Final Report Summary - SEE BAT (STIMULATION OF ENERGY EXPENDITURE AND BROWN ADIPOSE TISSUE IN HUMANS)

In this project we investigated energy expenditure in relation to obesity. Obesity and its associated metabolic complications, such as type II diabetes, form a major and so far unresolved global health burden. Treatment strategies for obesity aim to alter the balance between energy intake and energy expenditure in such a manner that weight loss is induced, thereby preventing the development of diabetes and its complications.

The most frequently used strategy is to decrease energy intake by diets and/or appetite decreasing medication, but these often fail. An attractive option would therefore be to induce weight loss and or prevent weight regain by increasing energy expenditure. Conversely, increased energy expenditure does not seem to result in as strong a compensatory response in terms of increased food intake, making strategies that increase energy expenditure an attractive method to combat obesity. However treatments that aimed to increase energy expenditure so far came with unwanted side effects (e.g. treatment with thyroid hormone and dinitrophenol). Brown adipose tissue (BAT) has been known for a long time to be the only tissue with the capacity to safely generate heat at the expense of energy by uncoupling fatty acid oxidation from adenosine triphosphate (ATP) generation, thus increasing metabolic rate (1). Therefore BAT is a thermogenic tissue, that safely converts produces heat via the unique uncoupling protein (UCP) 1. Thus activation of BAT has the potential to induce weight loss. Recently there has been a re-awakened interest in brown adipose tissue (BAT) in humans due to the finding that BAT has been shown to be present and active in adult humans. Prospective studies, using fluorodeoxyglucose positron-emission tomography scans (FDG-PET CT), showed that cold exposure activates BAT in lean, healthy individuals (2, 3). Moreover, very recent biopsy studies have shown the presence of BAT in humans (4, 5). This revived attention for brown adipose tissue (BAT) in humans may lead to therapies that increase energy expenditure.
FDG-PET-scans showed BAT signal in predilection areas such as the supraclavicular region (2, 3). The chance of detecting active BAT on non-temperature controlled FDG-PET CT was shown to be predicted by multiple factors such as young age, female sex and low body mass index (BMI). The use of beta-blockers was associated with a lower chance of detecting BAT presence on FDG-PET scans, compatible with adrenergic mediated activation of BAT. Importantly, the outdoor temperature at time of scan is negatively associated with the chance of detecting active BAT, indicating the effect of cold on BAT activation.

Activation of brown adipose tissue in humans
Cold exposure activates BAT generally as follows (1): when skin and mucous membranes of the gastrointestinal tract are exposed to cold, activation of transient receptor potential channels (TRPs, i.e. TRPA1) in nerve endings follows (6). The TRP cation channel superfamily is a diverse family of 28 cation channels that have varied physiological functions, including thermal and other sensations (7). The cold signal then reaches the hypothalamus via afferent neuronal pathways. Via activation of certain cerebral nuclei, a sympathetic response is produced that reaches end organs such as BAT, skin and skeletal muscle (figure 1) (8). This may be mediated by the extreme-cold receptor transient receptor potential channel member A1 (TRPA1) that is present in the skin and mucosal lining of the gastro intestinal tract and activated, as suggested by its name, cold exposure (4). The important role of TRPA1 in human cold mediated perception and signalling is highlighted by patients with the Familial Episodic Pain Syndrome. These patients harbour a gain-of-function mutation in the TRPA1 gene (chromosome 8q12–8q13) and experience pain to various stressors

Project information

SEE BAT

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including cold (9).

The transient receptor potential cation channel subfamily V member 1 (TRPV1) is another TRP (7). TRPV1 agonists have an dual effect: i.e., the stimulation of heat loss (sweating) and heat production (thermogenesis) (10). This makes it an interesting TRP to study next to TRPA1. TRPV1 is present in the central nervous system and many other tissues. Activation of the above receptors then affects the beta 3 adrenergic receptor via the autonomic nervous system finally increasing BAT activity (12, 13).
In addition cold exposure has been shown to increase glucose and lipid availability for thermogenic purposes (14). Where as animal studies showed increased secretion and hydrolysis of triglycerides (TG), it was shown in humans that acute exposure to cold increases plasma and interstitial glycerol concentration with also enhanced non-oxidative disposal of fatty acids (i.e., TG/FFA cycling) (14). Finally, cold exposure (depending on its magnitude) induces vasoconstriction and increases blood pressure (15). In fact, mild cold exposure vasoconstriction is also a thermoregulatory mechanism to decrease heat loss (15).
BAT may very well have an impact on energy balance. It has been suggested that the lack of BAT activation in FDG-PET CT imaging in obese subjects may be partly responsible for the weight gain in these obese individuals (2). However, it is difficult to study the contribution of BAT activity to energy expenditure in different settings due to a lack of appropriate imaging techniques. Even more, a recent biopsy study showed that BAT may be present in more individuals who do not reveal BAT on FDG-PET CT (4); this finding shows the importance of including such individuals in studies that investigate the BAT induced increase in energy expenditure.

Currently, imaging of BAT in humans relies on PET-CT, which has several limitations. High radiation exposure prevents sequential imaging in longitudinal studies, and rendering analyzing effect of interventions on BAT activity impossible. Also BAT needs to be activated to be imaged by PET-CT (so far done by cold exposure). However, BAT could be present, not being visible on FDG- PET CT, depending on its abundance (4).

These limitations indicate the need for an alternative way of imaging BAT and several lines of basic research show promising results. Infrared thermography is potentially a good method to image activity of BAT, since it visualizes the primary product of brown adipose tissue: heat. Moreover, it is a simple and fast method with relatively low costs and can be used in the same individual as often as necessary (not dependent on a large scanner and allowing rapid analysis of BAT activity under different circumstances). Infrared thermography during cold exposure shows that the skin overlying the largest BAT depot in adult humans (the supraclavicular area) remains warmer compared to a mediastinal control region that does not contain BAT (16). Moreover, differences in skin temperature of the subcutaneous (overlying BAT) and sternal area correlate with the change in energy expenditure after cold exposure (pilot data, see figure 2).

Objectives
In this proposal we want to develop BAT activation protocols using different thermogenic stimuli. The research objectives of this proposal are as follows:

A) To assess differential effects of BAT activating stimuli (cold, activation of TRPA1 and/or TRPV1) on thermogenic response, BAT, glucose/lipid metabolism and cardiovascular changes in healthy lean individuals.

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During the first year of the fellowship we have made great progress already in the project by fortifying our pilot work and executing large parts of Objective A (see objectives A and C under “APPROPRIATENESS OF RESEARCH METHODOLOGY AND APPROACH”. In this year we have strengthened the pilot data. Here we have included extra numbers of subjects. By doing so we were not only able to optimize the cold exposure protocol (see below), but also the thermogenic nutrient protocol (see

below).Hereafter we have started the study in which healthy volunteers were studied on three occasions up to now (in thermoneutrality, cold and thermogenic nutrient exposure). See full details below in the section “APPROPRIATENESS OF RESEARCH METHODOLOGY AND APPROACH”.
In appendix A to this report the results are depicted. Since the mustard (in capsules) unfortunately was not effective enough we decided in the second year to repeat the measurements after ingestion of mustard without capsules to expose the mucosal lining of the mouth and throat to the AITC. Although this led to a mild stress response (see cortisol data in appendix 1), we found no increase in energy expenditure. Therefore we did not repeat the experiment in obese subjects. However, we investigated energy expenditure and its variability in our volunteers that were studied 4 times and in a weight loss cohort.

B) To test energy expenditure inducing protocols (derived from objective A) in obese/insulin resistant subjects with respect to thermogenic response, BAT, glucose/lipid metabolism and cardiovascular changes.

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C) Develop infrared thermography to be used repetitively in humans for accurate measurement of the thermogenic response of BAT.

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As for objective A, we have initially strengthened the pilot data. Here we have included extra numbers of subjects. By doing so we were again able to optimize the infrared thermal imaging protocol.

Here after we have included (see details below) subjects that have underwent infrared thermal imaging in thermoneutrality, cold and thermogenic nutrient exposure. An important point is that we have embarked on a MRI based imaging technique in contrast to FDG-PET CT scans to visualize brown adipose tissue. We have started this since an MRI technique enables nonradiation visualisation that can be used under multiple stimulative conditions (repetitive) without harmful side effects for the subject. However, the noise to signal ratio for this latter technique was not strong enough. The results on thermography are depicted in appendix 1.

Interdisciplinary/multidisciplinary aspects of the proposal
The current proposal has a multidisciplinary approach with the participation of basic and clinical researchers within the Addenbrooke’s Hospital (University of Cambridge). The proposal is founded on the results of BAT oriented research that has been conducted during recent years within the Institute of Metabolic Science (Addenbrooke’s Hospital). The studies will be conducted in the Wellcome Trust Clinical Research Facility (WTCRF) within the Addenbrooke’s Centre for Clinical Investigation (ACCI). WTCRF staff members are already familiar with techniques described in the proposals as they are also used in other ongoing projects within the Facility. The institute of metabolic science has developed a track record in the brown adipose tissue research and we aim to translate this acquired knowledge to the clinical phase combining a metabolic (patho)physiological approach and imaging.

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We have been very successful in implementing the study protocol in the Welcome Trust Clinical Research Facility (CRF) of the Institute of Metabolic Science -

Addenbrookes Hopspital in Cambridge. Here we work closely together with different professionals.
There has been extensive interaction with the nursing staff in the day to day clinical care for the subjects both during the afternoon/evening/night before the experiment as well as during the experiment.
Moreover the applicant has been able to work closely together with the clinical scientists of the CRF who are very experienced and involved in the collection of indirect calorimetry, heart rate variability, body composition, core body temperature data etc. This has enabled the applicant to increase his knowledge and capability to carry out the studies depicted in the protocol.
Multiple research assistants have been contributing to the projects. Additionally (see also under “B2 TRAINING”), the applicant has been able to interact with different scientist within the Institute of Metabolic Science besides Professor A. Vidal-Puig.
All in all, the applicant has been well embedded and supported in both the IMS and the CRF.

APPROPRIATENESS OF RESEARCH METHODOLOGY AND APPROACH

General aim: Investigate activation of thermogenesis in BAT in humans using cold and activation of TRP activators (TRPA1/TRPV1) in vivo in humans following three objectives.
Hypothesis: Activation of BAT via stimulation of transient receptor potential channels in humans results in increased energy expenditure.

Objective A) To assess differential effects of BAT activating stimuli (cold, activation of TRPA1 and/or TRPV1) on thermogenic response, BAT, glucose/lipid metabolism and cardiovascular changes in healthy lean individuals.
As discussed above, cold, activation of TRPA1 and/or TRPV1 have been shown (in animal studies) to activate energy expenditure via BAT (6). Allyl-isothiocyanate (AITC) is potentially useful since it causes BAT activation via activation of the extreme-cold receptor transient receptor potential channel member A1 (TRPA1) (6). AITC mimics physiological cold and is found in common food ingredients such as mustard and wasabi, making it a safe method to stimulate brown adipose tissue. Using indirect calorimetry (figure 3) we found an increase in energy expenditure of 10.3% in response to mild cold exposure (16ºC) and in increase in energy expenditure of 7.7% 30 minutes after AITC ingestion (10 grams Colmans Mustard containing 10.6 mg AITC). In objective A we will study the thermogenic response to control and AITC (10 gr. mustard) under thermoneutrality (24ºC) in 8 subjects during 4,5 hours.

As a proof of involvement of the autonomic nervous system, experiments will be repeated after beta-blockade (propanolol 20 mg with the highest affinity for the beta 3 receptor involved in BAT activation). To assess to what extend the effects of AITC actually mimic the cold effect, studies will be repeated under mild cold exposure (18ºC).
Volunteers will be studied in the Wellcome Trust Clinical Research Facility of the Addenbrooke’s Hospital. This facility has metabolic rooms that can a- be acclimatised to the requested temperature and b- analyse resting energy expenditure via indirect calorimetry. Subjects come the evening before the experiment for admission and have a standardised meal. The following morning (0800hrs) they will be acclimatised at 24ºC during 2 hours. Hereafter they will have either control, AITC (10 grams Colmans Mustard), AITC plus propranolol or cold exposure and measurements for another 2,5 hours. Skin temperature will be measured using infrared thermography as described above and via direct skin thermography. Core body temperature will be continuously measured by temperature pill (encapsulated thermometer that is orally ingested) and the signal will be detected by a pocket-receiver. Blood samples will be drawn at baseline and each 30 minutes during the experiment AITC concentrations (analysed via high performance liquid chromatography (HPLC), stress hormones (e.g. catecholamines/cortisol) and substrates (glucose, free fatty acids). Glucose and lipid turnover will be assessed by

state of the art stable isotope techniques (not radioactive) using primed, continuous infusion of [6,6-2H2]glucose (glucose turnover and clearance rates) and [U-13C]palmitate (palmitate turnover and oxidation (13CO2 breath analyses). Cardiovascular measurements include bloodpressure, ECG tracing and heart rate variability (Actigraph). Finally the presence of imagable BAT will be confirmed by FDG-PET CT under cold circumstances.
To further dissect the responses to cold and AITC (TRPA1 stimulation) we will investigate stimulation of the thermogenic reponse by activating TRPV1. Indeed TRPV1 stimulation by capsaicin or a capsinoid (nonpungent capsaicin-related substances found in all tested variants of the Capsicum genus of plants, red pepper) increases energy expenditure and weightloss in humans (11). Experiments will be repeated (same study design as described for AITC) using capsinoids. In short: Capsicum Anuum L. [Solanacae (pepper fruit)] variety CH-19 Sweet will be used to extract capsinoid oil as described earlier (11). This will consist of capsiate, dihydrocapsiate, and nordihydrocapsiate and the respective concentrations will be analyzed a priori by HPLC (11).

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-Fortification pilot data
First, we have fortified the pilot data. Here we have included extra numbers of subjects. We have paid attention to the optimal control of our cold rooms as well as the optimal placement, calibration and setup of the calorimeters. We have set out to do so to minimize variation in data obtained during indirect calorimetry.
Also, in these studies we have investigated the different ways of AITC (mustard) administration. Since it is difficult to administer mustard blinded (e.g. taste) we have investigated the differential effects of mustard in capsules or by a spoon. No differential effects seemed to be present. By carrying out these additional pilot studies we were not only able to optimize the cold exposure protocol but also to optimize the thermogenic nutrient protocol.

-Study (control/cold/mustard)
After the pilot data we have started the official study. We have included 11 subjects whom (except for one) have been studied four times under the following conditions (balanced assigned): control (thermoneutrality, 24 degrees Celsius), mild cold (18 degrees Celsius) and the addition of a thermogenic nutrient (10 grams of Colemans mustard, packed in veggi capsules or simly on a spoon, containing AITC). Under control and cold conditions, subjects did receive a placebo that was matched for the composition of macronutrients and calories.

During the study visits of the subject (three visits taking two consecutive days per study visit) we have assessed the following parameters to characterise subjects metabolically (metabolical phenotyping) in order to place the results in an adequate clinical context.

Study day 1 - First, after entrance on the CRF (and subsequent informed consent), body composition was assessed by using both DXA and BODPOD techniques. In order to measure oxidative capacity, an exercise test was done to assess VO2max after taking an electrocardiogram.
In the early evening, subjects received a standardised (calories) meal as well as chest electrodes for the measurements of interbeat intervals (IBI, heart rate) to assess heart rate variability as a measure of para- and orthosympathetic tone. Patients entered the metabolic room at 2000 hours in the evening (at thermoneutrality) after which the collection of calorimetric data started. Here subjects were normally active (reading, television or computer) but no exercise was permitted. A period of ~8 hours of sleep has been custom (2300-0700 hours).

Study day 2 - After being woken up early morning (when the collection of overnight whole room calorimetric data was finished), bedside indirect calorimetry was performed after the administration for infrared imaging labels, skin and core body temperature sensors. Normal observations for blood pressure, tympanic

temperature, pulse rate, weight and visual analogue scores for temperature and hunger scores were also taken. Infrared thermography was used to measure skin temperature over the reference and brown adipose tissue predilection areas. Additionally blood samples were taken for different metabolites, hormones and other lab tests.
After these baseline measurements, subjects were balanced assigned to control (thermoneutrality, 24 degrees Celsius), mild cold (18 degrees Celsius), a thermogenic nutrient (10 grams of Colemans mustard in capsules in thermoneutrality) or a thermogenic nutrient (10 grams of Colemans mustard on a spoon in thermoneutrality). This “thermogenic intervention ”lasted for two and a half hours during which all measurements were repeated each 30 minutes. After we finished tha mustard in capsules, the thermogenic effect did not prove to be significant. Therefore we investigated the response when mustard mustard was in direct contact with the mucosa of the mouth and throat. Direct contact of the AITC (mustard) with the mucosa might be of importance for the thermogenic response.
To assess the effects of the thermogenic interventions on appetite and satiety, subjects received an ad lib meal during which the amount and speed of eating (appetite and satiety respectively) were assessed using a universal eating monitor. Unfortunately, we were not able in the end to find significant effects of the mustard either given as capsule or on a spoon. Although this is a negative result, the performed studies provided us with valuable biological data on the physiology of energy expenditure and cold exposure.

Analysis of study data – At this moment, all collected data during the study days have been analysed and interpreted by the applicant. All plasma samples that have been stored so far are analysed by the laboratory of clinical chemistry. We expect to publish the forthcoming results in the end of the summer or early autumn. Here we plan to publish results on the cold exposure and the thermogenic food intervention separately. For this final report, the data have been added to this final report as appendix (incorporating combined data for cold, thermogenic nutrient and the infrared thermal imaging).
Again, we were not able in the end to find significant effects of the mustard but the performed studies provided us with valuable biological data on the physiology of energy expenditure and cold exposure and hence will be published.

Objective B) To test energy expenditure inducing protocols (derived from objective A) in obese/insulin resistant subjects with respect to thermogenic response, BAT, glucose/lipid metabolism and cardiovascular changes.
When we have met the aims of objective A we will proceed to test the optimal protocol in 12 obese (but not diabetic) subjects (BMI 25-30 kg/m2, HbA1c IFCC <48 mmol/mol, no comorbidity) and 12 glucose tolerant matched (BMI, body composition and age) controls. Lean subjects will participate in thermoneutrality experiments only but obese subjects will participate in three experiments: a- placebo, b- optimised BAT activation protocol (derived from objective A) under thermoneutrality and c- under cold circumstances. Experiments will be performed as described in objective A.

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Objective B would be carried out according to the results of objective A. However since these results were negative (no effect of mustard on energy expenditure) we have amended the objective B. Here we were able to determine the response of weight loss with respect to energy expenditure, substrate oxidation and various clinical and scientific parameters. To this end we analysed 60 obese subjects that were allocated to weight loss via either diet, exercise of sibutramine therapy. In this study we were able to identify patterns of weight loss in relation to these various parameters. This was an objective that was not originally planned for, but proved to be very fruitful.
During this weight loss trial subjects were followed up intensively, but here we report on the visits at the start and after 4 and 12 weeks of the study. We assessed body composition, weight, energy expenditure (including substrate oxidation) and various plasma parameters such as acylcarnitines that are indicative of substrate oxidation

and energy expenditure. IN this project we were primarily interested to analyse certain plasma metabolites (acylcarnitines) in relation to the clinical parameters described above and energy epxenditure in particular.
Here we found that these plasma metabolites (that are thought to reflect substrate oxidation at the mitochondrial level) correlate with plasma fatty acids and not so much with rates of energy expenditure or substrate oxidation. For clarity and full description and nature of this project we refer to the appendix where we have added the draft of the manuscript that will be offered for publication in due time.

Objective C) Develop infrared thermography to be used repetitively in humans for accurate measurement of the thermogenic response of BAT.
Within objectives A en B, we develop a method that permits repetitive use in short experiments/periods (i.e. no radiation exposure). During the experiments described objects A and B, infrared thermography will be performed continuously. Skin temperature wil be assessed in three pre-defined areas: supraclavicular region (overlying BAT), sternal region (overlying bone, used as reference for temperature range) and skin temperature of the hand (that is prone to vasocontriction). As shown above, our pilot data show good correlation of the difference in supraclavicular-sternal temperature with the rise in energy expenditure during cold. Finally, we will validate the infrared thermography against FDG-PET CT scanning that currently is the golden standard to asses BAT activation.

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Fortification pilot data
In the first year, we have fortified the pilot data. Here we have included extra numbers of subjects. We have paid attention to the optimal control of the camera with respect to distance to the object, air humidity and temperature. Also we have assessed different confounding effects on thermal imaging (such as changing posture e.g. reclining versus standing position).
Additionally, we have performed all imaging studies in the AITC (mustard) intervention as well to assess effects on brown adipose tissue predilection areas and skin vasoconstriction.

Study (control/cold/mustard) performed in year 1 and 2.
Here, we have started the official study as outlined in objective A (and its Mid-term report). As described above, we have included 11 subjects whom all have been studied four times under control, cold and AITC conditions. Here I refer to the description that has been detailed under objective A. And the first part of the appendix added to this report.
An important point is that we have embarked on a MRI based imaging technique in contrast to FDG-PET CT scans to visualize brown adipose tissue. We have started this since an MRI technique enables nonradiation visualisation that can be used under multiple stimulative conditions (repetitive) without side effects for the subject. However, technical reasons made it difficult to detect changed in brown adipose tissue temperature and this led to termination of this project.

Please see also the attachments to this report.

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