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Citation for published version (APA):

van der Lans, A. A., Wierts, R., Vosselman, M. J., Schrauwen, P., Brans, B., & van Marken Lichtenbelt, W. D. (2014). Cold-activated brown adipose tissue in human adults: methodological issues. *American Journal of Physiology-regulatory Integrative and Comparative Physiology*, 307(2), R103-R113. <https://doi.org/10.1152/ajpregu.00021.2014>

Document status and date:

Published: 15/07/2014

DOI:

[10.1152/ajpregu.00021.2014](https://doi.org/10.1152/ajpregu.00021.2014)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

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

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Submitted 14 January 2014; accepted in final form 20 May 2014

van der Lans AJ, Wierds R, Vosselman MJ, Schrauwen P, Brans B, van Marken Lichtenbelt WD. Cold-activated brown adipose tissue in human adults: methodological issues. *Am J Physiol Regul Integr Comp Physiol* 307: R103–R113, 2014. First published May 28, 2014; doi:10.1152/ajpregu.00021.2014.—The relevance of functional brown adipose tissue (BAT) depots in human adults was undisputedly proven approximately seven years ago. Here we give an overview of all dedicated studies that were published on cold-induced BAT activity in adult humans that appeared since then. Different cooling protocols and imaging techniques to determine BAT activity are reviewed. BAT activation can be achieved by means of air- or water-cooling protocols. The most promising approach is individualized cooling, during which subjects are studied at the lowest temperature for nonshivering condition, probably revealing maximal nonshivering thermogenesis. The highest BAT prevalence (i.e., close to 100%) is observed using the individualized cooling protocol. Currently, the most widely used technique to study the metabolic activity of BAT is deoxy-2-[¹⁸F]fluoro-D-glucose ([¹⁸F]FDG)-positron emission tomography/computed tomography (PET/CT) imaging. Dynamic imaging provides quantitative information about glucose uptake rates, whereas static imaging reflects overall BAT glucose uptake, localization, and distribution. In general, standardized uptake values (SUV) are used to quantify BAT activity. An accurate determination of total BAT volume is hampered by the limited spatial resolution of the PET image, leading to spillover. Different research groups use different SUV threshold values, which make it difficult to directly compare BAT activity levels between studies. Another issue is the comparison of [¹⁸F]FDG uptake in BAT with respect to other tissues or upon with baseline values. This comparison can be performed by using the “fixed volume” methodology. Finally, the potential use of other relatively noninvasive methods to quantify BAT, like magnetic resonance imaging or thermography, is discussed.

brown adipose tissue; energy expenditure; PET/CT imaging; cooling; humans

BASED ON HISTOLOGICAL CRITERIA Heaton et al. (15) showed already in 1972 that there is a wide distribution of brown adipose tissue (BAT) in humans. In the field of nuclear medicine the presence of active BAT was shown by among others, Cohade et al. (8); an evaluation of 359 positron emission tomography/computed tomography (PET/CT) examinations, after the administration of 2-deoxy-2-[¹⁸F]fluoro-D-glucose ([¹⁸F]FDG) showed in 4% of all patients [¹⁸F]FDG uptake in fat tissue. This technique enables the study of metabolic active tissues on a glucose substrate. This fat tissue was called “USA-fat” of unknown etiology, which might cause confusion with tumor and/or metastases. These findings indicated that BAT is present in at least a significant fraction of human adults; however, its physiological importance required further investigation (30).

The relevance of BAT in human adults was eventually demonstrated in 2009. Dedicated studies using cold exposure

revealed active BAT depots in 46–100% of young healthy subjects (38, 50, 55). This showed that BAT in human adults, like in rodents and human infants, is linked to the cold-induced metabolic response. Besides the visual data (PET/CT imaging), the three groups confirmed the presence of BAT with positive staining for uncoupling protein-1 (UCP1) in biopsies from the supraclavicular region. UCP-1 is a hallmark of BAT as it uncouples the electron transport chain and thereby heat is produced (3). Since the discovery of the relevance of BAT depots in human adults, at least 20 dedicated studies have confirmed that human adults possess active BAT depots (Fig. 1 summarizes all dedicated studies on cold-activated BAT quantified by PET/CT imaging in humans). Cold-induced human BAT is usually found in the neck, in acromial-clavicular, supraclavicular, para-aortic, axillary, paravertebral, and in perirenal depots (Fig. 2).

Based on cell-specific markers UCP1-positive cells from human adipose tissue depots can be classified as “classical” (42) or “inducible” (beige/brite) (44). Seale et al. (42) showed in 2008 that “classical” BAT cells derive from a myf5+, a muscle-like lineage, whereas “inducible” cells derive from a myf5-cell lineage, a fat-like lineage (42). “Classical” BAT

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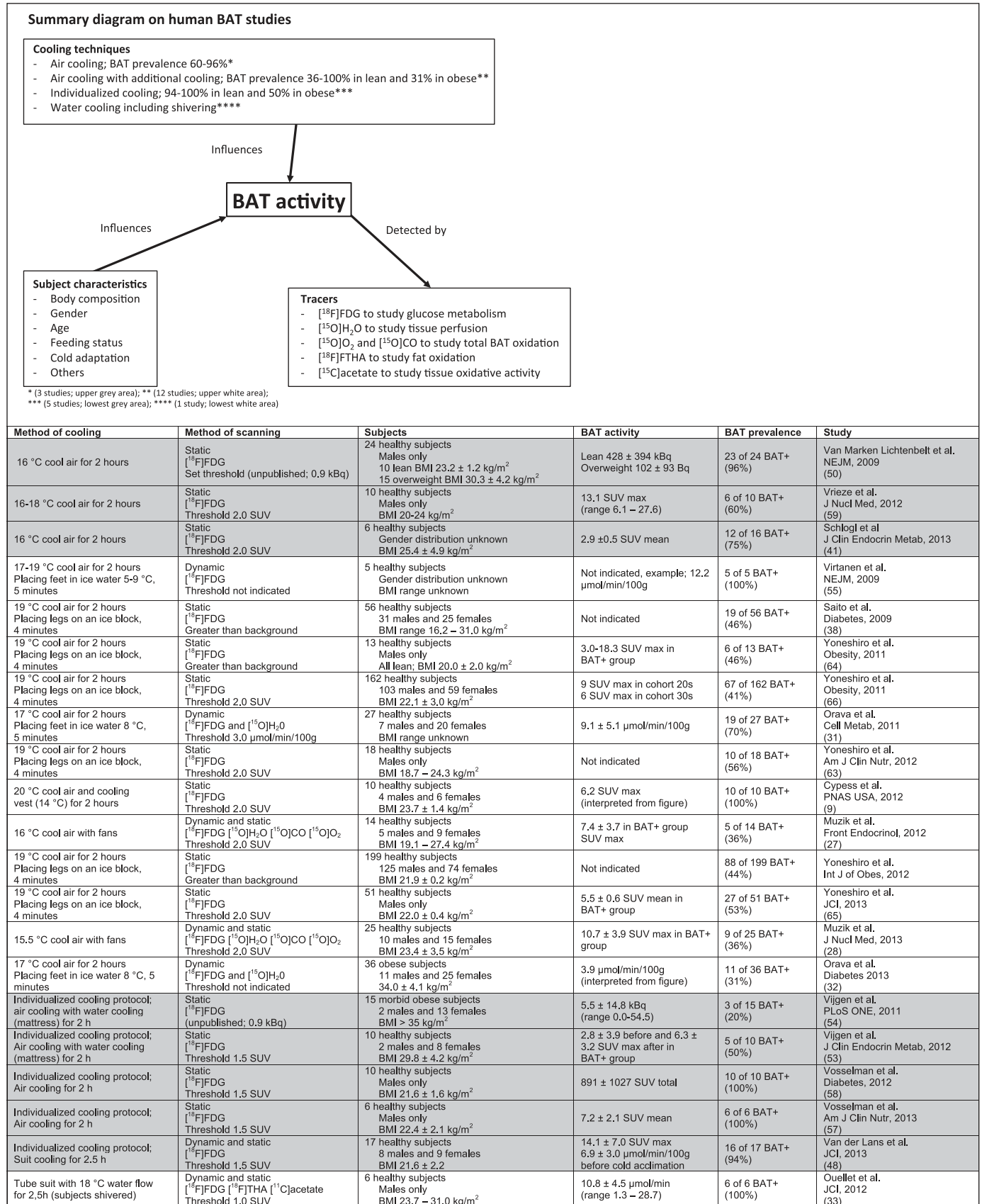


Fig. 1. All studies published since 2009 that studied cold-activated BAT in humans with PET/CT imaging are shown and the diagram summarizes the studies. The use of an individualized cooling protocol to activate BAT is preferred due to the highest probability to detect BAT. The effect of body composition,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

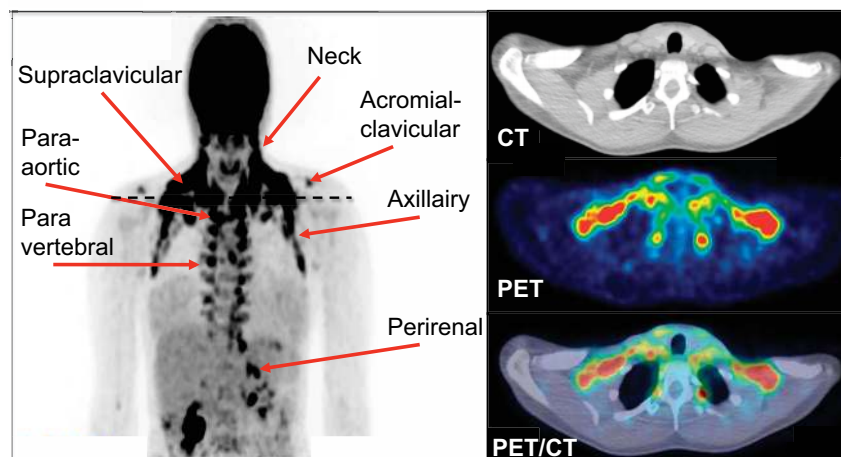


Fig. 2. Uptake of deoxy-2- $[^{18}\text{F}]$ fluoro-D-glucose ($[^{18}\text{F}]$ FDG) in brown adipose tissue (BAT). *Left*: $[^{18}\text{F}]$ FDG-positron electromagnetic tomography (PET) image of a subject showing BAT activity. Arrows indicate depots where human BAT is usually found. The dashed line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

cells express high levels of UCP1 under basal conditions, whereas clusters of “inducible” cells can only be recognized in white adipose tissue (WAT) after stimulation (14). Their co-expression of UCP1 makes it likely they both reveal the same functional role in adaptive thermogenesis. For an extensive review on the development and function of both “classical” and “inducible” fat, we refer to Harms and Seale (14). It is known that human depots show a mixed composition of both “classical” and “inducible” cells (11, 20, 24). Current imaging techniques cannot distinguish between the two BAT cell types. Therefore, classification is currently only possible based on biopsy material. Irrespective of their classification, only depots with increased glucose uptake compared with surrounding tissue are visualized with $[^{18}\text{F}]$ FDG-PET/CT imaging. $[^{18}\text{F}]$ FDG is the most commonly used radiotracer to quantify human BAT activity. But other tracers, like labeled fatty acids (33) to study fatty acid metabolism or labeled water (31) to study BAT perfusion, are used as well. To quantify human BAT activity without the use of radioactive tracers, scanning techniques like magnetic resonance imaging (MRI) (18, 51) and thermography (23, 47) are also explored.

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

BAT Activation

Cold exposure activates the sympathetic nervous system to secrete norepinephrine (NE), which activates brown fat cells (Fig. 3) (3). NE binds to the β_3 -receptor on the cell surface, after which intracellular signaling cascades lead to the degradation of triglycerides into free fatty acids (FFA) (5). FFA interact with UCP1 and this leads to respiration uncoupled from ATP synthesis (30). The energy from the combustion of substrates is therefore directly released as heat. Mitochondria

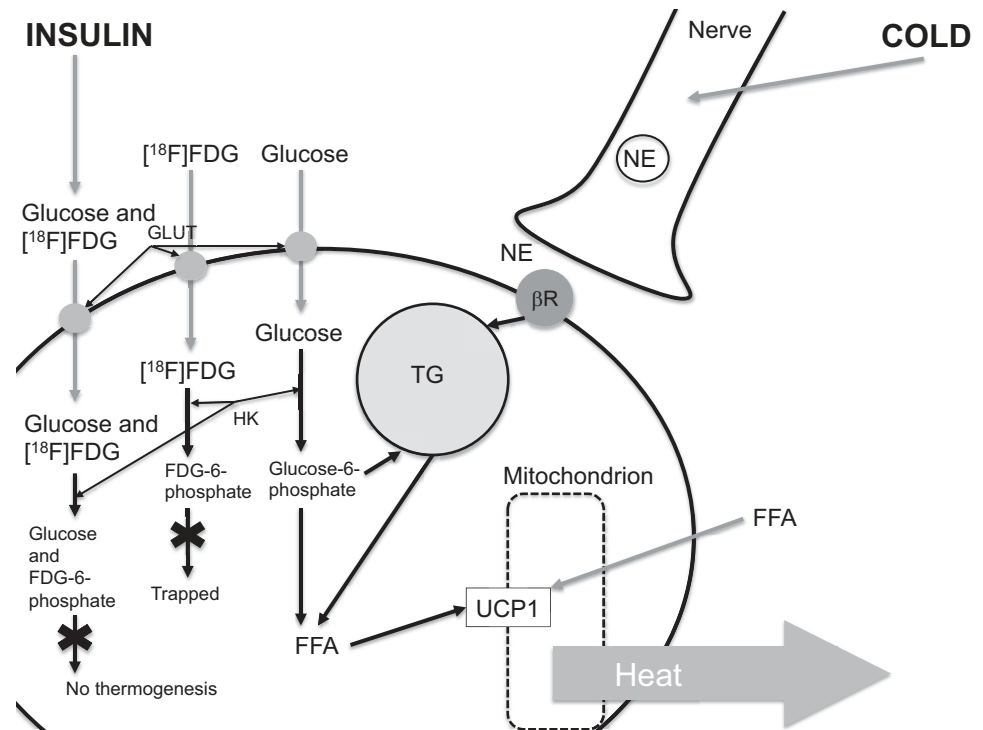
in BAT oxidize apart from internal stored FFA, glucose and FFA from the circulation. Besides cold, insulin might also stimulate glucose metabolism in BAT. BAT is a highly insulin-sensitive tissue, possibly due to a high expression of GLUT4 (3). Insulin infusion resulted in an increased glucose uptake in BAT, comparable with uptake in skeletal muscle (31). However, no increased perfusion was found. This indicates that insulin under these circumstances does not increase thermogenesis. Therefore, the authors state that insulin is not a significant player in the NE-driven cold activation of BAT. On the contrary, cold exposure decreases plasma insulin concentrations (31). The activation of BAT by cold and the uptake of glucose and $[^{18}\text{F}]$ FDG are schematically described in Fig. 3.

Tissues that take up glucose are visualized with $[^{18}\text{F}]$ FDG-PET imaging. $[^{18}\text{F}]$ FDG is a glucose analog, therefore, $[^{18}\text{F}]$ FDG-PET imaging provides information about glucose uptake and phosphorylation. After phosphorylation by hexokinase, FDG-6-phosphate is trapped in the cell (Fig. 3). Radioactivity values represent tracer uptake values. As illustrated in Fig. 3, glucose uptake is increased in activated BAT and therefore active BAT depots are visible as hotspots on the PET image. Thus BAT activity values reported in several articles represent glucose uptake values. The question remains if a glucose tracer is an adequate tool to study BAT oxidation. Recent work from Shabalina et al. (43) showed that “brite” cells utilize glucose from the circulation for oxidation, confirming increased glucose metabolism in activated cells. However, the proportion of glucose oxidation to total BAT oxidation is currently unknown in humans.

Mild cold exposure may induce nonshivering thermogenesis (NST). Rodent studies revealed that BAT is the main contributor to nonshivering thermogenesis (3). Therefore, most studies investigate BAT in humans by cold activation. So far, cold is the strongest and most widely tested activator of BAT in humans, for other activators we refer to the review of Harms and Seale (14). Since BAT is involved in NST, the studies using cold exposure are most representative to obtain insight on BAT prevalence and activation.

Retrospective studies showed that active BAT depots could be detected in $\sim 6\%$ of adult population (10, 34). These studies included images of patients that are scanned for diagnostic

Fig. 3. BAT activation and thermogenesis. Upon cold exposure brown fat cells are stimulated via norepinephrine (NE) released from the sympathetic nervous system. NE binds to the β_3 -receptor and intracellular signaling processes lead to degradation of triglycerides (TG) into free fatty acids (FFA). FFA interacts with uncoupling protein-1 (UCP1), which leads to respiration uncoupled from ATP synthesis. All energy from the combustion of substrate is therefore directly released as heat. Besides intracellular FFA released from TG, mitochondria in the brown fat cell also oxidize glucose and FFAs from the circulation. Insulin also results in glucose and [^{18}F]FDG uptake via GLUT4 activation, but no heat is produced. Both [^{18}F]FDG and glucose are taken up by members of the glucose transporter family (GLUT). After phosphorylation by hexokinase (HK), FDG-6-phosphate cannot be further metabolized and is trapped in the cell and is visualized by PET imaging. Adapted from Refs. 4, 5, 30, and 31.



reasons in which no active cooling protocol is applied. Dedicated studies that investigated human BAT using cooling protocols reveal a BAT prevalence of 20–31% in morbidly obese subjects (32, 53) and 40–100% (9, 27, 38, 50, 55, 58, 63, 64) in lean healthy subjects. Although the variation is still high, these results indicate that the prevalence of BAT in humans is much higher than previously appreciated. Besides individual variation, group (e.g., age, gender), and differences in ethnicity, the cooling protocols themselves may affect the probability to detect BAT and thus its presence and activity, as described below.

Shivering and nonshivering thermogenesis. Upon cold exposure internal body temperature can be maintained by means of increased heat production [shivering thermogenesis (ST) and/or NST] by reducing heat loss via vasoconstriction in the extremities or a combination of both (49). When assessing the contribution of BAT to energy expenditure (EE; heat production), it is necessary to distinguish between ST and NST. Rodent studies revealed that ST is due to involuntary muscle contractions, whereas for NST the extra heat is mainly produced in BAT. Whether this applies to humans is currently unknown. NST is defined as the “thermogenesis that replaces shivering” (3), which is totally UCP dependent (12). When rodents are adapted to cold environmental temperatures, shivering gradually diminishes while EE remains elevated, illustrating the increased NST.

Human BAT metabolism should be studied in the absence of shivering. Otherwise, active skeletal muscles (shivering is due to muscle contractions) would take up the [^{18}F]FDG, leaving less tracer available for other tissues. Moreover, no distinction can be made between ST and NST. To this end, most studies check the absence of shivering by visual inspection and/or a subjective measure (by asking the subject), sometimes combined with electromyography (EMG). However, not all re-

search groups use absence of shivering, as illustrated by Ouellet et al. (33), who reported that $1.6 \pm 0.5\%$ (range 1.1 to 2.4% of maximal voluntary contraction) shivering occurred, which makes it difficult to distinguish NST from ST. However, it should be noted that other studies have relied on subjective measurements, and it is our personal experience that subjects not always indicate shivering correctly. The use of EMG to measure the onset of shivering is complicated by individual variation in the muscles used for shivering. Therefore, we advise the combination of asking subjects, visual inspection, and EMG to check for the absence shivering during cooling protocols where maximal NST is studied.

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

BODY COMPOSITION. A thicker subcutaneous fat layer results in a higher insulation. As a consequence, obese subjects have a later onset of shivering (54). Indeed, after a “fixed” cooling protocol (16°C for 48 h) obese subjects show a blunted NST (62). It is possible that the cold stimulus given in this protocol was not enough to induce maximal NST in the obese subjects. It is likely that the use of an individualized cooling protocol is required to induce NST in obese subjects. Upon weight loss environmental temperatures for maximal NST condition increases, moreover an increased BAT activity was found (53), and in this study an individualized cooling protocol was used.

GENDER. On average, lower temperatures are needed to induce shivering in males compared with females. The higher BAT prevalence in females reported in retrospective studies (2, 10, 36) can therefore be biased by indoor temperatures in hospitals, which may be low enough to induce NST in females but not in males. Indeed the thermoneutral zone, a range of environmental temperatures that does not result in regulatory changes in evaporative heat loss or changes in metabolic heat

production, is in general higher for females compared with males (22). Only a few dedicated studies investigated cold-activated BAT activity in both females and males. Interestingly, two studies showed similar levels of cold-activated BAT for both sexes (9, 48). On the other hand, Orava et al. (32) and Muzik et al. (27, 28) reported, after fixed cooling protocols (see Fig. 1 for description of cooling protocols used), higher BAT prevalence in females (Orava et al.: 87% females in BAT+ group, 58% females in BAT- group, Muzik et al.: BAT+ group 8F/1M, BAT- group 7F/8M). However, both groups used a "fixed" cooling protocol, therefore, it is possible that the cold stimulus in both studies was too mild for the male subjects.

AGE. Based on [^{18}F]FDG-PET/CT imaging there is only one dedicated study that showed that elderly show a gradual decrease in BAT activity levels with increasing age (65). Pfannenbergl et al. (36) showed in a retrospective study that BAT activity declines with increasing age. Both studies are in line with a decline in cell-specific markers for BAT with increasing age found in biopsy material (15, 67). A decrease in BAT with increasing age is in line with the blunted or absence of NST in elderly found in other studies that investigated EE and not BAT activity (46, 52). With respect to the decreased BAT activity in elderly, Nedergaard et al. (29) speculate that ageing results in lower sex hormones levels, which might lower BAT activity and favors expansion of WAT.

FEEDING STATUS. Because of the lower BAT activity found in subjects with higher body mass index, it is suggested that BAT is involved in body weight regulation. Normally, human BAT is investigated in fasted subjects. Cold-exposed healthy male subjects showed lower BAT activity in the fed state compared with the fasted state (59). Intake of a high-caloric, high-carbohydrate meal showed that BAT is active in the postprandial phase; however, the contribution of BAT to diet-induced thermogenesis requires further elucidation (57). No BAT activation was found upon a 24-h 200% overfeeding protocol (41). However, it is possible that BAT activity was underestimated due a diet-induced change in availability of the tracer. The contribution of BAT on diet-induced thermogenesis and body weight regulation remains further elucidation.

COLD ADAPTATION. Huttunen et al. (19) already showed in 1981 that necropsies taken from neck arteries and pericardium have a higher occurrence of BAT in outdoor workers compared with control subjects. Indeed, recent [^{18}F]FDG-PET studies confirmed that cold acclimation increases BAT activity in parallel with an increased NST (48, 60).

Besides the above-mentioned interindividual variations, BAT presence and activity and NST vary within groups. This is illustrated by the wide range in BAT activity and NST, both in morbidly obese subjects and lean healthy men (33, 54) (BAT activity: 0–54.5 kBq, EE: 35.5–51.1 J/s and 1.3–28.7 $\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{ g}^{-1}$, EE 2.3–4.6 kcal/min, respectively). The reason for this variation is unclear yet possible explanations could be the degree of cold adaptation or genetic variation in for example UCP-1 (66). Besides the above-mentioned individual differences, the cooling protocols themselves have an influence on NST and BAT activity levels found, as described below.

Fixed cooling protocols. The first studies on cold-activated BAT in human adults used "fixed" cooling protocols to activate BAT. Cold exposure consisted of placing subjects in an envi-

ronmental temperature of 16°C for 2 h (50) or a slightly higher environmental temperature (19°C) with leg cooling in ice water (38, 55, 64). BAT prevalence in these studies ranged from 46% to 100%. Other groups used in addition air fans (28) or a cooling vest (9). The use of "fixed" cooling protocols with an ambient temperature below 17°C results in highest probability to detect BAT in lean subjects. The probability to detect active BAT depots is decreased when subjects are placed in an ambient temperature of 19–20°C. In those cases additional air fans did not increase the probability (28), whereas the use of a cooling vest resulted in 100% BAT detection (9).

Individualized cooling protocols. When using a fixed low temperature protocol an obese subject will experience less intensive cold than a lean subject. This is due to extra subcutaneous adipose tissue and lower surface-to-volume ratio, which results in a higher insulation (39). This was confirmed in pilot experiments where obese subjects had a lower onset temperature of shivering (54). From that moment on our research group uses individualized cooling protocols. Subjects are cooled until shivering and then temperature is set slightly (1–2°C) above the temperature that causes the onset of shivering. With this protocol BAT activity is measured during maximal nonshivering thermogenesis, most likely representing maximal BAT activity. We found that by means of an individualized cooling protocol the probability to detect BAT is nearly 100% (94–100%) in lean young subjects (48, 57, 58).

Air versus water cooling. BAT activation can be achieved by means of air cooling (cold room or tent) and/or water cooling (water-perfused suit). The latter has the advantage that it is suitable for simultaneous cooling and imaging inside the PET/CT scanner. Absolute temperatures for both thermoneutral and mild cold condition differ between both techniques because of the differences in thermal properties of the media. For example, environmental temperature for a thermoneutral measurement in healthy young men (dressed in sweatpants, T-shirt, and socks; 0.58 clothing-insulation value) positioned on an air-permeable stretcher was $24.3 \pm 0.2^\circ\text{C}$, whereas when wrapped in a water-perfused suit thermoneutral condition was obtained with a suit temperature (average of inlet and outlet) of $30.0 \pm 2.3^\circ\text{C}$. Stable nonshivering conditions were reached with an environmental temperature of $14.3 \pm 1.1^\circ\text{C}$ during air cooling, whereas the average suit temperature was $24.6 \pm 0.9^\circ\text{C}$. Nevertheless, these apparent different temperatures result in similar metabolic conditions, which is explained by the greater conductive heat loss during water cooling. When using individualized cooling protocols, both air- and water-cooling protocols result in high BAT detection (94–100%) in lean subjects (48, 57, 58).

In summary, temperatures for both thermoneutral and mild cold conditions differ among people and also depend on the cooling method used. This might result in differences in BAT activation and thus in the BAT activity values found. To study subjects in similar metabolic conditions (maximal NST), we advise the use of individualized cooling protocols. When obese subjects are studied, air cooling down to 9°C is not sufficient to reach maximal NST conditions (54). Therefore, for obese subjects we recommend the use of water-cooling protocols. Moreover it is important, when the contribution of BAT on NST is investigated, to avoid ST completely. In Fig. 1 all dedicated studies on cold-activated BAT by means of PET/CT imaging are summarized.

BAT Measurements

Nowadays, the assessment of [^{18}F]FDG uptake is the most commonly used method to visualize BAT activity in human adults. As indicated in Fig. 3, [^{18}F]FDG cannot be metabolized after phosphorylation by hexokinase and is therefore trapped inside the cell (30). Visualization of [^{18}F]FDG uptake therefore provides a measure of glucose uptake in the depots.

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

Dynamic imaging. In contrast to static imaging protocols, where cooling, tracer administration, and uptake take place before the PET/CT scan, during dynamic scanning tracer administration is performed at start of PET acquisition. During dynamic acquisition, tracer distribution is obtained as a function of time, which results in time-activity curves (TACs). Therefore, cold stimulation is required during the actual scan. This can be achieved by cooling with a water-perfused suit (33, 48). Another reported method used to cool subjects during the scan is placement of the leg in ice water (31, 32, 55). The first method ensures whole body cooling that strictly regulates skin temperatures. It can be speculated that leg cooling results in less intense cold stimulation since only local body parts are actively cooled and that this might result in less BAT activation. This is consistent with the lower BAT prevalence (70%) reported by Orava et al. (31) compared with the higher BAT prevalence found by van der Lans et al. and Ouellet et al., 94% and 100%, respectively (33, 48). From the TACs, a quantitative measure of glucose uptake rate (expressed in $\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{ g}^{-1}$) can be determined using Patlak curve fitting (35) using a lumped constant of 1.14 (56). The lumped constant accounts for the difference between [^{18}F]FDG and glucose with respect to tissue uptake and phosphorylation kinetics. Our research group performed both a dynamic and static PET scan in 16 young, healthy volunteers and we found a good correlation between glucose uptake rates and SUV mean values (Fig. 4, $R^2 = 0.77$; $P < 0.001$). BAT activity values obtained with static imaging (expressed in SUV) provide semiquantitative information on activity levels and represent overall tracer accumulation. A decreased total BAT activity can be observed when BAT activity decreases or when tracer availability is

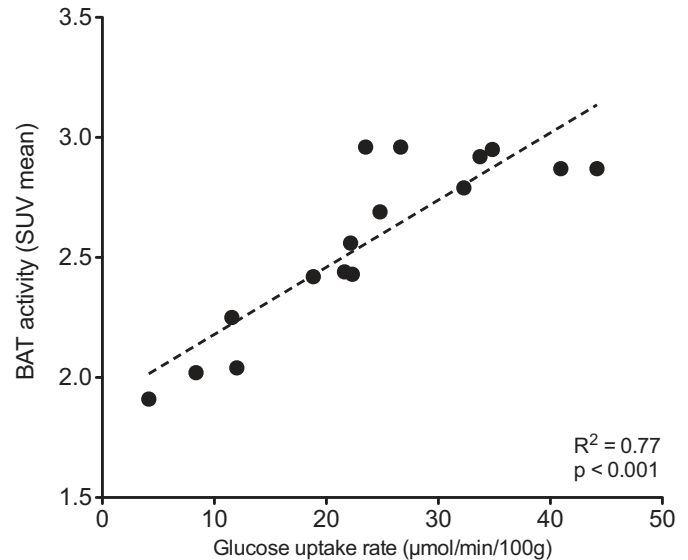


Fig. 4. Correlation between glucose uptake rate in supraclavicular depot and standardized uptake values (SUV) mean. A significant correlation was found between cold-activated BAT activity values obtained by means of dynamic scanning (glucose uptake rate) and static scanning (SUV mean). 16 young, healthy subjects were included, of which were 8 females and 8 males, age 23 ± 3.2 yr, and body mass index 21.6 ± 2.2 . Data was obtained from BAT-positive subjects before cold acclimation (48).

affected due to competitive tracer uptake by other tissue. For example, after a meal, $\sim 80\%$ of glucose is taken up by muscle tissue (45). This influences [^{18}F]FDG uptake in BAT and other tissues. Our research group showed a decreased [^{18}F]FDG uptake in the brain, which is anticipated to have a constant metabolism after a meal (57). By means of dynamic imaging a quantification of glucose uptake can be obtained due to the use of an arterial input function. This means that changes in tracer availability do not affect glucose metabolic rate values (21). So, depending on the research question, either a dynamic or a static scanning protocol should be performed, or both.

Thresholds. To quantify BAT activity, accurate segmentation of BAT depots is essential. When a tracer is distributed homogeneously in a certain volume, a SUV of 1.0 is found. Since glucose metabolism is increased in metabolic active BAT, tracer uptake is elevated ($\text{SUV} > 1.0$) compared with surrounding tissue. Tissues that take up relatively high levels of glucose have high SUVs, e.g., brain has a SUV of > 5.0 (57). WAT (38), which is relatively metabolic inactive, has a $\text{SUV} < 0.5$ (58). Maximal SUV values for BAT range from 3.0 to 28.0 (59, 64). Therefore, BAT segmentation on [^{18}F]FDG-PET images is often performed using SUV thresholding techniques. Our research group uses a SUV threshold of 1.5 (58), whereas other research groups use SUV values of 1.0 SUV (33) or 2.0 SUV (9, 28, 48, 59, 63). Obviously, with a lower threshold more activity and volume is included and assigned to BAT, in part, explaining the different outcomes between different research groups.

Not only SUV thresholding provides information on BAT activity, CT Hounsfield units (HU) might give additional information. The HU of a certain tissue is defined as the difference in attenuation coefficient of this tissue normalized to water. Water has a HU of 0. Tissues denser than water have a positive HU and tissues less dense than water have a negative

HU. Ahmadi et al. (1) found that based on CT HU a distinction between BAT and WAT can be made. Voxels with HU -10 to -87 can be assessed as BAT, whereas voxels with HU -88 to -190 can be quantified as WAT. However, Ouellet et al. (33) showed that cold exposure increases HU of active BAT, indicating that active BAT oxidizes internally stored fatty acids. If HU can be used to assess BAT activity, the technique of Ahmadi et al. needs validation with PET imaging.

BAT volume and activity. Despite the large number of dedicated studies investigating cold-activated BAT in humans, BAT volume levels are reported in only four studies. This is possibly due to the many difficulties concerning volume determination, which are discussed in this section. van Marken Lichtenbelt et al. (50) report an average BAT volume of 130 ± 98 ml in lean subjects and 77 ± 69 ml in overweight/obese subjects. Another study from our research group found an increase of BAT volume from 14 ± 22 to 85 ± 84 ml after gastric bypass-induced weight loss in obese subjects (53). Ouellet et al. (33) found an average BAT volume of 168 ± 56 ml, with a range of 31 to 329 ml. Ouellet et al. used both a SUV threshold (as mentioned above), in combination with a CT threshold. The latter ensures inclusion of fat tissue only. Values within -200 and -10 HU on the CT scan represent fat tissue. A much higher BAT volume is reported by van der Lans et al. ["detectable" BAT volume 665 ± 451 ml (48)]; in this study BAT volume determination was PET based (only SUV threshold was used, CT HU were only used to verify the presence of fat tissue at the location of increased [^{18}F]FDG uptake). Because of the limited spatial resolution of the PET image, spillover of the PET signal from the high active BAT regions into the surrounding tissue occurs. Therefore, defining BAT regions based on an absolute, fixed SUV threshold only results in relatively large BAT volumes, this is visualized in Fig. 5. Thresholding based on SUV only can lead to BAT volume overestimation, whereas thresholding based on SUV and HU can on the other hand lead to an underestimation of total BAT activity. It is evident that when both information on BAT activity and BAT volume are needed, different analyzing techniques need to be used. We suggest the use of a SUV-based threshold technique to study total BAT activity. How-

ever, when BAT volume is needed the use of HU is an alternative (1). Additionally, the use of MRI can provide additional information on BAT anatomy (see *Alternative Imaging Techniques*).

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

Fixed volume methodology. Upon an intervention, subjects are scanned repeatedly, and therefore comparisons need to be made between BAT activity values before and after the intervention. When a thresholding technique is used, problems arise when volumes of interest change. For example, "detectable" BAT volume after intervention increases due to addition of areas at the border of the region of interest with SUV values now reaching levels just above the threshold. Therefore, total BAT activity will increase only slightly, whereas volume changes can be relatively large. As a result BAT activity values expressed per volume ($\mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{ g}^{-1}$) remain equal or can even decrease. To overcome this issue, we propose the use of a methodology using fixed volumes. In the BAT depot confirmed as brown fat tissue based on both CT and PET images, a box with fixed dimensions ($8 \times 8 \times 8 = 152\text{ mm}^3$) is centered at SUV max location of the PET image (57), without applying a threshold. In our opinion, when compared with SUV max, this technique is less affected by artifacts present in the image, since the SUV max value is based on the value of a single voxel. For example, cold acclimation significantly increased BAT activity (48); however, with the "threshold" technique no increase in glucose uptake rate for total supraclavicular BAT was found [before: 6.9 ± 3.0 ; after: $7.6 \pm 2.5\ \mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{ g}^{-1}$ ($P > 0.05$) (48)]. On the contrary, using the "fixed volume" methodology a significant increase in glucose uptake rate was found [before: 22.5 ± 12.5 ; after: $33.5 \pm 16.8\ \mu\text{mol}\cdot\text{min}^{-1}\cdot 100\text{ g}^{-1}$ ($P < 0.05$)]. The "fixed volume" methodology can also be used to compare activity values between different tissues (e.g., white adipose tissue,

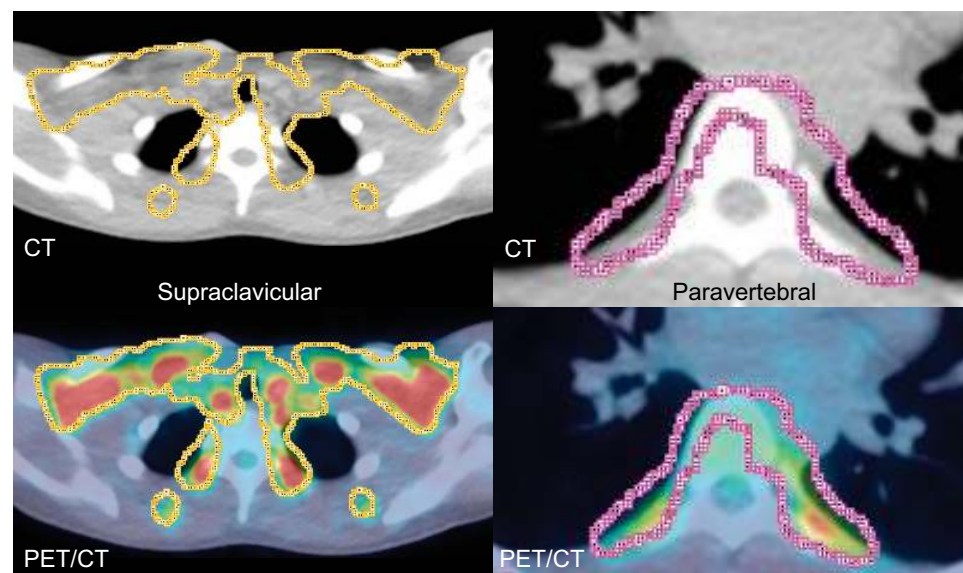


Fig. 5. Transversal PET/CT fusion (*top*) and CT (*bottom*) slices of the supraclavicular (*left*) and paravertebral (*right*) region demonstrating [^{18}F]FDG uptake. Volumes of Interest drawn are PET based (threshold 1.5 SUV), visually demonstrating that PET-based "detectable" BAT volume overestimates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

liver, brain, skeletal muscle) (57). For these tissues, the location of the “fixed volume” can be chosen using anatomical information based on the CT image.

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

Alternative Imaging Techniques

By means of [^{18}F]FDG-PET/CT imaging, tissue glucose uptake (rate) is measured. However, this tracer does not provide information about glucose oxidation or glycogen storage or brake down. With respect to the oxidative metabolism of active BAT, internal stores of fatty acids are likely important, which are also not measured by the glucose tracer. It is well known that BAT oxidizes fatty acids as well. A study in animals showed that fatty acid oxidation amounts to 90% of total oxidation (25). Thus studying BAT activity with a glucose tracer does not provide information on total BAT oxidative metabolism. The relative contribution of glucose and fat oxidation on total BAT oxidative metabolism is currently unknown in humans. In that perspective, fatty acid tracers provide important additional information. Ouellet et al. (33) used the fatty acid tracer [^{18}F]THA ([^{18}F]fluoro-thiaheptadecanoic acid) to study fat oxidation and [^{11}C]acetate to determine tissue oxidative activity. The results were compared with [^{18}F]FDG scans to study fat and glucose oxidation in cold-activated human BAT (33). The fatty acid tracers cannot identify the contribution of oxidation of internal stored fats, so the actual relative contribution of glucose and fatty acids on total BAT oxidation still awaits further investigation. The tracers [^{15}O]CO and [^{15}O]O₂ provide information on total BAT oxidation (obtained via oxygen consumption), and with the tracer [^{15}O]H₂O additional information on BAT perfusion is obtained, therefore, Muzik et al. (27, 28) compared these tracers with [^{18}F]FDG scans. These studies showed that at least part of the glucose transported into BAT undergoes oxidative metabolism. The labeled water tracer [^{15}O]H₂O was also used by Orava et al. to define BAT perfusion (31, 32). These studies showed that upon cold exposure BAT perfusion doubles and that cold-stimulated glucose uptake rate and perfusion are correlated. Moreover, cold-activated BAT perfusion correlated with total energy expenditure. This confirms that BAT is highly vascularized and that BAT plays a role in human metabolism.

Nowadays, the labeled glucose tracer [^{18}F]FDG is the most commonly used tracer to study BAT in human adults. Additional information on fatty acid and total BAT oxidation is obtained by the use of fatty acid and labeled oxygen tracers. It is currently unknown which proportion of total BAT oxidation is derived from glucose combustion. Therefore, further mechanistic studies on BAT oxidation are warranted.

All above-mentioned studies used radioactive tracers to investigate BAT in humans. Because of the ionizing radiation, the number of scans for each participant is limited. There is an urgent need for a technique that investigates BAT without

radiation penalty. In that perspective, several research groups explored the feasibility of MRI for BAT quantification. BAT has higher mitochondrial content, multiple intracellular lipid droplets, and a greater vascularization than WAT. This results in different magnetic properties of BAT and WAT. These differences can be used by several MRI-scanning techniques to distinguish BAT from WAT. Mice studies revealed fat/water-signal differences (17, 24, 51) and different relaxation times (6, 13). Moreover, functional MRI techniques can potentially be used to study blood perfusion (40) differences. All above-mentioned studies support the capability of MRI to detect and anatomically characterize human BAT in vivo. Hu et al. (18) identified BAT in a human infant by means of chemical-shift MRI and Holstila et al. (16) used DUAL spectral presaturation inversion recovery (SPIR) MR to identify BAT in three adult humans. Our research group explored the feasibility of MRI to study BAT in 11 human adults, and we showed a lower fat fraction in BAT depots compared with subcutaneous WAT depots (62.5 ± 7 vs. $81.5 \pm 5.4\%$, $P < 0.05$). Additionally, dynamic T2*-imaging revealed signal fluctuations that were sensitive to BAT activation (51). The results so far are promising but need further development before they can be applied.

The main function of BAT is heat production. Therefore, measuring the temperature in BAT could be a valid method to define whether it is active or not. BAT temperature can be assessed by placing temperature probes into BAT (7, 26) or by thermography of the skin overlaying BAT depots (61). Because of practical considerations, the first method is difficult to implement in humans. The use of infrared thermography was explored by several research groups (23, 37, 47). All showed a larger increase at BAT sites (supraclavicular, neck, and upper back) compared with non-BAT sites (mediastinum) upon cold stimulation (23, 47) or after ephedrine injection (37) in human adults. Therefore, the authors state that thermal imaging can be used to quantify thermogenesis in BAT regions. However, it should be noted that this technique measures skin temperature and not heat production in BAT. The heat produced in BAT is used to maintain core body temperature. Consequently, most of the produced heat will be transported by perfusion to core body sites. Some of the heat might nevertheless be transported toward the skin. The question is whether the change in supraclavicular skin temperature due to this heat leak is representative for BAT heat production. Symonds et al. (47) performed pilot experiments and no increase in supraclavicular skin temperature was found at higher environmental temperatures. In that perspective, thermography could potentially be used to show BAT activity in a qualitative way.

Another noninvasive method to measure BAT thermogenesis could be the use of near-infrared spectroscopy to measure regional blood oxygen saturation. Light from the near-infrared spectrum penetrates through several centimeters tissue and thereby oxygenated hemoglobin can be measured noninvasively (28). A significant correlation between blood oxygen saturation of BAT compared with muscle (not of BAT compared with abdominal WAT) and metabolic rate of oxygen fluxes (obtained by dynamic PET scanning) was found only in subjects with activated BAT. Therefore, the authors suggest that near-infrared spectroscopy can be used in subjects with activated BAT to measure oxidative metabolism noninvasively (28). However, no correlation was reported with the absolute blood oxygen saturation in BAT, only the correlation with the

ratio of BAT to muscle is reported. Thus the ratio of BAT to muscle oxygen saturation might be used as an alternative method of BAT thermogenesis.

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

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

Perspectives and Significance

There is an urgent need for standardization of BAT activating protocols and analyzing techniques. Based on the above-mentioned studies, we advise the use of individualized cooling protocols to activate BAT and to maximally activate BAT in all subjects. In particular, when obese subjects are studied, the use of water cooling is recommended. To compare BAT activity values using [¹⁸F]FDG-PET/CT imaging between the different research groups, the same threshold values and analyzing techniques should be used. Therefore, we propose that all researchers studying BAT in humans report the results based on the using a threshold of 1.5 SUV. Besides using a threshold of 1.5 SUV, every research group, of course, can add their own BAT analyses. This would be beneficial to compare activity values between the several research groups investigating human BAT. To compare activity values upon an intervention and between different tissues we recommend the use of the “fixed volume” technique.

GRANTS

This work is financed by The Netherlands Organization for Scientific Research (TOP 91209037 to W. D. van Marken Lichtenbelt) and by the EU FP7 project DIABAT (HEALTH-F2-2011-278373 to W. D. van Marken Lichtenbelt).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.A.v.d.L. prepared figures; A.A.v.d.L. and W.D.v.M.L. drafted manuscript; A.A.v.d.L., R.W., M.J.V., P.S., B.B., and W.D.v.M.L. edited and revised manuscript; A.A.v.d.L., R.W., M.J.V., P.S., B.B., and W.D.v.M.L. approved final version of manuscript.

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