Chronic ephedrine administration decreases brown adipose tissue activity in a randomised controlled human trial: implications for obesity

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Abstract

Aim

Activation of Brown Adipose Tissue (BAT) may have therapeutic potential to combat obesity. Acute treatment of mice with sympathomimetic drugs activates BAT thermogenesis, and chronic treatment increases BAT thermogenic capacity. It has previously been demonstrated that human BAT is acutely responsive to oral administration of the sympathomimetic ephedrine. This study aimed to determine whether chronic treatment with ephedrine could mimic adaptive thermogenesis in humans.

Methods

Twenty-three healthy young men were recruited via general advertisement from Melbourne, Australia to participate in a randomised, placebo-controlled, parallel group trial. Recruited individuals were unmedicated, non-smokers, physically inactive and had no prior history of either cardiovascular disease, insulin resistance or diabetes. They were allocated to either a placebo (*n*=11; 22±2 years, 23±2 kg/m^2) or 1.5 mg/kg/day ephedrine (active group; n=12, age 23±1 years, BMI 24±1 kg/m^2) treatment group for twenty-eight days. Body composition was measured before and after the intervention by dual energy x-ray absorptiometry. BAT activity, measured before and after the twenty-eight day intervention period, via ^{18}F-fluorodeoxyglucose positron emission tomography-computed tomography (^{18}F-FDG PET/CT) in response to a single dose of 2.5mg/kg ephedrine, was the primary outcome measure.

Results

After twenty-eight days of treatment, the active treatment lost significantly more total body fat (placebo 1.1± 0.3 kg, ephedrine -0.9 ± 0.5kg; $p<0.01$) and visceral adipose tissue (placebo 6.4 ± 19.1g, ephedrine -134 ± 43g; $p<0.01$), with no change in lean mass or bone mineral content, compared with the placebo group. In response to acute ephedrine, BAT activity (change in mean standardised uptake value: placebo -3 ± 7 %, ephedrine -22 ± 6%) and the increase in systolic blood pressure were significantly reduced ($p<0.05$) in the active group compared with placebo.

Conclusion

Chronic ephedrine treatment reduced body fat content, however, it was independent of an increase in BAT activity. Rather, chronic ephedrine treatment suppressed BAT glucose disposal, suggesting that chronic ephedrine treatment decreased, rather than increased BAT activity.

Student declaration

I hereby certify that all material presented in this thesis is my original work unless otherwise specified, towards a Master of Philosophy Degree. No other person's work has been used without due acknowledgement. I have received assistance in the preparation of the thesis from Dr Andrew Carey and Prof. Bronwyn Kingwell.

I duly acknowledge individuals who made a direct contribution to the writing of this thesis

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Preface

The respective recognition of writers and collaborators to the publication included within this thesis is as follows:

Renata Pajtak, Andrew Carey and Bronwyn Kingwell we primarily responsible for the conception of the study and design of the protocol and experiments, conducting statistical analyses and writing of the manuscript.

Nina Eikelis, Gavin W. Lambert, Melissa F. Formosa, Bruce Van Every, David A. Bertovic, Mitchell J. Anderson, Victor Kalff, Stephen J. Duffy, and Martin H. Cherk contributed to aspects of study design, data collection, data analysis and manuscript review.

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1. Introduction

1.1 Obesity

1.1.1 A growing concern

The Australian population comprises one of the highest rates of overweight and obese persons in the developed world, with the prevalence of obesity increasing by 19% between 1995 to 2018.[1] A staggering 63.4% of Australian adults were overweight or obese in 2014-2015.[2] Australia is not alone in its growing problem. The United States accounts for a third of the world's obesity, with regions of Europe, Western Pacific and parts of Africa and Asia closely trailing.[3] This places obesity as one of the leading causes of preventable death worldwide.[4] Each year 2.8 million people die as a result of being overweight or obese, and an estimated 35.8 million global disability-adjusted life years (DALYs) are caused as a direct result.[3] Also, of concern, are the associated rise of obesity in children and adolescents and its persistence into adulthood and subsequent generations. The current prevalence of obese children and adolescents rose to 14 million in 2016, more than ten times higher than in 1975, and a further 213 million children and adolescents were classified as overweight.[5-7]

Obesity results from a consistent imbalance between energy intake and expenditure. This imbalance is accentuated by an environment which enables easy access to high energy-content food in combination with a sedentary lifestyle. A commonly utilised scaling system to indicate an individual's body habitus, and therefore the risk of developing health complications, is the body mass index (BMI). Calculated as [weight (kg)/height (m^2)], individuals are categorized relative to population-based norms (**Table 1**).

Independent of BMI classification, individuals who accumulate mesenteric and visceral adipose tissue have been noted to have the highest risk of obesity-related complications.[8, 9] Accumulation of adipocytes surrounding the viscera is considered an independent risk factor for disease processes occurring in other important metabolic regulatory organs such as the liver, skeletal muscle, immune cells and the pancreas.[10]

Table 1. Body Mass Index Scale kg/m^2	
BMI range (kg/m^2)	**Classification**
<18.5	Underweight
18.5-24.9	Normal range
25-29.9	Overweight
30-34.9	Obesity I
35-39.9	Obesity II
40	Obesity III

Excessive weight gain in any form, however, is a major contributing factor to increased morbidity and mortality associated with type 2 diabetes, coronary heart disease, stroke, hypertension, sleep apnoea, depression, metabolic syndrome and a variety of cancers (**Figure 1**).[11] In Australia alone, obesity-related expenses are costing the healthcare system $56 billion dollars annually with a projected rise to $87.7 billion in additional direct and indirect costs to Australia accumulated across the ten years to 2025.[12]

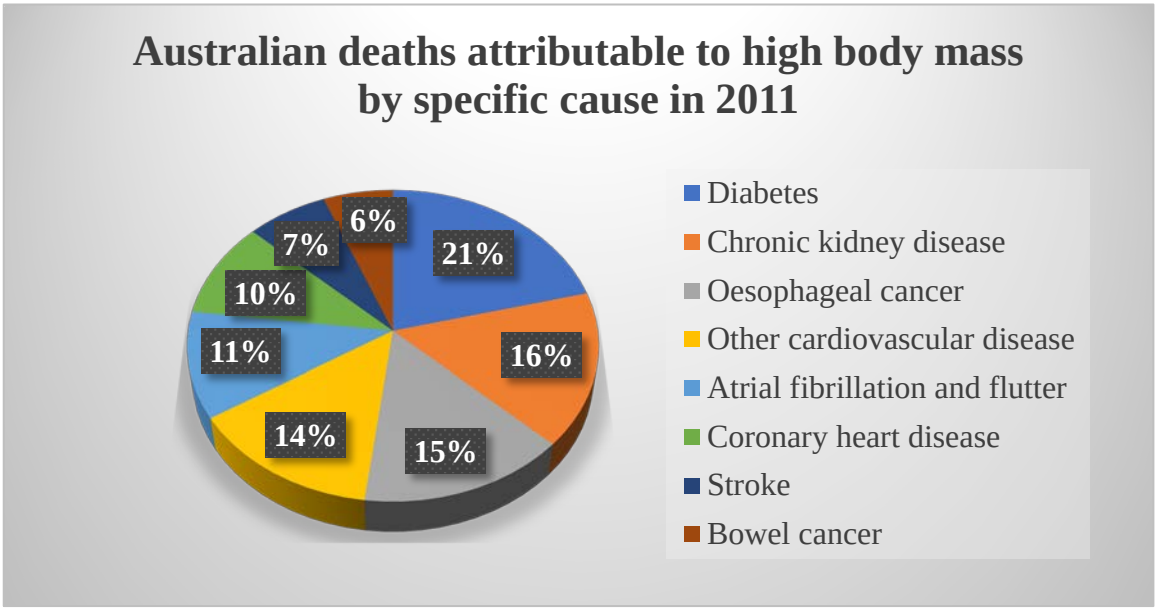

Figure 1. Australian deaths attributable to high body mass by specific cause in 2011. *The proportion of Australian deaths in 2011 related to diabetes, chronic kidney disease, oesophageal cancer, other cardiovascular disease, atrial fibrillation and flutter, coronary heart disease, stroke and bowel cancer, in individuals with a high body mass.*[13]

1.1.2 Benefits of weight loss

After major weight loss (35-40% of body weight)[14, 15] approximately two-thirds of individuals with type 2 diabetes no longer require treatment and return to normal fasting blood glucose and serum insulin levels.[16] Sixty per cent of individuals with hypertension revert to healthy-range blood pressure, sleep apnoea resolves, and depression improves.[17] In general, an individual's quality of life is restored to pre-obesity status, and their overall life expectancy improves.[18] Given these outcomes, weight loss should be a significant priority of those committed to good health care, with a primary focus on developing novel strategies to combat weight gain.

1.2 Current therapies to combat obesity

Current clinical approaches for obesity are based on creating a shift towards negative energy balance. Diet and exercise remain first-line recommendations with alternative options including a select number of drugs and bariatric surgery for the morbidly obese.

1.2.1 Lifestyle modification: diet, exercise and behavioural modification

Restrictions on dietary intake remain first-line therapy for the treatment of obesity. A modest weight loss of 5% of initial body weight through diet restriction, has been reported to delay and reduce the development of coronary heart disease, hypertension, type 2 diabetes and other degenerative diseases in obese patients.[19] Low energy diets (LEDs), which restrict daily intake to 3347-6276 kJ/day, have resulted in weight loss of 8% of total body weight over six months.[20] Very low energy diets (VLEDs) which restrict intake to 1845-3280kJ/day have reported weight loss to range from 17.8 ± 0.6kg after 12 weeks.[21]

LEDs were initially recommended over VLEDs because they have been proven to be equally effective after 12 months, with less risk of nutritional deficiency.[22] Mustajoki & Pekkarien,[23] directly compared outcomes of VLED and LED diets between varying timeframes of 8-16 weeks duration and concluded the there was no evidence that VLED programs lead to different outcomes when compared to programs with low energy or other dietary approaches.[23, 24] These findings were supported by a literature review conducted by the National Health and Medical Research Council (NHMRC) and incorporated into the current practical guidelines of the management of overweight and

obese individuals in Australia.[25] While short-term outcomes of dietary restriction are positive; long-term adherence is difficult. A comprehensive literature review by Saris,[26] reported weight re-gain following a VLED to range between -7% to 122% one year after treatment. Although various weight maintenance strategies have been investigated including meal replacements, high-protein diets, low glycaemic index diets, low-fat diets, green tea extracts, prolonged re-feeding or fasting periods[27] the majority of individuals re-gain weight within five years.[28]

As with appropriate diet, regular physical activity imparts a myriad of health benefits including protection from cardiovascular disease, type 2 diabetes, dementia and cancer.[29-31] Logically, this increase in energy expenditure should assist loss of bodyweight by leading to an energy deficit and weight loss. The World Health Organization (WHO) currently recommends individuals engage in regular physical activity to the amount of 60 minutes a day for children and 150 minutes per week for adults.[32] Aerobic exercise of moderate to vigorous intensity seems to have the greatest impact on reducing visceral adipose tissue with >250min/week of moderate to intense physical activity being associated with significant weight loss.[33] Indicating it is possible to obtain a decrease in body weight through exercise alone in people who are overweight or obese.

On closer examination, 12-month intervention studies of participants partaking in 176 mins/week of aerobic exercise yielded total body fat loss of 1%.[34] Additionally a 12 month randomised controlled trial of participants partaking in 60min/day, six days a week of exercise showed a total fat mass reduction of -1.9kg in women and -3kg in men respectively.[35] These data suggest that while statistically significant weight loss can be achieved through exercise alone, losses are not clinically significant due to concomitant increases in energy intake. Energy restriction through diet alone has been found to be superior to exercise alone regarding weight loss.[36, 37]

Lifestyle interventions such as energy restriction and physical activity exert independent effects on different markers of metabolic health. Therefore combinatorial treatments encompassing both diet restriction alongside physical activity could be additive on crucial health endpoints. A Cochrane systematic review, which included 43 studies reporting results from 41 randomised controlled trials, including 3476 participants showed that exercise combined with diet resulted in greater weight reduction than diet alone (mean

difference -1.0kg). They also noted that further increasing exercise intensity, increased the magnitude of weight loss (mean difference -1.5kg).[38] Although the combination has been successful for weight loss, there has been a small increased risk of complications including gallstones and micronutrient deficiency in these individuals.[39]

To address barriers to compliance, behavioural therapy was introduced as an adjunct to diet and exercise. Individuals were educated on principles including reinforcement. Combination strategies using diet, exercise and behavioural modifications have been shown to be more effective than diet or exercise alone[40] with lifestyle education promoting long-term weight maintenance of approximately 56% two years after VLED treatment.[41] However, irrespective of the lifestyle intervention plan, there is a continued high weight regain rate in obese individuals. Weight regain of 50% by one year has been reported and modest weight losses of <5% observed by 2-4 years.[42, 43]

Energy restriction induces an energetic compensation through reduction of energy expenditure, enhancing the difficulty of achieving sustained weight loss. Leibel et al[44] demonstrated that maintenance of a body weight reduction of 10% or greater of initial weight was associated with a mean reduction in total energy expenditure of 3-9kcal/kg in non-obese and 3-12kcal/kg in obese subjects.[44] Additionally, Weigle et al[45] demonstrated that a 10% reduction in body weight in individuals who were obese was accompanied by at 20-25% decline in 24-hour energy expenditure.[45] Additional reasons for the long-term failure of dietary intervention include setting unrealistic goals and expectations,[46, 47] poor adherence,[48] and weight regain due to a return to previous old habits.[49] Thus while changes to lifestyle are simple to prescribe, they rarely achieve sustainable weight-loss outcomes.[50]

1.2.2 Drug therapy

The ideal anti-obesity drug would induce a sustained weight loss, with minimal side effects in overweight and obese individuals.[51, 52] A number of compounds have been investigated for their potential to induce weight loss however current pharmacological approaches are limited.[53]

Based on the theory that weight loss can be achieved through reduced energy intake or increased energy expenditure, drugs targeting an increase in the basal metabolic rate (BMR) including dinitrophenol and thyroid hormones (TH) have been investigated, from as early as the 1930s in the case of dinitrophenol.[54, 55] Shortly after their release the unfavourable outcome of heart failure was discovered amongst its users, and they were immediately withdrawn.[56] Amphetamines and sympathomimetics which reduce energy intake and increase locomotor activity had been used successfully since the 1930s but were likewise abandoned because of their cardiovascular side effects and addictive properties.[57, 58]

Drugs targeting energy intake were thought to hold more promise. Nitramine a serotonin and noradrenaline re-uptake inhibitor-controlled appetite by producing a feeling of satiety. An average weight loss of 4.5kg per year (95% CI, 3.6-5.3kg) was reported in obese patients.[59] However, its side effect of hypertension, raising the risk of heart attack and stroke led to its removal from the consumer market in 2010.[60]

Rimonabant, a central[61] and peripheral[62] acting cannabinoid CB_1 (cannabinoid receptor type 1) receptor antagonist was shown to effectively treat obese individuals and their co-morbidities via decreasing activity of the endocannabinoid system preventing weight gain.[63-66] After extensive testing, however, it was deemed an unsafe drug due to increased incidence of anxiety, depression and suicidal ideation.[65] Its termination led to the rapid discontinuation of several other CB_1-antagonist-based anti-obesity drug development programs.[67]

Lorcaserin is a novel anti-obesity addition. Its mechanism of action is as a serotonin 5-hydroxytryptamine 2C receptor (5HT-2C) agonist increasing satiety by binding to 5HT-2C receptors on anorexigenic proopiomelanocortin (POMC) neurons in the hypothalamus.[68, 69] Initially rejected owing to concerns about spontaneous tumour growth in pre-clinical studies, it has now been approved for distribution at an altered dose. A multicentre randomised trial involving 883 patients demonstrated an average weight loss of 5.8 ± 0.2 kg.[70] Along with its success, the emergence of anticipated side effects has ensued, including a headache, dizziness, nausea, constipation[71] and less commonly reports of psychiatric disorders, bradycardia, haematological changes, and pulmonary

hypertension. Its effect on breast cancer risk also remains unclear and requires further monitoring.[72, 73]

Qysmia, a combination drug of phentermine and topiramate, was approved alongside Lorcaserin. As a dual combination drug, its effects are likewise elicited via dual mechanisms. Phentermine, suppresses appetite through modulation of catecholamines in the satiety centres of the hypothalamus,[70] and Topiramate, although the mechanism remains unclear, is thought to at least partially enact its effects through antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainite receptors.[74, 75] The CONQUER, EQUIP and SEQUEL clinical trials of Qysmia demonstrated consistent effects of 5% or greater weight loss in obese individuals within a 12-week period.[76] While adverse events are rare, they include paraesthesia, headache, constipation, dizziness, insomnia, depression and blurry vision, with several clinical trials reporting dropout rates of ~19%.[77-79] Two other compounds Cetilistat a monotherapy[80] and Tesofensine a polytherapy have reached phase III testing but have not been approved for use as yet.[81]

Contrave is an extended-release tablet combining naltrexone, an opioid receptor inhibitor slowing weight gain, and Bupropion a norepinephrine reuptake antagonist.[82] The COR-1 trial demonstrated that 42% in the Contrave group compared to 17% in the placebo group achieved a clinically significant 5% reduction in body weight.[83] However, much like its predecessors Contrave has safety concerns inducing hypertension, depression and seizures and naltrexone acute opioid withdrawal should be considered.[84]

The treatment of diabetics with glucagon-like peptide 1 (GLP-1) receptor agonists has also been a subject of interest to patients experiencing substantial weight loss while receiving the diabetic treatment. GLP-1 is an endogenous gut peptide that was initially identified as an incretin hormone[85] and later found to be part of a satiety cascade.[86] Trials of higher dose GLP-1 receptor agonist, Liraglutide have demonstrated weight loss of up to 10kg in trials of up to 2 years duration.[87, 88] Phase III trials have identified potential risks of pancreatitis, cholecystitis and its use is currently contraindicated in pregnancy and hypersensitivity individuals.[89] Thus, despite the introduction of four novel anti-obesity drug therapies due to persistent side effects Orlistat remains the only widely utilised anti-obesity drug therapy.

Orlistat has been one of the most successful anti-obesity drugs to have come out on the market. It induces an average weight loss of 2.9kg per year of treatment (95% CI, 2.3-3.5kg)[59] through long-acting gastrointestinal lipase inhibition, directly blocking the absorption of fat.[90, 91] Simultaneously, it decreases low-density-lipoprotein (LDL) cholesterol, blood pressure and hyperglycaemia, producing multiple health benefits in patients.[92, 93] However 20% of patients reportedly develop side effects such as diarrhoea, flatulence, bloating, dyspepsia, faecal incontinence and urgency, preventing its widespread prescription.[63, 94]

Unfortunately thus far anti-obesity drug development has been largely unsuccessful with Rodgers et al[58] several years ago summarising the field as: "the history of anti-obesity drug development is littered with false starts, failures in clinical development, and withdrawals due to adverse effects that were not fully appreciated at the time of launch."[58] There have been no new and successful drugs dedicated to weight loss to have come to the market within the past ten years.[95] To succeed in developing effective anti-obesity drugs significant effort to identify novel targets along with rethinking current development and treatment strategy paradigms are required including strategies to overcome the extensive list of side effects.

1.2.3 Bariatric surgery

Bariatric surgery (BS) currently prevails as the most effective and sustainable method of weight loss and treatment for morbid obesity.[96, 97] Its availability in Australia is restricted to patients with either a BMI of 40kg/m^2 or higher or a BMI greater than 35kg/m^2 with obesity-related comorbidities.[98] Currently the most frequently performed procedures globally are adjustable gastric banding 42%, Roux-en-Y gastric bypass 39%, and sleeve gastrectomy 5% (**Figure 2**).[99, 100] However, there is variation in the preferred procedure between countries, often reflecting local regulatory and insurance factors. In Australia, laparoscopic adjustable gastric banding accounts for two-thirds of all primary procedures,[101] followed by sleeve gastrectomy and roux-en-y gastric bypass (RYGB).[102, 103]

Meta-analyses have recorded weight loss of more than 50% of pre-surgical body weight, regardless of the type of bariatric surgery performed.[96, 104, 105] On closer examination of the most common bariatric surgical procedures, where weight loss is reported as the mean percentage of excess weight loss (weight loss/ excess weight) x 100, where excess weight refers to total preoperative weight minus ideal weight, has been recorded as 47.5% for gastric banding, 68.2% with RYGB, 61.6% with gastric bypass and 70.1% for biliopancreatic diversion.[96] Interestingly, the ASERNIP-S systematic review demonstrated that although initial weight loss in the first two years may differ, the weight loss outcomes are not significantly different between procedures at three and four years post operatively. [106]

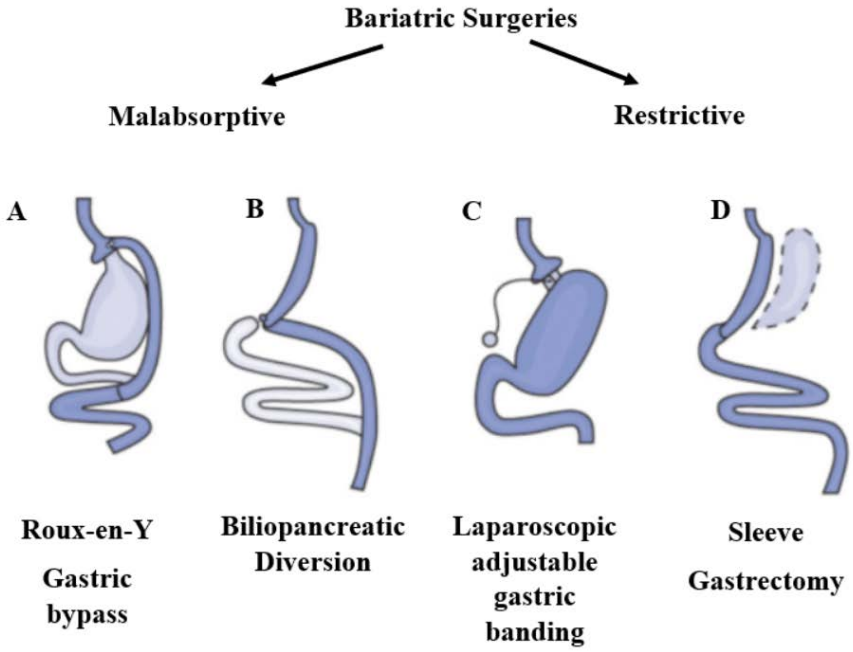

Figure 2. Bariatric Surgeries. *There are two primary subsets of bariatric surgery, malabsorptive and restrictive. The malabsorptive subtypes include the Roux-en-Y gastric bypass and biliopancreatic diversion with duodenal switch. The restrictive subtypes are laparoscopic adjustable gastric banding (LAGB) and sleeve gastrostomy.* **A** *Roux en Y gastric bypass involves the construction of a small pouch and biliary limb, which bypasses 95% of the stomach, entire duodenum and portion of the jejunum.* **B** *The biliopancreatic diversion with duodenal switch consists of a partial gastrectomy and intestinal bypass.* **C** *LAGB involves the placement of an adjustable band around the proximal stomach forming a small pouch.* **D** *Sleeve gastrectomy involves partial resection of the greater curvature of the stomach and is comparable to the malabsorptive procedures.*[107]

Alongside weight loss, individuals who have undergone bariatric surgery are observed to have a 41% reduction in long-term all-cause mortality compared to non-operated obese controls.[108-110] Multiple studies have demonstrated after significant weight loss, more than two-thirds of patients with type 2 diabetes return to having no clinical evidence of the disease, and have normal fasting blood glucose, serum insulin and HbA1C levels.[96, 111] Hypertension,[96, 112] dyslipidaemia,[96, 112, 113] reflux oesophagitis,[114] asthma,[115] depression,[116] non-alcoholic steatohepatitis,[117] obstructive sleep apnoea,[96, 118] and polycystic ovary syndrome have all also been identified as improved or resolved in the post-operative period.[119, 120]

Bariatric surgery has repeatedly been shown to result in greater quantity and longer duration of weight loss than either lifestyle modifications and drug therapies. The Swedish Obese Subjects (SOS) trial compared surgically treated patients to lifestyle-modified controls and found that the surgical group experienced a body weight loss of 23% at 2 years, 17% at 10 years, 16% at 15 years and 18% at 20 years post-surgery compared to the life-style modified controls yielding 0-1% body weight loss at this time.[96, 97, 121] The STAMPEDE trial demonstrated increased weight loss in the bariatric surgery group as compared with the medical therapy group (RYGB –29.4 ±9.0 kg, SG –25.1 ±8.5 kg, medical therapy –5.4 ±8.0 kg, P<.001).[122]

Bariatric surgery is not without adverse events and complications. The bariatric outcomes longitudinal database and the longitudinal assessment of bariatric surgery showed a perioperative mortality risk of 0.1% and 0.3% respectively.[123, 124] Maggard et al[105] reported a pooled mortality of 0.02% for LAGB, 1.0% for RYGB indicating that while the type of bariatric surgery is not statistically significant regarding weight loss outcomes, it is important for patient mortality outcomes.[105] A recent meta-analysis conducted by Chang et al[125] however, demonstrated that the mortality associated with bariatric surgery is generally low ranging from 0.08-0.31%, [125] while the complication and reoperation rates remain high at up to 17% and 7% respectively. [125]

The major surgical complications include deep vein thrombosis, infection, gastric leaks, fistulas, small bowel obstructions which are seen in up to 40% of cases.[126] Also there is a high need for revisional procedures. For LAGB the need for revisional procedures has been recorded to be as high as 30%.[127] Long-term complications include nutritional

deficiencies, weight regain and hyperinsulinemic hypoglycaemia.[16] Patients are also required to commit to life-long follow-up[128] which not only inconveniences patients but also burdens the health care system.[129] Furthermore, bariatric surgery is not always readily accessible, especially to those who are more likely to benefit, such as individuals from lower socioeconomic backgrounds.[130, 131] On a population scale, certain countries have an overweight and obesity prevalence of 60% and 25% respectively, and this is continuing to rise. Thus surgery alone is neither a viable nor efficient approach.[53, 132]

1.2.4 Future directions for weight loss therapy

Lifestyle modification remains the first line approach for reducing the burden of obesity, however, given poor adherence medical intervention remains essential. Recent and currently available drug therapies are of limited efficacy and/or have unacceptable side-effect profiles. Bariatric surgery remains promising regarding efficacy, however, due to poor economic viability, conducting such surgeries on a majority of the already large and increasing quantity of obese individuals worldwide is untenable.

Novel therapies are required as adjuncts to currently available options. The principle of increasing energy expenditure to prevent or reduce body fat content remains a possibility. Brown adipose tissue (BAT) has been proposed as a potential target to increase energy expenditure for several decades.[133] More recent conclusive evidence of its presence and function in adult humans[134, 135] has reinvigorated this concept. Further studies in humans are required to understand the therapeutic viability of BAT-directed therapy.

1.3 The adipose family

There are three main types of adipose tissue which can be distinguished in mammals, white (WAT), classic brown (BAT), to which discussion to this point has generally referred) and beige[136] or brite[137] (BeAT) adipose tissues. White adipose tissue (WAT) stores excess energy in the form of triglyceride along with possessing important endocrine functions integral to the regulation of energy storage and expenditure. BAT is specialised in heat production which is associated with high energy consumption. BeAT has only been described extensively in the past 5-10 years and is highly adaptable, and in mice, appears to fluctuate between BAT and WAT appearance and function depending on requirement.[138]

1.3.1 White adipose tissue

White adipose tissue (WAT) is a loose connective tissue designed to store large quantities of triacylglycerol and fat-soluble substances.[139] Each cell contains a single large lipid droplet, a nucleus on the lateral edge of the cell and a small number of thinly elongated mitochondria. In humans, WAT is distributed throughout an almost continuous subcutaneous (sWAT) layer as well as visceral, or mesenteric (vWAT) adipose within the abdominal cavity surrounding the viscera.[140] sWAT can be further divided into deep and superficial layers, particularly in the abdominal and gluteal regions.[140] Interestingly, accumulation of adipose in abdominal (android obesity) compared to gluteal (gynoid obesity) regions has been associated with increased risk of metabolic disease. However, the mechanisms responsible for the different functions of these tissue depots have not been well elucidated.[140]

The role of white adipose tissue in energy homeostasis is well defined, as both a storage depot and sensor of energy storage status. Once consumed, certain nutrients induce the secretion of insulin, which instructs adipocytes and myocytes to transport and store nutrients as lipids and glycogen, respectively.[141, 142] The storage of lipids in WAT acts as a long-term fuel reserve which can be mobilised during an energy deficit (e.g. food shortage), through the release of fatty acids for oxidation in other organs.[143, 144]

Concurrently with lipid storage, adipocytes sense energy storage and secrete a myriad of hormones including leptin, to initiate a feedback loop with the central nervous system, reducing food intake and activating the sympathetic nervous system (SNS).[145] Activation of the SNS results in the release of adrenaline and noradrenaline from nerve terminals in adipose tissue, activating β-adrenergic receptors on adipocytes increasing the rate of lipolysis and thermogenic processes.[146]

In obesity, adipocytes expand in both size and number to accommodate the need for increased lipid storage. However, they ultimately reach a threshold, upon which the adipose tissue will initiate an inflammatory stress response leading to various interrelated disease processes.[147]

1.3.2 Brown adipose tissue

BAT is a highly specialised tissue, containing a centrally placed nucleus, a dense population of mitochondria and numerous discrete lipid droplets stored in multiple vacuoles. The primary function of BAT is to maintain core body temperature in response to cold stress by generating heat in a process known as thermogenesis.[139]

1.3.2.1 The function of BAT: non-shivering & adaptive thermogenesis

BAT evolved in mammals to allow for survival primarily during cold exposure, but also in some mammals during periods of hibernation or when consuming diets low in protein. It achieves these functions through its ability to both store energy primarily as lipid and when activated to produce heat in a process called non-shivering thermogenesis.[139]

When an animal is acutely exposed to environmental temperatures below thermoneutrality (the environmental conditions at which an endothermic organism is not required to increase energy expenditure above the resting level to produce heat to maintain core temperature), it requires additional heat production to compensate for heat loss in order to maintain body temperature. The initial compensatory increase in energy expenditure to produce additional heat is referred to as facultative thermogenesis.[148] Facultative thermogenesis is highly variable and is comprised of multiple elements including the facultative part of diet-induced thermogenesis which varies with composition and amount of food,[149] cold-induced thermogenesis consisting of skeletal muscle shivering,[150] non-exercise activity thermogenesis and non-shivering thermogenesis.[151]

With chronic cold exposure (weeks-months), an animal will gradually cease to shiver, and BAT will take over the burden of heat production to sustain core temperature. This process is called non-shivering adaptive thermogenesis and involves an increase in brown adipocyte proliferation, differentiation, mitochondrial density, sympathetic nerve branching, local tissue angiogenesis and content of uncoupling protein-1 (UCP-1), a protein unique to BAT which is essential to its thermogenic function.[152-154] Whilst recent studies have demonstrated that in genetically modified UCP-1 knock-out mice, skeletal muscle (via sarcolipin) thermogenesis compensates for loss of BAT activity,[155] in wild-

type, non-genetically modified mice, BAT has been postulated to be the predominant, and potentially sole, contributor to any adaptive increase in energy expenditure.[139, 156]

When BAT stimulation via cold is not maintained the tissue reverts to its warm-adapted state, in which it loses most of the features described above,[157] a phenomenon that has been demonstrated in humans.[158] It is because of this ability to undergo an adaptive increase in energy-expending capacity that BAT is recognised as having the potential to play a key role in response to obesity.

1.3.2.2 Sympathetic-adrenergic activation of BAT

The principal regulator of BAT facultative and adaptive thermogenesis is the sympathetic nervous system (SNS).[139] SNS signals to BAT are conducted mainly through β-adrenergic receptors (ARs), which are members of the broad class of G-protein coupled receptors. Although all of β1, β2 and β3-ARs are required to induce the full thermogenic response,[159] the β3-AR is primarily responsible for activating mature brown adipocytes.[159, 160] Thus the amount of BAT sympathetic nerve activity, β3-adrenergic receptor binding and noradrenaline release to brown adipocytes, determine the level of thermogenesis in BAT by regulating both the activity of lipases which provide the immediate fuel for BAT mitochondria and determine the level of expression of BAT UCP-1.[139]

In response to activation signals, e.g. cold, peripheral receptors and/or endocrine factors direct an afferent signal to the dorsomedial hypothalamus and dorsal hypothalamic area (DMH/DHA).[161] This, in turn, stimulates a prominent location of BAT sympathetic premotor neurons in the rostral ventromedial medulla, centred in the rostral raphe pallidus (rRPa) and extending into nearby raphe magnus nucleus and over the pyramids to the parapyramidal (PaPy)region.[162] Glutamate and serotonin in these regions provide an excitatory drive to BAT sympathetic preganglionic neurons in the thoracolumbar spinal cord which in turn excites sympathetic ganglion cells innervating peripheral BAT.[163]

Noradrenaline is released from synaptic terminals of sympathetic neurons on the surface of brown adipocytes, binding to β3-ARs on the surface of BAT. Downstream signalling, activation of adenylate cyclase, increase in intracellular cyclic adenosine monophosphate (cAMP) levels and activation of cAMP-dependent protein kinase A (PKA).[139, 164, 165] Activated PKA results in a cascade of signalling events which converge to result in

activation of processes increasing thermogenesis, along with those which increase the functional components contributing to adaptive thermogenesis (**Figure 3**). Increased lipolysis within brown adipocytes is believed to be the primary mechanism responsible for activation of UCP-1 (described subsequently) through increasing availability of intracellular fatty acids, although recent evidence suggests this mechanism is more complex.[166-168] Upon activation, brown adipocytes oxidise fatty acids and glucose, mainly releasing expended energy as heat, to be systemically distributed, through the action of UCP-1.[139] UCP-1 mRNA does not produce heat.[139]

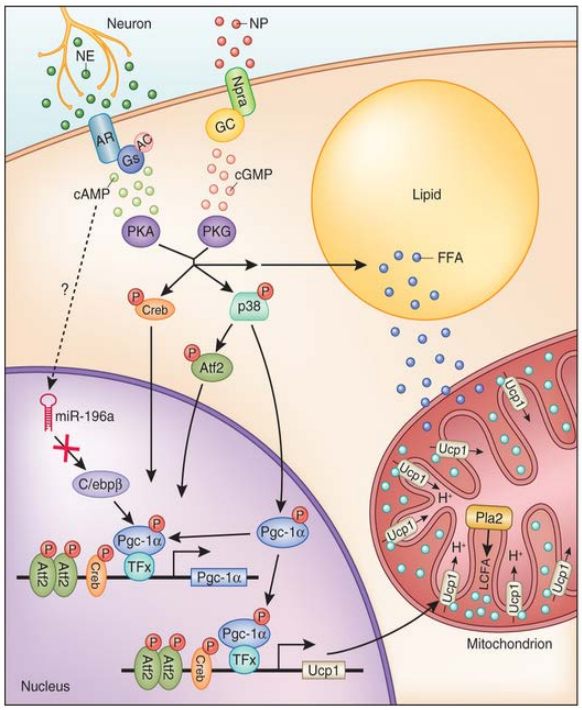

Figure 3. Noradrenaline-induced stimulation of thermogenesis in brown adipocytes. *Noradrenaline binds β-3 adrenoreceptors triggering G-protein signalling, activation of adenylyl cyclase, increase in intracellular cAMP levels, and activation of cAMP-dependent protein kinase A. Activated PKA phosphorylates pathways to activate UCP-1 and facilitates the breakdown of triglycerides to produce free fatty acids. The released fatty acids activate UCP-1 and are oxidised in the mitochondria to serve as an energy source for the production of heat. Noradrenaline (NE), Cyclic adenosine monophosphate (cAMP), Hormone Sensitive Lipase (HSL), Triglycerides (TG), Free fatty acids (FFA), Beta Oxidation (β-ox), Citric Acid Cycle (CAC).*[139]

1.3.2.3 Uncoupling-Protein 1 (UCP-1)

UCP-1 is the protein responsible for the unique thermogenic capacity of BAT and is located on the inner membrane of mitochondria in brown adipocytes.[169] The mechanism by which UCP-1 produces heat is through offering an alternate route for the dissipation of the proton gradient generated by the mitochondrial respiratory chain, rather than the usual coupling of H^+ protons to adenosine triphosphate (ATP) synthesis (**Figure 4**). The requirement for ATP for normal cell function remains, therefore as long as UCP-1 remains active, the drive to sustain energy expenditure for normal cell function remains,

and uncoupled thermogenesis will continue at a rate directly proportional to the quantity of active UCP-1.[170] Interestingly, if UCP-1 quantity and activity are great enough the requirement for ATP production to support coupled respiration can exceed the capability of mitochondrial respiration to provide ATP, and the cell will die.[171]

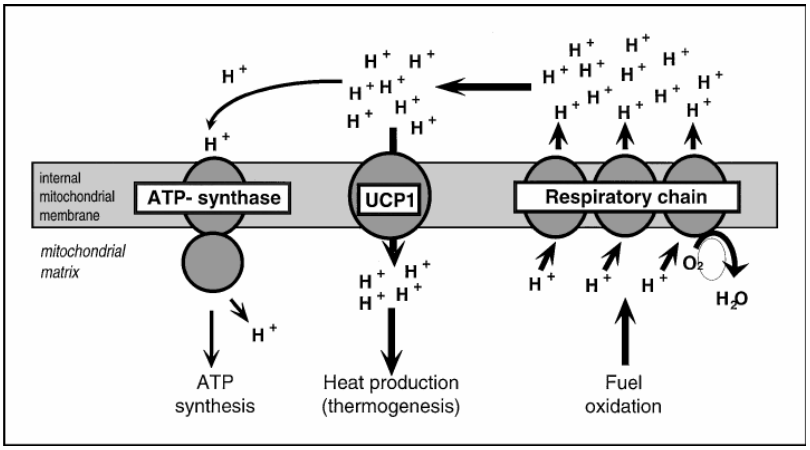

Figure 4. Uncoupling protein-1 (UCP-1) proton gradient. *UCP-1 produces heat in BAT by offering an alternate route for the dissipation of the proton gradient generated by the mitochondrial respiratory chain, as opposed to the usual coupling of H+ protons to ATP synthesis. Adenosine triphosphate (ATP), Hydrogen (H^+).*[172]

1.3.2.4 The presence of BAT in adult humans

The presence of BAT in rodents and certain large mammals has been well documented.[139, 173] In humans, however, it was initially thought that BAT was present in infancy, gradually decreasing with age, as the requirement for heat generation decreased, and was absent, or at least greatly diminished and non-functional, by adulthood.[138, 174-176] In 1996 symmetrical uptake of ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) in the supraclavicular region of adults was first reported.[177] However, it was only after the introduction of positron emission tomography-computed tomography (PET/CT) fusion technology, that this focal uptake was confirmed to be brown adipose tissue.[178, 179]

Retrospective analysis of PET/CT scans leads to the detection of BAT at discrete anatomical sites in adults, especially in the areas of the cervical, supraclavicular, paravertebral, pericardial, mediastinal and mesenteric areas.[135, 170, 180] However, the prevalence of BAT was in a minority (5-10%) of scanned subjects. These studies were

conducted under ambient temperatures, not designed to active BAT, resulting in a low reproducibility and false-negative results.[181]

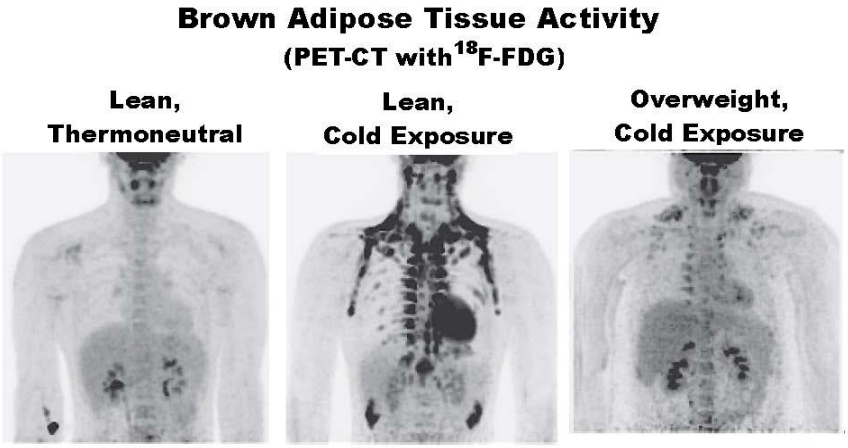

Figure 5. Cold-activated brown adipose tissue detected by ^{18}F-FDG-PET/CT. *^{18}F-FDG uptake into adipose tissues at the supraclavicular and paraspinal regions is negligible at room temperature 27^{o}C, but increases greatly after exposure to cold at 19^{o}C for 2 hours. ^{18}F-FDG uptake decreases with increasing BMI. ^{18}F-Fluorodeoxyglucose positron emission tomography-computed tomography (^{18}F-FDG PET/CT).*[135]

In a series of dedicated BAT activation trials, where healthy volunteers were exposed to mild cold at 16-19^{o}C with light clothing for 2 hours prior to PET/CT imaging, active BAT was observed in ~30-90% the cohorts, demonstrating that BAT activity could potentially be an important part of metabolic control **(Figure 5)**.[135, 182, 183] However, humans are not routinely exposed to prolonged cold stress and BAT is therefore not subject to regular and sustained activation. Due to the principle of adaptive thermogenesis, the absence of BAT activation results in atrophy and tissue dormancy. Whether BAT can be rescued from dormancy into a functional state is a key issue for its future therapeutic application.[184]

1.3.3 Beige adipose tissue

The concept of transitional adipose tissue between brown and white adipose tissue is not new, particularly in reference to "browning." Browning refers to the acquisition of functional brown adipose tissue characteristics including proliferation, differentiation and recruitment in any adipose tissue.[154] The term is most frequently associated in reference

to the appearance of brown-like adipocytes in WAT, termed beige, or 'brite' (brown in white) adipocytes (BeAT).[185] A hallmark of beige adipocytes is their potential to take on a thermogenic phenotype in response to various stimuli including cold, chemical compounds or genetic factors. Similar to classic brown adipocytes, beige adipocytes which have undergone "browning" appear to have multilocular lipid droplet morphology, high mitochondrial content and the expression of brown-fat specific proteins including UCP-1.

BeAT adipocytes can be distinguished from BAT on a number of features. Firstly, they differ in their anatomical location with beige adipocytes emerging from WAT depots, and BAT adipocytes arising from bona fide BAT typically located in interscapular regions of human infants.[186] Secondly BAT originates from precursor cells in the embryonic mesoderm, and express the transcription factor embryonic Myf5 (encoding myogenic factor 5-expressing).[187-189] In contrast, beige adipocytes are believed to arise from an Myf5 negative lineage, though this remains to be confirmed.[186]

In vivo mouse models have demonstrated that increases in energy expenditure protective against obesity are associated with activation of both BAT and BeAT.[190, 191] This is evidenced by weight loss recorded upon beige adipocyte recruitment in WAT of in vivo mice models[192] and the promotion of an obesogenic phenotype upon specific loss of beige adipocytes.[137, 192] Despite these novel data it is likely that BAT remains the primary[137] and only[193] physiologically relevant contributor to thermogenesis.

1.3.4 Brown adipose tissue in obese humans

The activity and prevalence of BAT are inversely related to body mass index, body fat and visceral fat.[181] PET/CT imaging of adult human's support this, indicating BAT depot size is inversely correlated with body mass index.[134, 182, 183]

In humans, numerous studies indicate that BAT activation in obese individuals is impaired in response to a variety of stimuli including cold exposure,[194, 195] insulin,[196] and sympathomimetics.[184] Although it has not yet been distinguished whether low BAT activity contributes to obesity, or conversely, whether obesity leads to diminished brown

adipose tissue function, it is clear that restoration of BAT may lead to prevention or reversal of obesity and metabolic disease through increased energy expenditure.[197]

Vijgen et al[198] were the first to analyse BAT activity in obese individuals who had lost weight through laparoscopic adjustable banding. The study demonstrated that evidence of BAT activity was observed in 50% of individuals, compared to 20% at baseline.[198] While the mechanism for impaired BAT activity in obesity and for BAT recruitment in a small number of individuals after weight loss in this study was not established, significant weight loss via gastrointestinal surgery may result in partial restoration of normal endocrine and cellular function, enabling BAT activation during mild cold stress.

1.4 Brown adipose tissue: a novel therapeutic target

Definitive identification of functional BAT in adults using novel imaging techniques along with the development of well-characterised immortalised human brown adipocyte cell lines[199, 200] has allowed for expanded opportunity to study the potential for manipulating BAT for therapeutic development.

Early studies attempted to estimate total potential energy expenditure for BAT in humans, however none involved direct measurement or accounted for all relevant tissue.[201] Subsequent studies in the past ~5 years using various PET/CT imaging techniques have estimated via direct measurement, the quantity of energy human BAT could potentially expand.[202, 203] Recent estimates suggest activated BAT could contribute an additional ~5% of energy expenditure above resting metabolic rate.[149] However no evidence has been produced to demonstrate the maximal capacity of the human BAT, and current methods to estimate total activity have most likely not accounted for the total volume of BAT.[204] Further, BAT activation in metabolic disease may be beneficial independent of weight loss.[205, 206] Therefore, BAT remains a viable option to therapeutically enhance energy expenditure to combat metabolic diseases.

1.5 Mechanisms of BAT activation

Whether BAT can be rescued from dormancy in obesity into a functional state is a key issue for its therapeutic application.[184] Means to maintain persistent thermogenic activation must be sustained to drive BAT browning, differentiation and recruitment.

Central to this process is to increase the quantity of its key functional components such as UCP-1 and other regulatory proteins such as those contributing to mitochondrial volume and function and adrenergic signalling, followed by mechanisms to maintain thermogenic activation. To this end, multiple pharmacological approaches have been studied in humans in recent years, predominantly using ^{18}F-FDG uptake and PET/CT imaging techniques.

1.5.1 Cold-induced BAT activation

Acute cold exposure increases BAT activity and regular daily cold exposure results in adaptive thermogenesis in a timeframe similar to mice (2-4 weeks).[154] It does this by activating the sympathetic nervous system[207] and modulating local thyroid hormone metabolism.[208, 209] After a period of adaptation in humans, an equivalent degree of mild cold exposure increases BAT activity, by approximately 25-60% more than before adaptation. This has been associated with doubling of BAT total oxidative metabolism, a promising observation in relation to potential weight loss.[210]

Additionally, chronic cold exposure studies support the hypothesis that increasing BAT activity may prevent or control type 2 diabetes.[211] A daily regime of cold exposure – either 2 hours at 17ºC daily for six weeks or up to 6 hours at 15-16ºC daily for ten days increased glucose uptake into BAT, and one study reported a 37% increase in estimated BAT volume.[212, 213] Prolonged cold exposure (5-8hrs) with a liquid-cooled vest (20ºC) increased resting energy expenditure (EE), whole-body glucose disposal and insulin sensitivity in adult humans with the active BAT.[214] In a cohort of over 65,000 patients, HbA1c was also shown to vary seasonally, with a fall during winter.[206] Moreover, mild cold exposure daily for two weeks increased BAT activity and insulin sensitivity in patients with type 2 diabetes.[205] This is promising evidence that human BAT is an active, highly plastic trainable tissue that is responsive to physiological stimuli.

While chronic cold exposure is the physiological stimulus which maximises adaptive thermogenesis in BAT; the associated discomfort renders the feasibility of cold 'training' as an obesity therapeutic as low. Secondly, in modern environments hyperphagia usually accompanies cold-exposure, negating any effects of BAT activation on energy balance. Thus, while chronic cold exposure is the natural stimulus for adaptive thermogenesis, it

is not feasible as a clinical therapeutic option.[184] It is therefore essential to identify pharmacological strategies which mimic cold-related signalling pathways to elicit adaptive thermogenesis and harness the benefits of BAT.

1.5.2 Thyroid hormones

Thyroid hormones (TH) play an important role in brown adipocyte differentiation[215-217] and the adaptation of BAT in response to the cold.[218] Cold exposure increases the expression and activity of tissue deiodinase 2 (DIO2), which stimulates the conversion of thyroxine (T4) to active triiodothyronine (T3) resulting in increased local T3 production from T4 and TH receptor agonism.[219] TH does not activate BAT through the classic adrenergic thermogenic pathway, rather, catecholamines and local T3 act synergistically to stimulate the BAT thermogenic gene expression program and thermogenesis.

Studies in humans examining the impact of TH on BAT facultative and adaptive thermogenesis have shown mixed results.[220, 221] Studies of individuals with hyperthyroidism (Grave's Disease)[220] or treatment of hypothyroidism with T4[222, 223] suggest that thyroid hormones increase BAT activity. However, this was not supported in follow-up studies.[221] All evidence on the influence of thyroid hormones on human BAT is based on studying patients with thyroid-related disease, so care should be taken when interpreting data and attributing weight gain and loss to BAT in these clinical scenarios. The BAT-directed therapeutic potential for TH thyroid hormones is limited, however, due to the widespread systemic effects and therefore side-effects.

1.5.3 Transient receptor potential channel agonists

Eleven transient receptor potential (TRP) channels have been identified in the 20 years since the first TRP channel, TRPV1 was identified.[224] Thermo- TRPs are responsible for sensing extremes of temperature and regulate events related to energy metabolism, including the differentiation and/or thermogenesis in brown adipocytes and energy expenditure mediated by sensory nerve-brain sympathetic reflexes.[225]

Studies in rodents have supported a role for TRP channel agonists such as capsaicin, capsinoids and catechins, which are natural food components as an avenue for BAT activation.[226] The proposed mechanism is that these compounds stimulate the transient

receptor potential cation channel subfamily V member 1 (TRPV1) located in the upper digestive tract, which in turn sends afferents to thermoregulatory centres in the central nervous system (CNS), resulting in sympathetic-mediated thermogenic activity and indirectly activating BAT.[226]

Studies in adult humans have shown that daily ingestion of capsinoids for six weeks increases cold-induced thermogenesis[213] and fat oxidation.[227, 228] However, whole-body energy expenditure is increased only in individuals with metabolically active BAT.[229] This may explain why in some studies of young healthy subjects, ingestion of capsinoids does not affect energy expenditure.[230] Additionally although TRV1 is expressed in brown adipocytes, achieving direct targeted therapy with capsinoids may be unlikely because orally ingested capsinoids are rapidly hydrolysed, and thus are usually undetectable in the circulation in humans. Further human studies are required to characterise the specificity, mechanism and magnitude of BAT activation and whether TRP channel agonists have efficacy for BAT activation in obesity.[225]

1.5.4 Adenosine

The purinergic transmitter adenosine[231] has been found to alter cyclic adenosine monophosphate (cAMP) signalling in various tissues.[232] Adenosine, had previously been noted to be released from BAT during sympathetic activation but was thought to inhibit thermogenesis, through inhibition of lipolysis leading to limited fatty acid availability.[233] However recent cross-species investigation of expression patterns of adenosine A_1, A_{2A}, A_{2B} and A_3 receptors, identified that human BAT cells primarily express A_{2A} receptors and can mediate thermogenesis through this mechanism.[234]

Adenosine is thought to have induced these effects via two main mechanisms, its release during sympathetic nerve stimulation together with norepinephrine and in a paracrine-autocrine manner in brown adipocytes. The released adenosine acts as a synergistic co-transmitter with noradrenaline to stimulate BAT activity.[235] Given the restricted tissue distribution of A_{2A} receptors, this promising development should now be further investigated in human clinical trials.

1.5.5 Thiazolidinediones

Thiazolidinediones (TZDs) including Rosiglitazone and Pioglitazone are known to promote adipocyte differentiation, adipogenesis and browning by activating peroxisome proliferator-activated receptor γ (PPARγ) in adipocytes.[236] Mice treated with PPARγ agonists have a profound expansion of BAT mass,[237], and those with adipose-specific PPARγ ablation demonstrate impaired BAT development.[238]

In adult humans, Rosiglitazone and Pioglitazone were widely prescribed for the treatment of type 2 diabetes until cardiovascular safety concerns with Rosiglitazone arose[239] leaving Pioglitazone as the predominant TZD in use.[240] The first trial examining BAT function after Pioglitazone treatment in humans demonstrated cold-induced BAT glucose uptake decreased post Pioglitazone treatment, contradicting previous in vitro and animal in vivo data.[138] This suggests that reduced BAT activity may contribute in part to the previously documented weight gain associated with Pioglitazone and other TZDs when used in humans.[241, 242]

1.6 Sympathomimetics and the use of ephedrine

Both in vitro and in vivo studies have established that BAT facultative and adaptive thermogenesis is initiated through adrenergic receptor signalling, highlighting its potential as a pharmacological target. Early studies using adrenergic receptor agonists provided inconclusive and conflicting findings of BAT function in humans.[243, 244]

Early trials in lean, healthy humans using CL-316,243, a highly selective β3-adrenergic agonist, in humans for eight weeks showed marked plasma concentration-dependent increases in insulin sensitivity, lipolysis and fat oxidation, without causing β1- or β2-mediated side effects.[245] A single dose of L-796568 in obese adults increased energy expenditure by approximately 8%.[246] These data support pharmacological approaches using β3-adrenergic agonists for BAT activation.

Recent data, however, has suggested that β3 agonists while successful in rodents, proved to be ultimately unsuccessful in clinical trials. The failure of early ligands was primarily attributable to low oral bioavailability and differences between rodent and human β3-ARs resulting in poor binding kinetics.[247-249]

More recently the β3-AR agonist Mirabegron has been shown to have higher in vitro binding affinity to human β3-AR[250], and a single dose increased ^{18}F-FDG uptake in supraclavicular BAT in humans.[251] It is unclear however whether Mirabegron stimulated ^{18}F-FDG uptake in BAT is linked to classic uncoupled thermogenesis distinct from receptor-stimulated glucose disposal, storage and/or coupled oxidation. In general, thermogenesis induced by β3 and pan β-AR agonists is lower in humans compared to mice, and their role in adaptive thermogenesis and weight/fat loss remains to be determined.

Ephedrine, its isomer pseudoephedrine, and other plant-derived ephedra alkaloids have been used in medications and dietary supplements for weight management and energy enhancement.[252] Weight loss through ephedrine intake is attributed to ephedrine's effects on the CNS, causing a reduction in food intake through central appetite suppression.[253] The principal mechanism by which ephedrine stimulates BAT is thought to be from the displacement and release of noradrenaline from storage vesicles in pre-synaptic neurons and direct effects on peripheral adrenergic receptors.[254]

Rothwell and Stock reported that a single oral dose of 1mg/kg ephedrine could increase skin temperature in the neck and upper back, suggesting BAT activation.[244] The study proved difficult to replicate with trials undertaken by Astrup et al[243] showing no changes in either perirenal nor interscapular BAT activity although more contemporary evidence demonstrated that the majority of BAT in adults was not found in these regions.[138] Recent studies using ^{18}F-FDG-PET/CT using treatment with the pan-β adrenergic agonist isoprenaline[255] or ephedrine[256] at doses expected to result in BAT thermogenesis based on prior evidence of whole body thermogenesis, likewise failed.

In a pilot study, Carey et al[184] replicated previous studies using 1mg/kg of ephedrine and observed no effect. However, dose escalation to 2.5mg/kg increased BAT activity.[184] The magnitude of the BAT response to ephedrine, however, was less than that for mild cold exposure. There was a two-fold increase in response to ephedrine, whereas mild cold increases BAT activity by greater than five-fold.[135, 183] Furthermore, the study confirmed previous data during cold exposure, demonstrating that BAT activation in obesity is impaired (**Figure 6**). While this study showed ephedrine stimulates BAT activity only in lean, healthy adult males, it may be that chronic dosing could mimic adaptive thermogenesis of BAT.

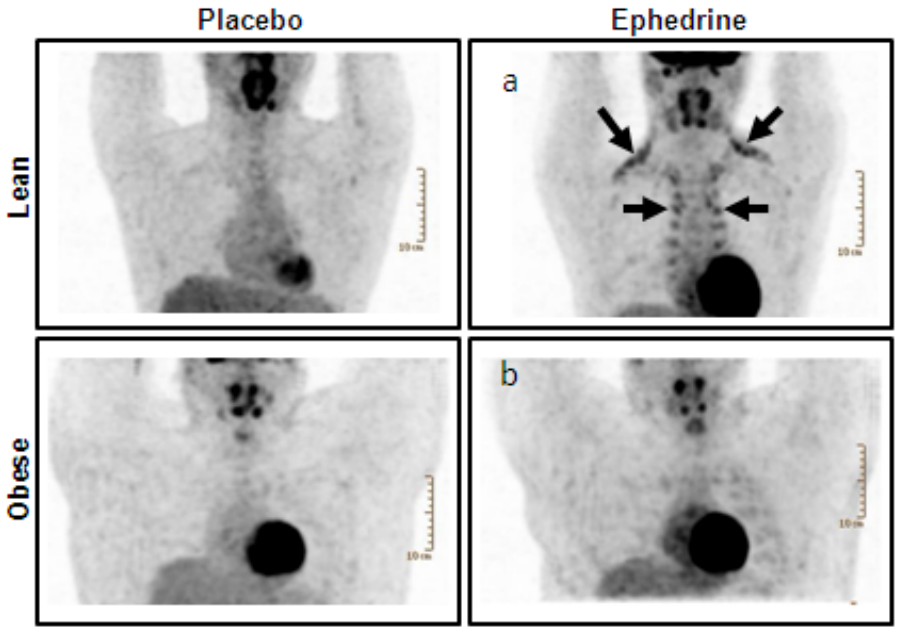

Figure 6. Pharmacological activation of BAT in lean but not obese individuals. *2.5mg/kg of ephedrine or placebo was administered to lean or obese participants and brown adipose tissue activity measured via ^{18}F-FDG PET/CT. This increased BAT activation in (a) lean but not (b) obese individuals. Arrows indicate active BAT in the supraclavicular and paraspinal regions.^{18}F-Fluorodeoxyglucose positron emission tomography-computed tomography (^{18}F-FDG PET/CT).*[50]

In vivo studies in rodents indicate that chronic ephedrine administration is associated with adaptive thermogenesis.[257] While not clinically indicated due to associated cardiovascular side-effects, the use of sympathomimetics provides an avenue for proof-of-concept studies regarding the potential for sympathomimetics to mimic BAT adaptive thermogenesis.

1.7 Hypothesis and aim of thesis

The aim of this thesis is to determine whether chronic sympathetic stimulation of brown adipose tissue with ephedrine will induce adaptive thermogenesis within brown adipose tissue in response to 4 weeks treatment with ephedrine.

The hypothesis of this thesis is that chronic treatment with ephedrine will result in increased brown adipose tissue activity in response to a single dose of ephedrine.

2. Publication

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ARTICLE

Chronic ephedrine administration decreases brown adipose tissue activity in a randomised controlled human trial: implications for obesity

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Abstract
Aims/hypothesis Brown adipose tissue (BAT) activation increases energy expenditure and may have therapeutic potential to combat obesity. The primary activating and adaptive signal for BAT is via β-adrenergic signalling. We previously demonstrated that human BAT is acutely responsive to oral administration of the sympathomimetic, ephedrine. Here we aimed to determine whether adaptive thermogenesis can be induced via chronic treatment with ephedrine.
Methods Twenty-three healthy young men, recruited from the general public in Melbourne, Australia, who were non-smokers, physically inactive and non-medicated with no prior history of cardiovascular disease or diabetes were recruited for this study. They were assigned to receive either 1.5 mg kg^{-1} day^{-1} ephedrine ('active' group; n=12, age 23±1 years, BMI 24±1 kg/m^2) or placebo (n=11; 22±2 years, 23±2 kg/m^2) for 28 days in a randomised (computer-generated random order sequence), placebo-controlled, parallel-group trial. Participants and all investigators were blinded to treatments. Body composition was measured before and after the intervention by dual energy X-ray absorptiometry. BAT activity, measured via ^{18}F-fluorodeoxyglucose positron emission tomography-computed tomography, in response to a single dose of 2.5 mg/kg ephedrine, was the primary outcome measure to be determined before and after the 28 day treatment period.
Results Twenty-eight individuals were randomised and consented to the study. Twenty-three completed the trial and only these participants were included in the final analyses. After 28 days of treatment, the active group lost a significant amount of total body fat (placebo 1.1±0.3 kg, ephedrine −0.9±0.5 kg; p<0.01) and visceral fat (placebo 6.4±19.1 g, ephedrine −134±43 g; p<0.01), with no change in lean mass or bone mineral content compared with the placebo group. In response to acute ephedrine, BAT activity (change in mean standardised uptake value: placebo −3±7%, ephedrine −22±6%) and the increase in systolic blood pressure were significantly reduced (p<0.05) in the active group compared with placebo.
Conclusions/interpretation Chronic ephedrine treatment reduced body fat content, but this was not associated with an increase in BAT activity. Rather, chronic ephedrine suppressed BAT glucose disposal, suggesting that chronic ephedrine treatment decreased, rather than increased, BAT activity.
Trial registration: ClinicalTrials.gov NCT02236962
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Abbreviations

AR	Adrenergic receptor
BAT	Brown adipose tissue
DEXA	Dual energy X-ray absorptiometry
NA	Noradrenaline
PET-CT	Positron emission tomography-computerised tomography
SUV	Standardised uptake value
UCP-1	Uncoupling protein-1
WAT	White adipose tissue

Introduction

The conclusive identification of functional brown/beige adipose tissue (BAT) in adult humans [1–5] and its functional impairment in obesity [3, 6, 7] has focussed attention on this tissue as an anti-obesity target. This impairment is due to the oxidative capacity of BAT, which, while relatively low and variable under basal conditions [8–10], can increase under conditions of chronic stimulation via a process called adaptive thermogenesis. Chronic cold exposure is the natural stimulus for adaptive thermogenesis, but mimicking cold-related signalling pathways also has the potential to elicit adaptive thermogenesis [8].

Cold-stimulated BAT adaptive thermogenesis in humans has recently been reported by four independent laboratories using different intermittent cold exposure protocols [11–13]. Prevailing views, therefore, suggested increasing cold stress by decreasing the ambient temperature in human dwellings and/or regular, purposeful 'cold-training' may reduce body weight, potentially via increasing BAT function and activity and decreasing body fat [14]. While there may be health benefits associated with regular intermittent cold exposure [15] it is unlikely to be effective for weight loss in humans. Prolonged cold exposure, regardless of intensity, will be difficult to sustain and, due to cold-stimulated hyperphagia, is unlikely to result in sustained weight loss and may actually promote weight gain [16, 17]. For the purpose of reversing obesity, identifying pharmacological agents that increase BAT function without concomitant central hyperphagic signals would be most effective [8].

Recent pharmacological (thyroxine) [18] and nutritional (capsinoid extract) [13] interventions have provided indirect evidence that BAT function may be enhanced by chronic treatment with orally bioavailable agents. Pharmacological studies in humans investigating BAT thermogenesis to date have focussed primarily on sympathomimetic agents [6, 19–21]. These agents signal via adrenergic receptors (ARs), thereby notionally replicating both the facultative and adaptive thermogenic central sympathetic signal to BAT in response to cold exposure. We recently reported that high doses of the sympathomimetic ephedrine can activate BAT in some lean young men [6]. This is consistent with the effects of acute ephedrine in mice, a species where chronic administration of ephedrine is associated with adaptive thermogenesis. This chronic action of ephedrine in rodent BAT contrasts with other tissues where responsiveness to adrenergic agonists is reduced with chronic stimulation [22, 23]. In this respect, ephedrine replicates some of the adaptive thermogenic effects of chronic cold exposure in rodents [24]. It is unknown, however, whether chronic treatment with sympathomimetic drugs can replicate cold-stimulated adaptive thermogenesis in humans. While chronic use of these agents is not advised in humans due to significant cardiovascular side effects, they represent a suitable drug class for proof-of-concept studies. With the advent of positron emission tomography-computerised tomography (PET-CT) imaging to semi-quantitatively and directly measure BAT activity, it is now possible to investigate this question. Accordingly, the aim of the present study was to determine whether or not chronic treatment with the orally bioavailable sympathomimetic ephedrine increases BAT activity in response to a single dose of this drug.

Methods

Twenty-three young male participants (no history/clinical evidence of cardiovascular disease and either diabetes, impaired fasting glucose or impaired glucose tolerance, unmedicated, physically inactive and non-smokers) took part in this study. The study was approved by the Alfred Hospital Ethics Committee and performed in accordance with the Declaration of Helsinki, Sixth Revision, 2008. A Consolidated Standards of Reporting Trials (CONSORT) checklist and flow diagram is presented in electronic supplementary material (ESM) Fig. 1. All patients provided written informed consent.

Study design This study was a randomised, double-blinded, placebo-controlled trial. Participants were randomised into two groups to receive either active treatment (ephedrine 1.5 mg kg^{-1} day^{-1}) or placebo (lactose). Dosing was selected based on pilot studies that determined 1.5 mg kg^{-1} day^{-1} to be the highest single dose that could be safely taken while unsupervised outside the laboratory. Sample sizes were based on power calculations determined from our prior studies [6, 25, 26]. Participants were randomised by The Alfred Hospital

Clinical Trials Pharmacy staff in blocks of 4–6 using Microsoft Excel (v2007) to generate a random order sequence.

Participants visited the laboratory three times, all located within the Departments of Cardiology and Nuclear Medicine at The Alfred Hospital. On visit 1, after obtaining informed consent a medical screen and body composition analysis (dual energy X-ray absorptiometry [DEXA]) were conducted. Visits 2 and 3 occurred before and after the treatment intervention, respectively, and included assessment of BAT activity (PET-CT), whole-body energy expenditure (indirect calorimetry) and blood variables (blood glucose, NEFA and noradrenaline [NA; norepinephrine]) in response to a single dose of ephedrine (2.5 mg kg^{-1}). These measurements were made using a protocol previously established by us [6]. The day after visit 2, participants began taking a single oral dose of ephedrine (1.5 mg kg^{-1}) or placebo between 09:00 and 11:00 hours daily for 28 days. Within 2 days of taking their final dose, participants returned to the laboratory (visit 3) and their body composition was reassessed via DEXA.

Previous studies indicate BAT activity varies seasonally [2, 27]. While we conducted experiments between March and October, participants were randomised in blocks of four to six to minimise the potential for seasonal variation between treatment groups. We did not observe any variation in ephedrine-stimulated BAT activity between groups during pre-intervention experimental trials (visit 2).

Outcome measures The primary outcome measure was change in BAT activity. Secondary outcome measures comprised changes in basal and ephedrine-stimulated energy expenditure, body composition, circulating hormones, lipids and other metabolites.

Experimental protocol

Screening Initial screening involved clinical history and examination by a physician and measurement of physical characteristics, including height, weight, waist:hip ratio, brachial artery blood pressure and 12-lead ECG. A fasting blood sample was drawn for measurement of lipid profile (total, LDL- and HDL-cholesterol and triacylglycerol), insulin, NA, HbA_{1c} and glucose. An OGTT was then performed. Briefly, participants consumed a 75 g glucose solution, after which blood glucose was measured at 60 and 120 min. Body composition (lean, bone and fat mass) was measured using DEXA.

BAT activation trials Before and within 24–48 h after the 4 week drug intervention, participants were given a standardised meal (3,180 kJ; 84% carbohydrate, 13% protein, 3% fat) to consume the evening prior to attending the laboratory (at 18:00–22:00 hours) on both experimental days. Laboratory temperature was 20–22°C. Upon arrival at 07:30–08:00 hours after an overnight fast and having abstained from vigorous exercise, caffeine, smoking and alcohol consumption for at least 2 days prior, participants voided and changed into standard hospital scrubs and socks. They then consumed a telemetric pill for recording of core temperature (Cortemp, HQ Inc, Palmetto, FL, USA) and a venous cannula was inserted into an antecubital vein. Brachial blood pressure (Philips Suresigns VS3; Philips Medical Systems, Andover, MA, USA) was measured every 15 min and heart rate (Cortemp) was continuously recorded. Participants then rested in a supine position for 2 h while covered with two blankets to ensure thermoneutrality.

After resting, energy expenditure was measured via indirect calorimetry, a blood sample was taken and participants then consumed 2.5 mg kg^{-1} ephedrine hydrochloride in a gelatine capsule with water. Blood samples were taken at 15, 30, 60 and 90 min after drug ingestion for subsequent analyses (described below). Participants were injected with an FDG tracer for BAT glucose uptake assessment via PET-CT 60 min after drug ingestion. Energy expenditure was again measured via indirect calorimetry 60–90 min after drug ingestion because this time was predicted to correspond to peak plasma NA concentrations and BAT activity [6, 19].

Indirect calorimetry Energy expenditure was measured with a ParvoMedics TrueOne 2400 metabolic analyser (ParvoMedics Inc, East Sandy, UT, USA). Mixed expired gases were measured after 10-min equilibration. Energy expenditure and respiratory exchange ratio were calculated and averaged over 20 min [6].

PET-CT imaging PET-CT imaging and analyses were conducted as previously described [6]. PET-CT variables resulted in an effective radiation dose of <7 mSv per scan, therefore the maximum radiation dose administered (including DEXA) was 14 mSv per participant. PET-CT images were acquired and reconstructed using a Philips Gemini Dual PET-CT scanner (Philips, Andover, MA, USA). Scans were analysed using 4 mm thick coronal slices on an Extended Brilliance Workstation (Philips).

Analysis focussed on the supraclavicular adipose tissue depot as this area has been consistently shown in numerous studies to demonstrate activity and/or molecular markers of BAT in humans [1–7, 9, 12, 13, 20, 28–30]. Recent evidence also suggests that in humans this tissue is likely to be predominately beige adipose tissue [29, 31–33]. For simplicity, hereafter this tissue will be referred to as BAT and activity in this region will be considered to represent 'BAT activity'. Tissue CT radiodensity in the established range of −180 to −10 Hounsfield units within this region was considered to represent adipose tissue [6, 20, 21, 34].

We quantified maximum standardised uptake value (SUVmax) in supraclavicular adipose (representative of BAT) and subcutaneous adipose (upper arm, representative of white adipose tissue [WAT]). Basal SUVmax of supraclavicular adipose is ~0.8–1.0, which is ≥2 standard deviations above that of subcutaneous WAT (~0.4) [6]. In order to better represent total activity in this region we also conducted volumetric analyses to encompass a larger tissue region. We did not, however, observe increased supraclavicular adipose SUVmax above basal levels in the majority of participants in response to acute ephedrine. In light of this finding, and given the prolonged interventional nature of this study, we conducted a 'fixed volume' analysis [35]. From three consecutive PET image slices (4 mm thickness) within the supraclavicular region, SUVmean from regions of interest of 100 mm^2 were determined on left and right sides. These data are, therefore, reported as SUVmean per 2, 400 mm^3. SUVmax and SUVmean are determined in units of g/ml, however neither our method of reconstruction and data analysis nor the formula to quantify these variables are quantitative. Data are, therefore, presented simply as 'SUVmax' and 'SUVmean' without units.

Biochemical analyses Where required, plasma was centrifuged and frozen for analyses. Plasma was measured for glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol, insulin, HbA_{1c} and NA as described [6]. Plasma NEFA levels were measured using a commercially available kit (Waco Diagnostics, Richmond, VA, USA).

Statistical analyses Physical characteristics between groups and change in body composition between groups as a result of treatments were compared using unpaired two-tailed Student's *t* tests. To determine effects of acute ephedrine treatment on energy expenditure, the change in response to the acute ephedrine treatment was determined both before (Pre) and after (Post) the chronic treatment in each group. The change in this value from Pre to Post was then determined and this change was compared between the two treatment groups using an unpaired two-tailed Student's *t* test. The same analysis was applied for comparison of haemodynamic, core temperature and circulating factor data, however the change in response to acute ephedrine treatment for each group and time-point was determined by subtracting the basal value from the mean of values obtained between 60–90 min after the acute ephedrine dose. As previously reported by us [6] and others [19], the peak in acute physiological responses to oral ephedrine varies between individuals but occurs within this period.

Effects on BAT activity were determined as described above for body composition data, however since PET-CT data were not normally distributed, a non-parametric Mann–Whitney *U* test was used to compare between groups. Analyses were conducted using SPSS (v15) and Microsoft Excel. Results are expressed as mean±SEM, and results were considered significant when $p \leq 0.05$.

Results

Baseline participant characteristics are presented in Table 1. Groups were not statistically different for all criteria. Table 2 shows body composition at baseline and in response to the 28-day intervention. The change in total mass (change placebo 1.1 ±0.3 kg; ephedrine −0.9±0.5 kg), body fat mass (change placebo 0.5±0.2 kg; ephedrine −1.1±0.3 kg), per cent body fat (change placebo 0.4±0.2%; ephedrine −1.2±0.3%) and estimated visceral adipose tissue mass (change placebo 6.4± 19.1 g; ephedrine −134±43 g) between groups were all significantly reduced after chronic ephedrine treatment only (Table 2; $p<0.01$). Lean mass and bone mineral content were unchanged.

BAT activity after chronic treatment was significantly lower in response to acute ephedrine in the chronic ephedrine

Table 1 Participant baseline characteristics

Patient characteristic	Placebo	Ephedrine	*p* values
n	11	12	
Physical characteristics			
Age (years)	22±2	23±1	0.44
Height (cm)	178±5	178±2	0.89
Weight (kg)	74±2	80±3	0.13
BMI (kg/m^2)	23±2	25±1	0.11
Body fat (%)	23±3	26±2	0.45
Waist:hip	0.88±0.39	0.88±0.20	0.92
Resting cardiovascular variables			
Systolic blood pressure (mmHg)	120±5	118±2	0.67
Diastolic blood pressure (mmHg)	74±4	73±2	0.61
Heart rate (bpm)	63±6	67±2	0.33
Fasting plasma hormones, metabolites and lipids			
NA (pmol/l)	936±134	975±180	0.56
Insulin (pmol/l)	49±17	72±31	0.50
Glucose (mmol/l)	4.9±0.2	5.0±0.1	0.43
HbA_{1c} (%)	5.3±0.2	5.2±0.1	0.59
HbA_{1c} (mmol/mol)	34±2	34±1	0.59
Total cholesterol (mmol/l)	4.4±0.8	4.1±0.2	0.37
HDL-cholesterol (mmol/l)	1.1±0.1	1.2±0.1	0.22
LDL-cholesterol (mmol/l)	2.7±0.6	2.4±0.2	0.32
Triacylglycerol (mmol/l)	1.4±0.7	1.1±0.1	0.36
NEFA (μmol/l)	621±54	533±30	0.18
OGTT			
60 min glucose (mmol/l)	6.0±1.3	6.3±0.5	0.64
120 min glucose (mmol/l)	4.9±0.7	5.3±0.3	0.39

Values are mean±SEM

$p>0.05$ for all (groups compared using an unpaired Student's *t* test)

Table 2 Body composition

Characteristic	Placebo			Ephedrine		
	Pre	Post	Change	Pre	Post	Change
Total mass (kg)	74.3±2.2	75.4±2.3	1.1±0.3	80.0±2.8	79.1±2.9	−0.9±0.5[a]
Lean mass (kg)	54.6±1.8	55.1±1.9	0.56±0.3	55.9±1.3	56.2±1.4	0.24±0.3
Fat mass (kg)	16.8±2.1	17.3±2.1	0.50±0.2	20.9±2.3	19.8±2.3	−1.1±0.3[a]
Fat mass (% total mass)	23.2±2.6	23.6±2.5	0.4±0.2	26.6±2.2	25.4±2.2	−1.2±0.3[a]
eVAT (g)	493±109	500±116	6.4±19.1	512±73	378±95	−134±43[a]
Bone mineral content (g)	2,915±95	2,917±95	1.6±94	3,150±94	3,132±94	−18±8

Values are mean±SEM

[a] $p<0.01$ for change between groups, ephedrine treatment significantly different compared with placebo (the pre–post change was compared between treatment groups using an unpaired Student's *t* test)

eVAT, estimated visceral adipose tissue

treated group but was unchanged in the placebo group when measured as change in SUVmax (Fig. 1a; mean change placebo −3±6%; ephedrine −13±7%, $p=0.03$) or SUVmean (Fig. 1b; mean change placebo −3±7%; ephedrine −22±6%; $p=0.01$). SUVmax in WAT was half that of BAT and was unchanged in both groups in response to acute ephedrine before chronic treatment (placebo pre 0.48±0.02, placebo post 0.45±0.02; ephedrine pre 0.45±0.02, ephedrine post 0.43±0.03).

Basal energy expenditure did not change as a result of chronic ephedrine treatment (Table 3). The increase in energy expenditure in response to acute ephedrine was unchanged after the chronic treatment period between groups (Table 3). The respiratory exchange ratio was not affected by either acute or chronic ephedrine treatment (Table 3).

The change in systolic blood pressure, heart rate, core temperature, blood glucose, plasma NA and plasma NEFAs in response to the acute dose of ephedrine are shown in Fig. 2 both before (Pre) and after (Post) the 28-day chronic ephedrine intervention. Compared with placebo, in the chronic ephedrine treatment group the change from pre- to post-intervention was significantly less for systolic blood pressure ($p<0.05$) and blood glucose ($p<0.001$), and trended towards a reduction for plasma NA ($p=0.06$). There was no difference in the response between groups for heart rate, core temperature and NEFA.

Discussion

In the present study, we hypothesised that chronic treatment with ephedrine would induce adaptive thermogenesis in BAT in adult humans, resulting in increased BAT activity. Contrary to this hypothesis, BAT activity was significantly reduced by 28 days of ephedrine treatment. This effect is likely to be due to ephedrine tolerance and may have implications for conditions of chronic sympathetic activation. Thus, chronic stress, hypertension and established obesity have all been associated

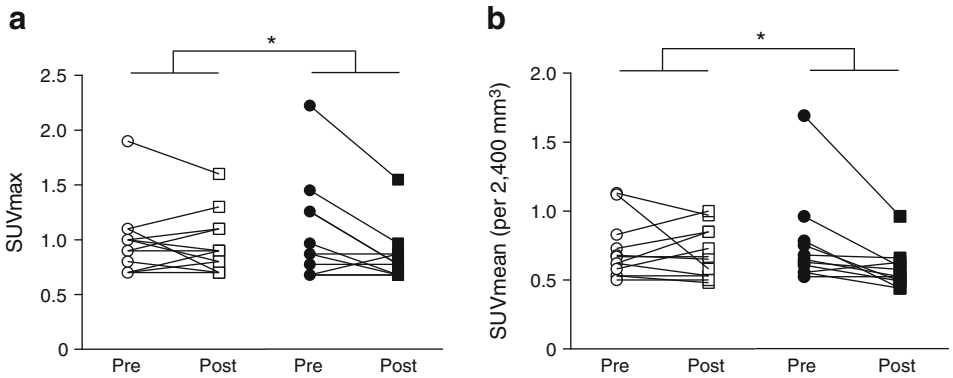

Fig. 1 (**a**) SUVmax and (**b**) SUVmean values plotted for each individual in supraclavicular brown/beige adipose tissue in response to treatment with a single dose of ephedrine (2.5 mg kg^{-1}), before (pre) and after (post) 28 days treatment with placebo or ephedrine (1.5 mg kg^{-1} day^{-1}). SUVmax group mean values: placebo pre (open circles) 0.98±0.09; post (open squares) 0.96±0.08; ephedrine pre (closed circles) 0.98±0.13; post (closed squares) 0.80±0.07. SUVmean group mean values: placebo pre 0.71±0.06; post 0.70±0.05; ephedrine pre 0.76±0.10; post 0.55±0.04. *$p<0.05$ for change from pre- to post-treatment between groups (the pre–post change was compared between treatment groups using a non-parametric Mann–Whitney *U* test)

Table 3 Indirect calorimetry

Characteristic	Placebo			Chronic ephedrine		
	Pre	Post	Change	Pre	Post	Change
Basal energy expenditure (kJ h^{-1})	287±17	285±8	−2±14	286±16	287±10	0.4±15
Ephedrine-stimulated energy expenditure (kJ h^{-1})	326±14	322±10	−4±13	340±11	323±11	−16±11
Basal RER	0.84±0.02	0.82±0.01	−0.02±0.02	0.84±0.02	0.81±0.01	−0.03±0.01
Acute ephedrine RER	0.82±0.01	0.82±0.01	0.01±0.02	0.85±0.04	0.84±0.02	−0.02±0.04

Whole-body energy expenditure and RER measured before (basal) and 70–90 min after (ephedrine-stimulated) ingestion of a single dose of ephedrine (2.5 mg kg^{-1}), measured before (pre) and after (post) 28 days treatment with either placebo or ephedrine (1.5 mg kg^{-1} day^{-1}). Change represents the difference between pre and post

Values are mean±SEM. (The pre–post change was compared between treatment groups using an unpaired Student's *t* test)

RER, respiratory exchange ratio

with sympathetic activation, which may lead to blunted sympathetic responsiveness in certain tissues [36]. Our findings may, therefore, contribute to an explanation for the observed reduction in BAT function in obese individuals.

BAT biopsies and other measures were not possible to directly measure BAT function or BAT sympathetic nerve activity. Nevertheless, a reduction in the increase in systolic blood pressure and blood glucose, and a trend towards a blunting of

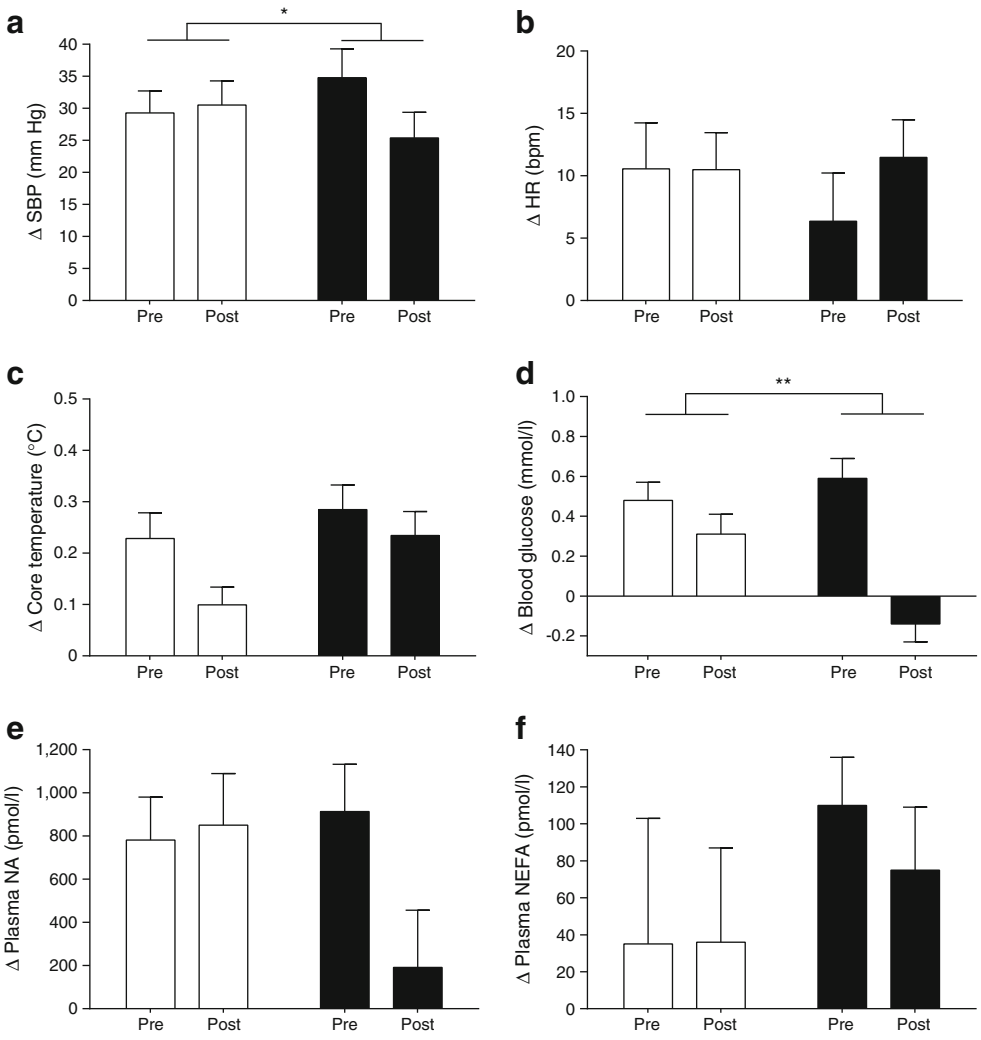

Fig. 2 Changes (Δ) in (**a**) systolic blood pressure (SBP), (**b**) heart rate (HR), (**c**) core temperature, (**d**) blood glucose, (**e**) plasma NA (p=0.06 for change from pre- to post-treatment between groups) and (**f**) NEFA from immediately prior to ingestion of a single dose of ephedrine (2.5 mg/kg) to the mean of values taken between 60 and 90 min after ingestion of the dose of ephedrine, before (pre) and after (post) 28 days treatment with placebo or ephedrine (1.5 mg kg^{-1} day^{-1}). Open bars, placebo; closed bars, ephedrine. *p<0.05 for the change from pre- to post-treatment between groups, **p<0.001 for change from pre- to post-treatment between groups (the pre–post change was compared between treatment groups using an unpaired Student's *t* test)

the rise in plasma NA in response to acute ephedrine in the chronic ephedrine treatment group support a blunting of sympathetic responsiveness to this protocol. The sympathomimetic action of ephedrine is based on increased endogenous NA release from sympathetic nerve terminals and blockade of its reuptake, thereby increasing and prolonging synaptic NA concentrations, spillover into circulation and subsequent exposure to cells [37]. Thus, the mechanism by which chronic ephedrine treatment downregulates adipose adrenergic sensitivity to acute ephedrine in humans is likely to be due to direct downregulation of β-AR on target tissues and/or altered synaptic NA release/reuptake via synaptic NA transporters. Blunting the rise in plasma NA response to acute ephedrine in the chronic ephedrine group indicates an alteration in the synaptic regulation of NA release and/or reuptake. Further, in rodents, chronic βAR stimulation with isoproterenol decreases β-AR sensitivity and receptor density [38], therefore both mechanisms are probably involved.

In many tissues, stimulation of β-ARs results in both short-term (hours) and chronic (days) downregulation of receptor sensitivity and density, respectively [23, 39]. Rodent studies, however, indicate that BAT β3-AR are unaffected, thus allowing adaptive thermogenesis to occur in response to persistent stimulation [22, 23]. Accordingly, chronic ephedrine treatment increases uncoupling protein-1 (UCP-1) and BAT activity in response to acute ephedrine treatment in mice [24], and similar results have been reported with β3-AR agonist treatment in adult dogs, which reportedly have functionally similar BAT to adult humans [40]. It is unknown which β-ARs are primarily responsible for human BAT thermogenesis, however, while expressed [4], β3-AR content is likely to be low [23] and to have little [41, 42] or no [43] involvement in human thermogenesis. The present data, therefore, support the notion that human BAT is functionally distinct from that of small rodents and other larger mammals such as dogs. Human BAT has recently been reported to be composed predominately of beige rather than classic brown adipocytes [29, 31–33], and a recent in vitro study highlighted that human and mouse BAT cells express UCP-1 in an opposing manner in response to all-*trans* retinoic acid treatment [44]. Moreover, chronic ephedrine treatment mimics adaptive thermogenesis in mice [24] and no study has reported a reduction in facultative or adaptive thermogenesis in classic brown or beige adipose depots in mice in response to sympathetic stimuli. Therefore, it is likely human BAT requires alternate stimuli to become responsive to adrenergic/sympathetic inputs, and this may contribute to the failure of β3-AR agonists to increase BAT activity in humans [45].

Mice require all β-ARs for full function of classic BAT, but are predominantly dependant on the β3-AR [22, 23, 46]. Since human BAT is not generally as well adapted as murine BAT it may require proliferative and adipogenic stimulation to precede browning to maximise adaptive thermogenesis. These processes may be more dependent on β1-AR than β3-AR signalling [23, 47], thus potentially explaining a desensitisation rather than adaptive phenomenon more representative of tissues that highly express β1/2-ARs. From an obesity therapeutic perspective, in future it would, therefore, be prudent to consider human BAT as genotypically and phenotypically distinct from murine BAT. Additionally, altered sympathetic function has been implicated as both a cause and consequence of obesity [36] and weight loss reverses dysregulated basal and glucose-stimulated sympathetic responsiveness [48, 49]. Neither reduced BAT activity nor sympathetic over-activity have been causatively linked to obesity. Since, however, BAT activity is reduced and basal sympathetic nervous system activity is increased in obesity, the present data connecting chronic sympathetic over-stimulation to reduced BAT activity strengthens the hypothesis that these observations are linked.

In our previous study, upper thoracic BAT activity, measured as SUVmax, in response to acute ephedrine was ~2.0, which was ~1.0 higher than the acute response to placebo [6]. In the current study, baseline values for SUVmax in response to acute ephedrine were lower those of our previous study at ~1.0. Nevertheless, chronic ephedrine treatment reduced SUVmax compared with placebo. Considering that SUVmax of deep upper thoracic BAT is significantly higher than subcutaneous WAT depots (~100% and >2 SDs in the present study and our previously reported basal levels [6]), it is clearly distinct in having higher metabolic activity. Accordingly, human upper thoracic BAT is now characterised as a distinct brown/beige fat depot and, therefore, may fluctuate in oxidative capacity. Nevertheless, this absolute level of BAT activity contributes only a small portion of whole-body energy expenditure [8]. While statistically significant, the small reduction observed here would be unlikely to contribute meaningfully to whole-body energy expenditure under the present experimental conditions. However, other physiological stimuli that activate BAT may result in greater differences of larger absolute magnitude, therefore it is not yet possible to conclusively evaluate the physiological relevance of these findings. Conversely, since we did not observe an increase in activity above a predetermined basal level, measurement of basal BAT activity before and after the present intervention would have been of limited value. Nevertheless, while ethical considerations related to radiation exposure prevented additional basal PET-CT scans in the current study, future intervention studies should aim to study the functional relevance of BAT under unstimulated conditions. The low proportion of participants for whom we observed acute ephedrine-stimulated activity approaching that of our previous study [6] could simply be related to only the smallest and/or leanest individuals being responsive; the lean group in our prior study (BMI 21 kg/m^2, body mass 66 kg, body fat content 17%) was significantly

lighter with lower body fat content than that of the present cohort (~24 kg/m^2, 76 kg, 25%).

Chronic ephedrine treatment resulted in a significant loss of body fat, particularly in the visceral compartment, with no loss of lean mass or bone mineral content. This highly desirable outcome was not attributable to any alteration in thermogenic function since neither basal nor ephedrine-stimulated energy expenditure changed in response to treatment. The likely explanation for the loss of body mass and fat is an increase in energy expenditure in combination with decreased energy intake. Ephedrine has a systemic half-life of ~4 h and single ephedrine doses of 1–2.5 mg kg^{-1} increase energy expenditure by ~10–15% for several hours post-treatment, putatively mainly via increased muscle thermogenesis [19]. Based on the present data indicating no change in basal or ephedrine-stimulated energy expenditure in response to chronic ephedrine treatment, tolerance does not develop with respect to the whole-body thermogenic response. Such shifts in energy balance may be compensated by increased gut and central orexigenic signals and, therefore, energy intake [50]; however, ephedrine is also an appetite suppressant [41]. We can only speculate as to the major contributing factor since body composition was not a primary outcome measure and food diaries (which are not particularly reliable) were not recorded.

While acute treatment with 2.5 mg kg^{-1} ephedrine did not result in increased BAT activity in the majority of participants in this study, and since we previously reported this dose would likely be the minimum necessary to activate BAT in lean adults [6], ideally we would have administered at least this dose daily in the present study. Due to the cardiovascular activation induced by this high dose, however, we opted to treat with a lower dose, hypothesising that the necessary adaptive adrenergic signalling events would occur in fat tissue whether or not significant thermogenesis was induced. Regardless, based on our observations, a higher dose would likely have only resulted in greater suppression of basal BAT FDG uptake. It is worth considering that the response to cold exposure after the chronic ephedrine intervention may differ to that of acute high-dose ephedrine. Cold exposure will activate BAT to a greater extent via physiological pathways that may result in differing signalling and/or substrate preference. This possibility remains to be investigated.

BAT function measured via FDG PET poses a number of technical limitations, including assessment of glucose uptake when lipids may be the predominant BAT substrate for thermogenesis. It is conceivable that in our studies of acute high-dose ephedrine treatment glucose is not a major BAT substrate. This is, however, unlikely, since chronic cold exposure studies report similar results whether using PET imaging with FDG [12, 13] or an acetate tracer to measure whole tissue metabolism [11]. Nevertheless, this possibility also remains to be studied via a technique that assesses whole tissue energy expenditure.

In the present study, we provide evidence that, contrary to our hypothesis and in contrast to mice, chronic treatment with a sympathomimetic decreases basal BAT activity and likely thermogenic responsiveness to adrenergic stimuli. This finding represents an important difference between mouse and human BAT and the differing function of human BAT should be carefully considered when assessing future BAT-dependant therapeutic targets for obesity. Importantly, while reduced BAT activity has yet to be causatively linked to obesity, our data suggest that the elevated sympathetic activity observed in obesity may perpetuate weight gain by further reducing BAT function.

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Contribution statement ALC, RP, MFF, BVE, DAB, MJA, VK, SJD, MHC and BAK were responsible for the conception and design of the study and experiments. ALC, RP, MFF, BVE, DAB, MJA, NE, GWL, VK, SJD, MHC and BAK acquired the data. ALC, RP, MFF, BVE, NE, GWL, VK, SJD, MHC and BAK analysed and interpreted the data. ALC, RP, MFF, BVE, DAB, MJA, NE, GWL, VK, SJD, MHC and BAK drafted and critically revised the manuscript. All authors approved this version of the manuscript. ALC is the guarantor of this work.

Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

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3. Extended discussion and conclusion

Since the publication of the manuscript in chapter two of this thesis, it remains that no pharmacological approaches have been shown to promote BAT adaptive thermogenesis in humans. However, several new pharmacological approaches to BAT activation have recently been trialled. The β3-AR agonist Mirabegron has high in vitro binding affinity for the human β3-AR and possesses satisfactory bioavailability.[250] A single dose has been demonstrated to increase ^{18}F-FDG uptake in supraclavicular BAT[251] however it is yet to be determined whether this uptake is reflective of classic uncoupled thermogenesis, or glucose disposal and whether long-term treatment with Mirabegron circumvents tolerance in the manner observed in the present study.

Further studies have investigated the role of glucocorticoids in BAT activation. Excess glucocorticoids are known to potentiate obesity. Ramage et al[258] demonstrated that three doses of prednisolone over 24 hours before cold-exposure augmented cold-stimulated BAT activity in humans. The trial also demonstrated that chronic glucocorticoid exposure led to BAT suppression. This was further supported in a double-blind, placebo-controlled trial in which one week of oral prednisolone decreased BAT activity measured via ^{18}F-FDG-PET/CT, infrared thermography, and increased whole-body lipid synthesis rate.[259] These studies suggest that similar to pan-β-adrenergic receptor agonists, glucocorticoids induce an acute increase in UCP-1 expression but suppress adrenergic signalling during long-term use.

Thiazolidinediones, a class of glucose-lowering drugs, have long been recognised as browning agents, based on preclinical experimental models.[260] Loh et al[138] recently examined this concept for the first time in humans in vivo with the use of the thiazolidinedione, Pioglitazone. Although effective at increasing in vitro browning and adipogenesis in human adipocytes, treatment for twenty-eight days in healthy adult humans resulted in the opposite effect, with BAT activity significantly reduced compared with placebo after the intervention.

Experimental limitations remain in detecting presence and function of BAT in humans. The development of non-invasive techniques to further understand the role of BAT in health and disease and to quantify BAT is warranted. The current gold–standard continues

to be ^{18}F-FDG PET/CT[261] which has been demonstrated to consistently identify areas of histologically-confirmed BAT[134, 182, 262] but it does not assess absolute BAT energy expenditure nor reliably identify BAT present in small systemic discrete depots.[204, 263] Additionally, glucose as a primary analogue provides no insight into BAT's primary energy substrate, lipids.[264] Furthermore there is increasing evidence that lipids are the preferential substrate for BAT in situations of prolonged stimulation[210] and pharmacological intervention.[265] Therefore, observed ^{18}F-FDG uptake might not reflect the true changes in total BAT thermogenic activity and energy expenditure in some cases.[266] An important step will be the development of a methodology which i) assesses total tissue substrate metabolism, ii) can measure BAT presence without the requirement for the tissue to be active, and iii) does not involve exposure to ionising radiation. Advances in imaging techniques including magnetic resonance spectroscopy,[267] contrast-enhanced ultrasound,[268] and thermography,[269] have been gained. Nevertheless, none of these techniques completely resolve current issues.

In conclusion, in the present study, we provided evidence that, contrary to our hypothesis and previous studies in mice, that chronic treatment with a sympathomimetic decreased basal BAT activity and likely thermogenic responsiveness to adrenergic stimuli. This finding highlights important differences in mouse models of BAT function and indicates a greater need for careful consideration when translating observations from preclinical animal models to humans, particularly in the development of future BAT-dependent therapeutic targets for obesity. Future studies should evolve in a bi-directional manner with both animal models and human studies informing each other of similarities and disparities between BAT distribution, function and signalling, so that clinically relevant findings may be uncovered.

Importantly, while reduced BAT activity has yet to be decisively linked to obesity, our data suggest that chronically elevated sympathetic activation observed in obesity may perpetuate weight gain by further reducing BAT function. Therefore, whether BAT can make a meaningful contribution to obesity through weight loss or rather through the management of metabolic disease remains to be determined. Whether cause or consequence, the well-established existence of BAT dysfunction remains a key co-factor in the context of human weight gain and obesity. Understanding the mechanisms

responsible and developing strategies to recruit BAT, therefore, remain important for research in this field.

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