 0.43 clo,¹²). Body composition (body fat%, fat mass (FM), fat free mass (FFM)) was determined by dual x-ray absorptiometry (DXA, Hologic, type Discovery A, USA). To determine body core temperature, a telemetric pill was orally ingested (CoreTemp, USA). Skin temperature was measured by applying iButtons at 14 ISO-defined skin sites.¹³ Next, subjects were placed in a specially adapted climate controlled tent (Colorado altitude training, USA). The temperature inside the tent was regulated by an air conditioner (accuracy of tent air temperature: $\pm 0.5^\circ\text{C}$). Energy expenditure was measured by indirect calorimetry using a ventilated hood system (Oxycon, Jaeger, Germany).

Personal cooling protocol and 18F-FDG-PET-CT

In all subjects we performed the personal cooling protocol described earlier.¹⁰ In short, subjects were placed in the climate tent. They rested semi-supine on a nephrodialysis chair, covered with a water-perfused mattress. To ensure maximal non-shivering thermogenesis, every subject was cooled until they reported shivering. Shivering was confirmed by electromyography of the m. pectoralis major. Upon the first signs of shivering the temperature was raised slightly (on average $1.8 \pm 0.6^\circ\text{C}$) to insure only non-shivering thermogenesis (NST). One hour of measuring under thermoneutral conditions (room temperature before weight loss; $22.8 \pm 0.6^\circ\text{C}$, after weight loss; $23.7 \pm 0.9^\circ\text{C}$, $P=0.079$, t_0 - t_{60}) was followed by two hours of individual mild cold (t_{60} - t_{180}). Laser Doppler Flowmetry (Perimed PF5000, Sweden) was used to determine if a subject was in its thermoneutral zone.¹⁴ At the end of the first hour of cooling (t_{120}), 10ml FDG was injected. After the second hour of cooling

Table 1A. Subject characteristics in ten patients (two male, eight female) with measurements before and after weight loss. BMI indicates Body Mass Index.

	Before	After	P-value
Age (yr)	40±9	41±9	
BMI (kg/m ²)	41.7±4.4	29.8±4.2	<0.001
Body mass (kg)	127.0±17.7	90.8±16.7	<0.001
Body fat (%)	48.6±5.2	34.8±7.9	<0.001
Fat mass (kg)	62.1±10.9	32.4±10.5	<0.001
Fat free mass (kg)	63.5±11.7	57.7±12.2	<0.001

Table 1B. Total brown adipose tissue (BAT) activity before and after weight loss. Group values (n=10) of BAT activity in kiloBecquerel (kBq), BAT volume (cm³), average BAT activity (kBq/cm³) and total and maximal Standard Uptake Value (SUV^{Total}, SUV^{Max}). In addition, values are shown for the group of five subjects that were indicated as BAT-positive (BAT+) based on their BAT-presence after weight loss.

	Before		After		P-value	
	Group	BAT+	Group	BAT+	Group	BAT+
BAT activity (kBq)	7.8±18.0	15.6±24.0	51.5±87.8	103.0±103.6	0.147	0.153
BAT volume (cm ³)	7.1±16.2	14.2±21.6	42.5±71.6	85.0±83.9	0.148	0.154
BAT activity (kBq/cm ³)	0.2±0.5	0.4±0.6	0.6±0.6	1.1±0.1	0.074	0.062
SUV ^{Total}	30.3±72.4	60.6±97.5	118.0±210.6	236.0±255.0	0.238	0.259
SUV ^{Max}	1.4±3.0	2.8±3.9	3.1±3.9	6.3±3.2	0.197	0.212

¹⁸F-FDG-PET-CT-imaging was performed to quantify metabolically active BAT. The scanning protocol, FDG activity (74 MBq) and data analysis were identical to our previous study.²

Follow-up

All subjects underwent LAGB without any perioperative or postoperative complications. One year after the LAGB procedure, all measurements were repeated.

Statistical analysis

Total BAT activity was expressed in kiloBecquerel (kBq) and Standard Uptake Value

Figure 1. Brown adipose tissue (BAT) activity before and after weight loss. (A) PET-images of five morbidly obese subjects before bariatric surgery (A) and after weight loss (B). (C) PET-image, CT-image and PET-CT-fusion-image of the subject that showed the largest increase in BAT activity before (C) and after weight loss (D). BAT activity was recruited in both supraclavicular and paravertebral areas, indicated by full and dashed arrows respectively. Full arrows indicate bilateral supraclavicular regions where BAT activity was observed after weight loss. Dashed arrows indicate bilateral paravertebral regions where BAT activity was observed after weight loss. (E) BAT activity in kiloBequerel (kBq) and Total Standard Uptake Value (SUVTotal, F) before and after weight loss for the five subjects depicted in (A) and (B). Abbreviations; CT: Computed Tomography, PET: Positron Emission Tomography. Baseline characteristics of subjects shown are from 10.

Figure 2. Relation between Brown Adipose Tissue (BAT) activity and Non-Shivering Thermogenesis (NST). (A) NST in BAT-positive (BATPos, $n=5$) and BAT15-negative (BATNeg, $n=5$) subjects before and after weight loss. (B) Non-shivering thermogenesis (NST) before and after weight loss. Full lines indicate subjects with BAT activity after weight loss (BAT-positive, $n=5$, one value not shown), dashed lines indicate subjects without BAT activity after weight loss (BAT-negative, $n=5$). * $P<0.05$

(SUV, as calculated by uptake (kBq/mL) / injected dose (kBq) / patient weight (g)). BAT activity of each region is determined by the average SUV uptake (SUV^{Mean}) times the volume of the region (cm^3), expressed as SUV^{Total} . Basal metabolic rate (BMR) correlated significantly to fat-free mass (FFM) before and after weight loss (respectively $r=0.912$, $P=0.001$ and $r=0.948$, $P<0.001$). Therefore, we corrected BMR for FFM using linear regression.¹⁵ Percentage of weight loss is expressed as $[(Beginning\ weight - Follow-up\ weight) / Beginning\ weight] \times 100$. Repeated measurements were compared using paired student's t-tests. Based on BAT presence after weight loss, subjects were categorized as BAT-positive or BAT-negative which was compared using unpaired student's t-tests.

Results and discussion

One year after LAGB mean body composition decreased significantly by 36.1 ± 8.3

kg with a mean percentage of weight loss of $28.65 \pm 6.23\%$ (Table 1A). BAT activity was measured after applying an individualised cooling protocol. Thus, maximal BAT activity is achieved by low environmental temperatures just above the temperature that causes shivering.¹⁰ Mean room temperatures during maximal NST were significantly higher after weight loss (WL) (before WL: 14.2°C (range 12-17), after WL: 15.7°C (range 12-21), $P=0.026$). Before surgery, we observed – albeit minimal – cold induced BAT activity in supraclavicular depots in two female subjects. Their mean BAT activity (39.0 kBq; range 23.6, 54.5 kBq) and volume (35.6 cm^3 ; range 22.5, 48.6 cm^3) were low (for group results see Table 1B, baseline characteristics based on ¹⁰). After WL, we observed BAT in five out of ten subjects, among whom were the two above mentioned female subjects (Figure 1). Of these five BAT-positive subjects (all female, the two male subjects did not show BAT activity), one subject increased her BAT activity (23.6 kBq to 228.3 kBq), three previously BAT-negative subjects were BAT-positive after WL and one female subject decreased her BAT activity (54.5 kBq to 13.8 kBq). In addition to supraclavicular BAT activity, the depots of active BAT also included paravertebral depots in three patients (Figure 1). There were no significant differences in body composition between subjects characterized as BAT-positive and BAT-negative after WL, neither before nor after WL.

The basal metabolic rate (BMR) showed a significant decrease after WL (95.3 ± 4.5 versus 88.8 ± 4.8 Watt, $P=0.028$). BAT-positive subjects (after WL) showed significantly higher NST compared to BAT-negative subjects (13.7 ± 7.7 versus $2.4 \pm 7.1\%$, $P=0.042$, Figure 2), in line with previous non-interventional studies on BAT and NST.^{10,11}

We show that the presence and activity of BAT in adult humans is dynamic and can increase after weight reduction. This important finding shows for the first time using functional tools (^{18}F -FDG-PET-CT and indirect calorimetry) that BAT recruitment in humans is possible. Reduced activity of BAT could potentially impair total daily energy expenditure and be of importance in the development of obesity. LAGB induces a caloric restriction that caused significant WL. It does not seem likely LAGB could be responsible for other mechanisms that could have increased BAT, since the digestive tract is not altered. For example, gut hormones do not change after LAGB.¹⁶

The increase in BAT presence after WL could be a direct effect of a decreased insulation due to loss of subcutaneous adipose tissue. WL significantly changed the thermal distribution, as demonstrated by the observed skin and core temperatures

Table 2. Core temperature (TCore), mean skin temperature (TSkin), proximal skin temperature (TProx) and distal skin temperature (TDist) during baseline measurements (Base) and cold exposure (Cold) before and after weight loss. The gradient between core and mean skin temperature is denoted as TCore-TSkin. Δ TCore and Δ TSkin denote differences between baseline measurements and cold exposure. Subjects are indicated as BAT-positive/BAT-negative based on their BAT-presence after weight loss. P-values shown are paired samples student's t-tests for group, five BAT-positive (BAT+) and five BAT-negative (BAT-) subjects before and after weight loss. *P<0.05, **P<0.001

		Before			After			P-value		
		Group	BAT+	BAT-	Group	BAT+	BAT-	Group	BAT+	BAT-
TCore (°C)	Base	37.2±0.3	37.3±0.3	37.1±0.4	37.0±0.3	37.1±0.3	36.9±0.3	0.176	0.532	0.103
	Cold	37.4±0.4	37.5±0.3	37.3±0.4	37.1±0.3	37.1±0.3	37.0±0.4	0.010*	0.142	<0.001**
TSkin (°C)	Base	31.6±0.6	31.6±0.8	31.6±0.5	33.3±0.5	33.1±0.4	33.4±0.5	<0.001**	0.013*	<0.001**
	Cold	27.7±1.5	27.6±2.0	27.9±1.0	29.6±1.5	30.0±1.7	29.2±1.3	<0.001**	<0.001**	0.044*
TCore-TSkin	Base	5.6±0.6	5.6±0.7	5.5±0.6	3.8±0.7	4.0±0.7	3.6±0.7	<0.001**	0.011*	<0.001**
	Cold	9.7±1.7	10.0±2.1	9.5±1.4	7.5±1.7	7.1±1.9	7.8±1.6	<0.001**	0.001*	0.026*
Δ TCore		0.2±0.2	0.3±0.1	0.1±0.2	0.01±0.1	-0.01±0.1	0.05±0.1	0.008*	0.008*	0.326
Δ TSkin		-3.9±1.3	-4.1±1.7	-3.8±0.9	-3.7±1.5	-3.1±1.6	-4.2±1.2	0.491	0.034*	0.401
TProx (°C)	Base	31.6±1.2	32.1±1.5	31.0±0.5	34.4±0.5	34.3±0.6	34.5±0.4	0.001**	0.011*	<0.001**
	Cold	28.3±2.4	28.8±3.4	27.8±1.1	31.0±2.0	31.4±2.6	30.5±1.4	<0.001**	0.006*	0.027*
TDist (°C)	Base	31.8±1.1	31.3±0.8	32.2±1.2	30.4±2.2	29.5±1.5	31.2±2.6	0.085	0.042*	0.517
	Cold	25.2±2.2	24.6±2.7	25.9±1.6	24.6±2.3	24.3±2.6	24.9±2.1	0.187	0.600	0.265

(Table 2). Obese subjects show a low gradient between proximal and distal skin temperature during cold exposure, indicating a low level of vasoconstriction.¹⁵ This gradient increased after WL (baseline; $0.2 \pm 1.9^\circ\text{C}$ before WL and $-4.0 \pm 2.4^\circ\text{C}$ after WL, $P=0.001$, cold exposure; $-3.1 \pm 1.9^\circ\text{C}$ before WL and $-6.4 \pm 2.2^\circ\text{C}$ after WL, $P=0.001$), suggesting increased skin vasoconstriction. Insulation of the body's core, as measured by the gradient between core and mean skin temperature, decreased significantly after WL (before WL; $5.6 \pm 0.6^\circ\text{C}$, after WL; $3.8 \pm 0.7^\circ\text{C}$, $P<0.001$). This indicates the insulation of the body's core decreased, despite increased vasoconstriction of the skin. Compared to BAT-negative subjects, BAT-positive subjects showed a significantly smaller increase in core temperature

(ΔT_{Core}) during cold exposure, accompanied by a smaller decrease in mean skin temperature (ΔT_{Skin}) (Table 2), suggesting less vasoconstriction.¹⁵ Hence, increased thermogenesis in BAT could be responsible for this difference.

It should be noted that the BAT activity we observed in this study is low in comparison to the levels observed in lean young men (51.5 ± 87.8 kBq versus 428 ± 394 kBq, $P=0.008$, ²). BAT activity is suggested to be lower in older subjects,³ which could possibly affect the energy balance. Therefore, further studies are needed to investigate the impact of BAT recruitment at this level for the long-term treatment of obesity. Nevertheless, this study provides the first evidence that BAT can be recruited in adults.

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Chapter 5

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**Increased skeletal muscle mitochondrial
function in morbidly obese subjects one year
after bariatric surgery**

The contents of this chapter are submitted.

Abstract

Background

Obesity and type 2 diabetes are associated with impaired skeletal muscle mitochondrial metabolism. As an intrinsic characteristic of an individual, skeletal muscle mitochondrial dysfunction could be a risk factor for weight gain and obesity-associated comorbidities as type 2 diabetes. On the other hand, impaired skeletal muscle metabolism could be a consequence of obesity. We hypothesize that marked weight loss after bariatric surgery increases skeletal muscle mitochondrial function in formerly morbidly obese subjects.

Methods

Skeletal muscle mitochondrial function as assessed by high-resolution respirometry was determined in eight morbidly obese subjects (BMI; 41.3 ± 4.7 kg/m², Body Fat; $48.3 \pm 5.2\%$) before and one year after bariatric surgery (mean weight loss: 35.0 ± 8.6 kg).

Results

ADP-stimulated respiration significantly increased after weight loss upon mitochondrial complex I-linked substrates (for malate+glutamate; 4.23 ± 1.38 versus 6.79 ± 2.03 O₂-flux, $P=0.029$; for malate+octanoyl-carnitine+glutamate (MOG); 4.19 ± 2.12 versus 8.93 ± 4.16 O₂-flux, $P=0.028$) as well as upon parallel electron input into both complex I and II (for malate+octanoyl-carnitine+glutamate+succinate (MOGS); 10.02 ± 3.23 versus 13.97 ± 6.63 O₂-flux, $P=0.031$).

Conclusions

We conclude that low skeletal muscle mitochondrial function is a consequence of obesity that significantly improves after marked weight loss induced by bariatric surgery. The pronounced amount of weight loss associated with bariatric surgery appears to be crucial since previous studies with limited weight loss remained without effect on skeletal muscle mitochondrial function. Future molecular studies should investigate the processes that are related to the observed change in skeletal muscle mitochondrial function upon bariatric surgery.

Introduction

Obesity is one of the Western world's primary health care issues and the increasing incidence of obesity is accompanied by an equal rise in comorbidities as type 2 diabetes, dyslipidemia and hepatosteatosi.¹ The excess amount of adipose tissue in obesity is associated with a dysfunction in several tissues; hepatosteatosi, which causes impaired liver function and increased lipid deposition in skeletal muscle is suggested to induce insulin resistance.^{1,2} In skeletal muscle from obese and obese type 2 diabetic subjects, a decreased mitochondrial function compared to lean and nondiabetic subjects has been reported. This could exacerbate the negative effect of IMCL accumulation on insulin sensitivity.³⁻⁷ However, although diet-induced weight loss decreased intramyocellular lipid content and has been shown to improve skeletal muscle insulin sensitivity in nondiabetic overweight subjects,^{8,9} skeletal muscle mitochondrial function (as determined by NADH-oxidase enzyme levels) *per se* did not change.^{8,10} This may suggest that reduced mitochondrial function in obesity is an intrinsic characteristic of obese subjects. However, it should be noted that weight loss in the above-mentioned studies was low (9-18 kg)⁸⁻¹⁰ in comparison to bariatric surgery (37-42 kg)¹¹ and it cannot be excluded that further weight reduction does improve skeletal muscle mitochondrial metabolism. Indeed, it has been shown before that proteins associated with mitochondrial biogenesis in skeletal muscle were enhanced and related to improved insulin sensitivity after bariatric surgery, suggesting that skeletal muscle mitochondrial function may be suppressed by excessive body weight.^{12,13} However, to our knowledge no study has evaluated the effect of bariatric surgery on skeletal muscle mitochondrial function *per se* and therefore these findings need further confirmation. To test our hypothesis that skeletal muscle mitochondrial function improves after bariatric surgery we investigated *ex-vivo* skeletal muscle mitochondrial function in morbidly obese subjects before and one year after laparoscopic adjustable gastric banding (LAGB) surgery.

Methods

Study protocol

Approval was obtained from the Maastricht University Medical Centre institutional review board. Written informed consent was received from two male and six female morbidly obese subjects who were awaiting laparoscopic adjustable gastric banding

(LAGB), with a mean BMI of 41.3 ± 4.7 kg/m² (Table 1). One female subject used levothyroxine for hypothyroidism and was euthyroid for several years. Diabetes mellitus was an exclusion criterion.

Body composition

Body composition (Fat Free Mass, Fat Mass) was determined under fasted conditions by dual x-ray absorptiometry (DXA, type Discovery A, Hologic, Bedford, USA).

Muscle biopsies

Several weeks before surgery, a muscle biopsy according to Bergström et al.¹⁴ was taken under fasted conditions at ~8:30 in the morning. Part of the muscle was directly used for the fresh preparation of permeabilized muscle fibers as described.³ The remaining part of the muscle was directly frozen in liquid nitrogen and stored at -80°C until further analysis.

To determine skeletal muscle mitochondrial capacity, high-resolution respirometry was performed in permeabilized muscle fibers at 37°C in a two-chamber-Oxygraph (OROBOROS® Instruments, Innsbruck, Austria), essentially according to Hoeks et al.¹⁵ Coupled (state 3) respiration, initiated by addition of 2mM ADP, was measured for complex I-linked substrate combination malate+glutamate, both in the presence and absence of the lipid substrate octanoyl-carnitine. Coupled respiration was then maximized with convergent electron input through complex I and complex II, by adding saturating concentrations of succinate (10mM). Subsequently, mitochondrial respiration uncoupled from ATP synthesis (state 4) was determined via addition of the ATP-synthase inhibitor oligomycin. In addition, the chemical uncoupler FCCP (carbonyl-cyanide-4-(trifluoromethoxy)-phenylhydrazone) was used to maximize oxygen flux to assess maximal mitochondrial capacity (state u respiration).

Normalisation for mitochondrial DNA content

To determine mitochondrial density, the mitochondrial DNA (mtDNA) copy numbers were determined according to Phielix et al.,³ and all muscle biopsy samples were run in the same analysis. For comparison, oxygen fluxes per wet-weight muscle mass were normalized for mtDNA. The mitochondrial DNA copy numbers did not differ significantly before and after weight loss (mtDNA copy number; 960.6 ± 425.1 before, 868.8 ± 386.0 after weight loss, $P=0.371$). All results showed similar trends

and significance when oxygen fluxes were not corrected for mtDNA (results not shown). Oxygen fluxes (O_2 flux) shown are expressed as picomoles $O_2 \times \text{seconds}^{-1} \times \text{milligrams of tissue}^{-1} \times \text{mtDNA} \times 10^6$.

Plasma values

Plasma TSH and FT4 were measured using an electrochemiluminescence immunoassay (Roche, Basel, Switzerland) and a fluoroimmunoassay (PerkinElmer, Massachusetts, USA). Plasma insulin, glucose, FFA and the HOMA-IR were calculated as described.¹⁶

Follow-up

All eight subjects underwent LAGB without any perioperative or postoperative complications. One year after LAGB a second muscle biopsy was taken.

Statistical analysis

Reported data were expressed as means $\pm$ SD. Statistical analyses were performed with PASW Statistics 18.0 for Mac OS X 10.6.4. Repeated measurements before and one year after bariatric surgery were compared using paired student's t-tests. A P-value below 0.05 was considered to be significant.

Results and discussion

Body composition was significantly altered one year after surgery (Table 1) with a mean weight loss of $27.9 \pm 6.6\%$. After a mean follow-up of 18 months, there was no difference in body composition compared to the one-year time point (body mass; 91.7 ± 18.8 versus 91.5 ± 18.3 kg, BMI; 29.8 ± 4.8 versus 29.7 ± 4.7 kg/m², $P=0.844$), which indicates patients were weight stable at the moment of the second muscle biopsy.

ADP-stimulated (state 3) respiration increased after weight loss upon complex I-linked substrates (for malate+glutamate; 4.23 ± 1.38 versus 6.79 ± 2.03 O_2 flux, $P=0.029$; for malate+octanoyl-carnitine+glutamate (MOG); 4.19 ± 2.12 versus 8.93 ± 4.16 O_2 flux, $P=0.028$) as well as upon parallel electron input into both complex I and complex II (for malate+octanoyl-carnitine+glutamate+succinate (MOGS); 10.02 ± 3.23 versus 13.97 ± 6.63 O_2 flux, $P=0.031$), although did this not reach statistical significance for malate+glutamate+succinate (MGS; 9.50 ± 2.43 versus 12.31 ± 5.50 O_2 flux, $P=0.086$, Figure 1). Leak respiration upon the ATP-synthase inhibitor oligomycin,

i.e. respiration not related to ATP synthesis (state 4 respiration), was determined as a marker for mitochondrial uncoupling, but remained unaffected by weight loss (6.13 ± 2.66 versus 5.42 ± 2.37 O₂ flux, $P=0.093$, Figure 1). The relative contribution of state 4 to state 3 respiration (uncoupling ratio; UCR) decreased significantly after weight loss (0.63 ± 0.19 versus 0.45 ± 0.07 , $P=0.049$, Figure 1). Maximally uncoupled mitochondrial respiration, as analysed after addition of FCCP in the presence of octanoyl-carnitine, did not increase significantly after weight loss (14.9 ± 4.45 versus 18.04 ± 8.18 O₂ flux, $P=0.212$, Figure 1).

In this study we investigated the effect of pronounced weight loss induced by bariatric surgery on skeletal muscle mitochondrial function in morbidly obese subjects. ADP-stimulated (state 3) respiration increased significantly one year after bariatric surgery. Our data suggest that low skeletal muscle mitochondrial function in morbidly obese subjects is reversible by bariatric surgery-induced weight loss.

Skeletal muscle fat oxidation is negatively related to BMI,¹⁷ resulting in an increased muscle fat accumulation in obesity.^{9,18} This impaired lipid oxidation is associated with skeletal muscle insulin resistance and thus suggests a pathophysiological basis for obesity-related type 2 diabetes.⁹ In line with this notion, skeletal muscle mitochondria from obese type 2 diabetics displayed a reduced

activity of the mitochondrial oxidative enzyme NADH-reductase, suggesting an impairment of mitochondrial function,⁵ which was confirmed by *in vivo* measurements.¹⁹ Furthermore, a detailed *ex vivo* analysis of mitochondrial function in permeabilized skeletal muscle fibres showed that the observed *in vivo* mitochondrial dysfunction in overweight type 2 diabetes (mean BMI; 28.9 kg/m^2) was associated with a decreased intrinsic mitochondrial capacity, i.e. ADP-stimulated (state 3) mitochondrial respiration corrected for mitochondrial density.³ Skeletal muscle mitochondrial state 3 respiration was also shown to be reduced in non-diabetic morbidly obese subjects eligible for bariatric surgery (mean BMI; 40 kg/m^2) compared to lean controls.⁶ To observe whether obesity is causative for a decreased mitochondrial function, several reports have studied the effect of weight loss on skeletal muscle mitochondrial function, in both type 2 diabetic and nondiabetics. A diet-induced weight loss of 10.6 kg did not induce changes in mitochondrial NADH-reductase activity in nondiabetic obese subjects (mean BMI before weight loss; 33.4 kg/m^2)⁸ and one report even observed a decrease in mitochondrial cytochrome c oxidase activity after weight loss (12-17 kg weight

Table 1. Subject characteristics for two male and six female morbidly obese subjects before and after weight loss. * $P < 0.05$, ** $P < 0.001$. BMI denotes Body Mass Index.

Characteristics	Before	After	P-value
Age (years)	40 $\pm$ 9	41 $\pm$ 9	<0.001**
Body mass (kg)	126.71 $\pm$ 19.98	91.71 $\pm$ 18.81	<0.001**
BMI (kg/m ²)	41.28 $\pm$ 4.75	29.82 $\pm$ 4.78	<0.001**
Body Fat % (BF%)	48.30 $\pm$ 5.21	34.11 $\pm$ 8.47	<0.001**
Fat Mass (kg)	61.45 $\pm$ 11.47	32.15 $\pm$ 11.79	<0.001**
Fat Free Mass (kg)	63.79 $\pm$ 13.02	58.98 $\pm$ 13.18	<0.001**

loss, initial BMI; 33–34 kg/m²).¹⁰ In contrast, after a more pronounced diet-induced weight loss in nondiabetics (13–32 kg weight loss, initial BMI; 33.8 kg/m²), an improved oxidative enzyme (succinate dehydrogenase) activity was observed.²⁰

Bariatric surgery results in pronounced weight loss and after Roux-en-Y-gastric-bypass and bilio-pancreatic-diversion surgery (42–63 kg weight loss, mean initial BMI; 45.9–53.5 kg/m²) proteins associated with mitochondrial biogenesis (PGC1 α and MFN2) were enhanced in skeletal muscle and related to improved insulin sensitivity as assessed by euglycemic-hyperinsulinaemic clamp.^{12,13} Finally, in subjects with a ~50 kg weight loss after Roux-en-Y-gastric-bypass, skeletal muscle fatty acid oxidation (as assessed *ex vivo* with ¹⁴C-palmitate) did not change and was decreased compared to lean controls.²¹ However, the postoperative BMI was relatively high (36.5 kg/m²) compared to the current study (30 kg/m²).

In contrast to the abovementioned weight loss studies using biochemical assessments of mitochondrial enzyme activities to assess skeletal muscle mitochondrial function we here performed a detailed characterisation of mitochondrial function via *ex vivo* mitochondrial respiration in permeabilized muscle fibers. In this study we show that ADP-stimulated state 3 respiration increased after a mean 35-kg weight loss. The mitochondrial density (as assessed by mtDNA copy numbers) did not change after weight loss and therefore the observed improvement in mitochondrial respiration is an intrinsic mitochondrial characteristic. The improvement in muscle mitochondrial metabolism suggests that the observed weight loss could ‘restore’ obesity-induced impairments in skeletal muscle mitochondrial function. Further studies should elucidate the molecular and clinical changes associated with the increase skeletal muscle mitochondrial function. All included morbidly obese subjects were non-diabetic and plasma values (Table 2) for plasma insulin showed a nearly significant decrease ($P = 0.064$). However, the homeostasis model for insulin resistance (HOMA-IR) did not change after weight loss ($P = 0.324$). Although fasting insulin levels tended to be lower after weight loss (mean reduction; 28.6%)

Figure 1. Skeletal muscle fibre respirometry before (white bars) and after weight loss (black bars). A: Oxygen consumption in the presence of endogenous substrates (state 2 respiration) was similar before and after weight loss ($P=0.310$). B: ADP stimulated state 3 respiration upon a lipid substrate for complex I (malate, octanoyl-carnitine and glutamate (MOG) and complex I&II substrates (malate, octanoyl-carnitine, glutamate and succinate (MOGS)). C: State 3 respiration upon complex I substrates (malate, glutamate (MG)) and complex I&II substrates (malate, glutamate and succinate (MGS)). D: Leak (state 4) respiration obtained by addition of the ATP synthase inhibitor oligomycin. E: The relative contribution of state 4 to state 3 respiration (uncoupling ratio; UCR). F: Maximal respiration upon FCCP (state uncoupled). O_2 flux shown: picomoles O_2 consumption $\times$ seconds $^{-1} \times$ milligrams of tissue $^{-1} \times$ mtDNA $\times 106$. Values shown are mean $\pm$ S.E.M., $n=8$. * $P<0.05$

Table 2. Plasma values before and after weight loss. Values shown for two male and six female morbidly obese subjects before and after weight loss. * $P < 0.05$, ** $P < 0.001$. TSH denotes Thyroid-Stimulating Hormone, FT4; Free Thyroxine, HOMA-IR; Homeostasis Model Assessment for Insulin Resistance, FFA; Free Fatty Acids.

	Before	After	P-value
TSH (mU/mL)	2.03 ± 0.7	1.85 ± 0.4	0.341
FT4 (pmol/L)	13.53 ± 1.94	14.85 ± 1.43	0.009*
Glucose (mM)	4.86 ± 0.38	5.22 ± 1.04	0.199
Insulin (μ U/mL)	25.44 ± 8.52	16.64 ± 5.16	0.064
HOMA-IR	4.90 ± 2.87	3.80 ± 1.13	0.324
FFA (μ M)	588.34 ± 234.34	567.0 ± 283.43	0.750

further studies in morbidly obese diabetic populations are necessary to determine the effect of weight loss on skeletal muscle mitochondrial function and its relation to obesity-induced insulin resistance.

In summary, this study shows an increase in *ex vivo* skeletal muscle mitochondrial function in morbidly obese subjects one year after LAGB-induced weight loss. The level of weight loss in this study was higher than previous reports that did not observe differences in mitochondrial function after weight loss. Possibly, the very pronounced weight loss after bariatric surgery is necessary to recover mitochondrial function in morbid obesity.

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Chapter 6

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**Increased oxygen consumption in human
adipose tissue from the “brown adipose
tissue” region**

The contents of this chapter are submitted.

Abstract

Background

Since the discovery of functional 'brown adipose tissue (BAT)' in adult man, there has been a renewed interest in the physiology of human BAT. Imaging studies using FDG-PET-CT have shown increased glucose uptake in adipose tissue regions assumed to be BAT in humans. However, so far this adipose tissue depot has not been characterized with respect to cellular and mitochondrial capacity. We here determine basal cellular oxygen consumption in human adipose tissue (AT) obtained from biopsies taken from the neck region, known to be FDG-PET-CT-positive in previous studies.

Methods

Mitochondrial respirometry in AT from the human BAT region was compared to the cellular respiration in subcutaneous white adipose tissue (WAT) taken from the neck of the same subject.

Results

Adipose tissue from the human BAT region displayed increased cellular mitochondrial respiration, on average three times higher compared to subcutaneous WAT. The difference in basal respiration was much more pronounced than the previous reported 10-20% higher mitochondrial respiration in visceral adipose tissue, and illustrates that adipose tissue from human BAT region is a distinct adipose tissue depot with high mitochondrial capacity, similar to studies performed in rodents. Presence of uncoupling protein 1 (UCP-1) was confirmed by immunostaining as performed before.

Conclusions

The results of this study suggest that human adipose tissue, taken from the region that was shown to be BAT-positive, can indeed be distinguished from subcutaneous WAT by the presence of UCP-1 and a significantly higher basal mitochondrial activity. Future studies should be directed to the further metabolic and bioenergetic characterization of this adipose tissue depot.

Introduction

Since the discovery of functional brown adipose tissue (BAT) in adult man,¹⁻³ there has been a vast increase in the scientific interest in the physiology of human BAT. PET-CT-studies (Positron-Emission-Tomography-and-Computed-Tomography) using the glucose tracer ¹⁸F-FDG (Fluorine-18-Fluoro-Deoxy-Glucose) have shown that cold-induced BAT activity is inversely related to body fat percentage and subjects with glucose uptake in BAT have higher thermogenesis.^{2,4,5} In addition, other studies have now indirectly measured *in vivo* BAT activity by means of different metabolic tracers.^{6,7} Collectively, these studies show increased metabolic activity in an anatomical region presumed to contain BAT in humans, located in the adipose tissue depot dorsal from the neck muscles. To date, adipose tissue in this region has not been characterized in great detail *ex vivo*, although some, mainly smaller studies have shown the presence of UCP1 and thermogenic preadipocytes in this adipose tissue.^{3,6,8} Very recently, we showed that adipocytes from this region can in fact be classified as beige fat cells; adipocytes genetically distinct from white and brown adipose tissue, but with the capacity to induce high levels of UCP1 expression as in BAT.⁹

Here we had the unique opportunity to further characterize perioperative neck adipose tissue (AT) biopsies taken from the area that is now recognized as human "BAT" based on FDG-PET-CT positive glucose uptake and determined cellular respirometry in comparison to subcutaneous WAT from the neck from the same subject. We aimed to investigate if the AT from the BAT region can indeed be distinguished from normal WAT based on mitochondrial capacity. It is important to mention that mitochondrial respiration measurements in adipose tissue are not trivial, and only a few reports describe respiration measurements in human WAT.^{10,11} One of these studies shows that visceral adipose tissue is characterized by a ~10-20% higher mitochondrial respiration when compared to subcutaneous adipose tissue.¹⁰ In this study we measured cellular oxygen consumption in small adipose tissue samples and determined basal mitochondrial respiration and compared it to subcutaneous WAT taken from the same region.

Methods

Approval was obtained from the medical ethical committee of the Maastricht University Medical Centre. Thirteen patients indicated for thyroid gland surgery (indications provided in Table 1) gave informed consent for perioperative AT from the BAT region and WAT biopsies. All subjects were euthyroid to rule out influence of thyroid hormone (Table 2).

Biopsy procedure

Previously successful UCP-1-positive adipose tissue biopsies were taken in the operative area involved in this type of surgery,² and were here repeated. During surgery, both the surgeon (N.B.) and the researcher (G.V.) visually inspected the exposed operative area for presence of small 'islands' (± 1 -2 cm) of AT from the BAT region along the sternocleidomastoid muscle and more cranially along the thyroid gland. These adipose tissue depots are typically light brown/beige and have a firm consistency compared to WAT. When AT from the BAT region was recognized a biopsy of 0.5cm³ was taken together with 0.5cm³ of WAT. AT biopsies showed both univacuolar and multivacuolar adipocytes, characterized as white adipocytes and brown/beige adipocytes, respectively (Figure 1).

Tissue processing

The tissue biopsies were directly placed in a tube with Phosphate-Buffer-Solution (PBS). Part of the AT from the BAT region and WAT ($\pm 10\%$ of the total biopsy) were snap-frozen in isopentane and stored at -80°C until further analysis. The remaining AT from the BAT region and WAT was placed in separate 2mL tubes with 10mM CaCl₂ solution and Collagenase solution (PBS, 1% Bovine-Serum-Albumin and 0.1% Collagenase-type-1) and then minced carefully. The tubes with minced AT from the BAT region and WAT were placed in a 37°C shaking water bath (75 RPM) for 30 minutes and then centrifuged for 5 minutes at 1200 RPM at room temperature. Then the tubes with tissue were loosely shaken manually to disrupt the pellet and centrifuged again. Subsequently, the separated top layer of both the AT from the BAT region and WAT was pipetted off in two separate tubes with 2.3mL MiRO5 respiration buffer (OROBOROS®, Innsbruck, Austria). The interphase was removed and the pellet was also added to the respiration buffer.

Table 1. Indications and surgical therapy per included subject. MENIIA-syndrome indicates the Multiple Endocrine Neoplasia syndrome type IIA, as was confirmed by genetic screening in subject families; a preventive, total thyroidectomy is indicated due to the high lifetime risk of developing thyroid gland cancer, mostly of the medullary type. Hyperparathyroidism indicates supraphysiological activity of the parathyroid gland, which is located adjacent to the thyroid gland itself. Parathyroidectomy indicates resection of the parathyroid gland, hemithyroidectomy indicates resection of the left/right half of the thyroid gland and total thyroidectomy indicates a total resection of the thyroid gland as a whole.

Subject	Indication	Surgery
1	hyperparathyroidism	parathyroidectomy
2	suspected thyroid gland mass	hemithyroidectomy
3	suspected thyroid gland mass	hemithyroidectomy
4	cyst left thyroid gland	hemithyroidectomy
5	MENIIA-syndrome	total thyroidectomy
6	suspected thyroid gland mass	hemithyroidectomy
7	suspected thyroid gland mass	hemithyroidectomy
8	MENIIA-syndrome	total thyroidectomy
9	MENIIA-syndrome	total thyroidectomy
10	struma	hemithyroidectomy
11	hyperparathyroidism	parathyroidectomy
12	struma	hemithyroidectomy
13	suspected thyroid gland mass	hemithyroidectomy

Table 2. Subject characteristics for eight male and five female subjects indicated for thyroid gland surgery. BMI denotes Body Mass Index; TSH denotes Thyroid-Stimulating Hormone (normal range 0.4-3.5 mU/L), FT4 denotes plasma free thyroxine (normal range 4.0-18.0 pmol/L).

Parameter	Mean $\pm$ S.D.
Male / Female	8 / 5
Age (years)	43 $\pm$ 19
Body mass (kg)	75.3 $\pm$ 14.9
BMI (kg/m ²)	25.7 $\pm$ 4.9
TSH (mU/L)	2.1 $\pm$ 1.4

The respiration buffer containing the tissue was poured in two separate respiration chambers. Since the chamber volume is ± 2 mL, the remaining 0.3 mL could be stored for determination of DNA and protein contents.

Respiration measurements

To determine oxygen consumption of AT from the BAT region and WAT, high-resolution respirometry was performed at 37°C by polarographic oxygen sensors in a two-chamber Oxygraph (OROBOROS® Instruments, Innsbruck, Austria). Basal respiration was measured once the tissue preparation was added

to the respiration chamber without addition of exogenous substrates for about 5-7 minutes. Once stable, mitochondrial respiration uncoupled from ATP synthesis (state 4) was determined via addition of the ATP synthase inhibitor oligomycin (1.0 μ L, 2mg/mL) for about 4-5 minutes. In addition, saturating concentrations (0.5 μ L, 10mM) of the chemical uncoupler FCCP (carbonyl-cyanide 4-(trifluoromethoxy)-phenylhydrazone) were used to maximize oxygen flux, which allows for assessment of maximal mitochondrial capacity (state u respiration). Baseline respiration, state 4 and state u were measured according to Hoeks et al.¹² After completion of respiration measurements, chamber-solutions were pipetted off and snap-frozen in liquid nitrogen and stored at -80°C until further analysis. To account for any differences in the amount of collagenase-liberated cells added to the respiration chambers, the results were normalized to the DNA content measured spectrofluorometrically in the tissue preparations collected after measurement.¹³ All raw oxygen fluxes (expressed as picomoles O_2 consumption $\times$ seconds⁻¹) for both WAT and AT from the BAT region were normalized for the DNA content measured in the cell suspensions derived from the oxygraph chambers (mg/mL). The DNA contents of these cell suspensions were not different for WAT and AT from BAT region (0.037 ± 0.014 mg/mL vs. 0.043 ± 0.021 mg/mL, $P=0.108$). Corrected oxygen fluxes are expressed as picomoles O_2 -consumption $\times$ seconds⁻¹ $\times$ (mg/mL)⁻¹. All results showed similar trends and significance when oxygen fluxes were uncorrected, or when corrected for cellular protein content (results not shown). For comparison of the relative decrease in oxygen consumption upon the addition of oligomycin, the oligomycin-insensitive leak-state-4 respiration was divided by baseline respiration. This uncoupling ratio (UCR) was not different in AT from the BAT region compared to WAT (AT from the BAT region; 0.83 ± 0.17 , WAT; 0.79 ± 0.20 , $P=0.501$). The relative contribution of baseline respiration and state 4 respiration to the maximally uncoupled respiration, as calculated by baseline respiration or state 4 respiration divided by state u, was higher in AT from the BAT region but this did not reach

Figure 1. Histology of adipose tissue taken from the neck region during thyroid gland surgery. A: 18F-FDG-PET-CT image of a lean young male subject after mild cold exposure. Black coloured areas indicate 18F-FDG glucose uptake. The red box indicates the anatomical adipose tissue area bordered by the medial edge of the sternocleidomastoid muscle, the lower edge of the thyroid gland and the jugulum. B: Hematoxylin-eosin (HE) section (5µm) of 'beige' coloured adipose tissue showing both univacuolar and multivacuolar adipocytes, characterized as white adipose tissue (WAT) and brown adipose tissue (BAT), respectively. C: Immunofluorescence-staining for uncoupling-protein-1 (UCP-1) was positive in the multivacuolar adipocytes, confirming the presence of brown adipocytes. BAT denotes brown adipose tissue and WAT denotes white adipose tissue. Shown histology derives from a perioperative biopsy taken during a preventive total thyroidectomy for MEN1A-syndrome in a 19-year old male subject with a BMI of 21.9 kg/m².

statistical significance (baseline / state u; AT from the BAT region; 0.83 ± 0.21 , WAT; 0.70 ± 0.33 , $P=0.292$, state 4 / state u; AT from the BAT region; 0.68 ± 0.17 , WAT; 0.54 ± 0.26 , $P=0.122$).

Statistical analysis

Reported data was expressed as means $\pm$ SD. Statistical analyses were performed with PASW Statistics 18.0 for Mac OS X 10.6.4. WAT and BAT respiration measurements within subjects were compared using paired student's t-tests. Comparisons between for age groups were made using unpaired student's t-tests. A P-value below 0.05 was considered significant.

Results

Presence of UCP-1 in adipose tissue biopsies

In the adipose tissue biopsies from the BAT region additional immunofluorescence staining for the brown/beige adipose tissue-specific uncoupling protein 1 (UCP-1, Figure 1) was positive in the multivacuolar adipocytes, confirming the presence of brown/beige adipocytes in this adipose tissue depot. UCP-1 immunofluorescence for the WAT biopsies was completely negative, confirming no UCP-1 was present in the subcutaneous WAT biopsies.

Adipose tissue respirometry in AT from the BAT region compared to WAT

Figure 2 shows the respiration levels of subcutaneous (WAT) and AT from the BAT region after normalization for DNA. Baseline respiration without addition of substrates was more than three times higher in AT from the BAT region compared to WAT (430.9 ± 410.3 vs. 132.6 ± 90.8 O₂ flux, $P=0.017$). After addition of oligomycin to achieve leak state 4 respiration, oxygen fluxes for both AT from the BAT region and WAT significantly decreased (baseline vs. state 4; AT from the BAT region; 430.9 ± 410.3 vs. 364.1 ± 367.7 O₂ flux, $P=0.002$, for WAT; 132.6 ± 90.8 vs. 104.6 ± 78.6 O₂ flux, $P<0.001$). Upon titration of FCCP oxygen fluxes increased to maximally uncoupled respiration (increase after FCCP; for AT from the BAT region; 364.1 ± 367.7 to 491.1 ± 423.8 O₂ flux, $P<0.001$, for WAT; 104.6 ± 78.6 to 189.4 ± 123.2 O₂ flux, $P=0.003$).

Age differences in respiration control ratios

The relative contribution of state 4 to state u respiration in WAT was significantly correlated to age ($r^2=0.466$, $P=0.010$, Figure 3). For AT from the BAT region, the ratio of state 4 vs. state u for AT from the BAT region was significantly higher for subjects aged under 50 years ($n=6$) compared to older subjects (≥ 50 years, $n=7$, state 4/state u; 0.78 ± 0.11 vs. 0.60 ± 0.17 O₂ flux, $P=0.042$, Figure 3).

Discussion

In this study oxygen consumption of adipose tissue from the human BAT region was compared to subcutaneous white adipose tissue, taken from the same anatomical region in the neck. The respiration in AT from the BAT region was on average

Figure 2. Cellular respiration levels of human white adipose tissue (WAT) and adipose tissue (AT) taken from regions shown to contain brown adipose tissue (BAT). Oxygen consumption in WAT and AT from the BAT region shown for baseline respiration, oligomycin-insensitive leak state 4 respiration upon addition of oligomycin and maximally uncoupled state u respiration after addition of FCCP. All uncorrected oxygen fluxes (expressed as picomoles O₂ consumption x seconds⁻¹) for both AT from the BAT region and WAT were normalized for the DNA content in chamber solutions (mg/mL). Values shown are means+S.E.M. *P<0.05 for comparison between AT from the BAT region versus WAT baseline, oligomycin and FCCP-stimulated oxygen fluxes, respectively. #P<0.05, ##P<0.001 for comparison between baseline vs. oligomycin and oligomycin vs. FCCP-stimulated oxygen fluxes within tissues for WAT and AT from the BAT region.

three times higher compared to subcutaneous WAT. These findings are in line with previous animal studies that show mitochondrial respiration in BAT is relatively high.¹⁴

Recently detailed metabolic imaging studies on BAT activity in man using PET-CT have been performed.^{3,6,7} Upon cold exposure BAT oxidative metabolism as determined using ¹¹C-acetate uptake increased $\pm 150\%$ whereas BAT perfusion using (¹⁵O)H₂O increased $\pm 200\%$ and glucose uptake using ¹⁸F-FDG increased $\pm 600\%$.^{6,7} The same parameters did not change significantly in WAT and baseline BAT oxygen uptake and perfusion were already doubled compared to WAT.^{6,7} Here we show that tissue taken from the region identified to be BAT already uses 300% more oxygen in the basal state and thus displays increased cellular respiration. This 300% higher basal respiration is much higher than the previous reported 10-20% higher respiration in visceral adipose tissue compared to WAT, indicating that in humans AT from the BAT region is a very special metabolic active adipose tissue depot.¹⁰ It is important to note that biopsies from the neck region do not solely consist of brown or beige adipocytes, as may be the case in more homogenous BAT depots in rodents. Rather, as observed in Figure 1 and before,¹⁵ the adipose tissue

of this region is a mixture of white and UCP1-positive adipocytes, with a varying composition between subjects. This indicates that mitochondrial respiration in pure brown/beige adipocytes is likely much higher, and illustrates that strategies that are able to induce human brown/beige adipocytes may effectively result in an adipose tissue depot with a very high metabolic potential. Due to the limited amount of human tissue available and the amount needed for respiration studies, we could not perform a further characterization of the neck adipose tissue, such as mitochondrial content, which most likely explains the higher basal metabolic activity. More important, it is generally thought that the main component of heat production (extra oxygen consumption) in BAT derives from UCP1-mediated uncoupled 'leak' respiration.¹⁶ The UCP-1 mediated thermogenesis needs to be activated by external stimuli, e.g. a sympathetically induced norepinephrine-release. Indeed,

Figure 3. Relationships between subject age and mitochondrial respiration states of WAT and AT from the BAT region. A: Relationship between age and the relative contribution of state 4 leak respiration to maximally uncoupled state u respiration in WAT. B: Relationship between age and the relative contribution of state 4 leak respiration to maximally uncoupled state u respiration in AT from the BAT region. C: Comparison of the relative contribution of state 4 leak respiration to maximally uncoupled respiration in AT from the BAT region from subjects below and above 50 years of age. * $P<0.05$

in mice maximal respiration in BAT upon addition of NE increases 7-8 fold over basal respiration,¹⁷ and thus much higher than the FCCP-induced respiration in our study. Unfortunately, we were unable to determine NE-stimulated mitochondrial respiration in the isolated adipocytes from the BAT region: initial attempts were not successful, and the limited amount of this valuable human tissue available did not allow optimization of the procedure. In that respect, it is also known from rodent studies that BAT from several (e.g. 6-8 mice) animals needs to be pooled to render isolated brown adipocytes that respond to NE *ex vivo*. Nevertheless, the reported basal respiration levels in AT from the BAT region, together with the fact that this tissue is inhomogeneous with respect to white and brown/beige fat cell composition, suggest that true respiration in pure human brown/beige adipocytes may be high.

Interestingly, the contribution of state 4 to state u in AT from the BAT region was higher in subjects <50 years, which would be consistent with the notion that younger subjects are suggested to possess more active BAT.¹⁸

Recent reports show that within WAT there is a subpopulation of cells that has the capacity to become thermogenic.^{19,20} These 'beige' or BRITE (brown-in-white) cells possess UCP-1 and are thus capable of thermogenesis upon sufficient stimulation.²⁰ Several studies have now reported the development of beige/BRITE cells upon diverse stimuli.²⁰⁻²³ *In vitro* cultured human preadipocytes that were isolated from adipose tissue taken in the same anatomical region as in this study became thermogenic adipocytes upon stimulation by both β -agonists and proliferator-activated-receptor-gamma (PPAR-gamma). Intriguingly, we recently showed that adipose tissue taken from the same area as in this report in fact is composed of such 'beige cells', genetically distinct from white and brown fat.⁹ Respiration analysis of cloned beige cells showed that respiratory activity of these cells is comparable to classical brown adipocytes.⁹ These findings suggest that the amount of "brown adipose tissue" in humans may be inducible when activated by the right stimulators, consistent with our recent finding of induction of human "BAT" after weight loss.²⁴ In summary, we here show that the basal mitochondrial respiration of adipose tissue from the region characterized as "BAT" can be distinguished from subcutaneous adipose tissue by a ~300% higher mitochondrial respiration. This illustrates that AT from human BAT region is a distinct adipose tissue depot with high mitochondrial capacity. Future studies should be directed to further study the metabolic and bioenergetic aspects of this interesting depot. Cultured adipocytes from this AT

depot may be of specific interest to identify molecular targets that are able to stimulate this tissue. Such studies could play an important role in obesity treatment by inducing BAT activity and increasing energy expenditure.

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Chapter 7

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Vagus nerve stimulated increased energy expenditure in relation to brown adipose tissue activity

The contents of this chapter are submitted.

Abstract

Background

Human brown adipose tissue (BAT) activity is inversely related to obesity and positively related to energy expenditure. BAT is highly innervated and it is suggested the vagus nerve mediates peripheral signals to the central nervous system, there connecting to sympathetic nerves that innervate BAT. Vagus nerve stimulation (VNS) is used for refractory epilepsy, but is also reported to generate weight loss. We hypothesize VNS increases energy expenditure by stimulating BAT activity.

Methods

Fifteen patients with stable VNS therapy (age: 45 ± 10 yrs; body mass index; 25.2 ± 3.5 kg/m²) were included. Ten subjects were measured twice, once with active and once with inactivated VNS. Five other subjects were measured twice, once with active VNS at room temperature and once with active VNS under cold exposure in order to determine maximal cold-induced BAT activity.

Results

Basal metabolic rate (BMR) was significantly higher when VNS was turned on (mean change; +2.2%). Mean BAT activity was not significantly different between active VNS and inactive VNS (BAT SUV^{Mean}; 0.55 ± 0.25 versus 0.67 ± 0.46 , $P=0.619$). However, the change in energy expenditure upon VNS intervention (On-Off) was significantly correlated to the change in BAT activity ($r=0.935$, $P<0.001$).

Conclusions

Activated VNS significantly increased energy expenditure. The observed change in energy expenditure was significantly related to the change in BAT activity. This suggests that the VNS-induced increase energy expenditure in humans is at least partly due to BAT activation. Chronic VNS might have a beneficial effect on the energy balance and play an important role in weight management.

Introduction

Obesity results from an imbalance between energy intake and energy expenditure and most applied obesity therapies are focussed on decreasing energy intake.¹ However, in addition to a reduction in energy intake, increasing energy expenditure could be an effective means to prevent or treat obesity. In rodents, activated brown adipose tissue (BAT) is the main contributor to the regulated energy expenditure.² Here, during cold exposure BAT activation increases energy expenditure by producing heat (thermogenesis) to prevent hypothermia.² Recently functional brown adipose tissue (BAT) was also shown in adult man by means of FDG-PET-CT (¹⁸F-fluorodeoxyglucose positron-emission tomography and computed tomography).^{3,4} Interestingly, BAT activity was inversely correlated with body mass index (BMI) and body fat percentage (BF%).^{3,5} Moreover, subjects with active BAT show a significantly higher energy expenditure compared to subjects without BAT activity.^{5,6} This suggests BAT is related to the development of obesity and increasing BAT activity could be a new treatment modality for obesity. Rodent studies and histological analyses of human tissue samples have shown BAT is highly sympathetically innervated.^{7,8} Stimulation of sympathetic β -receptors on brown adipocytes was already suggested as a possible target for the treatment of obesity several years ago⁹. More recent studies report stimulation and suppression of BAT activity in rodents which is mediated through the vagus nerve.^{10,11} It is suggested the vagus nerve mediates peripheral signals to the central nervous system (CNS), which in turn connects to the sympathetic nerves that innervate BAT.¹¹ Interestingly, electrical stimulation of the cervical part of the vagus nerve (vagus nerve stimulation (VNS)) generates weight loss in rodents.^{12,13} Weight loss was also reported as a secondary outcome of VNS in humans, where it is used to treat refractory epilepsy.¹⁴⁻¹⁹ The weight loss induced by VNS may be the result of increased energy expenditure through BAT stimulation. However, most human trials are primarily focused on the effect of VNS on epilepsy from a neurological viewpoint and to date no prospective study has investigated the effect of VNS on energy expenditure. Therefore, this study aims to define the relation between VNS energy expenditure and BAT activation in a patient cohort on chronic stable VNS therapy for refractory epilepsy. We hypothesize that VNS increases energy expenditure by stimulating BAT activity.

Methods

Approval for the study protocol was obtained from the ethics committee of the Maastricht University Medical Centre. The study was registered in the Clinical Trial Register under the ClinicalTrials.gov Identifier NCT01491282. Written informed consent was obtained from 15 patients on stable VNS therapy using the Vagus Nerve Stimulation Therapy System (VNS Therapy®, Cyberonics Inc., Houston, Texas, USA) for refractory epilepsy. All included subjects had stable therapy (i.e. no recent adjustments in VNS settings or epilepsy medication (Table 1) and were aged between 18 and 65 years. Exclusion criteria were daily epileptic seizures, pregnancy, ketogenic diet, mental retardation and psychological instability.

Principle of vagus nerve stimulation

VNS is an approved procedure for refractory epilepsy patients. The therapeutic goal of VNS is a reduction in seizure frequency, seizure severity, and an improvement in general well-being. The VNS Therapy® System consists of a programmable pulse generator implanted subcutaneously at the level of the upper left chest. A helical bipolar electrode connected to the left vagus nerve transmits electric signals generated by the pulse generator. The stimulation parameters are adjusted via telemetric control to an optimal value for every individual patient. Standard starting parameters are: output current 0.25 mA, pulse width 250-500 msec, frequency 30 Hz, On-period 30 sec, Off-period 5 min. If the effect is suboptimal, the output current is increased in steps of 0.25 mA to a maximum of 3 mA. The most common standard therapeutic setting is: output current 1-2 mA, pulse width 250 msec, frequency 30 Hz, On-period 30 sec, Off-period 5 min. A second option is the introduction of Rapid Cycling, where the On-period is 7 sec and the subsequent Off-period 20 sec. Patients responding to Rapid Cycling are thought to need this extra stimulation to acutely suppress an upcoming seizure in order to obtain a reduction in their seizure frequency.

Study protocol

Energy expenditure was measured using indirect calorimetry and BAT activity was assessed by means of FDG-PET-CT during actual VNS and when VNS was inactivated. This was possible since the anticonvulsive effect of VNS is considered chronic and short-term inactivation does not lead to an acute increase in seizures. In addition, we compared BAT activity during VNS and during mild cold stimulation. The mild cold

intervention served as a control since it is known to activate BAT.^{3,5} Ideally, one would like to compare the effect of active/inactive VNS with and without cold exposure, which would result in four FDG-PET-CT measurements in each subject. However, due to ethical considerations a maximum of two FDG-PET-CT measurements was allowed. Therefore, ten patients were measured in thermoneutral (TN) conditions with active (VNS-On) and inactive VNS (VNS-Off) respectively. In addition, five subjects were measured with active VNS in TN conditions (VNS-TN) and during mild cold exposure (VNS-Cold) respectively (Table 2). Subjects were measured under fasted conditions (no food intake from 10 p.m. the night before, only water consumption was allowed) from 9 a.m. to 2 p.m. under supervision of a specialized research nurse (L.L.). VNS-On and VNS-Off took place on separate occasions

Table 1. Medication use per patient.

Subject	Medication
1	carbamazepine, lamotrigine, lacosamide
2	carbamazepine, clonazepam
3	levetiracetam, oxcarbazepine, clobazam
4	carbamazepine, valproic acid, lamotrigine, lacosamide
5	lamictal, levetiracetam, calciumcarbonate, domperidon, paracetamol, folic acid, hydroxy-chloroquin, bisacodyl, tramadol, zolpidem, pantoprazol, estradiol/norethisteron
6	gabapentine, clobazam, levetiracetam
7	levetiracetam, carbamazepine, desogestrel
8	clorazepine, gabapentine, topiramate
9	oxcarbazepine, calcium supplements
10	carbamazepine, lacosamide, L-carnitene
11	clobazam, retigabine, lacosamide, sertralin, desogestrel
12	levetiracetam, lamotrigine, clemastine, cinnarizine, omeprazol
13	pipamperon, lacosamide, clobazam, carbamazepine, levetiracetam
14	clobazam, lamotrigine, gabapentin, carbamazepine, perindopril/indapamide, dipyridamol/ acetyl salicylic acid, bisoprolol, nifedipin, atorvastatin
15	fenytoin, clonazepam, oxcarbazepine

within 14 days. During VNS-Off the system was inactivated (output current 0 mA, magnet function 0 mA) at 9:30 a.m. prior to the measurements. At the end of the test day (2:00 p.m.) the VNS system was re-activated. During VNS-Cold the settings of the VNS system were not adjusted. VNS-TN and VNS-Cold were also

performed within a 14-day period. Body composition (body fat%, fat mass (FM), fat free mass (FFM)) was determined by dual x-ray absorptiometry (DXA, type Discovery A, Hologic, Bedford, MA, USA).

During the experiments the subjects wore standardized clothing (socks 0.02 clo (clothing insulation factor), shirt 0.09 clo, sweatpants 0.28 clo, underwear 0.04 clo, total clo factor 0.43 clo).²⁰ A catheter was placed in the left antecubital vein for blood sampling during measurements and to inject ¹⁸F-Fluoro-Deoxy-Glucose (FDG) for FDG-PET-CT (for further detail on scanning protocol see below). Body core temperature was monitored by a telemetric pill that was orally ingested (CoreTemp, HQ Inc., Palmetto, FL, USA). iButtons were placed at 14 ISO-defined skin sites to measure skin temperatures.²¹ For comparison between proximal and distal body temperatures, the proximal skin temperature was defined as the mean of the iButtons placed on the chest, abdomen, shoulder and lower back. The distal skin temperature was defined as the mean of the iButtons placed on the wrist and foot. Next, subjects were placed in a semi-supine position on a nephrodialysis chair covered with a water-perfused mattress (necessary for the cold exposure protocol, see below) in a specially equipped tent (Colorado Altitude Training, Louisville, CO, USA) where energy expenditure was measured by indirect calorimetry using a ventilated hood system (Omnical, Maastricht Instruments BV, Maastricht, the Netherlands). Laser Doppler Flowmetry (PF5000; Perimed, Jarfalla, Sweden) was used to determine skin blood flow gradients dorsal of the medial malleolus and at the glabrous skin of the ventral hand thenar to monitor whether a subject was in its thermoneutral zone as reported.²² The VNS-On/Off (n=10) and VNS-TN (n=5) measurements were performed under thermoneutral conditions (average room temperature VNS-On; 24.9±0.8°C, VNS-Off; 25.2±1.0°C, VNS-TN; 25.2±0.9°C, P=0.7260). After the first hour of measuring under thermoneutral conditions (t0-t60) 10ml FDG was injected. After the second hour (t60-t120), FDG-PET-CT imaging was performed to quantify metabolically active BAT. The scanning protocol, FDG activity (74 MBq) and data analysis were identical to our previous studies on BAT activity.^{5,23} During VNS-Cold a personal cooling protocol was used as described earlier in detail.⁵ In short, after measuring under thermoneutral conditions (t0-t30), the subject was cooled until they reported shivering. Upon the first signs of shivering the temperature was raised slightly (on average 2.3±0.6°C) to

ensure only non-shivering thermogenesis (NST).⁵ After one hour of stable cooling situation (t30-t90), 10ml FDG was injected, followed by the second hour of cooling (t90-t150) and subsequent FDG-PET-CT imaging.

FDG-PET-CT analysis

Tissue activity on FDG-PET-CT was expressed in standard uptake value (SUV; as calculated by uptake (kilobequerels per ml) per injected dose (kilobequerels) per patient weight (grams)) using the PMOD Biomedical Image Quantification tool (PMOD Technologies Ltd., Zurich, Switzerland). Tissue activity of each region was determined by the mean SUV uptake (SUV^{Mean}). For comparison, standard cube volumes-of-interest (VOIs) were placed in the tissue type studied: Brown Adipose Tissue (BAT), deltoid muscle (Deltoid), biceps muscle (Biceps), triceps muscle (Triceps), erector spinae muscle at the level of vertebrae C7 (C7), T8 (T8), and L3 (L3), the mean of all analyzed muscles (Muscle), subcutaneous white adipose tissue (WAT Sc), visceral white adipose tissue (WAT Visc), the liver (Liver) and the brain at the level of the cerebellum (Brain) (Figure 1). The volume of these three-dimensional cubes was 1.33 cm^3 (i.e. three consecutive slices of 0.44 cm^3 thickness). For BAT the placement of one cube in supraclavicular adipose tissue often interferes with adjacent muscle and hence the cube volume in BAT was composed of three separate slices of $1.0 \times 1.0 \times 0.44 \text{ cm}$. When drawing the cubes, attention was paid to the Hounsfield Units (HU) on CT to define the specific tissue type and to avoid interference with adjacent tissue. For example, adipose tissue has a HU of -200 to -10 and muscle 10 to 100. Finally, FDG-PET-CT imaging during the Off-measurement failed in one subject due to a critical image reconstruction error. Consequently, values shown are for nine subjects in the VNS-On/Off group and five subjects in the VNS-TN/Cold group.

Statistics

Reported data were expressed as means $\pm$ S.D. Statistical analyses were performed with PASW Statistics 18.0 for Mac OS X 10.6.4. Repeated measurements (VNS-On versus VNS-Off, and VNS-TN versus VNS-Cold) were compared using paired student's *t* tests. Comparisons between groups (VNS-On/Off versus VNS-TN/Cold) were made using one-way ANOVA with a post-hoc Bonferroni correction. A *P*-value below 0.05 was considered significant.

Results

Subject characteristics

Ten male and five female patients with a mean age of 45 ± 10 years and a mean Body Mass Index (BMI) of 25.2 ± 3.5 kg/m² that were (successfully) treated with VNS for refractory epilepsy were included (Table 1). VNS implantation was on average 59 ± 19 months (range; 22-89 months) ago and all subjects did not have any recent adjustments in their VNS settings or medication (last adjustment >1 month ago, VNS output current; 1.58 ± 0.55 mA, Table 2). Pre-VNS treatment body weight and BMI were retraceable for 11 subjects and were not significantly different from weight and BMI during the study (implant weight and BMI; 71.2 ± 12.5 kg, 24.7 ± 3.4 kg/m², current weight and BMI; 72.9 ± 11.6 kg, 25.2 ± 3.5 kg/m², $P=0.414$). The subject characteristics were not different for the On/Off ($n=10$) versus the TN/Cold group ($n=5$) (Table 2).

Energy expenditure

Basal metabolic rate (BMR) decreased significantly when VNS was turned off (68.6 ± 7.9 J/s versus 67.2 ± 8.1 J/s, $P=0.038$, mean change; 2.2%, range; -3.1 to 7.8%, Table 3, Figure 1). All but one subject decreased their BMR; the male subject with increased BMR was very restless during the Off-measurement due to personal affairs. Exclusion of this subject revealed energy expenditures of 68.6 ± 7.9 J/s (VNS-On) and 66.6 ± 8.31 J/s (VNS-Off) ($P=0.005$). The subject's data remained included in the further presented analyses.

Upon cold exposure BMR increased as compared to thermoneutral conditions (TN; 68.2 ± 1.5 J/s versus Cold; 75.1 ± 7.7 J/s, $P=0.107$, Table 3). BMR in the VNS-TN group was similar to BMR in the VNS-On group ($P=0.919$).

Core and skin temperatures

Body core and mean skin temperatures did not change significantly when VNS was turned off (core temperature; 37.0 ± 0.3 °C versus 36.9 ± 0.4 °C, mean skin temperature; 33.5 ± 0.8 °C versus 33.6 ± 0.7 °C, $P=0.738$, Table 2). When

skin temperatures were divided in proximal and distal zones ²¹, the distal skin temperature was significantly increased during the Off-measurement (31.7 ± 1.6 °C versus 33.0 ± 1.7 °C, $P=0.016$, Table 2).

During cold exposure skin temperatures decreased significantly (TN: mean skin temperature; 33.2 ± 0.4 °C versus Cold: 29.0 ± 0.8 °C, $P<0.001$, Table 2).

Skin perfusion

Perfusion of the medial foot and ventral hand did not change during VNS (foot; 8.2 ± 3.9 Arbitrary Units (AU) versus 13.4 ± 21.4 AU, $P=0.396$, hand; 54.5 ± 35.3 AU versus 81.7 ± 70.5 AU, $P=0.149$, Table 3). Cold exposure significantly decreased hand but not foot perfusion (hand; 25.1 ± 11.4 AU versus 6.0 ± 1.4 AU, $P=0.029$, foot; 9.6 ± 8.0 AU versus 6.8 ± 4.4 AU, $P=0.396$, Table 3).

BAT activity and FDG uptake in other tissues

Figure 2 shows representative images of FDG-uptake on PET-CT in the studied groups. The mean SUV for BAT showed no statistical difference during VNS (BAT SUV^{Mean}; 0.55 ± 0.25 versus 0.67 ± 0.46 , $P=0.619$, Table 2, Figure 3). In different muscles analyzed, the triceps muscle had a significantly increased FDG-uptake when VNS was turned off (Triceps SUV^{Mean}; 0.51 ± 0.14 versus 0.70 ± 0.16 , $P=0.024$, Table

Table 2. Subject characteristics for all subjects, the intervention group with Vagus Nerve Stimulator (VNS) On and Off ($n=10$) and for the group with VNS during thermoneutral (VNS-TN) conditions and cold exposure (VNS-Cold) ($n=5$). P-values shown for unpaired t-tests between VNS-On/Off and VNS-TN/Cold. * $P<0.05$

Characteristics	Group	VNS-On/Off	VNS-TN/Cold	P-value
n	15	10	5	
Sex (Male/Female)	10 M / 5 F	4 M / 6 F	1 M / 4 F	
Age (yrs)	45 ± 10	42 ± 10	49 ± 8	0.203
Height (cm)	170 ± 10.0	168.5 ± 8.4	173 ± 13	0.464
Mass (kg)	73.0 ± 11.6	70.1 ± 11.7	78.6 ± 10.4	0.194
BMI (kg/m ²)	25.2 ± 3.5	24.6 ± 3.0	26.6 ± 4.4	0.316
Fat Free Mass (kg)	49.8 ± 9.0	48.8 ± 9.5	51.7 ± 8.5	0.568
Fat Mass (kg)	21.9 ± 6.4	20.4 ± 5.3	25.0 ± 8.0	0.199
Body Fat (%)	29.5 ± 6.9	28.5 ± 6.4	31.4 ± 8.2	0.459
VNS output current (mA)	1.58 ± 0.55	1.55 ± 0.59	1.65 ± 0.52	0.752
VNS implant time (months)	59 ± 19	64 ± 15	50 ± 24	0.185
VNS implant mass (kg)	71.2 ± 12.5	69.8 ± 13.9	72.8 ± 12.0	0.717
VNS implant BMI (kg/m ²)	24.7 ± 3.4	24.9 ± 3.1	24.5 ± 4.1	0.861

Table 3. Energy expenditure, core and skin temperatures, skin perfusion and Standard Uptake Values (SUV^{Mean}) for Brown Adipose Tissue (BAT), all muscles analyzed (Muscle), deltoid muscle (Deltoid), biceps muscle (Biceps), triceps muscle (Triceps), erector spinae muscle at the level of vertebrae C7 (C7), T8 (T8), L3 (L3), subcutaneous white adipose tissue (WAT Sc), visceral white adipose tissue (WAT Visc), the liver (Liver) and the brain at the level of the cerebellum (Brain). AU denotes Arbitrary Units for skin perfusion. *P<0.05, **P<0.001

Parameter	On	Off	P-value	TN	P-value
Energy expenditure (J/s)	68.6±7.9	67.2±8.1	0.038*	68.2±1.5	0.107
Core temperature (°C)	37.0±0.3	36.9±0.4	0.738	36.9±0.2	0.877
Mean skin temperature (°C)	33.5±0.8	33.6±0.7	0.686	33.2±0.4	<0.001**
Proximal skin temperature (°C)	34.6±1.1	34.6±0.5	0.996	34.0±1.3	0.012*
Distal skin temperature (°C)	31.7±1.6	33.0±1.7	0.016*	31.7±2.5	<0.001**
Perfusion Foot (AU)	8.2±3.9	13.4±21.4	0.396	9.6±8.0	0.553
Perfusion Hand (AU)	54.5±35.3	81.7±70.5	0.149	25.1±11.4	0.029*
SUV ^{Mean} BAT	0.55±0.25	0.67±0.46	0.619	0.65±0.29	0.012*
SUV ^{Mean} Muscle	0.57±0.14	0.65±0.11	0.100	0.78±0.14	0.012*
SUV ^{Mean} Deltoid	0.57±0.16	0.62±0.10	0.281	0.74±0.11	0.031*
SUV ^{Mean} Biceps	0.62±0.17	0.69±0.17	0.390	0.89±0.14	0.130
SUV ^{Mean} Triceps	0.51±0.14	0.61±0.13	0.024*	0.70±0.16	0.260
SUV ^{Mean} C7	0.62±0.22	0.66±0.13	0.572	0.85±0.23	0.209
SUV ^{Mean} T8	0.63±0.12	0.72±0.10	0.081	0.79±0.16	0.475
SUV ^{Mean} L3	0.46±0.15	0.60±0.16	0.057	0.75±0.29	0.389
SUV ^{Mean} WAT Sc	0.19±0.09	0.24±0.08	0.194	0.24±0.09	0.742
SUV ^{Mean} WAT Visc	0.36±0.16	0.55±0.30	0.111	0.47±0.13	0.881
SUV ^{Mean} Liver	1.60±0.46	2.04±0.45	0.038*	2.44±0.41	0.405
SUV ^{Mean} Brain	6.58±2.07	6.82±1.65	0.730	7.45±1.68	0.183

3). However, for all muscles together there was no significant change in activity (Muscle SUV^{Mean}; 0.57±0.14 versus 0.65±0.11, P=0.100, Table 3). The FDG-uptake in the liver significantly increased during VNS (Liver SUV^{Mean}; 1.60±0.46 versus 2.04±0.45, P=0.038, Table 3). After cold exposure, all subjects showed increased BAT activity (BAT SUV^{Mean}; 0.65±0.29 versus 3.40±1.63, P=0.012, Table 3, Figure 3). Deltoid muscle activity was significantly decreased in the cold (Deltoid SUV^{Mean};

Figure 1. Basal metabolic rate (BMR) during active and inactive VNS in relation to BAT activity. A. Individual responses upon VNS intervention. Notice all but one (marked line) subjects decreased energy expenditure upon deactivation of the VNS (subject that increased energy expenditure also described in Results section). B. Mean group BMR upon VNS intervention. C. The change in energy expenditure (Δ Energy Expenditure) upon VNS intervention (On/Off) correlated to the change in BAT activity. One subject (open dot) showed a high increase in energy expenditure upon VNS intervention. This subject was confirmed as an statistical outlier in the exponential growth equation shown and therefore excluded from the equation. Values shown are means $\pm$ S.E.M. Significance shown for paired t-test in VNS intervention group consisting of 4 males and six females. * $P < 0.05$

0.74 $\pm$ 0.11 versus 0.65 $\pm$ 0.14, $P = 0.031$, Table 3, Figure 3) and also activity for all muscles together decreased upon cold exposure (Muscle SUV^{Mean}; 0.78 $\pm$ 0.14 versus 0.72 $\pm$ 0.14, $P = 0.012$, Table 3, Figure 3).

Correlations

Energy expenditure during VNS-On and VNS-Off measurements was not related to BAT activity, activity of other tissue (Muscle, WAT), skin perfusion, core and skin temperatures or any other study parameter. However, the change in energy expenditure upon VNS intervention (from VNS-On to VNS-Off) was positively

Figure 2. FDG-PET-CT images of intervention group and cold exposed subjects. Transaxial and coronal CT, PET and PET-CT fused images of VNS-On and VNS-Off from a subject in the VNS-On/Off group and images from a subject in the VNS-TN/Cold group during thermoneutral conditions (TN) and cold exposure (Cold). PET-CT image shown with and without Volume-Of-Interest (VOI) cubes for determination of activity. The white line in the coronal image indicates the transaxial slice shown above.

correlated to the change in BAT activity (exponential curve fitting, $r=0.935$, $P<0.001$, Figure 1). VNS output current was not related to BAT activity or energy expenditure.

Discussion

This study shows that even short-term intervention of VNS therapy by turning off the VNS for only several hours significantly decreased energy expenditure in a cohort of treatment-stable VNS patients. In addition, the change in energy expenditure upon inactivation of VNS was significantly related to a change in BAT activity. Therefore, VNS possibly positively influences energy balance by increasing energy expenditure through involvement of BAT in humans. This might induce weight loss and as such VNS might contribute to weight management.

Comparison with previous studies on VNS therapy

An effect of VNS on metabolism had already been suggested by the authors of a

Figure 3. FDG-PET-CT activity of different tissue types upon VNS intervention. SUVMean (Standard Uptake Value)w values for Brown Adipose Tissue (BAT), all muscle (Muscle), deltoid muscle (Deltoid), biceps muscle (Biceps), triceps muscle (Triceps), erector spinae muscle at the level of vertebrae C7 (C7), T8 (T8), L3 (L3), subcutaneous white adipose tissue (WAT Sc), visceral white adipose tissue (WAT Visc), the liver (Liver) and the brain at the level of the cerebellum (Brain). A. Comparison of activity during VNS-On and VNS-Off. B. Comparison of activity between VNS-On, VNS-Off and VNS-Cold. Values shown are mean+S.E.M. * $P < 0.05$ for paired t-tests between VNS-On and VNS-Off. # $P < 0.05$ for one-way ANOVA with post-hoc Bonferroni correction between VNS-On, VNS-Off and VNS-Cold.

brief report where weight loss was observed in 17/27 patients with VNS for epilepsy (2002).¹⁷ Pardo et al. studied a morbidly obese cohort of 14 VNS patients treated for depression (weight 91 ± 27 kg, BMI 43 ± 5 kg/m²) with VNS settings comparable to the present study (>24 months VNS implant, Output current; 0.25-1.5 mA, Freq; 30 Hz, Pulse; 500- μ s, On-period; 30s, Off-period; 5 min, VNS current study; Output current; 1.58 ± 0.55 mA, Freq; 30 ± 0 Hz, Pulse; 266.7 ± 64.5 μ s, On-period; 23.9 ± 10.5 s, Off-period; 3.7 ± 2.1 min) and observed a significant BMI-related weight loss after VNS therapy.¹⁶ The short-term intervention in normal weight subjects in the current study already showed a significantly increased energy expenditure during VNS. Although the mean difference in BMR was small, the weight loss observed by Pardo et al. in morbidly obese subjects suggests long-term VNS results in increased energy expenditure and could be important in weight maintenance.

Another study reported 33 obese patients (BMI; 30.1 ± 3.6 kg/m²) receiving VNS for depression and investigated the response to a food stimulus upon active and inactive VNS.¹⁹ Short-term VNS intervention significantly altered food craving. Duration of VNS intervention was not specified, but seems to have taken place within several hours, which is similar to the present study.¹⁹

All currently published data on the effect of VNS on metabolism in humans are retrospective. However, in animals a prospective study in overfed minipigs undergoing bilateral VNS (implanted 5 cm above the diaphragm) with stimulation parameters comparable to our study (VNS minipigs; Output current; 2mA, Freq; 30Hz, Pulse; 500- μ s, On-period; 30 s, Off-period; 5 min, VNS current study; see above) has revealed that weight gain was prevented in the VNS-treated minipigs and not in controls.²⁴ VNS in these minipigs significantly decreased food intake, especially carbohydrate consumption ('sweet craving') as assessed by the respiratory quotient (RQ).²⁴ In the present study we did not observe a difference in RQ after VNS intervention (RQ On; 0.82 ± 0.03 versus RQ Off; 0.82 ± 0.04 , $P=0.8774$) and the mean RQ of ± 0.8 confirms the prescribed overnight fast preceding the BMR measurements. However, possibly RQ does show a significant difference in man when studying VNS in longer time periods, for example before and after VNS implantation.

Finally, in a study on the effect of VNS on metabolism Sobocki et al. used a crossover design in pigs with activation and de-activation, each in a 4-week period and found that VNS prevented weight gain but did not affect metabolic rate as assessed by indirect calorimetry.²⁵ However, metabolic rate was determined under general anesthesia, which probably affected BMR measurements. Possibly BMR did change under awake conditions, as was observed in the current report, which

would explain the significant differences in weight gain observed by Sobocki et al.

Mechanism of VNS increased energy expenditure

Possible mechanisms by which VNS affects energy expenditure have been suggested in several studies. From an anatomical viewpoint, it is interesting to note that the majority of fibers present within the vagus nerve are afferent fibres (74%) and only a minority (26%) are efferent, indicating that the vagus nerve is both an afferent nerve and efferent nerve as well.²⁶

Within the hypothalamus, several areas have been studied in respect to the regulation of energy balance. The hypothalamic arcuate nucleus receives vagus signals and projects them to the paraventricular nucleus (PVN), which in turn signals to the sympathetic nervous system and to the thyroid axis.²⁷ Vagotomy blocks these responses and alters energy homeostasis.²⁷ In the ventromedial nucleus of the hypothalamus (VMH), an area adjacent to the PVN, both parasympathetic and sympathetic neurons are present, and it is suggested the VMH regulates the 'balance' between sympathetic and parasympathetic nerve activities. Therefore, VMH stimulation may affect sympathetic mechanisms. Electrical stimulation of the VMH increased energy expenditure and decreased RQ in rats, suggesting an increased fat oxidation.²⁸ Indeed, VMH stimulation, but not PVN-stimulation, increases norepinephrine turnover and thermogenesis in interscapular BAT in rats, and this effect is completely inhibited by blockage of sympathetic ganglions.^{29,30} Interestingly, vagotomy has been demonstrated to decrease NE-turnover in BAT,¹¹ suggesting that vagus signals may influence BAT thermogenesis. In the present study, we observed a significant change in energy expenditure that was related to changes in the activity in BAT and not to changes in any of the other tissues that we have measured. Despite the fact that mean BAT activity did not increase upon VNS, the change in BAT activity explained a large part of the change in energy expenditure. To our opinion, this suggests at least part of the effect of VNS intervention on energy expenditure can be explained by BAT activity.

BAT presence and activity in refractory epilepsy

Since BAT activity is reported to decrease with age³¹ and can be affected by several pathophysiological conditions,³² we considered the possibility of reduced presence of BAT in (these) patients with refractory epilepsy. Therefore, we investigated maximal BAT activity in VNS patients after mild cold exposure. Mild cold exposure is known to induce BAT activation in 100% of lean young subjects (age; 24.3 ± 3.6 years, BMI;

23.2±1.2 kg/m²) and to be related to BMR and cold-induced thermogenesis.^{3,5,6} Despite their relatively high mean age (49±8 years) but comparable BMI (26.6±4.4 kg/m²), cold-induced BAT activity was present in all five patients. After mild cold stimulation BAT presence and activity were significantly higher than under TN conditions (VNS-Cold; BAT SUV^{Mean} 3.40±1.63 versus VNS-TN; BAT SUV^{Mean} 0.65±0.29, P=0.012), but slightly lower as compared to previously studied healthy young subjects (Age; 23±2 years, BMI; 23.1±1.7 kg/m², BAT SUV^{Mean}; 7.19±2.09 versus current study BAT SUV^{Mean}; 3.40±1.63, P=0.010, Vosselman et al., unpublished data). We believe this may be attributed to an age-related decrease in BAT activity.³¹ Therefore, BAT thus does not appear to be decreased in patients with refractory epilepsy.

Effect of VNS on muscle and liver metabolism

In contrast to the significant increase in energy expenditure that was related to BAT activity, we observed a decrease in liver and triceps muscle glucose uptake on FDG-PET-CT upon VNS. This is in line with the observation that in rodents cannabinoid receptor 1 (CB1) antagonism is known to induce sympathetic nervous system (SNS) activity and increase energy expenditure and BAT activity (as measured by the temperature in BAT itself), but not liver and muscle activity.³³ In addition, CB1 antagonism also induces fatty acid oxidation,³⁴ which could be the effect of the increased BAT activation, since BAT was recently shown to be able to combust lipids.³⁵ Moreover, it is known increased fatty acid oxidation can lead to inhibition of glucose uptake (the so-called Randle effect),³⁶ which would decrease activity on FDG-PET-CT imaging since this selectively depicts glucose uptake. Therefore, although there is a decrease in FDG-PET-CT-activity in liver and muscle, there could still be increased metabolism upon VNS in these tissues resulting from increased fatty acid oxidation. This would be consistent with an increase in BAT fatty acid oxidation and increased energy expenditure upon VNS. However, in this study we were not able to study fatty acid metabolism since FDG-PET-CT is a glucose tracer. In this regard, more research focused on liver and muscle function as well as fatty acid metabolism upon VNS is needed.

Conclusion

In summary, the present study demonstrates for the first time that VNS is accompanied by an increase in whole body energy expenditure, compared to short term deactivation of VNS. Moreover, the increase in energy expenditure was positively related to the change in BAT activity. This suggests that the VNS-induced increase in energy expenditure is at least in part due to changes in BAT activity. Therefore, chronic VNS might have a beneficial effect on energy balance and might play a role in weight management. However, the observed mean differences in energy expenditure are small, probably to a large extent because of the short duration of the intervention, and future, long-term, prospective studies should point out if VNS has a clinically and therapeutically significant effect on whole body metabolism. In addition, detailed metabolic imaging studies should assess the effect of VNS on BAT and other tissues in order to identify new possible targets in the treatment of obesity.

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Chapter 8

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General Discussion

Introduction

Heat production in brown adipose tissue (BAT) serves a vital function in small mammals and human newborns in order to prevent hypothermia upon cold exposure.¹ Recently, BAT has also been shown to be present and active in adult humans.²⁻⁴ In this thesis, we studied the physiology of human BAT from a cellular level to effects on whole body metabolism. In man, BAT activity is negatively related to the degree of obesity and positively related to energy expenditure.^{2,4,5} Since obesity results from a disturbed energy balance, it is interesting to study the physiology of human BAT. To increase the understanding of the function of human BAT in obesity, in this thesis we investigated BAT activity under pathophysiological conditions in morbid obesity. Moreover, we investigated if human BAT can be stimulated upon electrical nerve stimulation. In this final chapter the main findings of this thesis will be discussed and interpreted. In addition, possible clinical implications and future directions of BAT in treating disease will be addressed.

Measuring human BAT on a cellular level

In **Chapter 6** for the first time cellular oxygen consumption was determined in surgical biopsies taken from the adipose tissue region known to contain BAT in man. When we compared the cellular oxygen consumption in this adipose tissue depot to subcutaneous WAT, we strikingly found a ~300% higher level of oxygen consumption in adipose tissue derived from the BAT region. These results thus confirmed the presence of metabolically active BAT in the neck. However, our measurements were performed under baseline conditions without the addition of stimuli to increase BAT activity as cold or sympathetic nervous stimuli. In pure brown adipocytes derived from mice it was possible to increase BAT activity 700-800% upon NE-infusion.⁶ Therefore, possibly the addition of adequate stimuli can even further increase human BAT activity. On top of that, adipose tissue from the BAT region does not solely consists of 'pure' brown adipocytes, but rather seems to be a mixture of several types of adipocytes that vary in size in mitochondrial content.⁷ To characterize these cells, it is important to understand the origin of BAT. Classically, it was thought that during development preadipocytes irreversibly differentiated into the brown or white phenotype, without being able to change throughout life. However, histological observations suggested there also were adipocytes that could 'turn into one another'.⁸ In 2007 it was shown that addition of rosiglitazone (an antidiabetic drug known to stimulate the transcription factor PPAR-gamma) to cultured WAT-precursors induced the development of

UCP1-containing thermogenic cells.⁹ These cells were genetically different from BAT, but able to generate a similar level of thermogenesis, and therefore baptized BRITE (brown-in-white) adipocytes. At the same time, the discovery of PRDM16, a zinc-finger protein, gave more insight into the development of BAT,¹⁰ and later it was shown PRDM16 and BMP7 are crucial factors in the development of BAT.^{11,12} Following the experiments tracing back the development of BAT, it was also shown that prostaglandins (more specific COX-2, which is interestingly inhibited by most anti-inflammatory drugs) were able to induce 'browning'.^{13,14} Moreover, preadipocytes capable of becoming thermogenic were also shown to reside in skeletal muscle and a specific exercise-induced myokine capable of 'browning' was discovered; irisin.¹⁵⁻¹⁷ In man, in cultured biopsies taken from the same adipose tissue region as described in **Chapter 6**, it was shown to be possible to reproduce the rosiglitazone-effect *in vitro*.¹⁸ In addition, molecular characterization was done on human adipose tissue biopsies from our center, similar to the biopsies studied in **Chapter 6**. The results showed the adipocytes present in these tissue depots could be characterized as 'beige' adipose tissue, and not BAT.¹⁹ Full-grown beige cells were shown to form stable adipose tissue depots after transplantation in mice.¹⁹ In the murine beige cells studied cellular respirometry was performed, similar to the method we used in **Chapter 6**. Murine beige cells showed a significantly higher baseline respiration and oligomycin-insensitive uncoupled respiration compared to WAT.¹⁹ Also, under unstimulated conditions, the contribution of uncoupled respiration to baseline respiration was 78% for BAT and 60% for beige cells, suggesting UCP1-induced mitochondrial uncoupling is already present without any stimulus. In **Chapter 6**, in our biopsies from the human BAT region, 83% of baseline respiration was derived from uncoupled respiration, which confirms findings observed in mammals.¹⁹ Unfortunately, we were not able to determine the effect of, for example, β -adrenergic stimulation on human biopsies, which has been shown to dramatically increase cellular respiration up to 700-800% of baseline.⁶

Measuring human BAT on a whole body level

The development of new imaging techniques as FDG-PET-CT allowed for detailed study of metabolically active tissue activity and in a prospective study under controlled conditions of cold exposure from our lab FDG-PET-CT showed active BAT in lean young adults in 2009.² Other reports that were published in the same period also showed consistent BAT activity in humans and therefore a renewed curiosity for investigating BAT in man was born.^{3,4,20} In this thesis, in **Chapter 3** we

found that - in combination with our group of previously studied lean subjects² - BAT activity was robustly related to BMI and percentage body fat. This suggests a sufficient level of BAT activity could play an important role in maintaining body weight. Chronic overfeeding previously showed large interindividual differences in weight gain, which were suggested to possibly result from undetermined genetic factors.²¹ Interestingly, genetic polymorphisms in the UCP1-gene have been shown to be related to the energy balance (**Chapter 2**). Moreover, recently it was shown that polymorphisms of the UCP1-gene and the gene for the $\beta 3$ -adrenergic receptor, both essential for BAT thermogenesis (**Chapter 2**), may be associated with visceral fat accumulation during life.²² Therefore, genetic factors predisposing for low BAT activity could possibly contribute to the development of obesity.

Intermezzo

Measuring human BAT using an individualized cooling protocol

From a methodological point of view, it is important to note that in Chapter 3 and 4 all morbidly obese subjects underwent an individualized cooling protocol in order to induce maximal NST. To adequately measure BAT activity using FDG-PET-CT imaging presently only cold exposure is known to be able to consistently promote FDG-uptake in BAT. However, the cold exposure needs to be strong enough to induce maximal NST, but low enough to prevent ST. This is necessary, since shivering in skeletal muscle would increase the muscular FDG-uptake and possibly decrease uptake in BAT resulting in an inadequate interpretation of BAT activity. Most dedicated studies on BAT activity use a standardized cooling level for each studied subject, not taking into account the interindividual differences in body composition and thermoneutral zone.²³ When exposed to a similar level of cold exposure, a morbidly obese subject will experience a less intensive cold stimulus compared to a lean subject through their difference in subcutaneous adipose tissue insulation.²⁴ Therefore, we cooled each subject until shivering occurred. Subsequently, we gradually increased temperature until shivering just stopped (Figure 1). This protocol ensures a maximal level of NST, and thus a maximal level of BAT activity for each subject, independent of body composition.

Figure 1. Individualized cooling protocol. During two hours of cooling maximal non-shivering thermogenesis (NST) is induced to stimulate brown adipose tissue (BAT) activity. Temperatures shown are room temperatures in degrees Celsius.

In **Chapter 4**, we observed an increase in BAT activity after a ~35 kg weight loss. In addition, subjects with absent BAT activity during morbid obesity showed recruitment of BAT activity upon weight loss in regions that contained BAT activity in the lean subjects studied before. These results suggest BAT is rather a consequence than a cause of obesity. After observing an increase in BAT activity after weight loss in **Chapter 4**, we also studied the effect of weight loss on skeletal muscle mitochondrial uncoupling in **Chapter 5**. Although we did not observe a change in skeletal muscle mitochondrial uncoupling, the ADP-stimulated skeletal muscle metabolism did significantly increase after weight loss. This suggests that in addition to ‘restoring’ the level of BAT activity, sufficient weight loss is also able to recover obesity-associated impaired skeletal muscle metabolism. Although we were unable to define the molecular mechanism responsible for the observed recruitment of BAT, the observed BAT recruitment provides hopeful evidence for future studies targeting BAT recruitment in man. Moreover, as discussed in **Chapter 2**, in addition to obesity other studies have now shown relations between a low BAT

activity and type 2 diabetes, hepatosteatosi and hypothyroidism, which even more emphasizes that BAT seems to be play a relevant role in both human physiology and pathophysiology.

Intermezzo

Functional imaging of human BAT

The first evidence of functional BAT in humans was shown using FDG-PET-CT imaging studies (Figure 2).^{25,26} Prospective studies using controlled cold exposure to maximize NST in BAT showed high levels of BAT activity which was related to body composition and energy expenditure.²⁻⁴ However, FDG-PET-CT relies on the concept of cellular uptake of radioactive glucose (FDG). Interestingly, detailed studies in the mammalian brown adipocyte have shown the main fuel for BAT are free fatty acids (FFAs).¹ Glucose uptake in BAT is considered low (~10%) and is suggested to function in order to maintain the mitochondrial membrane proton gradient or to be part of the glycerol component of FFAs (Chapter 2). Estimations made from glucose clearance rates in dynamic FDG-PET-CT studies, assuming glucose contributes for 10% and FFAs 90% to BAT metabolism, suggest a metabolic contribution for BAT of ~5% to the basal metabolic rate.²⁷ However, this is a rough estimation since FFA-metabolism was not quantified and most

Figure 2. FDG-PET-CT-scan of morbidly obese subject. The PET-image (A) shows FDG uptake supraclavicular on the right (1) and left (2) side. The combined PET-CT (B) shows activity in adipose tissue (3 and 4), confirming this is BAT.

FFA-lipolysis in BAT is performed intracellular.¹ Therefore, although FDG-PET-CT has facilitated the discovery of functional BAT in man, ideally one would like to use other metabolic tracers to determine the metabolic capacity of BAT. Interestingly, fatty acid uptake in human BAT was recently shown by using the fatty acid tracer ¹⁸FTHA.²⁸ During cold exposure, fatty acid uptake in BAT was significantly higher compared to WAT. The increase in energy expenditure in this report was high (up to 80%), but possibly resulted to a large extent from skeletal muscle shivering due to the strong cold exposure in this study. Another study showed a significant relation between BAT perfusion (as measured by radioactive labelled water (¹⁵O) H₂O) and whole-body energy expenditure,²⁹ confirming the relation found using FDG,² but unfortunately did not allow for determination of the contribution of BAT to NST. The most recent dedicated report measured oxygen consumption in BAT during cold exposure by dynamic oxygen (¹⁵O) PET imaging, a possibly very adequate marker for BAT metabolism.³⁰ Remarkably, the authors conclude the contribution of BAT is only ~1% of the basal metabolic rate, much lower than reported above (~5%). However, the cooling period in this study was very short (20 min), whereas previously it was shown maximal NST is not generated until at least 60-80 minutes of cold exposure.³¹ In summary, no robust estimation of the contribution of BAT to the human energy balance has been shown yet, but prospective studies using adequate tracers and cooling methods can potentially determine the metabolic value of BAT in man.

Stimulating BAT in man

The above-discussed results suggest human BAT recruitment is possible and future studies should point out the effect of stimulating BAT differentiation *in-vivo* and implanting *in-vitro* developed tissue grafts. At this moment the most powerful and reproducible physiological stimulator of BAT is cold exposure, which increases sympathetic nervous system activity that is suggested to β -adrenergically induce mitochondrial uncoupling in BAT (**Chapter 2**). In 1985, it was found that the β -agonist ephedrine could not induce BAT activity in humans.^{32,33} However, the available methods for the assessment of BAT activity were limited to measuring tissue temperature with thermoprobes and therefore this experiment was recently repeated in combination with FDG-PET-CT studies.³⁴ Although increased heart rate, blood pressure and energy expenditure showed the SNS was already clearly activated by a relatively high dose of ephedrine (1.0 mg/kg), BAT remained inactive in subjects that possessed active BAT upon cold exposure.³⁴ A comparable study

using isoprenaline to induce β -adrenergic activation also did not show BAT activity.³⁵ However, a high level of systemic ephedrine infusion (2.5 mg/kg) indeed induced BAT activity in lean but not obese subjects.³⁶ Nonetheless, the BAT activity was low compared to cold-induced BAT activity and the high heart rate (~75 BPM) and increased blood pressure (~160 mm/Hg) that are accompanied with such high levels of ephedrine should not be underestimated. Therefore, possibly mimicking the effect of cold exposure on BAT could be performed by direct peripheral or central nervous stimulation (CNS). Interestingly, in 1989 electrical stimulation of the ventromedial hypothalamus (VMH) in the CNS of rats showed increased norepinephrine (NE) turnover in BAT in rats.³⁷ This suggests electrical stimulus could possibly induce BAT activity in man. In practice, electrical stimulation of the CNS by vagus nerve stimulation (VNS) is performed in refractory epilepsy patients.³⁸ VNS decreases seizure frequency, possibly due to alterations in central nervous system activity.³⁸ However, the exact mechanism of action of VNS is not known yet. Interestingly, VNS treatment has been reported to be associated with weight loss.^{39,40} Animal studies have also shown significant weight loss, associated with a decreased food intake.⁴¹⁻⁴³ Based on this literature, in **Chapter 7** we investigated the effect of VNS on energy expenditure and BAT activity in a treatment stable cohort of refractory epilepsy patients. Strikingly, we found that a short-term deactivation (~4 hours) already significantly decreased energy expenditure, which suggests VNS indeed increases energy expenditure and thus promote weight loss. On top of that, we observed a relation between the change in energy expenditure upon VNS and the change in BAT activity as observed by FDG-PET-CT. Although mean BAT activity did not change significantly upon the short period of deactivation, this shows that the increase in energy expenditure upon VNS could be facilitated by changes in BAT activity. All studied patients with refractory epilepsy showed increased BAT activity upon cold exposure, suggesting potentially high levels of active BAT could be induced by VNS. Therefore, the results described in **Chapter 7** justify a prospective study that observes VNS before and after implantation to unravel the long-term effect of VNS on BAT activity. Subsequently, the effect of VNS could be studied in diseases other than refractory epilepsy associated with low BAT activity. In addition, possibly direct sympathetic stimulation could provide a more direct induction of BAT activity. However, electrical sympathetic nervous system stimulation has never been performed and animal studies should be done first. In addition, other mechanisms to alter BAT activity should be studied, since VNS is an invasive surgical procedure with unknown long-term effects.

Does BAT matter?

In the sections above, we discussed the high metabolism of human BAT under *ex-vivo* conditions and the large differences in human BAT activity *in vivo* in the context of the energy balance. On top of that, this thesis showed VNS increased energy expenditure, which could partly be explained by changes in BAT activity. Together, these results suggest human BAT could be of significant physiological value. To be able to estimate the actual contribution of human BAT to human energy expenditure, several assumptions can be made based on the results of this thesis. In **Chapter 7**, the basal metabolic rate (BMR) decreased significantly when VNS was turned off, with a mean change of 2.2% up to a maximum of 7.8%. After weight loss, BAT-positive morbidly obese subjects showed a mean NST of 13.7% (**Chapter 4**) compared to a mean NST of 2.4% in BAT-negative subjects. Therefore it is very likely that a large part of the ~10% difference in NST between BAT-positive and BAT-negative subjects was induced by active BAT. This again corresponds with the previous suggestion that NST in BAT could increase energy expenditure in man by ~5-20% of BMR.²⁷ However, the static FDG-PET-CT imaging used in this thesis is not able to directly measure the metabolic activity of BAT, but recent dynamic imaging studies using FDG and other metabolic tracers have suggested BAT activity contributes up to 1-4.5% of BMR.^{3,30,44} BMR accounts for 55-65% to the total daily energy expenditure,²⁷ which would make the contribution of BAT ~2.5% (~5% of BMR = ~2.5% of total daily energy expenditure) of the total daily energy expenditure (Figure 3). In summary, the results obtained in this thesis show, in combination with other reports, human BAT is related to human energy expenditure. Future studies should unravel methods to induce high levels of human BAT activity comparable to observations in rodents. In addition, possibly BAT metabolism could be positively altered in pathophysiological conditions that are accompanied with disturbed BAT activity, which would open the door for using BAT activation as a therapeutic tool in medicine.

Concluding remarks and future directions

Since Conrad Gesner in 1551 first described BAT as a tissue type he could not clearly classify as adipose tissue or muscle, the knowledge on BAT dramatically increased throughout the centuries. Especially in the last decades extensive information has emerged from rodent studies that investigated BAT into great detail.¹ In this thesis BAT activity was shown to be strongly and inversely related to obesity, suggesting BAT is important in the pathophysiology of overweight. Moreover, possessing active

BAT was affiliated with a significantly higher level of NST. This is highly interesting, since NST is a relevant component of the human energy balance and thus low NST and low BAT could be risk factors for developing overweight. Furthermore, this thesis shows that high levels of weight loss that radically change body composition are accompanied with an increase in BAT activity. This is important since it shows that low BAT activity is a consequence of obesity rather than a cause. The observed BAT recruitment upon weight loss provides hope for therapeutic options to stimulate BAT activity when low or absent. Therefore, possibly increasing or maintaining sufficient BAT activity could be beneficial for health. Future studies targeting BAT activity in obese patients could point out if stimulating BAT could be used to treat obesity and other diseases associated with low BAT activity. In addition, in this thesis human BAT was studied on a cellular level in order to give more insight in the metabolic value of human BAT. It is very likely the human 'BAT' as studied in **Chapter 6** is 'beige', which provides a strong potential for a therapeutic increase in thermogenic cells in man since culturing and transplantation of beige cells was shown to be successful in rodents. If the successful transplantation of beige depots performed in mice could also be executed in man, this would provide a therapy for

Figure 3. Estimation of the contribution of human BAT to total daily energy expenditure based on the results obtained from this thesis. Possibly, future studies targeting the factors shown in the cloud depicted in the middle could increase the contribution of human BAT.

increasing BAT in man. Finally, this thesis investigated the possibility of stimulating BAT by electrical vagus nerve stimulation (VNS). VNS was shown to significantly increase energy expenditure, which could partly be explained by changes in BAT activity. Further research on the effect of VNS on energy expenditure and BAT activity could point out if VNS has a clinically and therapeutically significant effect on the energy balance.

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Summary of results

Summary of results

The number of people suffering from obesity and its associated comorbidities is rapidly increasing and therefore it is necessary to find a therapy for this global health problem. Here, in the last decade the interest in brown adipose tissue (BAT) has increased since BAT stimulation might serve a role in the treatment of obesity. Naturally, BAT has the ability to produce heat when a subject is exposed to a cold environment. When BAT could be stimulated to produce heat, the body's energy expenditure increases and with a similar energy intake this could lead to weight loss. The activity of human BAT can be visualized by using FDG-PET-CT scans. This is an advanced scanning technique that depicts the uptake of radioactive glucose (FDG; 2-deoxy-2-18F-fluoro-D-glucose) by means of PET-CT scanning (PET-CT; positron-emission-tomography-and-computed-tomography). In this thesis, FDG-PET-CT was used to measure human BAT activity. This thesis describes studies on human BAT activity in three specific groups of patients. The first group of patients was suffering from morbid obesity, an amount of overweight that causes high health risks. Therefore, these patients underwent laparoscopic adjustable gastric banding (LAGB) surgery to restrict food intake and to induce weight loss. BAT activity was measured before and one year after surgery in **Chapter 3 and 4**. In **Chapter 6** we studied the activity of human brown and white adipocytes in a second group of patients that underwent thyroid gland surgery. We here took biopsies of adipose tissue in the areas that previously showed BAT activity on FDG-PET-CT scans. Finally, in **Chapter 7** we studied patients with a vagus nerve stimulator for medically refractory epilepsy. In these patients we observed the effect of vagus nerve stimulation on energy expenditure and BAT activity.

The review in **Chapter 2** showed that BAT is present and active in humans and possibly serves an important physiological role. Moreover, from a clinical point of view the relation between BAT activity and energy expenditure suggests the stimulation of BAT activity might be a new therapeutic target to treat obesity. Furthermore, evidence from *in vivo* and *in vitro* studies showed that altered BAT activity could also be relevant in the pathophysiology of the metabolic syndrome (type 2 diabetes, dyslipidemia, hepatosteatosis), thyroid disease, pheochromocytoma, cancer cachexia, hibernoma and the immune system. Based on the literature available, therapeutic suggestions are made to stimulate BAT by cold, insulin, the central nervous system, bile acids and gut hormones. A summary is provided of molecular studies that show possibilities for the development of *de novo* BAT derived from other types of adipocytes or their progenitors. Finally,

targets for future studies on the role of BAT in disease are discussed. In **Chapter 3** we studied BAT activity in morbidly obese subjects and showed that - in combination with our previous findings in lean subjects - BAT activity was strongly and inversely related to body mass index (BMI) and body fat percentage (BF%). The morbidly obese subjects showed no BAT activity (BAT-negative) or lower BAT activity compared to lean subjects (BAT-positive). There was a significantly lower increase in energy expenditure upon cold exposure (non-shivering thermogenesis (NST)) in BAT-negative compared to BAT-positive subjects. This is important, since it may indicate that BAT is involved in human energy expenditure upon cold exposure. To unravel the observed low BAT activity in obesity in **Chapter 3**, we studied a group of morbidly obese patients selected for bariatric surgery in **Chapter 4**. In this group, we investigated the effect of weight loss induced by bariatric surgery and found an increased BAT activity after weight loss. Strikingly, after weight loss BAT became active in regions that were BAT-negative before weight loss, showing for the first time recruitment of BAT in man. Again, after weight loss BAT-positive subjects had a significantly higher level of NST compared to BAT-negative subjects. These findings BAT could be important in weight management in obesity. Most dedicated studies on BAT activity show BAT becomes active after cold exposure and suggest an important contribution of BAT to non-shivering thermogenesis (NST).⁶ Interestingly, in man NST has also been associated with skeletal muscle mitochondrial uncoupling.⁷ Therefore, we investigated the effect of weight loss induced by bariatric surgery on skeletal muscle metabolism in **Chapter 5**. Our results show the level of skeletal muscle mitochondrial uncoupling remains unaffected by weight loss induced by bariatric surgery. This is in line with previous reports that did not show changes in skeletal muscle metabolism after diet-induced weight loss. However, in **Chapter 5** we showed skeletal muscle mitochondrial ADP-dependent state 3 respiration significantly increased after high levels of weight loss as observed after bariatric surgery. This suggests that, in addition to a beneficial effect on BAT activity and NST, bariatric surgery positively affects skeletal muscle metabolism but not thermogenesis.

Both studies performed in **Chapter 3 and 4** used FDG-PET-CT imaging to non-invasively quantify BAT activity. In **Chapter 6**, we determined cellular oxygen consumption in human adipose tissue obtained from the neck region that is known to be FDG-PET-CT-positive for BAT. Strikingly, in these tissue biopsies we observed an on average three times higher cellular mitochondrial respiration compared to subcutaneous white adipose tissue (WAT) from the same subject, measured under unstimulated conditions. These results show that adipose tissue from the

human BAT region has an increased metabolism even without external stimuli. To therapeutically induce BAT activity, in **Chapter 7** we explored the effect of vagus nerve stimulation on BAT activity in refractory epilepsy patients. Our results show that during active VNS there is a significantly increased energy expenditure. The observed increase in energy expenditure upon VNS was related to the change in BAT activity, suggesting the effect of VNS on energy expenditure is partly due to changes in BAT activity.

In the last years the knowledge on human BAT has strongly expanded. In this thesis, human BAT activity was studied and we observed a relation between BAT activity and obesity. This suggests low BAT activity could be important in the development of obesity. Fortunately, BAT activity increases after weight loss and therefore BAT could possibly be meaningful in weight management and health. Future studies aiming for increasing human BAT activity could possibly provide new options for the treatment of obesity.

Dutch summary

Samenvatting

Bruin vet is bekend sinds 1551 toen de Zwitserse bioloog Conrad Gessner bij het ontleden van marmotten een weefsel vond dat hij “noch als vet, noch als vlees” beschreef. In de eeuwen daarna werd er steeds meer bekend over bruin vet en in 1972 volgde de eerste gedetailleerde anatomische omschrijving van menselijk bruin vet. Bruin vet zit bij volwassenen rond de grote vaten en spieren in de hals, boven het sleutelbeen, langs de wervelkolom en rond de nieren.

Bruin vet verschilt duidelijk van wit vet; de witte vetcel heeft de capaciteit om overvloedige energie vast te houden en bestaat met name uit een grote vacuole (blaas) waarin vetzuren worden opgeslagen voor gebruik in tijden van energietekort. Wanneer er sprake is van een overmatige hoeveelheid wit vet spreken we van overgewicht of obesitas. De bruine vetcel heeft een cytoplasma (celvloeistof) met daarin een grote hoeveelheid mitochondriën (energiecentrale) en bevat meerdere kleine vacuoles (blaasjes) gevuld met vetdruppels. De mitochondriën in bruine vetcellen zijn donker gekleurd en geven bruin vet zijn karakteristieke kleur, die in de praktijk varieert van karamelkleurig tot bruin/donkerrood. Naast een kleurverschil zijn er een aantal belangrijke eigenschappen die bruin vet onderscheiden van wit vet. De functie van bruin vet is het belangrijkste verschil met wit vet. Het is inmiddels aanvaard dat bruin vet energie gebruikt om warmte te produceren, in tegenstelling tot wit vet dat vooral functioneert als opslag van energie. Bruin vet kan die warmte produceren door een uniek proces dat plaats vindt in het mitochondrium van de bruine vetcel. Dit proces heet mitochondriële ontkoppeling, mogelijk gemaakt door het ontkoppelingseiwit 1 (uncoupling protein 1 (UCP1)). Tijdens de mitochondriële ontkoppeling in de bruine vetcel wordt warmte geproduceerd uit energie die in andere cellen gebruikt zou worden als bouwstof voor het lichaam. In de bruine vetcel wordt deze energie niet als bouwstof benut, maar gebruikt om het lichaam te verwarmen in een koude omgeving. Ten slotte is bruin vet ten opzichte van wit vet zeer goed doorbloed en heeft een rijke sympatische zenuwvoorziening.

Omdat overgewicht een steeds grotere rol speelt in onze samenleving is de interesse in het functioneren van bruin vet de laatste jaren toegenomen. Hierbij wordt met name gedacht aan een rol voor bruin vet stimulatie als behandeling van overgewicht. Als bruin vet wordt gestimuleerd tot warmteproductie wordt er meer energie gebruikt door het lichaam; bij een onveranderd eetpatroon zou dat kunnen zorgen voor gewichtsverlies.

De activiteit van bruin vet kan bij de mens zichtbaar worden gemaakt door bij milde koude een FDG-PET-CT scan te maken. FDG-PET-CT is een geavanceerde

scantechniek, waarbij radioactieve glucose (suiker) wordt ingespoten. De stofnaam voor deze radioactieve glucose is FDG (2-deoxy-2-18F-fluoro-D-glucose), waarbij PET-CT (positron-emission-tomography-and-computed-tomography) de scantechniek is om de locatie in het lichaam van het opgenomen FDG af te beelden. Deze techniek hebben we gebruikt om de activiteit van bruin vet te meten. Dit proefschrift bevat studies naar de activiteit van bruin vet in gezonde proefpersonen en patiënten met een bepaald ziektebeeld. In dit proefschrift worden de resultaten beschreven van onderzoeken gedaan bij drie groepen patiënten. De eerste groep patiënten had ernstig overgewicht (morbide obesitas), waarbij er zoveel overgewicht was dat er sprake was van een groot gezondheidsgevaar. Daarom werd bij deze patiënten operatief een maagband geplaatst om te zorgen voor een beperking in voedselinname om af te vallen. De activiteit van bruin vet bij deze patiënten wordt voor de operatie en een jaar na de operatie beschreven in **hoofdstuk 3 en 4**. In **hoofdstuk 6** onderzochten we bij een tweede groep patiënten de activiteit van bruine en witte vetcellen, afgenomen op plekken in de hals waar in eerdere onderzoeken op de FDG-PET-CT scan bruin vet activiteit werd gezien. We hadden bij deze patiënten de mogelijkheid om bipten (stukjes weefsel) van bruin en wit vet te nemen omdat ze een schildklieroperatie ondergingen. Daarbij is het nodig spieren en vet in de hals bloot te leggen om de schildklier goed te kunnen zien; daardoor hadden we de mogelijkheid om bruin en wit vet bipten te nemen in het operatiegebied. Als laatste wordt in **hoofdstuk 7** de derde onderzoeksgroep beschreven met patiënten die een elektrische nervus vagus zenuwstimulator hebben om epilepsie te behandelen. Wij onderzochten bij deze patiënten of zenuwstimulatie bruin vet kan activeren. Bruin vet activiteit werd met FDG-PET-CT scans gemeten nadat de stimulator aan en uit stond.

In dit proefschrift wordt in **hoofdstuk 2** een literatuur overzicht gegeven van eerdere klinisch georiënteerde onderzoeken naar bruin vet in de mens. De belangrijkste uitkomsten zijn: bruin vet activiteit laat een positief verband zien met de toename van energieverbruik in een koude omgeving en bruin vet activiteit is negatief gerelateerd aan van overgewicht. Daarnaast zijn er aanwijzingen dat bruin vet activiteit een rol speelt bij ziektes als type 2 diabetes, vetstofwisselingsstoornissen, vervette lever, schildklierziekten, kanker en afwijkingen in het immuunsysteem. Bruin vet zou mogelijk gestimuleerd worden door langere tijd in een koude omgeving te verblijven, door de zenuwen die bruin vet activeren elektrisch of met medicatie te stimuleren of door hormonen toe te dienen die de activiteit van bruin vet in cel- en dierstudies verhoogden. **Hoofdstuk 3** beschrijft ons onderzoek naar bruin vet activiteit bij obesitas. De resultaten laten zien dat bruin vet activiteit zeer

laag of zelfs afwezig is bij morbide obesitas en bij de patiënten zonder bruin vet activiteit was de warmteproductie in een koude omgeving veel lager. Nadat deze patiënten een maagbandoperatie hadden ondergaan en gemiddeld 36 kilogram slanker werden onderzochten wij opnieuw de bruin vet activiteit in **hoofdstuk 4**. Een jaar na de operatie werd er bij vier van de tien patiënten een toename van bruin vet activiteit gezien. Dit suggereert dat ernstig overgewicht de koude activatie van bruin vet voorkomt. Mogelijk kan dit van invloed zijn op het handhaven van een gezond gewicht. Naast bruin vet kan ook spierweefsel zijn mitochondriën voor ontkoppeling gebruiken. Hierdoor wordt in een koude omgeving in de spieren warmte geproduceerd zonder te rillen. In **hoofdstuk 5** onderzochten wij in dezelfde morbide obese patiënten de functie van mitochondriën in spiervezels voor en na gewichtsverlies door een maagbandoperatie. De resultaten laten zien dat de activiteit van spiermitochondriën toenam na gewichtsverlies. Dit suggereert dat gewichtsverlies door middel van een maagbandoperatie naast een toename in bruin vet activiteit ook de spierstofwisseling kan laten toenemen. In **hoofdstuk 6** werd het zuurstofverbruik van bruine vetcellen vergeleken met witte vetcellen van dezelfde persoon. Deze cellen waren afkomstig uit het halsgebied van patiënten die een schildklieroperatie ondergingen. Er werd weefsel afgenomen uit het gebied dat in de onderzoeken in **hoofdstuk 3 en 4** op de FDG-PET-CT scan bruin vet activiteit liet zien. Omdat er nog eerder onderzoek is gedaan naar de activiteit van menselijke bruin vetcellen moest er een protocol worden ontwikkeld, waarbij gebruik gemaakt werd van de kennis opgedaan uit onderzoek naar menselijk wit vet en bruin vet van dieren. De resultaten die werden verkregen met gebruik van het ontwikkelde protocol laten zien dat het zuurstofverbruik in vetweefsel dat werd afgenomen op de plek die eerder op FDG-PET-CT scans bruin vet activiteit liet zien drie keer hoger is dan in witte vetcellen van dezelfde patiënt. Dit suggereert dat de menselijke bruine vetcel buiten het lichaam, zonder de toevoeging van stoffen die bruin vet activeren, een hoge stofwisseling heeft. **Hoofdstuk 7** beschrijft het effect van zenuwstimulatie (nervus vagus stimulatie) op het energieverbruik en bruin vet activiteit. Er werd gezien dat zenuwstimulatie het energieverbruik verhoogt. Ook was er een relatie tussen de stijging van het energiegebruik en bruin vet activiteit. De stijging in energieverbruik kan dus voor een deel veroorzaakt zijn door bruin vet activatie.

Sinds Conrad Gessner in 1551 bruin vet voor het eerst beschreef is de kennis over bruin vet sterk toegenomen. Met name in de afgelopen jaren is er veel informatie verkregen uit studies in cellen en muismodellen. In dit proefschrift werd bruin vet activiteit onderzocht in de mens en werd er een relatie gezien tussen bruin vet

activiteit en overgewicht. Dit suggereert dat een lage bruin vet activiteit belangrijk kan zijn bij het ontstaan en in stand blijven van overgewicht. Mogelijk is een lage bruin vet activiteit een risicofactor voor het ontwikkelen van overgewicht. Gelukkig is er een toename in bruin vet activiteit na fors gewichtsverlies. Behandelingen gericht op het stimuleren van bruin vet kunnen mogelijk een positieve invloed hebben op het gewicht en de gezondheid. Toekomstige studies gericht op het verhogen van bruin vet activiteit bij mensen met overgewicht kunnen mogelijk nieuwe opties geven voor het behandelen van obesitas.

Dankwoord

Dankwoord

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List of publications

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Grants and awards

2011 Collegium Chirurgicum Neerlandicum Travel Grant

2010 Kootstra Talent Fellowship

Curriculum Vitae

About the author

Guy Vijgen was born on the 13th of June 1985 in Geleen, the Netherlands. In 2003 he finished secondary school at the Graaf Huyn College in Geleen. Consecutively he started his medical training at the Maastricht University Medical Center. After graduating medical school in 2009 he started working in a combined function as a resident in bariatric surgery and PhD-student at the Department of Surgery in the Maastricht University Medical Center under the supervision of dr. N.D. Bouvy, dr. W.D. van Marken Lichtenbelt and prof. dr. P. Schrauwen. After obtaining a Kootstra Talent Fellowship in 2010 he became a fulltime PhD-student at the Department of Human Biology and the Department of Surgery. In July 2012, he started as a resident in the Department of General Surgery at the Sint Franciscus Gasthuis in Rotterdam under supervision of dr. G.H.H. Mannaerts.