CALORIC RESTRICTION & LONGEVITY

Ad-Libitum ▶ ▶ ▶ ▶ ▶ ▶ 2 Years Caloric Restriction

Fat
Carbohydrate
Protein

O_2 →

← O_2

ROS

ROS ↓

ATP
+
CO_2
+
ROS
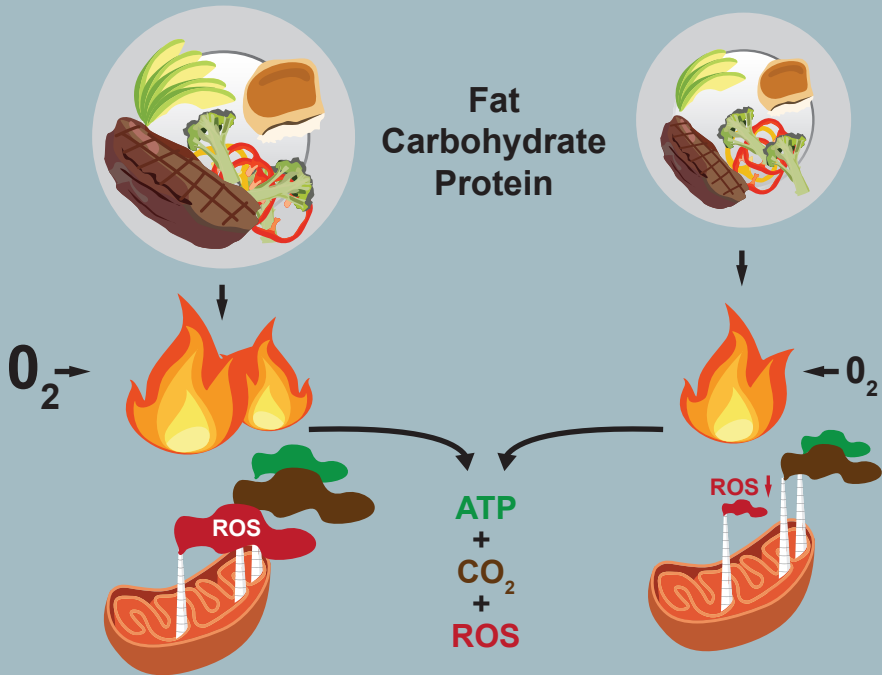
Highlights
- Calorie restriction (CR) extends maximum lifespan in most species
- Young, healthy individuals achieved 15% CR and 8 kg weight loss over 2-years
- Energy expenditure (24-hour and sleep) was reduced beyond weight loss
- Oxidative stress was also reduced supporting two longstanding theories of aging

eTOC Blurb

The rate of living theory of aging posits that individuals most efficient at utilizing energy should experience the greatest longevity. In a randomized controlled trial, Redman et al. tested whether sustained calorie restriction, a nutrition intervention hypothesized to extend lifespan, alters energy metabolism and oxidative damage in healthy, non-obese humans.

Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Supports the Rate of Living and Oxidative Damage Theories of Aging

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SUMMARY

Calorie restriction (CR) is a dietary intervention with potential benefits for healthspan improvement and lifespan extension. In 53 (34 CR and 19 Control) non-obese adults, we tested the hypothesis that energy expenditure (EE) and its endocrine mediators are reduced with a CR diet over two-years. Approximately 15% CR was achieved over two-years resulting in an average 8.7 kg weight loss whereas controls gained 1.8 kg. In the CR group, EE measured over 24-hours or during sleep were approximately 80-120 kcal/d lower than expected on the basis of weight loss indicating sustained metabolic adaptation over two years. This metabolic adaptation was accompanied by significantly reduced thyroid axis activity and reactive oxygen species (F2 isoprostane) production. Findings from this two-year CR trial in healthy, non-obese humans provides new evidence of persistent metabolic slowing accompanied by reduced oxidative stress which support the rate of living and oxidative damage theories of mammalian aging.

INTRODUCTION

For the past 40 years, aging research has focused on the mechanisms underlying the beneficial health impact of a sustained reduction in caloric intake below usual levels, while maintaining adequate intake of essential nutrients. Observations in a variety of laboratory animals indicate that calorie restriction (CR), beginning early or in mid-life and sustained for a substantial portion of the lifespan, increases longevity in a wide variety, but not all, species (Speakman and Mitchell, 2011). While the field of CR research eagerly awaits final lifespan data from the two remaining colonies of CR primates (Colman et al., 2009; Mattison et al., 2012), despite differences in study designs, current data support the observation that sustained CR extends life without chronic disease and promotes a more youthful physical and mental functionality (Mattison et al., 2017). In terms of CR in humans, few controlled clinical trials exist and much of what is known has been derived from observational and cross-sectional studies of individuals who are long-lived such as the centenarians who reside in Okinawa, Japan (Willcox et al., 2006) or individuals self-practicing CR who are members of the calorie restriction optimal nutrition (CRON) society (Fontana et al., 2004).

A variety of mechanisms have been proposed as mediators of the effects of CR on lifespan. Old, but arguably one of the most prevailing theories supporting lifespan extension with CR is a hybrid between two longstanding hypotheses of aging; the "rate of living" and the "oxidative damage" theories of aging. Pearl (Pearl, 1928) in 1928, proposed the idea that mammalian longevity is inversely related to their metabolic rate per unit of tissue mass, hence "rate of living". Several decades later, Harman proposed the oxidative damage theory of aging and suggested that reactive oxygen species (ROS) − byproducts of oxidative phosphorylation in mitochondria − damage DNA, lipids and proteins, all leading to accelerated biological aging (Harman, 1956). There are data from studies in rodents (Hambly and Speakman, 2005), non-human primates (Blanc et al., 2003; Ramsey et al., 1997a) and humans (Heilbronn et al., 2006) indicating that CR results in a decrease in metabolic rate that is greater than that expected on the basis of loss of tissue mass (Heilbronn et al., 2006; McCarter and Palmer, 1992a). This phenomenon referred to as metabolic adaptation, was associated with less oxidative damage to DNA in our 6-month pilot study of CR in humans (Heilbronn et al., 2006). The CR field has also focused on the ability for CR to attenuate age-related changes in physiological and endocrine factors that are known to change with age such as core body temperature, plasma insulin, DHEAS and thyroid hormones (Roth et al., 2002b) as well as endocrine mediators of metabolic slowing such as plasma leptin (Rosenbaum et al., 2005).

Phase 1 CALERIE or the Comprehensive Assessment of the Long term Effects of Reducing Intake of Energy studies were the first randomized controlled trials to test the metabolic effects of CR in non-obese humans (Das et al., 2009; Heilbronn et al., 2006; Weiss et al., 2006). Then, the phase 2 CALERIE study, a 2-year 25% caloric restriction prescription in non-obese volunteers, was shown to be safe and without any untoward effects on quality of life (Martin et al., 2016; Rickman et al., 2011; Rochon et al., 2011; Romashkan et al., 2016). Importantly, the study confirmed the presence of a CR-induced decrease in total daily energy expenditure measured by doubly labeled water after 12 and 24 months (measured CR was 12% on average) indicating a decrease in physical activity and/or a metabolic adaptation. However, in the CR group compared to the control group, resting metabolic rate adjusted for loss of fat-free and fat masses was only lower during the weight loss phase, i.e. at 12 months of intervention (Ravussin et al., 2015) but not a year later. Furthermore, reductions in core body temperature were noted in the CR group but were not different from the controls and changes in oxidative damage were not assessed.

As an ancillary study of the multi-center CALERIE phase 2 trial, individuals studied at Pennington Biomedical Research Center underwent additional procedures to assess changes in the different components of sedentary energy expenditure measured in a metabolic chamber (more precise measure of daily energy expenditure including sleeping metabolic rate) after 1 and 2 years of CR. Importantly, we also measured changes in potential metabolic mediators and biomarkers of aging mediators such as core temperature, thyroid hormones, leptin and insulin as well as downstream effectors including lipid peroxidation and DNA damage, markers of oxidative stress. We hypothesized that after one year, energy expenditure would be lower than that expected on the basis of the loss in energetically active tissues (fat-free mass and fat mass) and that this metabolic adaptation will still be present after another year of sustained CR and weight stability as previously shown in obese individuals (Rosenbaum et al., 2008). Furthermore, following from our 6-month pilot CR study, we hypothesized a reduction in oxidative damage after 1 and 2 years of CR. Taken together, such results would speak in favor of the long-standing hypotheses of biological aging stating that prolonged CR enhances energy efficiency at rest and therefore results in less ROS production and reduced oxidative damage to tissues and organs, thus a combination of the "rate of living" and the "oxidative damage" theories of aging. To test this hypothesis, we delivered a highly controlled and intensive behavioral intervention targeting a 25% CR diet over 2 years and obtained reliable measurements of the most robust component of daily sedentary energy expenditure, i.e. energy

metabolism during sleep, measured in a room calorimeter. Hormonal mediators of metabolism were measured along with urinary F2 isoprostane excretion as an index of oxidative damage.

RESULTS AND DISCUSSION

This paper summarizes the findings of the first randomized clinical trial to test whether CR in young, healthy individuals sustained over two years provides support for the rate of living and oxidative damage theories of aging.

Study subjects and throughput

Out of 80 eligible subjects, 73 consented to the ancillary study and 2 subjects did not complete the baseline metabolic chamber measurement **(Figure 1)**. During the course of the 2-year study, 3 subjects failed to complete all 3 chamber stays (2 in the CR group and 1 in the control), 7 subjects dropped (6 in the CR group and 1 in the control), leaving a group of 60 subjects with complete data available for analysis. Based on adherence to the prescription (weight change), the final data analyses were performed on 53 subjects (36 females, mean age=40±6y), of which 34 subjects were randomized to the CR intervention and 19 subjects to the *ad libitum* control group. The 53 participants were objectively selected on the basis of adherence to their assigned treatment groups whereby the criteria for adherence was >5% weight loss at either Y1 or Y2 for CR participants (3 excluded) and ≤5% weight loss at either Y1 or Y2 for control participants (4 excluded). Per design, all subjects were healthy with 22 (41.5%) being normal weight and 31 (58.5%) slightly overweight at screening. The cohort was 39.8±6.3 years of age at randomization and majority were female (67.9%) and White (73.6%).

Prescribed Two Year CR Resulted in Sustained Weight Loss

As anticipated by the study design and use of a mathematical model to guide weight loss during the CR intervention (STAR Methods), after Y1, the CR group achieved a 16.5±1.5% reduction in energy intake (or CR) from baseline with an overall 14.8±1.5% CR over the entire 2-year intervention **(Figure 2A)**. Despite a slight tendency to gain weight, there was no change in energy intake in the Control group from baseline at either time point (Y1: -1.8±2.0, Y2: -1.7±2.0 %CR). Subjects in the CR group experienced a significant weight loss at Y1 (-9.4 ± 0.4 kg) which was maintained at Y2 (-8.7 ± 0.4 kg, **Figure 2B**). Subjects in the Control group essentially maintained weight during the 2-year period **(Figure 2B)**. In the CR group, the majority of weight loss (70.7%) was fat mass (Y1: -6.7±0.3; Y2: -5.9±0.3, kg, *p*<.0001 from baseline, within group

effect), however a significant loss in fat-free mass (Y1: -2.9±0.2; Y2: -3.1±0.2, kg, *p*<.0001 from baseline, within group effect) was also observed from baseline at both time points **(Figure 2B)**.

CR for Two Years Resulted in Metabolic Adaptation Measured in a Metabolic Chamber

Sedentary 24-hour energy expenditure (24hEE) was significantly reduced from baseline in both the CR and Control groups at Y1 and Y2 **(Table 1)**, whereas SleepEE was reduced from baseline only in the CR group at both time points **(Table 1)**. In response to the reduced body weight, we observed an approximate 10% drop in absolute energy expenditure during sleep. After taking the loss of metabolic tissues (fat-free and fat masses) into account, SleepEE was still reduced by ~7% in the CR group indicating a metabolic adaptation in comparison to the control group (**Figure 3A**, *p*<.02). Similarly, 24hEE adjusted for changes in body composition (FFM and FM) was significantly decreased from baseline at Y1 and Y2 but not differently from the control group **(Figure 3B,** *p*>.55).

An ongoing debate among metabolism, obesity and aging investigators is whether a chronic deficit in energy intake leads to metabolic slowing or a decreased "rate of living". This phenomenon which has been termed "metabolic adaptation," defines a reduction in energy expenditures that is larger than expected for the metabolic mass of the organism following a reduction in the respiring mass due to a caloric deficit (Heilbronn and Ravussin, 2003). It is thought that the rate of biological aging may be delayed by prolonged CR through a reduction in the "rate of living," (Sacher and Duffy, 1979) leading ultimately to reduced oxidative damage. Together these theories imply that increased metabolism (above what is required to support the respiring mass) and the resultant increased production of reactive oxygen species (ROS) leads to a shorter life span unless these ROS are removed by antioxidant mechanisms.

Support for the rate of living hypothesis as a mechanism which may extend lifespan in mammals is debated (Speakman and Mitchell, 2011). Indeed some of this conflict is due to discrepancies in the different degrees and duration of the imposed CR, the timing of the energy expenditure evaluation following the initiation of CR, and whether energy expenditure was measured under basal or free-living conditions, which includes physical activity. However, probably the most likely source of conflicting evidence is owed to conclusions being drawn from energy expenditure measurements without appropriate statistical approaches used to account for the losses of metabolic tissues, which also are reduced with CR (Ravussin and Bogardus, 1989; Speakman et al., 2002). For instance, regarding the observations in rodents, the early seminal work of McCarter showed that the basal metabolic rate in rats following a 40% CR for six months (McCarter et al., 1985) and across the entire life span (McCarter and Palmer, 1992b)

was comparable when compared to rats fed *ad libitum*. These data were however, soon after criticized for the assumption and statistical consideration that oxygen consumption should be adjusted for functional metabolic mass (Lynn and Wallwork, 1992). In contrast to McCarter's study, the evaluation of 24h energy metabolism in rats after 11 weeks of moderate (25%) and severe (50%) CR showed a decrease in oxygen uptake (Ballor, 1991), that after adjustment for the change in body mass, was only observed in severe CR. In agreement with the necessity for more severe CR in rats, a 14% lowering of the metabolic rate (adjusted for body size) was observed following a 60% CR for six weeks, in comparison to counterparts fed *ad libitum* (Gonzales-Pacheco et al., 1993). Of the three non-human primate colonies, a 30% CR diet had no impact on oxygen consumption (measured over 36 hours) after 1 year in comparison to controls (Kemnitz et al., 1993). However, energy expenditure (night time and 24h) examined in the same cohort after 30 months was significantly lower in CR monkeys (Ramsey et al., 1997b). This agrees with a reduction in total daily energy expenditure (adjusted for fat-free mass) observed in a different colony undergoing a 26-31% CR for more 10 years. Yet, despite a consistently lower 24h energy expenditure in a third colony exposed to a 30% CR, the reduced EE was not different from the control animals (Lane et al., 1995).

In our earlier pilot study, which achieved 19% CR but across only 6 months, we observed a significant metabolic adaptation of energy expenditure (24h and Sleep) measured in a metabolic chamber which was paralleled by a reduced oxidative damage to DNA (Heilbronn et al., 2006). The multi-center CALERIE trial in 218 individuals, using a measure of resting metabolic rate with a bed-side indirect calorimeter (ventilated hood), observed a metabolic adaptation after 1 year in comparison to the controls (*ad libitum* diet) but this adaptation was no longer significant after another year of weight stability (Ravussin et al., 2015).

Believed to be a mechanism for energy conservation, metabolic adaptation has been the focus of weight loss (not specifically CR) in overweight or obese individuals undergoing intensive dietary interventions (Johannsen et al., 2012; Knuth et al., 2014; Rosenbaum et al., 2008). Weight-loss induced metabolic slowing has been reported in overweight/obese individuals with weight loss maintenance persistent for up to 7 years (Rosenbaum et al., 2008). However, the weight loss literature has not focused on quantifying the downstream effects on oxidative damage, which is the other key assumption in a reduced energy flux for delaying biological aging and potentially extending lifespan.

Physical activity

Recent investigations in non-human primates (Yamada et al., 2013) allude to an increased physical activity at a lower metabolic cost with sustained CR. We observed no significant treatment effect for activity-related EE (AREE; p=.20). AREE in the CR group was not changed at Y1 (-101±55 kcal/d, p=.07, within group effect) but was significantly decreased from baseline at Y2 (-119±55 kcal/d, p=.03, within group effect). In the control group, there was no change in AREE from baseline at either time point. The mean change in spontaneous physical activity (SPA, kcal/d) measured in the metabolic chamber was not different between the CR and Control groups (p=.46, treatment main effect). SPA was significantly decreased from baseline in the CR group at both Y1 and Y2 (Y1: -29±9; Y2: -30±9, kcal/d, p<.01 for both, within group effect), suggesting a reduced energy cost of activity in the chamber.

The lack of effect of CR on physical activity related energy expenditure was echoed in our preliminary 6 month study (Redman et al., 2009). Although the measurement of spontaneous physical activity in the metabolic chamber alludes to a possible reduction in activity with CR, physical activity was unfortunately not objectively measured in the present study while participants were free-living.

Mediators of Energy Metabolism, Biomarkers of Aging and Relationship with Metabolic Adaptation

As expected from the differential changes in fat mass between the treatment groups, there was a significant treatment effect for the change in leptin (p<.0001, treatment main effect) with significant reductions from baseline in the CR group at both Y1 and at Y2 and no observed changes in the Control group (**Figure 4A**). T3 concentrations were significantly decreased from baseline at Y1 and Y2 in the CR group (Y1: -23.5±2.7; Y2: -29.9±2.7, ng/dL, p<.0001 for both, within group effect) and that was significantly different from the change in the Control group (p<.01, treatment main effect). Similarly, there was a significant treatment effect observed for the change in T4 concentrations (**Figure 4B**, p=.02) and the post-hoc comparison revealed that the change in T4 concentration from baseline in the CR group was only significant at Y2 (p<.0001). This reduced activity of the thyroid axis was not supported by a change in TSH (Y1: -0.15±0.09; Y2: -0.16±0.08, uIU/mL, p<.10 for both, within group effect) or reverse T3 (data not shown). Sympathetic nervous system activity assessed through excretion of urinary catechomines over 24-hours during the chamber was not changed from baseline for epinephrine or norepinephrine in either group at Y1 or Y2. Core temperature recorded over 24-hours was not changed during the weight loss phase (Y1: -0.01±0.03 ^{o}C, p=.88, within group

effect), however with weight maintenance and sustained CR (Y2), there was a trend for core temperature to be decreased from baseline (Y2: -0.06±0.03 ^{o}C, p=.07, within group effect). When day-time (0800-2200h) and night-time (0200-0500h) temperature were considered separately, no change in day-time temperature was observed in the CR or Control at either time point and therefore the reduced 24h core temperature in the CR group at Y2 was attributed to a reduction in core body temperature recorded at night (Y1: -0.05±0.05, p=.30; Y2: -0.10±0.05, ^{o}C, p=.05, within group effect).

While there was no observed treatment group effect on the changes in fasting concentrations of DHEAS, there was a significant interaction (diet intervention group by time) for fasting insulin (p<.05), with a significant reduction in insulin concentration in the CR group at Y1 which was no longer evident at Y2 (Y1: -1.5±0.4, p<.001; Y2: 0.15±0.4, μIU/mL, p=.70, within group effect). Furthermore, there was a significant increase in adiponectin concentrations (HMW) from baseline in the CR group (Y1: 1188±275, Y2: 1185±271, ng/mL, p<.001 for both, within group effect) which was different from Control group (Y1: 23±363; Y2: -722±363, ng/mL, within group effect) and at both time points (treatment main effect, p<.001).

During the weight loss phase (Y1), the metabolic adaption in SleepEE (SleepEE residual) was associated with greater reductions in leptin (**Figure 4C**, r=0.35; p=.01), but not in the thyroid axis activity (T3, T4, TSH or rT3). At Y2, however when weight loss was maintained, the relation between metabolic adaptation in SleepEE and leptin was no longer significant (p=.22), but the metabolic adaptation was correlated with the reduction in T4 concentrations (**Figure 4D** r=0.33; p=.02).

In comparison to the Control group, significant reductions in hormonal mediators of energy metabolism including leptin, and thyroid hormones (T3 and T4) and an increase in adiponectin were observed in the CR group. However, the relationship of the changes in these hormones to the metabolic adaption differed in relation to CR during weight loss (Year 1) compared to CR during weight loss maintenance (Year 2). The CR group also demonstrated attenuation in two well-described biomarkers of aging; night-time core body temperature and fasting insulin.

There appear to be two distinct hormonal mechanisms during CR that potentially influence the development and sustenance of the metabolic effects. During the weight loss phase, we observed a 28% reduction in fat mass and a parallel reduction in leptin. We have previously shown that leptin is a determinant of metabolic adaptation during CR (Lecoultre et al., 2011), independent of the changes in fat-free mass and fat mass. Indeed, the change in leptin after 1 year of CR was significantly associated with the metabolic adaptation during sleep, however this relationship was no longer evident after year 2, when weight and fat mass loss were maintained

but the metabolic adaptation still present. The mechanisms linking leptin to metabolic adaptation are unclear even if leptin replacement in obese individuals who had undergone a 10% weight loss rescued in part the metabolic adaptation and reduction in thyroid hormones (Rosenbaum et al., 2002). With prolonged CR and weight loss maintenance the hormone milieu changes with less contribution from obesity-related hormones (insulin, leptin) and greater contribution from metabolic hormones (T3 and T4). A reduction in thyroid axis activity is a hallmark feature of the hypometabolic state with weight loss and has been described as a biomarker aging (Roth et al., 2002b). In the non-human primate colonies (DeLany et al., 1999; Lane et al., 1996; Ramsey et al., 2000; Roth et al., 2002a) exposed to CR diets and in individuals either naturally-exposed to CR or self-practicing CR diets (Fontana et al., 2006; Soare et al., 2011), reduced thyroid hormones and lower core body temperatures are also reported. Whether these biomarkers are necessary drivers for maintaining metabolic adaptation or a consequence is unknown and cannot be discerned from our study.

Markers of oxidative stress

Urinary F2-isoprostane excretion (2,3-dinor-iPF(2α)-III) was significantly reduced from baseline at Y1 and Y2 in the CR group (Y1: -0.42±0.12; Y2: -0.49±0.12 ng/mg Cr, $p<.01$ for both, within group effect) and not changed in the Control group ($p>.5$ for both time points, data not shown). The pairwise comparison showed that the difference in 2,3-dinor-iPF(2α)-III concentrations was significant between CR and Control at Y2 ($p<.05$). Similarly, the three additional isomers of F2-isoprostanes (iPF(2α)-III, iPF(2α)-VI, and 8,12-iso-iPF(2α)-VI) were significantly reduced from baseline in the CR group at Y2 ($p<.05$, within group effect) and not changed in the control group, but no treatment effects were observed. The change in 2,3-dinor-iPF(2α)-III concentrations from baseline to Y2 (expressed as percent change) within the CR group was associated with 24hEE metabolic adaptation (r=0.33, $p=.05$) and percent CR achieved (r=-36, $p=.05$). Serum protein carbonyl concentrations were not changed from baseline at Y1 or Y2 in either the CR or Control group.

Urinary F2-isoprostane excretion including four isomers of (F2-isoprostanes) was significantly reduced following the weight loss phase and remained significantly lower than baseline after year 2. Importantly, the drop in 2,3-dinor-iPF(2α)-III, our primary measure of oxidative damage was associated with metabolic adaptation in 24-hour energy expenditure and the degree of calorie restriction. In contrast, no changes in plasma protein carbonyl levels were found in either group. This discordance has also been observed in previous studies. However urinary F2-isoprostanes are thought to be more sensitive biomarkers of oxidative stress in both

animals (Kadiiska et al., 2005) and humans (Il'yasova et al., 2010) and are sensitive to changes in age and caloric restriction (Ward et al., 2005), whereas protein carbonyls are less sensitive to oxidative assault (Il'yasova et al., 2009; Kadiiska et al., 2005).

The "free radical theory of aging" or "oxidative stress" hypothesis is a well-supported theory of aging. It is widely accepted that the metabolic rate of an organism is a major factor in the rate of aging and is inversely related to its lifespan (Sohal and Allen, 1985). Additionally, since 1% to 3% of consumed oxygen is associated with the production of reactive oxygen species (ROS), namely superoxide ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2^{\bullet-}$), and the hydroxyl ion ($OH^{\bullet-}$) (Alexeyev et al., 2004), the production of these highly reactive molecules is thought to be proportional to the metabolic rate of an organism. Numerous studies have shown that modulation of the oxidative stress of an organism through prolonged CR retards the aging process in various species, including some mammals (Sohal and Weindruch, 1996; Weindruch et al., 1986) and possibly humans (Heilbronn et al., 2006). In support for a CR-induced metabolic slowing and reduction in oxidative stress, the CR group in this investigation had a significant reduction in urinary 2,3-dinor-iPF(2 α)-III isoprostane excretion after both 1 and 2 years and three other F2 isomers after 2 years. The reductions in isoprostane excretion were significantly associated with metabolic slowing (metabolic adaptation) in 24-hour energy expenditure at Year 2. This finding may emphasize the necessity for long-term studies of sustained CR in humans. Interestingly, reduced levels of oxidative damage have not been reported in centenarians (Klapcinska et al., 2000; Paolisso et al., 1998). Furthermore, in a large study of nonagenarians residing in Louisiana, we did not find an association between a reduced age-related decline in energy metabolism and oxidative damage to DNA (Frisard et al., 2007). This could bring into question the validity of the oxidative damage theory of aging particularly as it relates to the rate of living, or alternatively it could point to technical limitations in the measurement of ROS with only indirect biomarkers including urinary protein carbonylation and/or F2-isoprostane excretion rates and/or measurement of energy expenditure (bedside calorimeter versus metabolic chamber). These methods may be either insensitive to the small changes to energy efficiency (metabolic adaptation) with CR, or alternatively ROS accumulation may not be attenuated with CR but buffered by the antioxidant defenses of the organism (Schachter et al., 1993). Indeed, observational studies in individuals who live to over 100 years have higher levels of several antioxidant molecules (Mecocci et al., 2000) including those individuals residing on Okinawa (Suzuki et al., 2010) who have been exposed to natural CR across most of their lifespan.

In the exploration of a potential mechanism linking the rate of living and oxidative stress theories to explain the benefit of prolonged CR on aging, it is not surprising that the work has

focused on mitochondria. Mitochondrial metabolism is the major endogenous source of ROS production (Fulle et al., 2004). However, the findings from the CR studies do not explain how changes in mitochondrial metabolism lead to lower ROS production. For example, CR in rats has been shown to reduce mitochondrial proton leak and production of hydrogen peroxide (Hagopian et al., 2005), although ROS generation is proportional to transmembrane potential and is down-regulated by proton leak (Brookes, 2005; Stowe and Camara, 2009). In monkeys and humans undergoing CR of a shorter duration, CR has been shown to induce robust increases in PGC-1 and mitochondrial biogenesis (Civitarese et al., 2007; McKiernan et al., 2012; Stein et al., 2012). As a result of mitochondrial biogenesis, CR results in improved mitochondrial function or mitochondrial efficiency, decreased total body oxygen consumption, and therefore decreased production of ROS (Civitarese et al., 2007). Indeed an increase in skeletal muscle work efficiency has been observed in obese individuals with a metabolic adaptation following weight loss (Rosenbaum et al., 2003). Furthermore, in our previous 6 month study (Heilbronn et al., 2006), we observed a reduced energy cost of physical activity after CR.

Overall Conclusions and Limitations of Study

In summary, according to the rate of living theory, those individuals who are the most efficient at utilizing energy should experience the greatest longevity. Observational studies of human aging have shown higher mass-adjusted metabolic rate (24hEE or Resting EE) is associated with disease burden (Fabbri et al., 2015; Schrack et al., 2014) and is a predictor of early mortality (Jumpertz et al., 2011; Ruggiero et al., 2008). Interventions with the capacity to induce a sustained slowing of energy metabolism such as calorie restriction should remain a focus of longevity research because randomized clinical trials and cohort studies are lacking. With careful phenotyping of energy metabolism, biomarkers of aging and oxidative stress, this modest, two-year study of human calorie restriction identified a reduction in the rate of living along with a reduction in systemic oxidative stress. The duration of imposed CR being for only two years clearly limits any extrapolation or speculation of the impact of CR on longevity in humans. Notably, many biomarkers of aging (that could be a consequence of the overall improved metabolic profile commensurate with adipose tissue loss) were also improved in these young, healthy individuals. There is a clear need for continued investigations of calorie restriction in humans since the non-human primate data is not entirely conclusive on the extension in the average and maximal lifespan but provides strong evidence for extensive health benefits including improved quality of life. The CALERIE study did not prescribe a

particular diet composition and thereby this research cannot be extrapolated to infer diet recommendations to promote healthier aging apart from a reduction in calories. Future research on CR for healthy aging would benefit from considering the diet quality of individuals who have successfully defied the aging process (e.g. antioxidant content). Finally, future studies of CR may benefit from a combined approach with use of a CR mimetic such as resveratrol or greater attention to the dietary prescription which would include foods that will increase antioxidant defense systems.

AUTHOR CONTRIBUTIONS

E.R. and L.M.R.: designed the study, obtained funding, conducted the research, interpreted the results and wrote the manuscript; S.R.S., C.K.M. designed the study, obtained funding, conducted the research; J.H.B. performed the statistical analysis. All authors reviewed and approved the final draft of the manuscript.

DECLARATION OF INTERESTS

The authors declare no financial conflicts of interest in association with the research described in this paper.

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FIGURE AND TABLE LEGENDS

Figure 1. Subject throughput from enrolment (N=71) to data analysis (N=53). Analyses were performed on 53 men and women who on the basis of an objective pre-analytical criterion (weight change) were determined to be adherent to their assigned treatment groups.

Figure 2. Percent of calorie restriction (A) achieved after 1 and 2 years of calorie restriction and the resulting change in fat mass (FM) and fat free mass (FFM), panel B. N=53; 34 CR, 19 Control. *P*-value for statistically significant treatment group effects, adjusted for multiple comparisons is shown. The change in weight, FM and FFM were all significantly different between the CR and control group ($p<.0001$ for all, treatment main effect).

Figure 3. A comparison of metabolic adaptation in sleep energy expenditure (panel A) and 24-hour energy expenditure (panel B) between the AL (control, n=19 ■) and CR (n=34, □) groups, after 1 and 2 years of calorie restriction. Metabolic adaptation was considered to represent the change in energy expenditure after adjusting for the changes in fat-free mass, fat mass, age and sex and the metabolic adaptation at baseline (see methods, Sedentary 24-hour Energy Expenditure, for calculation). *P*-values for statistically significant treatment group effects, adjusted for multiple comparisons are shown.

Figure 4. A comparison of changes in the potential mediators of metabolic adaptation; leptin (panel A) and thyroxine (T4, panel B), and the association between metabolic adaptation in Sleep EE with percent change from baseline in leptin concentrations at year 1 (Y1) (panel C) and percent change from baseline in thyroxine concentrations (T4) at year 2 (Y2), panel D. AL (control, ■) and CR groups (□). *P*-values for statistically significant treatment group effects, adjusted for multiple comparisons are shown. Scatterplots show the linear regression model with 95% confidence interval. N=53; 34 CR, 19 Control.

TABLE 1. Physical characteristics of 53 men and women during weight maintenance at baseline and following 1 and 2 years of calorie restriction. Baseline data is presented as mean ± SD. Change from baseline data is the adjusted LS mean ± SE from the mixed linear models which include the baseline value as a covariate. * denotes significant within group change from baseline ($p<.05$). *Abbreviations*: Ad lib, *ad libitum* control group; CR, calorie restriction group; BMI, body mass index; kcal, kilocalories; d, day; 24hEE, 24-hour energy expenditure; SleepEE, energy expenditure during sleep (0200h-0500h); T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

The dataset pertaining to the current study is available upon written request. Resources will be shared in accordance with appropriate data use agreements and IRB approvals for secondary analyses. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Leanne Redman (leanne.redman@pbrc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study Design: CALERIE 2 (Rochon et al., 2011) was a two year multi-center, parallel-group, randomized controlled trial that recruited healthy individuals to receive an intervention aimed at reducing energy intake by 25% (CR group) or to maintain habitual energy intake on an *ad libitum* basis (control group). Two hundred and twenty individuals from Pennington Biomedical Research Center (Baton Rouge, LA), Washington University (St. Louis, MO) and Tufts University (Boston, MA) were randomized in this multi-center study (NCT00427193) for which Duke University, (Durham, NC) was the coordinating center (Rochon et al., 2011). The present ancillary study (NCT02695511) was approved by the IRB of the Pennington Biomedical Research Center and offered only to the 80 individuals enrolled in the parent study at this site. Interested individuals provided written informed consent for the additional visits and procedures. Following baseline assessments, participants were randomized to adhere for two years to a diet that targeted 25% calorie restriction (CR group) or calorie intake *ad libitum* (AL; Control group) according to a 2:1 allocation in favor of the CR group. Randomization was stratified by study site, sex and BMI dichotomized into normal weight (22.0≤ BMI<25.0 kg/m^2) and overweight (25.0≤ BMI<28.0 kg/m^2). Ancillary testing included an additional outpatient visit and a 24-hour stay in a metabolic chamber at baseline, and after 1 year (Y1; 12 months) and 2 years (Y2; 24 months) of intervention. The Clinic staff involved in the collection of study outcomes was blinded to the treatment group assignments.

Participants: Men and women were aged 20 to 50 years and 20 and 47 years, respectively, and had body mass index (BMI) between 22.0 to 27.9 kg/m^2 at the initial screening visit. Potential participants in the ancillary study were excluded for claustrophobia, contraindications to MRI and history of blood clotting disorders. The CONSORT diagram summarizing throughput of participants in the study is provided in **Figure 1** and the characteristics of the participants at baseline is summarized in **Table 1**.

METHOD DETAILS

Study Interventions: From day 1, the CR intervention targeted a sustained 25% restriction of energy intake prescribed on the basis of the energy requirements determined from two, 14-day doubly labeled water measures at baseline (Redman et al., 2014; Rickman et al., 2011). The goal for the intervention was adherence to a mathematically predicted weight loss trajectory that reached 15.5% below baseline weight after one year of intervention followed by maintenance of this weight over the second year (Pieper et al., 2011). Participants received a weekly weight loss graph that showed a targeted weight range which was used as the primary tool to maintain adherence during the intervention. Because of the variability in projected weight loss needed to achieve 25% CR, participants were also provided with guidance indicating a "zone of acceptable weight loss" which ranged from 12 to 22%. Nutritional and behavioral guidance was customized and modified to decrease the degree to which weight change differed from the target. Adherence to 25% CR was further fostered by provision of meals for the first 27 days of the study. Participants were fed their assigned caloric prescription in the form of three, 9-day diets. The food provision was used to educate on portion size, energy content and anticipated diet changes necessary to maintain 25% CR with different types of dietary patterns. The behavioral intervention included delivery of a structured curriculum in regular group and individual meetings with interventionists (clinical psychologists and nutritionists) from a standardized treatment manual developed specifically for the study (Rickman et al., 2011). Participants randomized to the control group were advised to continue their current diets on a completely *ad libitum* basis. No specific level of physical activity was required or recommended for either group. All participants received a multivitamin (Nature Made Multi Complete, Pharmavite LLC, Mission Hills, CA) and calcium supplement (1000mg/d, Douglas laboratories, Pittsburgh, PA) to foster nutritional adequacy of the self-selected diets.

Energy Intake and Calorie Restriction: Energy intake was calculated at baseline by total daily energy expenditure (doubly labeled water) and during the trial between baseline and Y1 as well as baseline and Y2 by the intake/balance method derived from total daily energy expenditure (doubly labeled water) and the changes in energy content of fat mass (9,300 kcal/kg) and fat-free mass (1,100 kcal/kg) from DXA (Racette et al., 2012). The percent reduction in energy intake (%CR) achieved during each interval was defined as; %CR = 100 x (Energy intake at baseline – EI during Interval) / Energy intake at baseline.

Total Daily Energy Expenditure: For each doubly labeled water (DLW) assessment, two baseline urine samples were collected before subjects consumed an oral cocktail containing 0.1 g of ^2H_2O at 99.98 atom % ^2H and 0.16 g of 100% ^{18}O per kg body weight. After dosing, participants were asked to void their bladder at approximately 1-3 h after ingestion (this sample was discarded) and to collect six additional, timed urine samples: two approximately 4.5h and 6h after dosing, two on day 7, and two on day 14. Measurement of hydrogen and oxygen isotope enrichments were measured by gas-isotope-ratio mass spectrometry at the USDA/ARS Children's Nutrition Research Center Stable Isotope Laboratory (Houston, TX) (Racette et al., 1994; Wong et al., 1992). Carbon dioxide production rate (VCO_2) was calculated from the fractional turnover rates of ^2H (k_H) and ^{18}O (k_O) (Racette et al., 1994) and converted to TDEE based on an energy equivalent of a liter of CO_2 to be 3.815/RQ + 1.2321 where the RQ was determined for each individual using food diaries and changes in body composition.

Anthropometrics and Body Composition: Metabolic body weight was measured (Scale Tronix 5200, White Plains, NY) in the morning after an overnight fast and voiding while wearing a surgical gown which was subtracted from the total weight. Body composition (fat, lean, and bone) was measured by dual X-ray absorptiometry (DXA; Hologic QDR 4500A; Hologic, Bedford, MA) according to a standardized protocol and all scans were analyzed at a centralized reading center (University of CA, San Francisco) using Hologic software version Apex 3.3.

Sedentary 24-hour Energy Expenditure: Participants entered a metabolic chamber at approximately 0800h after an overnight fast for measurement of 24 hour sedentary energy expenditure (24hEE) and sleeping energy expenditure (SleepEE). Meals were prepared by the metabolic kitchen and served according to a fixed schedule. At baseline, the energy intake provided was estimated according to an equation and adjusted during the day on the basis of actual measured energy expenditure of the first 7 hours of measurement (Nguyen et al., 2003). For the subsequent chambers, the energy content of the food was held constant for control participants and was 75% of measured baseline 24-hour energy expenditure for CR participants. SleepEE was assessed between 0200-0500h for those minutes that activity is less than 1%. During their stay in the chamber, no exercise will be allowed. The change in 24hEE and SleepEE is expressed as the residual EE which is the difference between the measured value and the value predicted for the EE measurement (on the basis of weight and body

composition) at each time point. The predicted values were derived from a linear regression at baseline for the 71 participants using fat-free mass, fat mass, age and sex as covariates;

1) 24hEE (kcal/d) = 1100 + 17.2 (fat-free mass, kg) + 4.6 (fat mass, kg) – 1.9 (age, y) - 167 (sex; 1=female, 0=male); R^2=0.70, p<.0001.
2) SleepEE (kcal/d) = 749 + 17.6 (fat-free mass, kg) + 3.2 (fat mass, kg) – 2.6 (age, y) - 58 (sex; 1=female, 0=male); R^2=0.70, p<.0001.

The difference in the residual EE (follow-up minus baseline) was then used as a marker of the extent to which energy expenditure adapted to calorie restriction independently from the changes in body mass and body composition with negative values indicating metabolic adaptation (Galgani and Santos, 2016).

Core body temperature: Core body temperature (VitalSense, Mini-Mitter, Bend, OR) was measured and recorded every minute during the energy expenditure measurement in the metabolic chamber. Mean temperature over 24-hours as well as mean day time (0800-2230h) and night time (0200-0500h) temperatures were calculated.

Physical activity: The energy cost of physical activity, termed activity related energy expenditure (AREE) was calculated as the cost of daily activities beyond sleep using linear regression model of total daily energy expenditure by doubly labeled water measures (TDEE) and SleepEE at baseline: TDEE (kcal/d) = 859 + 1.1 (SleepEE, kcal/d) + 4.1 (age, y) - 340 (sex; 1=female, 0=male); R^2=0.66, p<.0001. AREE is positive for subjects with higher physical activity than average and negative for subjects with lower physical activity than average (Redman et al., 2009). Second, the calories expended in spontaneous physical activity (SPA) were determined by microwave motion detectors and indirect calorimetry in the metabolic chamber.

Oxidative Stress: Our primary measure of oxidative damage, urinary 2,3-dinor-iPF(2α)-III was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Shimadzu 20A series LC and Applied Biosystems API 4000 QTrap MS/MS instruments as previously described (Il'yasova et al., 2010). We also measured three additional isomers of F2-isoprostanes; iPF(2α)-III, iPF(2 α)-VI, and 8,12-iso-iPF(2α)-VI as exploratory variables. Urine specimens were diluted to 0.65 mg/mL creatinine, and samples with creatinine levels equal to or below this value were analyzed without dilution. Sample preparation included addition of internal

standards [iPF(2α)-III-d4, 8,12-iso-iPF(2α)-VI-d11, iPF(2α)-VI-d4] and 10 μL 1M HCl; washing of samples (500 μL) with 1 mL hexane; extraction of the analytes by ethyl acetate/hexane mixture (3/1, v/v); evaporation of the liquid and resuspension of the residue in 150 μL of a mixture containing 70% mobile phase A (0.1% formic acid in water) and 30% methanol. Using LC-MS/MS, 100 μL of sample were injected into two solid core C18 columns (Phenomenex Kinetex C18, 150 x 4.6 mm) in series to achieve chromatographic separation of the F2-isoprostane isomers. The mass spectrometer was operated in negative mode with the following MRM transitions (m/z): 353/193 [iPF(2α)-III], 357/197 [iPF(2α)-III-d4], 325/237 [2,3-dinor-iPF(2α)-III], 353/115 [iPF(2α)-VI and 8,12-iso-iPF(2α)-VI], 364/115 [iPF(2α)-VI-d11], and 357/115 [8,12-iso-iPF(2α)-VI-d4]. Calibration samples covering the expected range of concentrations were prepared by adding pure material into pooled human urine, injected before and after the patient samples. Lower limits of quantification (LLOQ, >80 % accuracy) were 0.007, 0.34, 0.25, and 0.12 mg/mL for iPF(2α)-III, 2,3-dinor-iPF(2α)-III, iPF(2α)-VI, and 8,12-iso-iPF(2α)-VI, respectively. As a complimentary measure of oxidative damage, serum protein carbonyls were determined using a modified 2,4-dinitrophenylhydrazine assay (Mates et al., 2000).

Clinical Chemistry: Fasting blood samples were collected and the following assays were performed at the CALERIE central biochemistry laboratory at Vermont University or at the Clinical Chemistry Core at Pennington Biomedical Research Center: thyroid stimulating hormone (TSH) and triiodothyronine (T3) by chemiluminescent immunoassay (ADVIA Centaur, Bayer Health Care, Deerfield, IL); thyroxine (T4) by particle-enhanced immunonephelometric assay, (BN II, Siemens, Deerfield, IL); reverse T3 by multiplex immunoassay (Bio-Plex, Bio-Rad Laboratories, Hercules, CA); leptin by multiplex immunoassay (Bio-Plex, Bio-Rad Laboratories, Hercules, CA); and insulin by chemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics, Indianapolis, IN). Nitrogen, creatinine, norepinephrine and epinephrine were measured in a 24 hour pooled urine sample collected during the chamber stay.

QUANTIFICATION AND STATISTICAL ANALYSIS
Sample size estimation: This study was powered on the ability to detect a significant adaptation in energy metabolism (24hEE, SleepEE) from baseline and to detect differences in this adaptation between the two diet groups (AL vs CR). Sample size estimates were derived from the data obtained in our 6 month pilot study where the standard deviation in EE was assumed to be 140 kcal/d. Anticipating that a maximum of 75 subjects (50 in CR and 25 in control) would
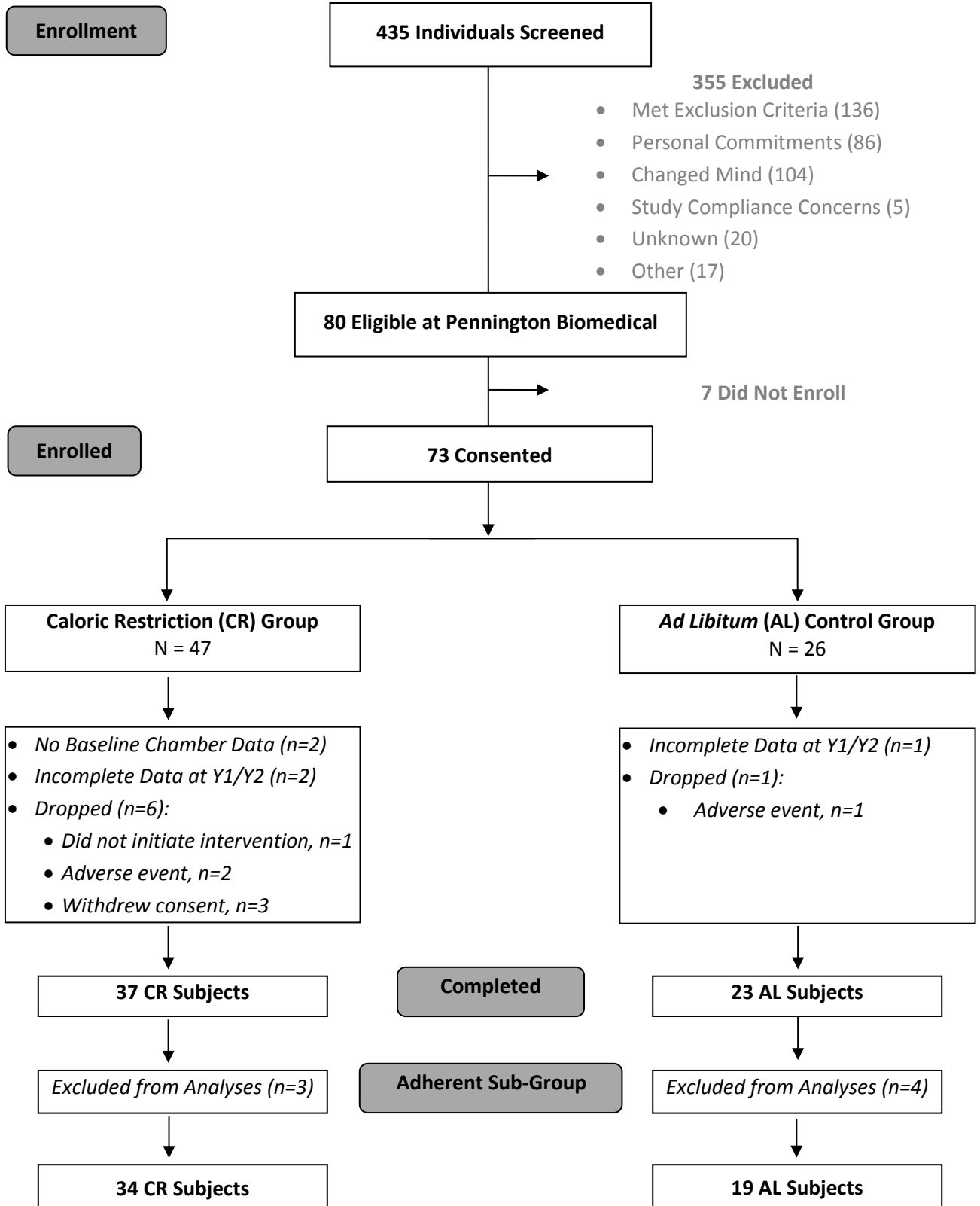
enroll in the ancillary study, the minimal detectable metabolic adaptation within groups is 60 kcal/d and between groups is 100 kcal/d to achieve a power ≥ 80%.
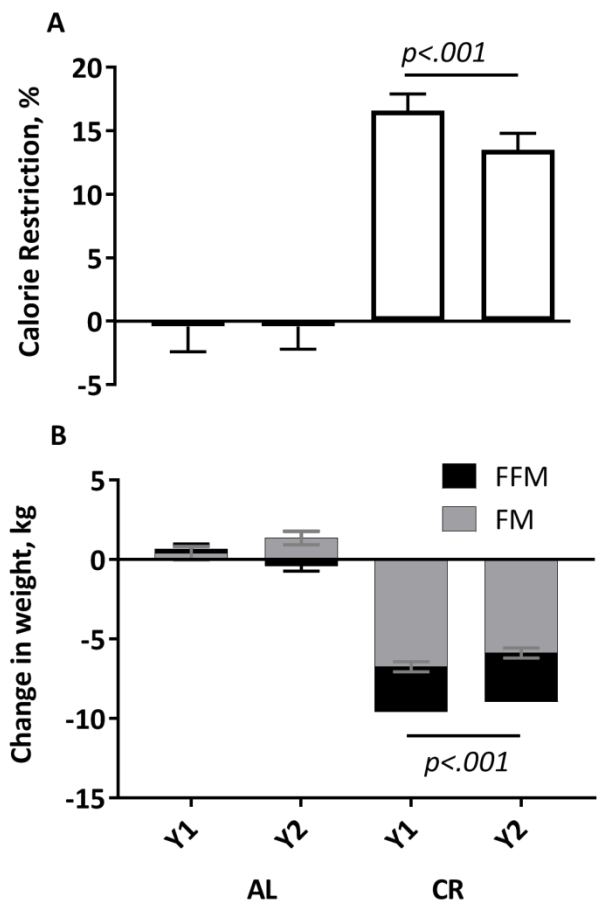
Statistical Analysis: All analyses were carried out using SAS/STAT software, Version 9.4 of the SAS System for Windows© (SAS Institute Inc. Cary, NC, USA) and tests were evaluated using significance level of α=.05. The per-protocol analysis (see "study subjects and throughput in the Results Section) comprised of computing the change from baseline to Y1 and Y2 in all outcomes which were investigated for fixed effects (treatment group, time) and a treatment-by-time interaction using linear mixed models for repeated measures. The models included the baseline outcome value as a covariate. A random subject effect was also included to account for intra-individual correlations over time. Two-sample t-tests derived from least squares means (LSM) were used to compare adjusted mean changes between treatment groups (AL vs CR) and to test for group differences in adjusted mean change at Y1 and Y2. This same method was used to model and assess differences in percent change from baseline. Finally, Pearson's correlation analysis was used to assess relationships between %CR and metabolic adaptation, change from baseline in clinical chemistries and for Spearman's correlation analysis was used to examine relationships between %CR, metabolic adaptation and isoprostane concentrations which were non-normally distributed.
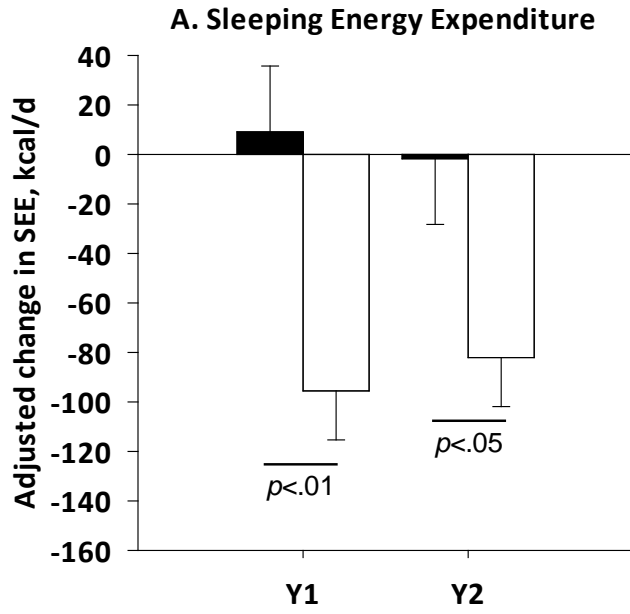
TABLE 1. Physical characteristics of 53 men and women during weight maintenance at baseline and following 1 and 2 years of calorie restriction.

	Ad Lib (Control, n=19)			CR (n = 34)		
	Baseline	ΔY1	ΔY2	Baseline	ΔY1	ΔY2
Age, *y*	39.0±5.4	-	-	40.2±6.7	-	-
Weight, *kg*	71.0±8.3	0.6±0.6	1.8±0.6	71.9±8.7	-9.4±0.4*	-8.7±0.4*
BMI, *kg·m²*	25.5±1.6	0.2±0.2	0.5±0.2	25.7±1.5	-3.4±0.2*	-3.2±0.2*
Body Fat, *%*	32.9±5.5	0.2±0.4	1.4±0.4*	34.2±6.6	-5.6±0.3*	-4.5±0.3*
Fat mass, *kg*	23.4±4.0	0.4±0.4	1.4±0.4*	24.7±5.0	-6.7±0.3*	-5.9±0.3*
Fat-free mass, *kg*	48.3±8.1	0.3±0.3	-0.1±0.3	47.8±8.7	-2.9±0.2*	-3.1±0.2*
Energy Requirement, *kcal/d*	1782±242	-	-	1747±248	-	-
24hEE, *kcal/d*	1893±250	-90±28*	-81±28*	1834±244	-209±21*	-186±21*
SleepEE, *kcal/d*	1523±219	12±25	-6±25	1530±197	-170±18*	-160±18*
T3, *ng/dL*	121.4±20.1	-9.1±3.6*	-13.3±3.6*	115.9±24.4	-23.5±2.7*	-29.9±2.7*
T4, ug/dL	7.6±0.9	0.01±0.22	0.14±0.22	7.1±1.5	-0.29±0.17	-0.73±0.17*
TSH, *μIU/mL*	1.63±1.25	-0.04±0.11	-0.26±0.11*	1.31±0.63	-0.15±0.09	-0.16±0.08
Leptin, *ng/dL*	183.6±146.0	-0.52±1.6	-0.63±1.6	193.9±171.5	-11.4±1.2*	-9.3±1.2*
Insulin, *μIU/mL*	6.5	0.7±0.5	0.5±0.5	5.1±2.4	-1.5±0.4*	0.2±0.4
2,3-dinor-iPF(2α)-III, *ng/mgCr*	2.17±1.03	-0.09±0.17	-0.07±0.17	2.16±0.87	-0.42±0.12*	-0.49±0.12*

Baseline data is presented as mean ± SD. Change from baseline data is the adjusted LS mean ± SE from the general linear models which include the baseline value as a covariate. * denotes significant within group change from baseline (*p*<.05). *Abbreviations*: Ad lib, *ad libitum* control group; CR, calorie restriction group; BMI, body mass index; kcal, kilocalories; d, day; 24hEE, 24-hour energy expenditure; SleepEE, energy expenditure during sleep (0200h-0500h); T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

Enrollment	**435 Individuals Screened**	

355 Excluded
- Met Exclusion Criteria (136)
- Personal Commitments (86)
- Changed Mind (104)
- Study Compliance Concerns (5)
- Unknown (20)
- Other (17)

80 Eligible at Pennington Biomedical

7 Did Not Enroll

Enrolled

73 Consented

Caloric Restriction (CR) Group
N = 47

***Ad Libitum* (AL) Control Group**
N = 26

- *No Baseline Chamber Data (n=2)*
- *Incomplete Data at Y1/Y2 (n=2)*
- *Dropped (n=6):*
  - *Did not initiate intervention, n=1*
  - *Adverse event, n=2*
  - *Withdrew consent, n=3*

- *Incomplete Data at Y1/Y2 (n=1)*
- *Dropped (n=1):*
  - *Adverse event, n=1*

Completed

37 CR Subjects

23 AL Subjects

Adherent Sub-Group

Excluded from Analyses (n=3)

Excluded from Analyses (n=4)

34 CR Subjects

19 AL Subjects

A

Calorie Restriction, %

B

Change in weight, kg

- FFM
- FM

Y1 Y2 Y1 Y2
AL   CR

p<.001

A. Sleeping Energy Expenditure

B. 24-hour Energy Expenditure

A.

Change in Leptin, ng/mL

Ad Lib
CR

p<.001

p<.001

Y1   Y2

B.

Change in T4, ug/dL

Ad Lib
CR

p<.01

Y1   Y2

C.

Metabolic Adaptation Sleep EE, kcal.d

r=0.35, *p*=0.01

Percent Change in Leptin

D.

Metabolic Adaptation Sleep EE, kcal.d

r=0.33, *p*=0.01

Percent Change in T4