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STATEMENT OF COMPLIANCE

The protocol will be carried out in accordance with International Conference on Harmonisation Good Clinical Practice (ICH GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812)

National Institutes of Health (NIH)-funded investigators and trial site staff who are responsible for the conduct, management, or oversight of NIH-funded trials have completed Human Subjects Protection and ICH GCP Training.
The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; an IRB determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

Précis

The balance between energy expenditure (EE) and energy intake ultimately determines body weight. Resting EE is the major component (60-75%) of total EE in an adult human being. Resting EE dynamically adapts to environmental changes such as ambient temperature. In our on-going study of environmental temperature changes within and around the thermoneutral zone, we observed that healthy young men can increase EE by 17 % of the basal metabolic rate through the process of non-shivering thermogenesis (NST). This capacity for NST is unexpectedly large as compared to prior reports of mild cold-induced thermogenesis (3 to 11%) and suggests that increasing NST could be explored as an intervention to combat obesity.

The aim of this study is to better understand the physiology of NST and to develop improved assays for evaluating the effect of drugs that alter EE. For example, only recently has it been realized that brown adipose tissue is functional in adult humans and that white adipose tissue can be converted to brown-adipose-like tissue to increase heat production during cold exposures. Moreover, skeletal muscle likely also plays a role in cold-induced thermogenesis even before overt shivering occurs. It is plausible that the mechanisms governing heat production for NST contribute to regulation of body weight and thus may be contributing to the current obesity epidemic: even small changes in EE, if not compensated by changes in food intake, can have long-term effects on body weight.

This protocol has two phases. The first uses a pharmacologic approach to investigate the mechanism of NST in young healthy lean males. Since the principal physiologic stimulus to BAT (and possibly muscle for NST) is via the sympathetic nervous system (SNS), β-adrenergic receptors may hold key roles in regulating human EE. We hypothesize that, by careful measurements of NST (at an individually-titrated cool environmental temperature, between 18-21ºC vs. at thermoneutrality of 27ºC) and using β-adrenergic drugs that differ in receptor specificity and agonist/antagonist properties, we will gain better understanding of the regulation of human NST.

The second phase of the study focuses on measuring of FDA-approved drugs' (such as anti-obesity drugs) potential effect on basal metabolic rate (BMR) under thermoneutral conditions. The rationale is that previous studies of drug effect on EE in humans have not always rigorously enforced the use of thermoneutral conditions, thus may have increased variability and underestimated the effect, contributing to inconclusive findings.

It is envisioned that this study will further our knowledge of the mechanisms that regulate the acute adaptive changes in resting energy expenditure and the effects of drug therapy targeting obesity in humans.

Abbreviations:

BAT	brown adipose tissue
BMI	body mass index
BMR	basal metabolic rate
Cmax	maximum plasma concentration
CT	computed tomography
DEOB	Diabetes, Endocrinology, and Obesity Branch
DXA	dual energy X-ray absorptiometry
EE	energy expenditure
EKG	electrocardiograph
FDG	[^{18}F]-fluorodeoxyglucose
GWAS	genome wide association study
IRB	institution review board
Ki	binding receptor affinity
MRI	magnetic resonance imaging
NST	Non-shivering thermogenesis
PET	Positron emission tomography
REE	resting energy expenditure
RQ	respiratory quotient
SNS	sympathetic nervous system
Ucp	uncoupling protein
VAS	visual analog scale
TNZ	thermoneutral zone

I. Introduction and Background: Energy expenditure and environmental temperature

Resting energy expenditure (EE) comprises 60-75% of the total EE in an average adult human. Resting EE is not static. Like other mammals, humans adjust EE (or heat production) to balance heat loss in order to maintain constant body temperature. In rodents, the relationship between EE and environmental temperature is shown graphically in figure 1. In an ongoing protocol (12-DK-0097), we are mapping this response in men and women to determine the effect of adiposity, age, and race on the response to a short-term and tolerable temperature manipulation.

Animals invoke physiological adaptations such as vasoconstriction and piloerection in response to the changing ambient temperature. The temperature range at which such adaptations are sufficient to maintain a minimum heat production to balance the heat loss is called a thermoneutral zone (TNZ). [1] [2] Within the TNZ, resting EE is known as basal metabolic rate (BMR). When these responses cannot sufficiently compensate for the increased heat loss associated with the further decrease in ambient temperature, additional EE is needed. Figure 1 shows this thermogenic response process is gradual which reflects the net heat balance of the animal. Moreover, when exposed to extreme cold, muscle shivering is recruited to increase heat production, but cannot be maintained for a prolonged period of time. In contrast, exposure to mild cold induces non-shivering thermogenesis (NST),[3] which may be sustainable and tolerable. NST has been studied to a limited extent in adult humans, with high variability in its estimates due to several potential factors (Table 1).

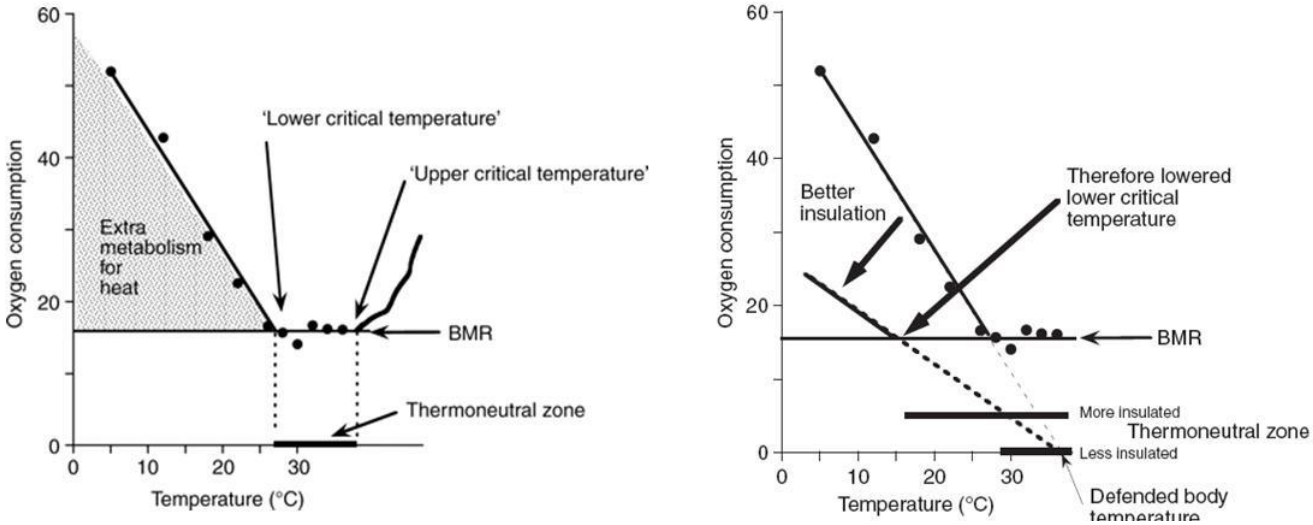

Figure 1. Graphs of the metabolic rate (oxygen consumption) vs environmental temperature relationships, Figures 1 and 4 in Cannon and Nedergaard [1]. BMR is basal metabolic rate (also known as resting EE in humans).

One source of variability is the actual definition of NST used in the studies. NST is the % change in resting EE at a defined cold temperature without shivering as compared to the BMR measured at thermoneutrality (within the TNZ). Individual TNZ is

variable, for which traditional measurements of resting EE (not the true BMR) often do not carefully control for this factor. The second cause may be the differences in testing conditions, including the ambient temperature used for cold exposure and the insulating properties of the clothing worn during testing (quantified by a commonly used term clo for measuring the thermal insulation of clothes, where zero [0] Clo corresponds to a naked person and one [1] Clo corresponds to a person wearing a typical business suit.) Other sources of variability in NST include food intake, posture and physical activity (Table 1) during the testing period.

It was recently rediscovered that brown adipose tissue (BAT) is functional in adult humans [4-7] and its presence and activity appear to be highly variable. The primary function of BAT is to maintain body temperature and may be an important contributor to NST. BAT releases the energy from substrate oxidation as heat via uncoupling protein 1 (UCP1), rather than using the energy to generate ATP [8]. Moreover, non-shivering heat generation may also occur in skeletal muscle, through browning of white adipose tissue, and by other factors that contribute to the high variability of the cold-induced thermogenesis in the literature. Taken together, there is great interest in understanding the underlying mechanisms governing NST contribution to total body energy homeostasis and body weight regulation. Understanding these control mechanisms will provide insight into the pathophysiology underlying the current epidemic of obesity and should provide insight into potential treatments.

Table 1. A brief summary of literature review of cold-induced thermogenesis.

% Increase	Metric	Temp range, C	Clo	Reference
7.0	24h resting	22-28	NA	Dauncey MJ (1981) [9]
11.0	day resting	22-28	NA	Dauncey MJ (1981) [9]
6.0	24h resting	16-22	0.71	van Marken Lichtenbelt (2002) [10]
4.9	24h resting	16-22	0.71-0.8	Wijers SL (2007) [11]
6.0	day resting	19-24	0.55	Celi FS (2010) [12]
3.5	day resting	16-22	0.8	Wijers SL (2011) [13]
For comparison:				
17.4	day resting	18-27	0.36	12-DK-0097, preliminary analysis

Within the scope of protocol 12-DK-0097, while defining the detailed relationship between resting EE and environmental temperature, we are defining the maximum amplitude or the capacity of NST. In brief, in that protocol the range of environmental temperatures being studied (16-31°C) is that routinely encountered during daily activity, including the comfort zone [14] and slightly lower. Importantly, the protocol is designed to minimize the contributions of physical activity, thermic effect of food, and behavioral changes (such as changes in body position) and standardizes the insulating properties of clothing worn for testing (clo=0.36). Thus the measured EE is expected to reflect NST in response to the environment. The protocol determines the following parameters

experimentally (see Figure 1 [1,15]): 1) the thermoneutral zone (TNZ), defined as the environmental temperature range in which EE is at a minimum and does not change with environmental temperature, 2) the shivering threshold, defined as the lowest environmental temperature before shivering occurs, and 3) the capacity for non-shivering thermogenesis, defined as the difference in EE measured at the shivering threshold and at thermoneutrality.

To our knowledge, 12-DK-0097 is providing the first rigorous measurement of the capacity for non-shivering thermogenesis in humans. In a preliminary analysis of the first subjects, a lean Caucasian cohort, EE at the shivering threshold was 17.4 ±3.7% (n=6) greater than BMR. We were initially surprised by this unexpectedly large capacity for non-shivering thermogenesis, as prior publications of mild cold-induced thermogenesis found increases of only 3 to 11% (Table 1). However, we believe that the 17% value as seen in our current study is plausible for the following reasons: 1) our measurements are in the fasting state, removing any masking of facultative thermogenesis by the thermic effect of food (a phenomenon already recognized by Rubner in 1902 [9]), 2) strict enforcement of no physical activity, 3) strict prohibition of postural and behavioral changes, and 4) actual measurement of the TNZ and shivering threshold, ensuring that the full span of NST is captured, compared to two fixed temperature points which were used by most previous studies. We note that a crucial innovation is the use of a relatively short time period for the EE measurements, allowing stricter control of the above variables, enabled by specialized analysis of the chamber data [16].

The closest estimation to our magnitude of NST was found by Claessens-can Ooijen et al., which a 17% increase in EE was detected in lean male subjects.[17] It is worth noting that the testing conditions were quite different in this study, where all measurements were conducted at 15°C. The thermoneutral state was define when the subjects wore standard clothing consisted of sweat shirts and pants (clo =0.71) and covered with a duvet (clo=6.8). When the duvet was removed, the 1-hr exposure was defined as mild-cold. It is possible that a steady-state EE was not achieved by this design. Davis [18] reported a 34% increase over resting following 31 days of exposure to 12 °C for 8 h per day where significant shivering at the first 20+days. While this is believed to be an acclimation effect but demonstrates a very significant potential capacity in healthy lean men. It was not understood at that time that brown fat was heat-producing,

In summary, the preliminary results from 12-DK-0097 demonstrate that 1) a suitable experimental system can be devised to measure the capacity for NST in humans and 2) the NST contribution to EE is greater than previously recognized.

The two major questions asked in this protocol are:

- ***What are the mechanisms driving human NST?***
- ***What are the effects of drugs on human BMR?***

I.1 Scientific and Clinical Significance

The high prevalence of obesity is a major public health concern. Small differences in energy expenditure, if not compensated by changes in food intake, would have long-term effects on body weight.[19 20] Mild cold environmental temperatures elevate energy expenditure in humans, likely via BAT activation [4]. It is plausible that an increasingly thermoneutral environment is contributing to the obesity epidemic. Conversely,

increasing heat production via lowering of environmental temperature could be a potential approach to combat obesity. This study will expand our understanding of the mechanisms underlying human thermal physiology in a temperature range that is close to those encountered in daily indoor living. Moreover, several FDA-approved drugs' (such as anti-obesity drugs) currently on the market may act through mechanism(s) that modulate BMR and/or NST, however, no previous studies have rigorously quantified their impact with precise measurements as we plan to accomplish in this protocol.

I.2 Mechanistic basis for NST

What are the physiologic mechanisms responsible for human NST? To answer this question, the contributions of adipose tissue and muscle must be considered as well as shared and unique signaling pathways, neurotransmitters and hormones. Experiments in mice suggest that non-shivering facultative thermogenesis occurs in at least two tissues. BAT is believed to be the major source of facultative thermogenesis via uncoupling protein 1 (Ucp1) [8,21]. (In this protocol, 'BAT' is used in the generic sense and includes both classic (typically interscapular in location) BAT and inducible BAT, also known as beige or brite adipose tissue [22]). Indeed, some data suggest that Ucp1 ablation cannot be compensated for by other mechanisms in sufficient magnitude to prevent shivering [23]. However, clinical data [13] and recent experiments in mice suggest that muscle can also contribute significantly to NST, likely via sarcolipin, SERCA, and/or RyR1 [24].

Assuming BAT is the chief source of facultative thermogenesis, one would like to quantitatively measure BAT activation (both tissue volume and metabolic activity per unit volume). However, the current standard technique for measuring BAT activity is FDG-PET/CT, which has two major limitations: (1) it is not suitable for multiple serial measurements due to radiation dose considerations, and (2) it only measures glucose uptake which is about 10% of the total substrates that is utilized by BAT [3]. We therefore have chosen a primarily pharmacologic approach to investigate the drivers of NST.

The principal physiologic stimulus to BAT (and possibly muscle for NST) is via the sympathetic nervous system (SNS). When activated, sympathetic nerves release norepinepherine which binds to α- and β-adrenergic receptors on adipocytes to stimulate heat production, with the β-adrenergic receptors driving thermogenesis. Indeed there are clinical reports that the β-adrenergic receptor blocker propranolol reduces the uptake into BAT during FDG-PET studies by 55 to 75% [25,26]. While adipose tissue shares β1- and β2-adrenergic receptors with many tissues, the tissue distribution of β3-adrenergic receptors is restricted and thus β3-adrenergic agonists could be used to selectively target adipose tissue, as is done in preclinical studies (eg [27]). However, the β-adrenergic receptors on adipose tissue do not appear to be interchangeable. While β3-receptors are typically assumed to be the major driver, β2-adrenergic receptors alone are sufficient for normal BAT morphology [28] and the β1-adrenergic receptor has been reported to be able to mediate most of the sympathetic stimulation of cold-induced thermogenesis [29]. α1-adrenergic agonists can also augment BAT stimulation, but do not contribute in the absence of β-adrenergic stimulation [30].

While the molecular details of muscle NST are not clear, it appears that in mice, dantrolene, via inhibition of the ryanodine receptor calcium channel, Ryr1, can inhibit

muscle NST [24]. Thus, dantrolene is a unique tool that may allow distinguishing of muscle vs BAT sources of NST.

In this protocol we will focus on the regulatory pathways of human NST via pharmacological modulations of the SNS receptors. The results of these studies will allow us to elucidate the receptors controlling NST in humans, and by inference, the contribution of BAT.

Propranolol

This study will target the β-adrenergic system contribution to NST. A non-specific β-adrenergic antagonist might be expected to inhibit NST via abrogation of sympathetic signaling to BAT. Propranolol is a β blocker approved for multiple conditions including hypertension, angina, atrial fibrillation, and others (Inderal label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/016418s080,016762s017,017683s008lbl.pdf). While propranolol is reported to inhibit the cold-stimulated uptake of glucose into BAT [25,26], it has also been reported that propranolol does not inhibit cold-induced thermogenesis [13]. There are several possible explanations to these previous findings. Propranolol is β1/β2-selective, with binding receptor affinity (K_i) for human β1-, β2-, and β3-adrenergic receptors of 1.8, 0.8, and 186 nM, respectively [31]. Based on the pharmacokinetics of propranolol, with a maximum plasma concentration (Cmax) at ~1-4 hours of ~150 ng/ml and t1/2 of ~4 hours (both parent and active metabolite) [32,33], the plasma concentration at Cmax is calculated as ~580 nM. For a 160 mg dose of regular propranolol (not sustained release), and after correcting for protein binding (90% bound [34]), the free plasma concentrations at Cmax are 32, 73, and 0.3 times the Ki of the β1-, β2-, and β3-adrenergic receptors, respectively. Thus, while this concentration should completely inhibit signaling via the β1- and β2-adrenergic receptors, its inhibition on the β3-adrenergic receptor is incomplete at best, with the level of inhibition dependent on the actual endogenous norepinepherine concentration. Since the actions of a 160 mg propranolol dose on suppression of the exercise-induce heart rate increase (a β1 effect) lasts 36 hours [33], a suitable wash-out interval is 48-72 hours. Note that based on the pharmacokinetics and receptor selectivity of propranolol, it is not surprising that 160 mg of sustained-release propranolol [13] is predicted to have a Cmax of of ~80 nM, which is 4.4-, 10- and 0.43-fold of the Ki of the β1-, β2-, and β3-adrenergic receptors, respectively. The inhibition at Cmax of glucose uptake into BAT by 20 mg [26] or 80 mg [25] of regular propranolol may suggest that BAT glucose uptake is largely via β1-and/or β2-, and not β3- adrenergic receptors.

The effect of 160 mg propranolol at thermoneutrality is expected to be slight. Propranolol's effect on EE has been studied multiple times, with three examples being a reduction of 4.1% (non-significant [35]), 1.6% (non-significant [36]), and 5.0% (p=0.002 [11]. At the low temperature, 160 mg should have a proportionally greater effect on reducing EE, although any prediction of the magnitude is largely an estimate.

Pindolol

A complementary test of the drivers of NST would be use of a selective β3-adrenergic agonist [37], but none is currently approved for this use in humans. However, pindolol (Visken, label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/018285s034lbl.pdf) is an

approved drug with properties suitable for 'clamping' the β-adrenergic tone. Pindolol is a mixed partial agonist and antagonist. At complete receptor occupancy, a partial agonist gives less response than that of the native ligand. In the presence of a full agonist, saturating partial agonist will reduce the receptor response.

Pindolol has pharmacokinetic and binding properties indicating that full receptor occupancy can be achieved with single oral dosing in humans. Specifically, pindolol has binding K_is of 2.6, 4.8, and 44.1 nM for the human β1-, β2-, and β3-adrenergic receptors, respectively [31]. The pharmacokinetics of a 20 mg dose of pindolol include a Cmax at ~1-2 hours of ~96 ng/ml, t1/2 of ~2.9 hours, and 38% plasma protein binding [38]. From the total plasma concentration at the Cmax of ~387 nM, one can estimate that the Cmax after a 20 mg dose of pindolol will yield free plasma concentrations that are 92-, 50-, and 5.4-fold the K_is of the β1-, β2-, and β3-adrenergic receptors, respectively. These data indicate that 20 mg of pindolol should saturate all three endogenous β-adrenergic receptors.

Pindolol is an antagonist at β1-adrenergic receptors [31,39] and a partial agonist at β3-adrenergic receptors [31,40]. It may either be a partial agonist [39] or antagonist [31] at the β2-adrenergic receptor. Interpreting reports of β-adrenergic receptor selectivity that antedate discovery of the β3-adrenergic receptor and of partial agonism that do not consider biased agonism [41] add some uncertainty. However these are moot, assuming that pindolol saturates all three β-adrenergic receptors.

If the predominant signaling to human NST mechanisms is via β-adrenergic receptors, one expects that by clamping β-adrenergic tone with pindolol, the same level of change in REE_{Low} and $REE_{27°C}$ will be observed. Since pindolol is a partial agonist, the prediction is that this clamped EE will be elevated moderately at a level between $REE_{27°C}$ and REE_{Low} with placebo.

Dantrolene

To differentiate NST from skeletal muscles, we will use the following two pharmacologic agents. Dantrolene (Dantrium, label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/017443s043s046s048s049lbl.pdf) is clinically indicated for treatment of muscle spasticity [42] and of malignant hyperthermia [43]. Dantrolene binds and inhibits the muscle ryanodine receptor (RyR1), which releases calcium from the sarcoplasmic reticulum [44,45]. A recent study in mice suggests that dantrolene blocks muscle NST, but has no effect on BAT NST [24].

Clinical dosing of dantrolene for spasticity is via titration from 25 mg QD to up to 100 mg TID, or even 100 mg QID (label). The pharmacokinetics of a single 100 mg oral dose of dantrolene include a Cmax at ~4.5 hours of 0.7-1.45 ug/ml (2.2-4.6 uM; Mr is 314), and t1/2 of 9-12 hours [46-48]. Single oral doses as high as 150 mg have been reported [47].

It is not clear what level of dantrolene is needed to maximally inhibit RyR1. Dantrolene is hydrophobic and highly bound to albumin [49], but we are not aware of any clinical measures of free drug levels. Thus, how an in vitro IC50 of 300 nM [45] corresponds to the exposure achieved with a single 100 mg oral dose of dantrolene is unknown.

We are not aware of prior studies of the effect of dantrolene on EE under conditions similar to those in this protocol. The prediction is that a single oral 100 mg oral dose of dantrolene will significantly inhibit muscle NST in humans.

Magnesium sulfate

Magnesium, a non-competitive inhibitor of inositol 1,4,5-triphosphate (IP3)-gated calcium channel and RyR1receptor is a mild muscle relaxant [50-53]. Although, the exact cellular mechanisms are unclear, intravenous magnesium in randomized controlled trials suppresses post-operative shivering (relative risk 0.38, 95% CI: 0.17-0.88) [54-56]. In healthy individuals, magnesium sulfate (80 mg/kg bolus followed by a maintenance infusion at 2 g/h) reduces shivering threshold, albeit minimally [57]. Nevertheless, intravenous magnesium sulfate is included in pharmacological anti-shivering regimens during therapeutic hypothermia [58]. In these clinical trials, serum magnesium concentrations increased by 31-84% (mean: 2.3 mmol) following a median cumulative intravenous magnesium dose of ~ 8 g [54]. Bolus intravenous magnesium (2 g) results in an acute but transient increase in serum magnesium (~ 2 mmol) with a t1/2 of ~ 4 h in individuals with normal renal function. However, steady state serum concentrations of ~ 2.2 mmol can be achieved by a maintenance infusion dose of 2 g/h [59]. Possible adverse reactions associated with intravenous magnesium include, nausea, flushing, mild hypotension, bradycardia, and minimal irritation at the infusion site [54].

Whether intravenous magnesium affects NST is not known. Given the inhibitory effects of magnesium on skeletal muscle excitation-thermogenic coupling and calcium handling, we predict that increasing magnesium concentrations significantly inhibits muscle NST in humans.

Mirabegron

Mirabegron is a selective β3-AR agonist approved by the FDA in June 2012 for treatment of overactive bladder at a dose of 50mg once daily. (Myrbetriq label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/202611s000lbl.pdf) According to the advisory committee report (FDA 2012), the peak mean concentration was achieved between 3 and 4.3 hours (p. A-81). At 200mg, the maximal mean increase in pulse was 10.3 -11.8 bpm (p. A-80) and in SBP and DBP, 11.6 and 7.7 mm Hg, respectively, as compared to placebo at hours 3 to 6 post-dose (p.A-81). Of note, the subgroup analysis by gender revealed that at a dose of 200mg, in females, mirabegron prolonged the QTc interval by a maximum of 10.42 msec, but there was no QTc prolongation in males (p. A-85). For this reason, the present study will recruit only healthy male volunteers with normal baseline QTc. No clinical effects of mirabegron atrial fibrillation or heart failure were detected (p. A87-A88).

Mirabegron has binding K_is for the human β1-, β2-, and β3-adrenergic receptors of 4200, 1300, and 40 nM, respectively. These values demonstrate a much greater β3-AR selectivity compared with pindolol , making mirabegron a promising candidate for adipose tissue-specific activation. In males, for 50 mg, the C_{max} and T_{max} were 23.7 ng/mL and 3.50 h, respectively, and for 200 mg, the C_{max} and T_{max} were 158 ng/mL and 2.68 h, respectively (FDA 2012). In our experience, as will be described in detail below, the C_{max} for a 200 mg dose was 274 ± 64 nM based on 65% protein binding, which is 6.9x the Ki of mirabegron for the β3-AR (40 nM). If the 50 mg dose achieves 18% of

200 mg, then the C_{max} would be 49 nM, which is 1.2x the Ki and 2.2x the in vitro EC_{50} of 22.4 nM (Takasu et al., 2007).

We have recently completed a pilot study of the effectiveness of a one-time dose mirabegron 200 mg to stimulate human BAT and increase energy expenditure in young (22.2±0.6 years), lean (22.7±0.5 kg/m^2), healthy men who were already known to have detectable BAT via PET/CT. The pilot study achieved its primary endpoint by demonstrating that mirabegron at 200mg significantly stimulated BAT metabolic activity and led to a 13% increase in RMR, demonstrating that a β3-AR agonist can stimulate human BAT thermogenesis and may be a promising treatment for metabolic disease.

The principal limitation of the pilot study is that the dose used (200mg) was higher than the approved dose of 50mg, and this difference was associated with clinically relevant adverse effects. At 3.5 hours after dosing with 200mg mirabegron, we observed increases in mean HR (14 ± 3 bpm) and systolic BP (11 ± 2 mmHg), but not diastolic BP (2 ± 1 mmHg). This is consistent with information provided in the Background Document for Meeting of Advisory Committee for Reproductive Health Drugs (FDA 2012); although, a slightly higher increase in diastolic blood pressure (+7.7 mm Hg) was reported by the FDA, which is greater than our previous experience with a 200 mg dose of the drug. These treatment-emergent adverse effects are thought to be a result of off-target binding of the drug to β1-ARs (Cypess 2015). Although very promising, these preliminary results indicate that mirabegron will not be useful clinically unless the cardiovascular stimulation can be limited while still stimulating NST and BAT.

To conduct future studies of mirabegron-induced NST, we will need to determine the optimal dosing strategy for mirabegron. In the first phase of this sub-study, we will perform a dose-response analysis of mirabegron at 0mg, 50mg, and 200mg, using mild cold exposure as a positive control. Should the 50mg dosage be sufficiently effective, this dose will be used in subsequent studies. If not, we will consider a second sub-study in which mirabegron will be combined with a selective β1-AR blocker, likely choosing pindolol or nebivolol.

Of note, the study will continue the use of pindolol in Cohort 1 (as described above). There are at least two reasons we have chosen to do so. First, its mechanism of action is quite different from mirabegron. It can serve as a negative control for β3-AR binding while having β1 and β2 binding. Second, 8 subjects (as of January, 2015) have already taken pindolol as part of Cohort 1, so it would be important to continue until the planned interim/final analysis before deciding to terminate its use early.

I.3 Assaying anti-obesity drugs for their effects on EE

Significance of improving assay of drugs that stimulate EE

When studied in detail in mice, it appears that the vast majority of centrally-acting drugs have dual beneficial effects of decreasing food intake and increasing metabolic rate [60,61]. Despite of the huge magnitude of facultative thermogenesis in mice, care is not always being taken to test drugs for metabolic effects at thermoneutrality.[62] While the effect of environmental temperature on metabolic rate was first described by Lavoisier in 1790 [63], studies of drug effect on EE in humans have not always rigorously enforced the

use of thermoneutral conditions. We propose to first explicitly demonstrate the need for measuring drug effect under thermoneutral conditions and then re-evaluate the effect of anti-obesity drugs on BMR employing our new knowledge of human capacity for NST under our optimized experimental conditions.

Caffeine

Caffeine has been chosen as the reference drug (positive control) for studying an increase in EE. Caffeine has multiple pharmacologic actions, with its effects at modest doses in humans being attributed to antagonism of the A_1R and $A_{2A}R$ adenosine receptors [64]. Caffeine was first shown to increase EE by Hoppe in 1857 (see [65]) and has been studied many times since. In an analysis of 12 studies using doses from 50 to 536 mg (published between 1915 and 2008 [66-77]) there was an approximately linear increase in EE with caffeine dose, with ~10% increase at a dose of 300 mg.

Caffeine is completely absorbed, with a Cmax of ~0.5 hours and a t1/2 of ~4-6 hours with multiple active metabolites [64]. Notably, there is attenuation of pharmacodynamic responses to caffeine with repeated dosing, which is avoided by keeping at least 72 hours between doses [78,79]. Possible adverse reactions are: frequent passing of urine, nervousness, restlessness, diarrhea, headache, and stomach upset. We propose dosing 300 mg caffeine, which is the same amount as in the Starbucks Grande size (http://www.cspinet.org/new/cafchart.htm). Actions of caffeine include some increase in blood pressure and heart rate [78] and increased alertness and reduced fatigue [80].

300 mg caffeine is predicted to increase EE at thermoneutrality by at least 10% (the effect will be larger if the prior studies were underestimations due to not using thermoneutral conditions). The effect on BMR by caffeine will serve as a positive control for the potential effects by other anti-obesity drugs.

Phentermine

Phentermine is a sympathomimetic drug approved for the short-term treatment of obesity (Adipex-P label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/085128s065lbl.pdf). The dose of 37.5 mg daily (30 mg free base) is twice the amount in the recently approved drug Qsymia (see below). The only publication known to us reporting the effect of phentermine on EE was of a 1.4% non-significant increase in resting metabolic rate with 8 mg TID x 5 days [81]. Phentermine has a Cmax at 2-4 hours and a t½ of 19 ±4 hours. These pharmacokinetics suggest that 4 days should be allowed after dosing for drug to be adequately cleared.

We propose dosing 37.5 mg phentermine (regular, not sustained release), the recommended daily dose. The effect of a single dose of 37.5 mg phentermine will be studied only at thermoneutrality. There may be attenuation of any EE increase with continued dosing, so it is difficult to know what to predict from a single dose compared to the prior experience with 5 days dosing.

Topiramate

Topiramate is a drug with multiple pharmacologic actions, including blockage of voltage-dependent sodium channels, augmentation at some GABA-A receptor subtypes, antagonism of AMPA/kainate subtype of the glutamate receptor, and inhibition of carbonic anhydrase (particularly isozymes II and IV) [82]. Topiramate is a component of

Qsymia (see below) and is also indicated for epilepsy, migraine prophylaxis (Topamax label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020505s042,020844s036lbl.pdf).

After topiramate 100 mg orally, the Cmax is 1.73ug/ml (5.1uM; Mr=339) and after 200 mg it is 10.9uM [83,84]. For single oral doses of 100-1200 mg, Tmax is 1.4-4.3 h; elimination t1/2 ~21 hours. The pharmacokinetics suggest that 4 days should be allowed after dosing for drug to be adequately cleared and that the Cmax after a single dose of 200 mg models the steady state of 100 mg daily dosing. We have not located any studies of the effect of a single dose of topiramate on EE. The interpretation of EE in the setting of chronic dosing [85,86] is complicated by changes in body weight.

Qsymia

Qsymia (label: http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/022580s000lbl.pdf) was approved by the FDA in July 2012 and marketed in September 2012. Qsymia is a combination of phentermine and topiramate, with the highest dose being 15 mg/92 mg. The most common adverse reactions are: paraesthesia, dizziness, dysgeusia, insomnia, constipation, and dry mouth. There are no studies of the effect of Qsymia on EE. Details of the constituent agents are in the preceding sections.

Naltrexone

Naltrexone (label: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2000/75-434_Naltrexone%20Hydrochloride_prntlbl.pdf) is an opioid antagonist used in the treatment of ethanol dependence at 50 mg orally daily, which may alternatively be given as 100 mg every other day or 150 mg every third day. Naltrexone is a component of Contrave (48 mg naltrexone SR, 400 mg bupropion SR), being developed by Orexigen for the treatment of obesity. Naltrexone is completely orally absorbed and undergoes first-pass metabolism. After single oral doses of 50, 100, or 200 mg, the AUC and Cmax were dose proportional and the Cmax occurred at ~1 hour for both naltrexone and the active 6β-naltrexol metabolite, with elimination t1/2s of ~4 and ~14 hours, respectively [87-90]. Doses of up to 300 mg orally, twice daily for three days produced a non-significant 4.6% increase in metabolic rate [91].

II. Research Objectives

This protocol builds on the experience from our current ongoing research (12-DK-0097, titled "Energy expenditure responses to a range of environmental temperatures around the thermal neutral zone". We plan to use the same assay conditions and inclusion/exclusion criteria as in that protocol, minimizing changes to accommodate the goals of this protocol, which are to address the following questions:

- What are the mechanisms driving human NST?
- What are the effects of drugs on human BMR?

These studies will be performed in healthy male subjects' age 18-35 years with a BMI between18.5 and 25 kg/m^2.

II.1 Primary Objectives

Cohort 1 – This Cohort has completed enrollment
1) To evaluate the effect of propranolol 160 mg PO on resting EE at a temperature just above the subject's placebo shivering threshold.

2) To evaluate the effect of pindolol 20 mg PO on the difference in resting EE (comparing thermoneutrality to a temperature just above the subject's placebo shivering threshold).

3) To evaluate the effect of dantrolene 100 mg PO on resting EE at a temperature just above the subject's placebo shivering threshold.

4) To evaluate the effect of magnesium sulfate (50 mg/kg bolus followed maintenance infusion at 2 g/h) on resting EE at a temperature just above the subject's placebo shivering threshold.

Hypotheses
1) Propranolol 160 mg PO will reduce the resting EE measured at a temperature just above the subject's placebo shivering threshold (REE_{Low}) by greater than or equal to 5% compared to placebo REE_{Low}. If propranolol-treated subjects shiver at the low temperature, then measurement of non-shivering EE is not possible in this protocol. Propranolol will be judged to increase the shivering threshold if significantly more subjects shiver with propranolol than with placebo treatment.

2) Pindolol 20 mg PO will lead to a similar difference in resting EE measured at thermoneutrality ($REE_{27°C}$) and REE_{Low}.

3) Dantrolene 100 mg PO will reduce the REE_{Low} by greater than or equal to 5% compared to placebo REE_{Low}. If dantrolene-treated subjects shiver at the low temperature, then measurement of non-shivering EE is not possible in this protocol. Dantrolene will be judged to increase the shivering threshold if significantly more subjects shiver with dantrolene than with placebo treatment.

4) Magnesium sulfate (50 mg/kg bolus followed maintenance infusion at 2 g/h) will reduce the REE_{Low} by greater than or equal to 5% compared to REE_{Low}.

Cohort 2
1) To evaluate the effect of caffeine (300 mg PO) on BMR (=$REE_{27°C}$).

2) To evaluate the effect of Qsymia (topiramate 92 mg CR, phentermine 15 mg PO) on BMR.

3) To evaluate the effect of phentermine (37.5 mg PO) on BMR.

4) To evaluate the effect of topiramate (200 mg PO) on BMR.

5) To evaluate the effect of naltrexone (100 mg PO) on BMR.

Hypotheses

1) Caffeine 300 mg PO will increase BMR by greater than or equal to 5% vs placebo.

2) Qsymia (topiramate 92 mg CR, phentermine 15 mg) PO will increase BMR by greater than or equal to 5% vs placebo.

3) Phentermine 37.5 mg PO will increase BMR by greater than or equal to 5% vs placebo.

4) Topiramate 200 mg PO will increase BMR by greater than or equal to 5% vs placebo.

5) Naltrexone 100 mg PO will increase BMR by greater than or equal to 5% vs placebo.

Cohort 3 – This Cohort has completed enrollment
1) To compare the effects of mirabegron at 0mg, 50mg, and 200mg on BAT metabolic activity.

2) To compare the effects of mirabegron at 0mg, 50mg, and 200mg on BMR.

Hypotheses

1) Mirabegron at 50mg and 200mg PO will each increase BMR and BAT activity more than placebo.

2) The increases in heart rate and systolic BP after a one-time dose of 50mg will be lower than that observed using 200mg.

II.2 Secondary Objectives

For all available conditions (temperature and drug/dosage), the following will be reported (when not already reported as a primary objective):

1) resting EE using metabolic chamber (Cohorts 1, 2 and 3)

2) difference in resting EE (ΔEE) between $REE_{27^{\circ}C}$ and REE_{Low} (Cohort 1),

3) comparison between placebo ΔEE (Cohort 1) and ΔEE for each drug (Cohorts 1, 2 and 3)

4) difference between drug and placebo resting EE (at the same temperature) (Cohorts-1, 2 and 3)

II.3 *Exploratory outcomes*
1) resting respiratory quotient (RQ): although it is a short-term measure that reflects net macronutrient utilization. Our current data in the protocol 12-DK-0097 suggest that this is also correlated with ambient temperature.

2) difference in resting RQ between $RQ_{27°C}$ and a temperature just above the subject's placebo shivering threshold RQ_{Low},

3) difference between drug and placebo resting RQ (at the same temperature)

4) skin and core body temperature

5) heart rate and its variability

6) shivering by surface electromyography and accelerometry

7) hormonal changes that occur with NST by characterizing the effect of placebo and drug regimens on the following analytes, measured fasting at the end of each chamber session.

Urine:
norepinepherine
epinepherine
free cortisol
creatinine
volume

Plasma:
norepinepherine
epinepherine
glucose
insulin
free fatty acids
β-hydroxybutyrate
T3
T4
TSH
Glucagon
Cortisol
Parathyroid Hormone (PTH)
Adiponectin
Ghrelin
Gastric inhibitory polypeptide (GIP)
Peptide YY (PYY)
Glucagon-like peptide-1 (GLP-1)
Leptin
Growth hormone
fribroblast growth factor 21 (FGF21)
irisin [92]
atrial natriuretic peptide (ANP) [93]
ventricular natriuretic peptide (BNP) [93]
Adrenocorticotropic hormone (ACTH)

III. Study Protocol

III.1 Subjects and Recruitment

We will limit this study to young healthy male subjects with a BMI of 18.5 to 25.0 and age 18-35 years. Studying females with the current protocol is not yet feasible due to its length which would cause inclusion of both phases of the menstrual cycle, a possible confounder. However, the data from this study and from 12-DK-0097 will be used to design a shorter study protocol to study broader populations including women, older and younger subjects in the future. Subjects who have participated in 12-DK-0097 are eligible for this protocol and individual data from that protocol may be used to inform choice of the low temperature. Similarly, subjects who participate in Cohort 1 are also eligible for Cohort 2 and/or Cohort 3. We will allow a wash-out time for the drugs (>48 hours) and recovery time for the blood draws (to be determined by volume obtained and NIH guidelines). There is no additional risk due to increased radiation exposure for subjects participating in Cohorts 1 and 2. Although we will use BMI as a screening tool for practical concerns, we will measure body composition using DXA.

This study is planned for 16 volunteers in each of Cohort 1 and 2, with enrollment to continue until 12 complete each Cohort. No interim analysis is planned due to the small study size and the low probability that futility would be reached for all of the multiple endpoints, the criteria required for early termination.

Power calculation: To estimate power, we used data from 12-DK-0097. We first determined that the within-subject difference in the response in BMR within TNZ was normally distributed with a standard deviation of 2.54%. The maximum NST in 9 young healthy male subjects was 17.4±3.7%. With 12 subjects completing the protocol, if the true difference in the mean response to treatment is 5.0% as we hypothesized for each drug treatment, we will be able to reject the null hypothesis that this response difference is zero with probability (power) of 0.98 at an alpha of 0.05 in either Cohort. Since this study is designed to use the pharmacological agents only to test the mechanisms of NST and BMR, thus we will not compare any pharmacological interventions to each other. However, in the most conservative interpretation, multiple comparisons adjustments will only be necessary for the number of paired comparisons between drugs and placebo within each Cohort. The result of such a conservative estimate of power range from 0.92 at an alpha value of 0.01 (0.05/5, the Bonferroni method) in Cohort 2 to 0.94 in Cohort 1 (alpha = 0.05/4). Even if the true difference was 3%, the power to detect with n=12 is above 0.83 in all analyses. To consider in potential dropouts (about 30%), we will recruit 16 subjects for each cohort (subjects can participate in both with a minimum of 2 weeks washout in between the studies), aiming to have 12 completers for each cohort.

In Cohort 3, the primary endpoint is the difference in detectable BAT activity (units of mL*SUVmean*g/mL) between the 0 mg and 50 mg doses of mirabegron. Based on our previous study (Cypess et al., 2015), BAT activity is right-skewed and not

normally distributed, so our sample size determination is based on log BAT activity, which is normally distributed. We found that log BAT activity after treatment with 0 mg mirabegron was 0.05 ± 0.62 and after treatment with 200 mg mirabegron was 2.00 ± 0.79. Since it is unknown what the effect of 50 mg mirabegron would be on BAT activity, we calculated a range based on lower and upper bound expectations about the reported PK/PD profile of mirabegron. For the lower bound, the 50 mg dose has been found to have a mean plasma C_{max} that is 18% of 200 mg. If there were a linear relationship between BAT activity and dose, then we would predict 50 mg to produce a mean log BAT activity of 0.36.

Using a different set of assumptions leads to a higher predicted effect, or the upper bound expectation. We found that after the 200 mg dose, the estimated mean peak plasma concentration (C_{max}) of mirabegron was 274 ± 64 nM based on 65% protein binding, which is 6.9x the Ki of mirabegron for the β3-AR (40 nM) (FDA 2012). If the 50 mg dose achieves 18% of 200 mg, then the C_{max} would be 49 nM, which is 1.2x the Ki and 2.2x the in vitro EC_{50} of 22.4 nM (Takasu et al., 2007). Therefore, 50 mg mirabegron could produce ≥50% of the effect seen with 200 mg, or ≥1.00 log BAT activity. Therefore, for 50 mg mirabegron, the effect on log BAT activity ranges from 0.36 to ≥1.00.

For sample standard deviation, the log BAT activity after treatment with 0 mg mirabegron was 0.05 ± 0.62 and after treatment with 200 mg mirabegron was 2.00 ± 0.79. Assuming a linear relationship between dose and standard deviation of the outcome variable, for the 50 mg dose of mirabegron, the standard deviation for log BAT activity is predicted to be 0.66.

In summary, we are planning a study of a continuous response variable from study subjects where each acts as his own paired control. Assuming the true difference between 0 mg mirabegron and 50 mg mirabegron is 0.36 with a standard deviation of 0.66, we will need to study 28 subjects to be able to reject the null hypothesis that this response difference is 0 with α=0.05 and power of 80%. However, if the response is 1.00, then we will need to study only 6 subjects. Given this wide range (6-28 subjects), we will plan to complete 28 subjects with an interim analysis after 12 subjects. If the results of the interim analysis suggest that the response is closer to the upper bound estimate, then we will be able to reject our null hypothesis at that point. A sample size of 12 was chosen because if 12/12 subjects show higher BAT activity with the 50 mg, then non-parametric paired comparison yields a P value of 0.001.

For these types of studies, we have experienced a screen-failure rate of 50% and a rate of detectable BAT of 66%. We therefore plan to enroll up to 84 subjects, perform Study Day A in 42, and expect 28 to complete all four study days. If we are able to complete the study after the interim analysis, then we would end up enrolling 36 subjects, initiate the screening protocol with 18, and have 12 complete all four study days.

III.2-1 Protocol design, Cohort 1 – This Cohort has completed enrollment

Cohort 1 is a single-blind, fixed order (for drug treatment), partially randomized (for temperature), cross-over study, with data analysis performed in a blinded manner

All study volunteers will undergo the screening visit evaluation as outlined in Table 2, including a medical and laboratory examination to determine study eligibility. Qualifying volunteers will be invited for the full study at the Metabolic Clinical Research Unit as inpatients. An initial one-day equilibration diet will be standard before metabolic testing. This equilibration period will also serve as an orientation/ acclimatization to the metabolic chamber and procedures.

Table 2. Screening tests:

Eligibility phone contact pre-screen questionnaire	**O**
1st REG H+P or Protocol Screen Progress note	**O**
Temp, Pulse, BP, RR, WT, HT	**O**
Nutritional consult	**O**
Informed Consent Version:	**O**
Consent documented in CRIS	**O**
Eligibility Criteria Documentation	**O**
Clinical Labs/Procedures	
EKG	**O**
CBC with Differential	**O**
Acute care + hepatic + mineral panels (Chem 20)	**O**
Lipid Panel	**O**
Thyroid (TSH)	**O**
Toxicology (urine)	**O**
Ferritin	**O**

To match the criteria from 12-DK-0097, we will recruit subjects who do not work in a cold environment and are not trained athletes. Drugs will be administered exactly at 8:00 am (+/- 15 minutes), (except dantrolene, which will be administered at 6:00 am (+/- 15 minutes), and bolus magnesium sulfate (~ 30 min infusion) at 7:30 AM (+/- 30 minutes) and maintenance infusion at 8:00 AM (+/- 15 minutes)), upon entering the metabolic chamber. We will measure REE at times close to the Tmax of the drugs (9:30-10:00 and 10:30-11:00).

During a 17-day admission (with 4 off days on pass) to the NIH Hatfield Clinical Research Center, study volunteers will undergo daily EE measurements (up to 4 hours) in a metabolic chamber with temperatures set between 18.0 to 21.0 and 27.0°C. Individualized determination of the lowest environmental temperature that does not cause shivering is important to maximize assay sensitivity. This will be accomplished by using the mini-adaptive design approach (Table 3): On Day 2, the first full inpatient study day, volunteers will be acclimatized to the metabolic chamber at 19.0°C, wear multiple body sensors, and undergo standard testing procedures that will be repeated throughout the study. If shivering occurs on Day 2 at 19.0°C, the chamber temperature will be set to 20.0°C on Day 3. If shivering occurs at 20.0°C on Day 3 the low temperature will be

21.0°C; if no shivering at 20.0°C on Day 3, the low temperature will be 20.0°C. If no shivering occurs on Day 2, then the chamber temperature will be set to 18.0°C on Day 3. If shivering occurs at 18.0°C on Day 3 the low temperature will be 19.0°C; if no shivering at 18.0°C on Day 3 the low temperature will be 18.0°C.

Table 3

Day 2 chamber temperature	**Day 2 result**	**Day 3 chamber temperature**	**Day 3 result**	**Low Temperature**
19C	Day 2 shiver	20C	Day 3 shiver	21C
			Day 3 no shiver	20C
	Day 2 no shiver	18C	Day 3 shiver	19C
			Day 3 no shiver	18C

Table 4 Protocol flow

day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
	Sat	**Sun**	**Mon**	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**	**Mon**	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**	**Mon**
drug		pbo	pbo	pbo	pbo	pro	pro	off	off	dan	dan	Mg	Mg	pin	off	off	pin
temp.		19	18 or 20	low	27	low	27			low	27	27	low	27			low
notes	admit						pass		return					pass		return	d/c
blood				X,X	X,X	X	X			X	X	X	X	X			X

Where pbo=placebo, pro=propranolol (oral 160mg PD), dan=dantrolene (oral 100mg PD), Mg=magnesium sulfate (IV), and pin=pindolol (oral 20mg PD).

On Days 4-17, chamber temperature will be varied as indicated in Table 4 and procedures will be repeated. Temperatures will be block randomized (one day at the low temperature and one at 27.0°C), with blocks being: Days 4,5; Days 6,7; Days 10,11; Days 12,13; and Days 14,17. Subjects will not be informed of the environmental temperature.

Oral medications are placebo on Days 2, 3, 4, and 5; regular propranolol (not sustained release) 160 mg on Days 6 and 7; dantrolene 100 mg on days 10 and 11, pindolol 20 mg on Days 14 and 17; and magnesium sulfate on Days 12 and 13. Since it is not possible for all medications to have the same appearance, drug or placebo will be administered by Clinical Center personnel without subjects being able to see or taste the medication. The off-days (weekends) serve as washouts for propranolol and pindolol. Since both dantralene and magnesium sulfate function as muscle NST inhibitors, minimum washout is needed. Finally, magnesium sulfate has a half-life of ~4 hrs, thus its impact on EE of the next day is minimum. This compact schedule is the result of balancing the rigorous study design and the feasibility for the subjects.

For each chamber study, volunteers will be fasted after midnight and during the up to 4-hour study period while they are in the chamber. During this time, all subjects will be asked to minimize their physical activity while staying awake in a reclining position in the designated chair in the chamber. Between 9:30-10:00 and 10:30-11:00 AM, the subject is required to lower the ambient lighting and recline without any physical activity, but remain awake. Clothing will be standardized and limited to the following: one pair of undershorts, one sleeveless form-fitting cotton shirt, one pair of

form-fitting shorts, and one pair of light socks (issued by the inpatient unit), for a combined thermal insulation value of 0.36 (clo).[94] After each chamber study, participants will return to the inpatient room on the 5SW inpatient unit at a comfortable range between 23-25°C for the remainder of the day until the next morning. All meals will be served in the patient rooms. Each afternoon, study volunteers will have a standard walking session from 15:00-15:30 on a treadmill. The pace of the walking will be self-selected but will be standard throughout the inpatient study period for each individual and reflect the subject's prior activity level.

Subjects will be allowed to go on pass on Cohort 1 Days 8/9, 15/16, but refrain from increased exercise, exposures to extreme temperatures, and all caffeine intake, and must return before dinner. If deemed necessary, at the discretion of the Principal Investigator, any of the pass durations may be increased.

During each of the stays in the metabolic chamber, we will record the following parameters and will perform these procedures:

1) Real-time energy expenditure, respiratory quotient
2) Continuous heart rate
3) Surface electromyography and spontaneous movements
4) Core (ear) and skin temperature
5) 4-hour urine collection (8:00-12:00) for urea, cortisol, creatinine, and catecholamines
6) Infra-red thermography (8:30, 10:00, and 12:00)
7) Thermal comfort and hunger questionnaire (visual analog scales, Appendix I)

Table 5: The detailed scheduled of events for Cohort 1.

DAY number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
ADMIT PHYSICAL/HISTORY (Wk 1)	O																
Weight and Height	O																
DXA + Anthropometrics	O																
Daily Progress Note		O	O	O	O	O	O			O	O	O	O	O			O
Daily Vital Signs	O	O	O	O	O	O	O			O	O	O	O	O			O
Metabolic Diet	O	O	O	O	O	O	O			O	O	O	O	O			O
Habitual Physical Activity by accelerometer	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Room Temperature Log	O	O	O	O	O	O	O			O	O	O	O	O			O
Continuous recording during chamber study																	
REE in Metabolic Chamber, up to 4hr Continuous Recording (08:00-12:00)		O	O	O	O	O	O			O	O	O	O	O			O
Skin Temp		O	O	O	O	O	O			O	O	O	O	O			O

Electrocardiography (Holter)		O	O	O	O	O	O			O	O	O	O	O			O
Surface Electromyography		O	O	O	O	O	O			O	O	O	O	O			O
At 08:00, 10:00, and 12:00 of Chamber study																	
Tympanic Temp, IR thermography		O	O	O	O	O	O			O	O	O	O	O			O
Thermal comfort and hunger questionnaire		O	O	O	O	O	O			O	O	O	O	O			O
Research Labs																	
4hr urine (08:00-12:00)				O	O	O	O			O	O	O	O	O			O
Blood samples (08:00)				O	O												
Blood samples (12:00)				O	O	O	O			O	O	O	O	O			O

III.2-2 Protocol design, Cohort 2

Cohort 2 is a randomized, double-blind, placebo controlled, 6-period cross-over study, with data analysis performed in a blinded manner. Oral medications, dosed exactly at 8:00 am (+/- 15 minutes) on entering the chamber, will be randomly assigned so that each subject receives each of the six treatments listed in Table 6. Since it is not possible for all medications have the same appearance, drug or placebo will be administered by Clinical Center personnel without subjects or study personnel being able to see the medication.

All Cohort 2 study volunteers will undergo the screening visit with the following tests which are identical to Cohort 1 (Table 2), a medical and laboratory examination to determine study eligibility. Qualifying volunteers will be invited for the full study at the Metabolic Clinical Research Unit as inpatients. Subject criteria are the same as in Cohort 1. Subjects will be studied with 7-14 days between admissions and complete the entire study (Cohort 2) within a 12-week time window to prevent the variability in outdoor environmental temperatures and potentially body weight which will confound the study.

During six 1-day stays at the NIH Hatfield Clinical Research Center, study volunteers will undergo daily BMR measurements (up to 4 hours) in a metabolic chamber while exposed to temperatures of 27.0°C. Chamber conditions and assays are exactly as described for Cohort 1 (Table 5).

Table 6. Medications in Cohort 2

Treatment	Dose	Route	Minimum washout
Placebo		PO	None
Caffeine	300 mg	PO	3 days
Qsymia	topiramate 92 mg CR phentermine 15 mg	PO	4 days

Phentermine HCl	37.5 mg	PO	4 days
Topiramate	200 mg	PO	4 days
Naltrexone	100 mg	PO	? Days

III.2-3 Protocol design, Cohort 3 - This Cohort has completed enrollment

Cohort 3 is a randomized, single-blind, placebo controlled study, with the quantifier of BAT activity blinded to the four different interventions. Volunteers will undergo up to four experiments to study the effects of mirabegron on BMR and BAT activity.

All Cohort 3 study volunteers will undergo the screening visit with the following tests which are identical to Cohorts 1 and 2 (Table 2), including a medical and laboratory examination to determine study eligibility. Qualifying volunteers will be invited for the full study at the Metabolic Clinical Research Unit as inpatients. Subject criteria are the same as in Cohorts 1 and 2. At least 48 hours will separate each of the study days, and all 4 study visits will be completed within a 12-week time window to prevent the variability in outdoor environmental temperatures and potentially body weight which will confound the study.

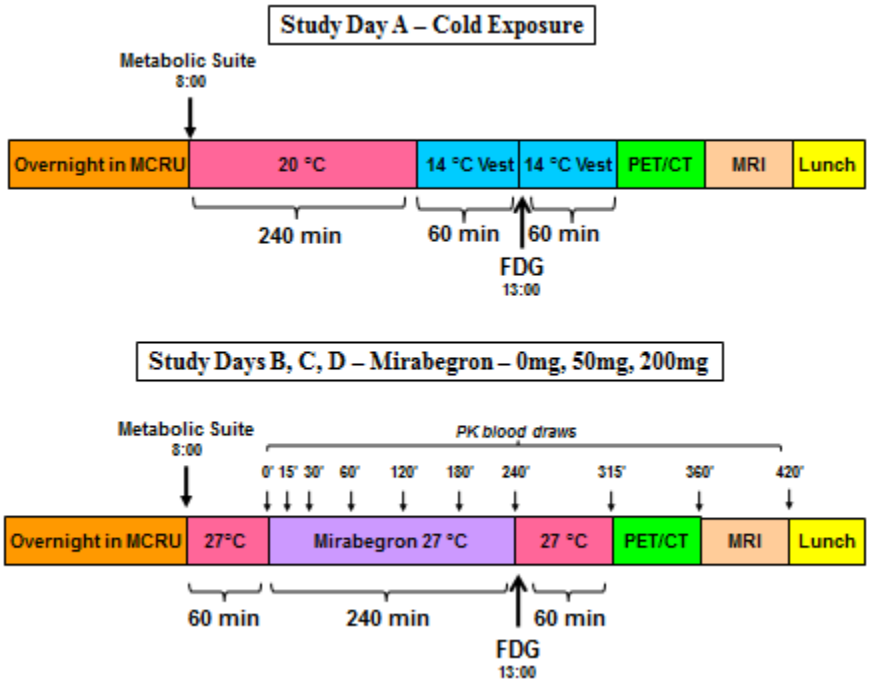

Figure 2. Schematic depicting the testing parameters and procedures for Cohort 3.

The first study visit will involve a 6-hour stay in the metabolic chamber. Each study volunteer will also be fitted with a cooling vest (CFA-9 vest, Polar Products, Stow, OH) while in the chamber. The first 240 minutes (4 hours) will be an acclimatization period with BMR measurement; the temperatures of the chamber and the water vest will be set at 20°C. At +240 minutes, the study team will reduce the temperature of the water

in the cooling vest to 14.0-16.0°C by turning on the vest pump which will be set up on the outside of the metabolic chamber. The tubing that circulates the chilled water through the vest will be placed through the access port of the chamber so that there is no disruption to the metabolic chamber data collection. Subjects may continue usual sedentary activities during this time. After 60 minutes of cooling, the volunteer will be injected with ^{18}FDG for PET scan. Those subjects with detectable BAT (by PET/CT) will then proceed to the additional study days in which they will receive oral mirabegron at 0mg, 50mg, and 200mg. If there is not sufficient detectable BAT after cold exposure, subjects will only complete the study day with mirabegron at 200 mg, not 0 mg or 50 mg. This will serve as a control for the differences in the amount of increase EE due to drug versus cold independent of BAT presence.

The second, third and fourth study visits will be conducted similarly. Volunteers will enter the metabolic chamber at 8:00 am for daily BMR measurements while exposed to a room temperature of 27.0°C. After a 60 minute acclimatization period, the drug or placebo will be administered in a randomized fashion at time -0- (9:00 am ± 15 minutes). Blood samples will be obtained at -0-, +15, +30, +60, +120, +180, +240, +315, +360, and +420 minutes. At +240 minutes of mirabegron administration, the volunteer will be injected with ^{18}FDG for PET scan.

For each study day (A, B, C, and D), a dose of [^{18}F]-fluorodeoxyglucose (FDG) will be given at 13:00 (+60 minutes of cold exposure or +240 minutes of mirabegron administration) and the volunteers will remain in the chamber for an additional 60 minutes to allow for uptake of the radiotracer. Volunteers will then exit the chamber and be transported via wheelchair to the Clinical Center Nuclear Medicine Department for a positron emission tomography/computerized tomography (PET/CT) scan at 14:30. The scan will take no more than 60 minutes. Immediately upon completion of the PET/CT, subjects may be transported to the Radiology Department for a Magnetic Resonance Imaging (MRI) study. This scan will take no more than 60 minutes, as well. Following completion of the PET/CT and MR imaging, volunteers will then return to their room on the Metabolic Clinical Research Unit where they may have a meal and then may be discharged. Volunteers will be instructed to return for the next study visit until testing is complete.

For each chamber study, volunteers will be fasted after midnight and during the 8-hour study period they are in the chamber followed by PET/CT and MRI. During this time, all volunteers will be asked to minimize their physical activity while staying awake in a reclining position in the designated chair in the chamber. Between 08:30-09:00 10:30-11:00, and 12:40-13:10, the volunteer is required to lower the ambient lighting and recline without any physical activity, but remain awake. Clothing will be standardized and limited to the following: one pair of undershorts, one short-sleeve hospital scrub shirt, one pair of hospital scrub pants, and one pair of light socks (provided by the inpatient unit), for a combined thermal insulation value of 0.4 (clo).[94]

III.3 Inclusion Criteria:

- Generally healthy
- Males between the age 18-35 years
- Written informed consent.

III.4 Exclusion Criteria:

- BMI less than 18.5 or greater than 25.0 kg/m^2
- History of cardiovascular disease such as congestive heart failure, heart block, clinically abnormal EKG as determined by investigators.
- History of a cerebrovascular accident (CVA) or seizures.
- History of liver disease or ALT serum level greater than two times the laboratory upper limit of normal
- History of kidney diseases or renal insufficiency or estimated creatinine clearance ≤ 50 mL/min (MDRD equation)
- History of cancer or bariatric surgery
- History of diabetes mellitus or fasting serum glucose > 126 mg/dL
- History of hypo- or hyper-thyroid or TSH >5.0<0.4 miU/L
- History of asthma, chronic obstructive pulmonary disease and glaucoma
- Psychological conditions, such as (but not limited to) claustrophobia, clinical depression, bipolar disorders, that would be incompatible with safe and successful participation in this study
- Weight change >5% in the past 6 months or a trained athlete
- Blood pressure greater than 140/90 mmHg or current antihypertensive therapy
- Iron deficiency (Hemoglobin <13.7 g/dL and Hematocrit <40.1%)
- History of illicit drug, opioids, or alcohol abuse within the last 5 years; current use of drugs (by history) or alcohol (CAGE≥2) [95]
- Current use of medications/dietary supplements/alternative therapies known to alter energy metabolism
- Current medications that may have interactions with study drugs as determined by the investigators
- Hypersensitivity and associated allergic reactions to mirabegron or similar drug substances or components of this medication
- Daily caffeine intake >500 mg (about 4 cups) and have withdrawal symptoms
- Current smoker or user of tobacco products
- Cannot commit to the schedule of visits to the Clinical Research Center (CRC) as required by the study timeline
- Have had previous radiation exposure within the last year (X-rays, PET scans, etc.) that would exceed research limits (please let us know if you have received radiation for research purposes)
- Have inflexible dietary restrictions
- Any other reason that the investigator thinks would make interpretation of the study results difficult.

- For subjects having an MRI (Cohort 3), history of pacemaker, metallic heart valves, aneurysm clip, pedicle screws, metallic foreign body in eye, or other metallic implant
- For subjects receiving mirabegron (Cohort 3). a diagnosis of bladder outlet obstruction or the use of antimuscarinic medications for the treatment of overactive bladder
- For subjects receiving mirabegron (Cohort 3). a history of hypersensitivity to mirabegron or other β3-Adrenergic Receptor Agonist medications.

All subjects will be fully informed of the aims, nature, and risks of the study prior to giving written informed consent.

III.5 Methods:

Pre-Screening
Prior to the official screening appointment, prospective subjects will be interviewed in person or by phone. Both general and protocol-specific recruitment scripting will be used to guide pre-screening interviews. Limited identifiable information pertaining to demographics, general health status, and contact information will be collected. We are requesting a waiver of consent for the pre-screening interviews since, per 45 CFR 46.116(d) (pre-2018 requirements):

1. The collection of limited identifiable information for pre-screening purposes involves no more than minimal risk to the subjects.
2. The waiver will not adversely affect subjects' rights or welfare. Pre-screening interviews will not impact subjects' patient care or ability to decline participation before or during the study.
3. Pre-screening interviews could not practicably be carried out without a waiver of consent as central recruiting services, utilized in the NIH Clinical Center, perform pre-screening activities for multiple studies and obtaining consent for each one is beyond their resources.
4. It is unlikely that any pertinent information about the pre-screening process will be discovered after subjects have completed it, but should that occur, we will provide subjects with that new information as appropriate.

Screening visit
Following prescreening interviews, all prospective subjects will be screened and counseled at the initial screening visit. After obtaining informed consent, the study volunteers will undergo history and physical examination and the following screening tests including Chem 20, lipid panel, CBC with differential, TSH, ferritin, EKG, and urinalysis. Subjects are required to fast for at least 12 hours prior to the laboratory testing. Study volunteers will be allowed to drink water during the fasting period. During the screening visit the study participants will be interviewed by a dietitian in order assess the calorie intake and food preferences of each study participant. Body weight, height, and anthropometrics (circumferences at the neck, waist, hip, arms and legs) will be performed.

Hospital stay-Cohort 1

Study volunteers will be housed in the Metabolic Unit of the NIH Clinical Center intermittently throughout the study period. Height will be measured. Daily afternoon physical activity consistent with prior physical activity levels will be devised in consultation with the dietitian in order to avoid deconditioning during the hospital stay. Sleep patterns and quality may influence REE and body temperature. Thus, all of the subjects will be asked to keep consistent sleeping habits (time, temperature, covers, no drinking or eating, etc) from three days prior to and throughout the entire inpatient study period, including any time on pass.

Hospital stay-Cohort 2

Study volunteers will be housed in the Metabolic Unit of the NIH Clinical Center on each admission. Height will be measured. Sleep patterns and quality may influence REE and body temperature. Thus, all of the subjects will be asked to keep consistent sleeping habits (time, temperature, covers, no drinking or eating, etc) from three days prior to and throughout the entire inpatient study period, including any time on pass.

Hospital stay-Cohort 3

Study volunteers will be housed in the Metabolic Unit of the NIH Clinical Center on each admission, which will be from 18:00 the night before the study until the imaging is completed - approximately 23 hours later. Sleep patterns and quality may influence REE and body temperature. Thus, all of the subjects will be asked to keep consistent sleeping habits (time, temperature, covers, no drinking or eating, etc.) from three days prior to the study period.

Diet- Cohort 1

Throughout the stay at the Metabolic Unit study volunteers will follow an isocaloric diet with the following macronutrient distribution: 55% carbohydrate, 15% protein, and 30% fat, as recommended by the American Dietetic Association. Subjects will be weighed (in triplicate), post-voiding each morning. Diet caloric content will be adjusted to maintain a stable body weight.

Diet- Cohort 2

Study volunteers will follow an isocaloric diet with the following macronutrient distribution: 55% carbohydrate, 15% protein, and 30% fat, as recommended by the American Dietetic Association.

Diet- Cohort 3

Study volunteers will be provided with an isocaloric dinner meal the evening prior to metabolic testing. It will consist of the following macronutrient distribution: 55% carbohydrate, 15% protein, and 30% fat, as recommended by the American Dietetic Association. A late night snack high in carbohydrates and protein will also be provided due to the prolonged fasting period the following day. Volunteers will be expected to consume their snack by midnight and then fast until testing is complete, approximately 16 hours. Then, he may resume a regular diet.

Body composition

A DXA scan (iDXA, GE Healthcare, Madison WI) will be performed at either Day 1 or 2 of the inpatient study stage. With this technique, one can determine total and

regional body fat and lean soft tissue masses, bone mineral content and density. DXA produces photons at two different energy levels, 40 and 70 KeV. The photons pass through tissues and attenuate at rates related to elemental composition. Bone mineral, with highly attenuating calcium and phosphorous, is readily distinguished from soft tissues. The different elemental profiles of fat and bone-mineral free lean components allows for the analysis of soft tissue fat content, so that bone mineral, fat, and bone mineral fat-free lean components may be resolved.

Environmental questionnaire

On Day 1, each subject will answer general questions about their typical thermal environment, such as the number of hours/day usually spent outside, clothing typically worn, environmental temperature at work and at home, and level of physical activity.

PET/CT (Cohort 3 only)

A PET/CT scan is the current standard for measuring BAT activation in humans. Study volunteers in Cohort 3 will be scanned following each stay in the metabolic chamber. At 13:00, a 5mCi dose of ^{18}FDG will be given intravenously to volunteers through the blood port of the metabolic chamber. At the end of the study (14:00), study volunteers exit the metabolic chamber and are then transported via a wheelchair to Nuclear Medicine Department of the NIH Clinical Center and a PET/CT scan will be performed on the Siemens mCT scanner. The PET/CT scan will expose the study volunteer to about 1.25 rem of total effective radiation exposure.

MRI (Cohort 3 only)

A MRI scan is a technology that may become extremely beneficial to measuring BAT activity. The MRI produces detailed images of tissues. In this study, it may also be useful in evaluating cell receptors associated with BAT activity. Study volunteers in Cohort 3 may be scanned following each stay in the metabolic chamber, depending on scanner availability. After completion of the PET/CT (described above), volunteers will be transported to the Radiology Department of the NIH Clinical Center; an MRI will be performed on the Siemens MRI T3 scanner. The MRI will not expose the study volunteer to additional radiation exposure.

BAT mass will be assessed using water-saturation efficiency and water-to-fat contrast ratio and the Dixon method, which separates fat from water by acquiring two images in the same scan, one at an echo time when fat and water signals are in phase and another when fat and water signals are out of phase. Several MRI sequences will also assess BAT activation by measuring changes in tissue blood flow and other physiological parameters.

Use of Fiducial Markers (Cohort 3 only)

The use of MR images to assess brown adipose tissue has not been proven as a useful technology to date. This study will explore this technique more thoroughly. In order to more effectively measure BAT, we will co-register the MR images with the PET/CT images, the standard method of measuring BAT activity. We will utilize fiducial markers to designate landmarks for the co-registration.

A fiducial marker is an object placed in the field of view of an imaging system which appears in the image produced, for use as a point of reference. The markers in this study will contain 1 μCi of Na-22. A total of up to 16 of these markers will be placed on the skin prior to the PET/CT scan. They will remain in place during the MRI that immediately follows. Subjects will be able to remove them after the MRI is complete.

Metabolic chamber

Study volunteers will enter the metabolic chamber at 08:00 AM after a minimum 8-hour fast and voiding. Superficial skin temperature probes will also be placed (see below). Drug or placebo will be administered (witnessed, blinded dosing) with 120 ml of water at exactly 08:00 (09:00 for Cohort 3). Study volunteers will wear standard clothing and will be instructed to be minimally active while staying awake in the metabolic chamber after the first 30 minutes. To help with the compliance and occupying the time in the chamber, each subject will be allowed to watch television (sports, movies, series, etc), play non-active video games, read, or perform deskwork. Details of the chamber have been published previously. [16,96] During the period of 08:00 to 12:00 (08:00 to 13:00 for Cohort 3), urine will be collected by the study volunteers, and then analyzed for total nitrogen, urea, cortisol, creatinine, and catecholamines.

The metabolic chamber is a specially constructed room to assess energy expenditure. Designed as a walk-in "pull" calorimeter, it is an open circuit unit that draws conditioned room air into the chamber at the same flow rate as it is extracted into the gas analysis system.[97] The room is equipped with toilet and sink with privacy screen, treadmill, bed, desk, and computer with access to television and other forms of entertainment. Physical activity level is measured continuously through a wall mounted monitoring device (microwave sensor). Calibration tests over the past three years at temperature settings from 15.3 to 32.1°C have demonstrated an accuracy of 98.4±2.5% for EE as compared to a weighed propane standard oxidized by combustion.

The relative humidity of each chamber is controlled between 30-50% for all temperatures. The overhead circulation fan unit mixes the air of the chamber uniformly. At the bed and the recliner where the subjects will be spending the 5 hour resting periods, the wind speed is less than 0.4 m/s (0.8 mile/hour), making the windchill and humidex (heat index) factors less than 0.2°C. We will measure chamber wall temperature using iButtons,[98] and calculate the exact "operational temperature" as defined previously[99].

The following parameters will also be recorded inside the metabolic chamber:

Continuous heart rate and heart rate variability

Heart rate will be measured and recorded using a portable ECG Holter monitor, which will be downloaded and analyzed for heart-rate variability using power spectrum analyses.

Muscle contraction and Shivering

Wireless surface electromyography (EMG) electrodes (Trigno, DelSys Inc, Boston MA) will be placed on upper arm (biceps brachii), chest (pectoralis major), neck

(upper fibers of the trapezius), and leg (rectus femoris) muscles to objectively measure the intensity and overall muscle contractions. Each surface sensor will include a triaxial accelerometer to simultaneously measure dynamic movements.[100,101] Signals will be transmitted to a laptop computer outside the chamber for data analysis to look for activity below the threshold of shivering, and for shivering. [102,103] To standardize the EMG measures, each EMG sensor is placed carefully in the same spot of the muscle with the edges clearly marked by surgical markers (water and sweat proof), and a five minute baseline data is collected before each chamber study at a constant temperature (23ºC) while the subject is reclined and rested under a blanket (no voluntary muscle contraction and no shivering). All EMG data during the chamber study will be normalized to this baseline period (as % of baseline) and compared between subjects. The normalized EMG data from the two resting periods (9:30-10:00 and 10:30-11:00) on the 27 ºC placebo day will be used as the baseline measurement. Form our preliminary data (12-Dk-0097) collected in the identical fashion, we found that EMG during cold exposure > mean + 2 x SD of the baseline period as the threshold closely compared to self-reported shivering, and we will use criterion of two spontaneously shivering muscle groups out of the four measured as the final determination of shivering.

Tympanic and skin temperature

We will measure core temperature using a handheld infrared tympanic thermometer (PRO4000 by Braun) as it has been shown to have the closest agreement with nasopharyngeal temperature measurements [104] but without being invasive. Tympanic temperature will be measured every 30 minutes by the study subjects after training by our study staff. Two measurements in the right ear will be taken each time. If the two data points are different by 0.3ºC, a third measurement will be taken with the median of the measurements averaged as the final result. All data points will be recorded immediately after each measurement.

Wireless probes (iButton, Maxim Inc., Sunnyvale CA) will record skin temperature and heat flux continuously with data reported at each minute. [48][105] The positions of the skin sensors at eight sites (anterior aspect of forearm, forehead, subscapular, triceps, pectoralis major, abdomen, anterior thigh, and calf) on the right side of the body will be according to the ISO standards.[94]

Thermal comfort and hunger questionnaire

Patients will be given a questionnaire to determine their level of thermal comfort and hunger using visual analog scales (VAS), as shown in Appendix I. The hunger VAS question has been characterized previously.[106] A computerized version of the questionnaire will be used.

Infrared thermography

Skin temperature can be monitored by infrared thermography (T400, FLIR, North Billerica MA) which has been described previously.[107] The camera will be positioned between 1-1.5 meters away from the subject in the chamber and used to take a picture at defined time periods (8:30, 10:00, and 12:00). This will be done in Cohorts 1 and 2 only.

Genetic testing

Genomic DNA will be isolated from peripheral mononuclear cells from a blood sample collected during screening. The purpose of obtaining genetic material is to perform genotype-phenotype association studies. Specifically, samples will be initially analyzed for the type-2 deiodinase gene Thr92Ala and -258 A/G,[108,109] and uncoupling protein-1 (UCP1) [110]polymorphisms, *i.e.* candidate genes for modulation of the metabolic response to changes in environmental temperature and should the literature indicate novel candidate genes/variants, for subsequent screening of candidate genes involved in obesity, endocrinology, metabolism, diabetes, and related diseases and conditions, as well as scanning for novel mutations in genes involved in metabolic pathways. Currently, we believe that obesity results from small influences on body weight from many genes and their complex interactions with environmental factors over time (years to decades). While future studies may be performed to explore such areas, the results of this testing are unlikely to have direct clinical relevance to the subjects. For example, whole genome/whole exome sequencing could be performed that may reveal novel findings in energy metabolism, fuel partitioning, and/or heat loss regulations in this study population. DNA samples may also be tested for variation that may affect drug pharmacokinetics.

With such broad range of genetic inquiry, it is possible that future testing could discover information (either directly related to the metabolic pathways under investigation or incidental genetic findings) that may be important to the subjects' health or the health of their offspring.

Because our genetic studies will take years to complete, we may not be able to reach all subjects in the event we find important health information. We do not intend to return results of genetic tests to subjects.

The information will not become part of the subject's hospital medical record since they are not CLIA-certified assays. For this reason, the DNA results gathered in this study are not appropriate for immediate clinical use. This information will be kept in a research data file separate from the subject's medical records. If the patient requests the results of the research files, those results will be discussed and clearly labeled as being research information only and not appropriate for clinical use. This is not a family study therefore genetic information about paternity is not applicable.

Other risks of participating in genetic research include anxiety about learning of possible disease association and insurance issues, including obtaining disability insurance and life insurance. The Genetic information Non-discrimination Act (GINA) generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate based on genetic information. This Federal law does not protect against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

In order to protect the privacy of potentially sensitive individual genetic data, steps will be taken to de-identify and assign a code to each subject's research sample.

Laboratory testing

A screening test (Chem 20, lipid panel, CBC with differential, TSH, ferritin, EKG, urinalysis) will be performed by the Clinical Center Department of Laboratory Medicine (DLM).

In Cohort 1, research blood samples will be drawn at 8:00 am on Days 4 and 5 and at 12:00 on 10 different days (Days 4,5,6,7,10,11,12,13,14,17) immediately after the

volunteer exits the chamber, for a total of 12 samples. For safety monitoring of medications being given, we will add a hepatic panel on Day 10, a mineral panel and potassium level on Days 11-14, and a creatinine clearance level on Day 11. On Day 17, one clinical sample (5 ml tube) will be added (Chem 20) to monitor any changes. The volume of each research blood draw will be approximately 24 ml, plus a one-time DNA sample of 10 ml. For the entire inpatient study, the total volume of blood sampling will be no more than 300 ml.

In Cohort 2, research blood samples will be drawn each Day at 8:00 am and at 12:00 immediately after the volunteer exits the chamber, for a total of 12 samples. The volume of each research blood draw will be approximately 24 ml, plus a one-time DNA sample of 10 ml. For the entire inpatient study, the total volume of blood sampling will be no more than 300 ml.

If a subject participates in Cohorts 1 and 2, we will make sure that sufficient recovery time between the tests such that no more than 550 ml blood will be drawn in any 8-week period.

In Cohort 3, research blood samples will be drawn each day at 8:00 am and at 1:00 pm, immediately before the ^{18}FDG injection. Pharmacokinetic blood samples for glucose, insulin and mirabegron levels will also be collected on study days B, C and D at ten time points: 0, +15, +30, +60, +120, +180, +240, +315, +360, and +420 minutes. Samples at +315, +360, and +420 minutes will be collected in the PET Department by trained technologists due to the radioactivity of the blood. A one-time DNA sample of 10 mL will also be collected. For the entire study (four visits), the total volume of blood sampling will be approximately 500 mL which is less than the NIH guidelines of 550 mL in 8 weeks for research subjects.

Research samples will be analyzed to measure changes in leptin, adiponectin, glucose, insulin, free-fatty acid, thyroid hormones (T3, T4, TSH), parathyroid, growth hormone, cortisol, adrenocorticotropic hormone (ACTH), catecholamines (epinephrine, norepinephrine), glucagon, ghrelin, peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and gastric inhibitory polypeptide (GIP), fibroblast growth factor (FGF) 21, irisin, ANP, BNP, and β-hydroxybutyrate. Subjects in Cohort 3 will also have blood analyzed for pyruvate, lactate and glycerol.

Research use, storage, and disposition of human subject's samples and data

Plasma and nucleic acid samples will be coded and stored in a -80° C freezer. The volume of the 4-hr urine collected each study day will be measured and recorded, then aliquoted into 3 10cc tubes and stored in a -80° C freezer. Study data will be kept in locked files, and subjects will be identified by codes. A registry of the samples' location will be kept in the PI's office. This will allow the PI and the AIs to analyze research data in relation to clinical data stored in the CRIS system. Data and samples will be retained for the foreseeable future in order to allow secondary analyses after the completion of the protocol. Such analyses include association studies for known mutations of genes involved in obesity, energy, carbohydrate, thyroid hormone and glucose metabolism, as well as gene scanning for novel mutations. Serum plasma and nucleic acids samples will be similarly utilized for future association studies of known and novel markers of obesity, energy, carbohydrate, thyroid hormone and glucose metabolism. Genomic DNA will be isolated from peripheral mononuclear cells using the QIAmp® system (QIAGEN) in Dr.

Cypess' laboratory or in the laboratory of another qualified AI. The PI will inform the IRB in case of loss of data/samples secondary to intrusion in the laboratory or database.

Portions of blood samples will be sent to the laboratory of Dr. Michael A. Kiebish at Berg, LLC for lipidomic, metabolomic, and similar screening platforms. The purpose of these studies is to screen for potentially physiologically relevant lipid and metabolic species in human plasma from subjects with known amounts of detectable BAT. We will also share samples with Evan Rosen, MD, PhD, at Beth Israel Deaconess Medical Center (BIDMC) to measure myostatin levels in human plasma from subjects with known amounts of detectable brown adipose tissue (BAT). Dr Rosen's lab at BIDMC has uncovered data that strongly suggest a direct link between BAT and muscle function.

To protect the privacy and confidentiality of the human subjects, the specimens that are sent to these outside laboratories will be coded and not contain any personal identifiers that could be traced to the actual human volunteer. The PI and LAI will maintain the code and it will not be shared with the collaborator. No additional consent is required; the Informed Consent signed by the subjects explicitly stated that "Specimens and/or data may be shared with investigators external to NIH in the event future collaborations are identified.

Non NIH Collaborators

Name	Institution	Project
Michael A. Kiebish, PhD	**BERG, LLC**	**Plasma samples analyzed for lipidomic, metabolomic, and similar screening platforms. The purpose of these studies is to screen for potentially physiologically relevant lipid and metabolic species in human plasma from subjects with known amounts of detectable BAT.**
Evan Rosen, MD, PhD	**Beth Israel Deaconess Medical Center**	**Human Plasma samples to measure myostatin levels from subjects with known amounts of detectable brown adipose tissue (BAT). Dr Rosen's lab at BIDMC has uncovered data that strongly suggest a direct link between BAT and muscle function.**

III.6 Possible Risks and Hazards:

Study medication-related risks are listed individually below. All these are Federal Drug administration (FDA)-approved medications for long term use. While we

summarize the possible risks and hazards as published by the FDA below, we also note that the general risks of taking these medications only once or twice (in two days) in healthy subjects and under resting conditions are considered to be low. In Cohort 1, the total dose per drug is two (once per day). Other research-related risks in this study include those associated with study procedures, namely blood drawing. We do not expect any significant risk connected to the modestly low or high environmental temperatures for the short exposure time at resting state. Shivering confounds measurement of resting metabolic rate so any subject who shivers will promptly be removed from the metabolic chamber and not be exposed to temperatures lower than those causing the shivering. If any medically significant findings are discovered incidentally, the subject will be referred to their primary care provider for follow up.

Study medications:

Propranolol

Propranolol will be administered as a pill by mouth once a day for two days at a dose of 160 mg in this study.

Side effects of Propranolol

The most common side effects of this medication include dizziness or lightheadedness, unusually slow pulse, diarrhea, drowsiness, nausea, unusual tiredness or weakness and numbness or tingling of the fingers and/or toes. Serious skin reactions can occur during treatment with this medicine, however, this side effect is very rare.

Black box warnings of exacerbated angina, MI, and ventricular arrhythmias are associated with abrupt withdrawal of medication after ongoing daily use; these reactions are not expected as we will be administering only two consecutive one time oral doses.

Pindolol

In this study, a single 20 mg dose of pindolol will be administered by mouth once a day for two days.

Side effects of Pindolol

More common potential side effects include insomnia (10%), muscle pain (10%), dizziness (9%), fatigue (8%), nervousness (7%), elevated liver enzymes (7%), joint pain (7%), edema (6%). Difficulty breathing (5%), wheezing, and tightness in chest (3%) are less common.

There are no black box warnings specifically for pindolol.

Dantrolene

In this study, you will be taking a dantrolene 100 mg capsule by mouth once a day for two days.

Side effects of Dantrolene

The most common side effects of dantrolene are drowsiness, dizziness, weakness, general malaise, fatigue, and diarrhea. About one-third of patients may experience drowsiness,

about 1 in 10 experiences dizziness and muscle weakness. Liver injury (1.8%), hepatitis (0.6%) and fatal hepatitis (0.3%) have been reported. These tend to occur more often at higher doses (800 mg and above) and longer duration of administration (greater than three months). Damage that includes fatal hepatitis has occurred in less than 2% of the patients. This medication can cause serious side effects affecting the blood, bone marrow, lungs and heart are but are usually seen at high doses or after taken for prolonged periods.

Black box warnings for this medication are for hepatoxicity as detailed in the previous paragraph.

Safety Monitoring
Safety measures will be done to monitor for potential, but uncommon side effects of muscle weakness and drowsiness/respiratory depression associated with taking dantrolene. To monitor for changes in muscle strength, we will perform a hand grip dynamometer measurement to be done during protocol week one (baseline) and then before and after chamber stays on protocol Day 10 and 11 to assess for any change after the subject has received dantrolene during protocol participation. More frequent respiratory rate will be assessed on protocol Day 10 and 11 during four hour chamber stay – hourly.

Mirabegron
This medication is a β3-adrenergic receptor agonist with sympathomimetic activity. It will be given one time to each volunteer at 50mg and 200mg.

Side effects of Mirabegron
The principal side effects of mirabegron are reported in chronic use at a 50 mg dose. These include: increased blood pressure (9-11%), headache (4%), dizziness (3%), increased heart rate (2%), constipation (2%-3%), diarrhea (2%), dry mouth (3%),urinary tract infection (3%-6%), back pain (3%), joint pain (2%), head cold (4%), sinusitis (3%), and flu-like symptoms (3%). In healthy adults, there are no known effects on the bladder or urination. We expect with a 200mg one-time dose, the effects may be the same or slightly more common and intense; in particular the effects on blood pressure, heart rate, and palpitations. Of note, only those three adverse effects were reported in our previous studies. None of the others were reported by healthy subjects with a one-time dose of mirabegron, including headache, dizziness, constipation, diarrhea, dry mouth, UTI, back pain, joint pain, head cold, sinusitis, and flu-like symptoms. It is possible that side effects with 200 mg may occur more frequently and become more symptomatic than those observed when mirabegron is prescribed at the standard dose approved for treatment, 50 mg.

Based on the effects of other related medications, high doses of mirabegron, well above what will be given in this study, may cause sweating, nausea or vomiting, tremor, weakness, dizziness, confusion, delirium, hallucinations, pallor, and difficulty breathing. None of these side effects were observed in healthy subjects in our previous studies with

mirabegron at a one-time dose of 200 mg. Screening for underlying susceptibility to mild catecholamine excess will be done during the medical history and ECG.

There is a potential connection between mirabegron use and hypersensitivity reactions. Specifically, in the clinical development program it was determined that there was not a consistent pattern to establish an association of mirabegron with nonimmediate, primarily noncutaneous hypersensitivity reactions such as hemolytic anemia and thrombocytopenia (1 patient) and neutropenia (1 patient). Similarly, an association could not be ruled out between mirabegron, particularly at doses ≥ 100 mg, with nonimmediate, primarily cutaneous hypersensitivity reactions such as urticaria, leukocytoclastic vasculitis, rash, pruritus, purpura, and lip and eyelid edema, as well as the serious adverse effects of cutaneous vasculitis (1 patient) and urticaria (2 patients).

Magnesium Sulfate
Magnesium will be given through an intravenous line directly into your vein for 3 hours in the morning for two days. (dosing: 50 mg/kg bolus followed by a maintenance infusion of 2g/h for 3 hours)

Side effects of Magnesium Sulfate
Magnesium can cause nausea, flushing, low blood pressure, slowing the heart rate, and minimal irritation at the infusion site may occur.

There are no black box warnings associated with this magnesium.

Caffeine
Caffeine 300 mg in capsule form will be given once by mouth.

Side effects of Caffeine
This medication can cause insomnia, (inability to sleep) nervousness and restlessness, stomach irritation, nausea and vomiting, increased heart and respiration rates.

There are no black box warnings associated with caffeine.

Phentermine
Phentermine will be given as a single dose of 37.5 mg as an oral pill.

Side effects of Phentermine
The common side effects are difficulty sleeping, dry mouth, and nervousness. Less common side effects include heart palpitations, increased blood pressure, chest pain, shortness of breath, dizziness, upset stomach, constipation, and itching.

There are no black box warnings for phentermine.

Topiramate
Topiramate 200 mg pill by mouth will be given just for one dose in this study.

Side effects of Topiramate
The most common side effects include nausea (6-10%), anxiety (2-3%), appetite loss (4-24%), dizziness (4-25%), double vision (1-10%), drowsiness (4-25%), fatigue (9-16%), tingling or burning sensations (1-11%), tremors (3-9%), and "metallic" taste in mouth. These side effects are more frequent with higher dosages of topiramate. Other reported effects include abnormal vision (2-13%) and recent FDA reports of acute myopia and secondary angle closure glaucoma syndrome associated with decreased visual acuity and/or ocular pain; typically occurring within one month of initiating topiramate.

There are no black box warnings for topiramate.

Qsymia
A single capsule containing 15 mg of phentermine and 92 mg of topiramate will be given once by mouth.

Side effects of Qsymia
Qsymia can cause increased heart rate (14-78%), mild dizziness (3-9%), anxiety and depression (3-8%), concentration, memory and speech problems (2-8%), headaches (10%), feeling tired or irritable (2-4%), constipation (8-16%), insomnia (5-9.4%), numbness or tingling feelings (4-20%), an altered or unpleasant sense of taste (1-9%), and dry mouth (7-19%). Less common (< 1%) but serious side effects include increased pressure in your eyes, acute angle-closure glaucoma and suicidal ideations. The incidence of side effects could be observed by or after 4 weeks of taking the medication daily, but can potentially occur as early as one week after starting the medication. The most common side effects are parasthesias, dizziness, an unpleasant sense of taste, insomnia, constipation and dry mouth.

Precautions while taking Qsymia include refraining from alcohol intake and not driving a car or operating heavy machinery. Qsymia can cause sleepiness and dizziness in combination with alcohol, and can slow your thinking and motor skills, as well as affect vision.

The company that manufactures Qsymia has put in place a REMS (Risk Evaluation and Mitigation Strategy) program since Qsymia is contraindicated in pregnancy for women due to an increased risk of birth defects, cleft lip and cleft palate. This program requires that information on this contraindication is given to all who receive the drug.

There are no black box warnings associated with phentermine/topiramate (Qsymia).

Naltrexone
A 100 mg tablet of Naltrexone will be given once.

Side effects of Naltrexone
This medication can cause nausea (10-33%), headache (3-25%), dizziness (4-13%), anxiety 2-12%), tiredness (4%), and trouble sleeping (3-14%) may occur with

Naltrexone. In a small number of people, mild opiate withdrawal symptoms (< 1%) may occur, including abdominal cramps, restlessness, bone/joint pain, muscle aches, and runny nose. Liver injury (13%) has been reported in individuals taking this drug. However, this appears to occur when the dose of naltrexone is higher (more than 300 mg once a day) and when taken for weeks. A single dose of naltrexone does not appear to cause liver injury.

Black box warning for hepatotoxicity with excessive doses beyond indicated dosing.

Metabolic Chamber (up to 6 hours per day)

Besides inconveniences that can reasonably be expected as a result of spending an extensive time (up to 6 hours) in the live-in room calorimeter, the serious risk to subjects' health is minimal.

Continuous EKG (up to 6hours per day)

A portable Holter monitor will be used to collect heart rate variability in the standard three-lead configuration (4 hypoallergenic disposable EKG electrodes). No known risks have been reported.

Tympanic and surface temperature measurements. (up to 6 hours per day)

A non-invasive tympanic thermometer (PRO4000 by Braun) will be used measured every 30 minutes by the study subjects after trained by our study staff. Wireless surface patches (VitalSense, Mini Mitter, Bend OR) will be used to measure the skin temperature by a portable receiver positioned close to the body. No known side-effect has been reported.

Surface Electromyography sensors with accelerometers (up to 6 hours per day)

The solid-state sensors, each measuring 1.5x1x0.6 inch, pick up electric signals generated by the muscle fibers, which are transmitted wirelessly to the computer. Sensors will be taped with thin hypoallergenic double-sided tape to the specific skin sites, and pose no risk to the subjects.

Infrared thermography

We know of no significant risks from taking pictures with a camera in the infra-red range.

Urine collection (up to 6 hours per day)

Besides the inconveniences that can reasonably be expected as a result of 4-5 hour urine collection during specific time periods, there is no health risk of this procedure.

DXA Scan (1 test)

The use of DXA scan apparatus may cause some minimal discomfort in claustrophobic subjects and may cause some minimal back pain in a small minority of the individuals.

PET/CT (4 scans)

The PET/CT scanner apparatus may cause some discomfort in claustrophobic patients and from lying in a supine position for an extended period of time. Volunteers may also experience minor discomfort and/or bruising at the site of the intravenous injection of the PET radiotracer.

MRI (4 scans)

The MRI scanner apparatus may cause some discomfort in claustrophobic patients and from lying in a supine position for an extended period of time.

Use of fiducial markers (4 times)

The placement of these markers (up to 16) is not expected to cause any pain to the subjects. Volunteers may experience some skin irritation at the site(s) of placement. They will be adhered to the skin surface.

Radiation Safety

The radiation exposure of a DXA scan is less than 1 mrem per session. A normal individual is exposed to 300 mrem/year from natural sources (background exposure).

Each PET/CT scan will use 5 mCi of ^{18}FDG. The radiation exposure of the PET/CT is an effective dose of 0.83 rem, 3.5 rem/year for 4 PET/CT scans, which is below the guideline of 5 rem per year allowed for research subjects by the NIH Radiation Safety Committee. A typical radiation dosage from a chest x-ray is 20 mrem.

The fiducial markers will contain 1 μCi of Na-22. This adds 0. 000085 rem per PET to the current radiation dosage. The effective dose for this study remains 3.5 rem/year.

Blood drawing

The placement of intravenous needles may cause transient pain, and rare infection or bruising at the insertion site.

Fasting over time

The discomfort of fasting daily from midnight until 13:00 (16:30 for Cohort 3) of next day can be difficult for some subjects. There is no known long term harm to the subjects as overall energy balance (dietary intake = energy expenditure) is preserved.

III.7 Adverse events

Adverse events, non-compliance both serious or continuing, protocol deviations both major and minor, as well as unanticipated problems are defined & described by the NIH Office of Human Subjects Research Protection policy #801, and will be reported in accordance with this policy.

Serious adverse events

The PI is responsible for summarizing all serious adverse events and adverse events at least possibly related to the research procedure and interventions at the time of Continuing Review. All deaths that have occurred among study participants since the previous review will be summarized at the time of continuing review.A serious adverse event is any experience that:

1. Results in death;
2. Is life threatening;
3. Results in hospitalization or prolongs hospitalization;
4. Results in persistent or significant disability/incapacity;
5. Results in congenital anomaly/birth defect; or
6. Results in a condition, which in the judgment of the investigator represents a significant hazard.

Clinical Monitoring

Study procedures will be subject to audits and/or monitoring visits to ensure compliance with the protocol and applicable regulatory requirements consistent with the NIDDK quality assurance program plan. Audit and/or monitoring visit results will be reported to the Principal Investigator for further reporting as appropriate. Study documents and pertinent hospital or clinical records will be reviewed to verify that the conduct of the study is consistent with the protocol plan.

Data and Safety Monitoring Plan

No DSMB will be instituted to record adverse events in Cohorts 1 and 2 since this study administers a single dose of FDA-approved medications. Thus the level of risk is minimal, and Dr. Muniyappa will serve as study monitor.

In Cohort 3, no DSMB will be instituted to record adverse events since this study is open label, and given the modest level of risk involved. For this sub-study, LAI, Dr. Cypess, will serve as study monitor.

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site manager. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. The PI and study team will review participant data on at least a monthly basis to review the data for safety and efficacy. Events meeting requirements for expedited reporting as described in HRPP Policy 801 will be submitted within the required timelines.

III.8 Recruitment of Women, children and minority individuals

We will actively encourage the participation of minorities as indicated. The study design precludes the recruitment of children since the differences in thermal biology and metabolic rate between adults and children would impede the interpretation of the data collected. Similarly, the durations of the study (each Cohorts) preclude studying women

due to the variation introduced by the phases of the menstrual cycle. However, our aim is to use data from young men of this study and our experiences with 12-DK-0097 (women and men of different ages) to design a shorter study protocol to study broader populations including women, older and younger subjects in the future.

Benefits

Study subjects will receive no direct benefit from participation in this study. A thorough medical examination and a series of diagnostic tests will be provided as part of the evaluation. Abnormal values will be discussed with the study volunteers and forwarded to their primary care physicians.

III.9 Remuneration

Cohorts 1 and 2
Patients will receive payments for time and discomfort connected with the visits/procedures according to the following scheme:

	Per unit	Cohort 1	Cohort 2
1. Daily compensation for inpatient stay	$40	x 14 = $560	x 6 = $240
2. Metabolic chamber stay	$40	x 12 = $480	x 6 = $240
3. Body sensor measurements	$20	x 12 = $240	x 6 = $120
4. Hunger and thermal questionnaires	$10	x 12 = $120	x 6 = $60
5. DXA and anthropometrics	$35	x 1 = $35	x 1 = $35
6. Blood draws	$20	x 12 = $240	x 6 = $120
7. Bonus for completing the study		$100	$100
Maximum compensation:		$1,775	$ 915

Cohort 3
Patients will receive payments for time and discomfort connected with the visits/ procedures according to the following scheme:

Intervention	**Per Unit**	**Number**	**Cohort 3**
Metabolic chamber stay	$40	X 4	$160
Body sensor measurements	$20	X 4	$80
Hunger and thermal questionnaires	$10	X 4	$40
DXA	$35	X 1	$35
Anthropometrics	$20	X 4	$80
PET/CT	$200	X 4	$800
Blood draws	$20	X 4	$80
MRI	$80	X4	$320
Bonus for completing the study	$100	X 1	$100
Maximum Compensation			$1695

III.10 Research Use, Storage and Disposition of Human Subject's Samples and Data

The following safeguards will be regularly employed to protect subject privacy and confidentiality:

- All medical records will be kept in the medical records department and that research records will be kept in a secure place in a locked file cabinet or in a password protected computer in the office of the Principal Investigator.
- Access to records and data associated with personal information will be restricted to the Principal, Co-, and Associate Investigators
- Samples (blood, fluids, or tissues) sent to outside laboratories for analysis and testing will contain coded numbers, without personal identifiers such as name or address.
- Although samples will be coded, we will retain the key to the sources in order to interpret the data in the context of additional tests performed after the completion of the active recruitment of study volunteers.
- Data and samples will be stored for the foreseeable future; research charts will be kept in the PI's office in a locked file cabinet. E-mail messages containing identifiers of the study participants will be encrypted, and samples will be stored in locked refrigerators.
- The PI will report to the IRB the loss of data or information in case the loss could result in dissemination of study participants' personal information or could threaten the validity of the study, i.e. inability of interpreting the data.

Consent process and documents

Before any screening or study procedures can begin, written informed consent will be obtained. The process of informed decision-making by research subjects will include discussion about the research study with the Principal Investigator and/or designee in language understandable to the subject. Because there are both clinical and technical areas that comprise this protocol, we will ensure both areas are fully addressed to the subjects by the research team with a clinical and technical member present during screening and consenting. Sufficient time and opportunity will be given to discuss the research, (minimizing or eliminating coercion or undue influence) as well as to answer any questions the subject may have. The current IRB-approved informed consent document will be signed by the subject and the Investigator. The investigator signature on the informed consent will be signed by the principal investigator or a designated clinical investigator. A copy of the consent will be given to the subject for future reference. The signed documents will be sent to the Medical Records Department for placement in the subject's permanent CC medical record. The consent process will additionally be documented in the electronic medical record (CRIS).

We anticipate the enrollment of NIH staff into this study and will provide the *NIH Information Sheet on Staff Research Participation* to NIH employees who are

considering participation on our protocol prior to obtaining consent. In addition, we will discuss the following applicable safeguards to NIH staff as a vulnerable class of subjects:

- Unbiased participation for protocol integrity and participant risk assessment.
- Ensure there is no perceived workplace pressure or expectation on either participation or deciding not to participate on the protocol in regards to a benefit or adverse effect on their NIH employment or staff position.
- Protection of privacy and confidentiality will be maintained , but also with acknowledgement of the limits due to sensitive information that may be in their NIH file.
- Discussion of time commitments of the study and compensation in accordance with NIH policy 2300-630-3, *Leave Policy for NIH Employees Participating in NIH Medical Research Studies*.

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Appendix I

The following survey will appear on a custom-programmed iPAD App, in the identical appearance as follows:

Comfort Questionnaire

Date of survey (MM/DD/YYYY): _ _ / _ _ / _ _ _ _

Time of survey (HH:MM): _ _ : _ _ AM PM (circle AM or PM)

How do you feel about the room temperature?

Very hot ——————————————— Very cold

How do you feel about the room humidity?

Very humid ——————————————— Very Dry

How do you feel about the room air flow?

Very drafty ——————————————— No air movement at all

Are you sweating?

Profusely ——————————————— Not at all

Are you shivering?

Constantly ——————————————— Not at all

How hungry do you feel?

I am not hungry at all ——————————————— I have never been more hungry

Other parameters (noted by investigator at time of VAS report; this section is not shared with subject):

Current Chamber Temperature: _____

Current Relative Humidity:_____

Previous day Chamber Temperature: _____

Current Outdoor Temperature: _____

Previous day Outdoor temperature (high): _____

Previous day Outdoor temperature (low): _____

Patient room temperature: _____

Other comments: