



Published in final edited form as:

*Ageing Res Rev.* 2017 October ; 39: 36–45. doi:10.1016/j.arr.2016.08.005.

## Calorie restriction in humans: an update

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### Abstract

Calorie restriction (CR), a nutritional intervention of reduced energy intake but with adequate nutrition, has been shown to extend healthspan and lifespan in rodent and primate models. Accumulating data from observational and randomized clinical trials indicate that CR in humans results in some of the same metabolic and molecular adaptations that have been shown to improve health and retard the accumulation of molecular damage in animal models of longevity. In particular, moderate CR in humans ameliorates multiple metabolic and hormonal factors that are implicated in the pathogenesis of type 2 diabetes, cardiovascular diseases, and cancer, the leading causes of morbidity, disability and mortality. In this paper, we will discuss the effects of CR in non-obese humans on these physiological parameters. Special emphasis is committed to recent clinical intervention trials that have investigated the feasibility and effects of CR in young and middle-aged men and women on parameters of energy metabolism and metabolic risk factors of age-associated disease in great detail. Additionally, data from individuals who are either naturally exposed to CR or those who are self-practicing this dietary intervention allows us to speculate on longer-term effects of more severe CR in humans.

### Keywords

Calorie restriction; Aging; Energy metabolism; Metabolic adaptation; CALERIE; Age-associated diseases

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### Declaration of interest

No conflict of interest has to be disclosed.

## Introduction

Calorie restriction (CR) without malnutrition is the most studied and robust non-genetic non-pharmacological experimental intervention for extending healthspan and lifespan in multiple animal models. In budding yeast, fruit flies and worms, CR can extend lifespan dramatically (2–3 fold). A 20 to 50% reduction in caloric intake, without malnutrition, in some strains of rats and mice prolongs median and maximal lifespan up to 50%, and prevents or delays the onset of many chronic diseases, such as obesity, type 2 diabetes, cancer, nephropathy, cardiomyopathy, neurodegeneration and multiple autoimmune diseases (Heilbronn and Ravussin, 2003). The effects and mechanisms through which CR modulates health and lifespan in simple model organisms and rodents are discussed in more detail in two separate review articles of this special issue of Ageing Research Review (ref #1, ref #2).

During the past two decades, the biology of aging scientific community has kept a close watch on studies of prolonged CR in non-human primates. As discussed in more detail by De Cabo et al. (ref#) in this issue, accumulating data from two long-term ongoing primate studies clearly indicate that 30% CR drastically reduces the incidence of glucose intolerance/type 2 diabetes, cardiovascular disease and cancer (Colman et al., 2009; Colman et al., 2014; Mattison et al., 2012). Moreover, in the Wisconsin National Primate Research Center (WNPRC) study, CR has been shown to slow down age-related sarcopenia, hearing loss and brain atrophy in several subcortical regions (Colman et al., 2009; Colman et al., 2014). For the time being, data on longevity are still discordant, but differences in study design, husbandry and diet composition likely contribute to the controversial findings between the primate colonies studies (Cava and Fontana, 2013).

While studies to elucidate the CR-mediated effects on disease prevention and life extension continue in experimental animals, evidence for the benefit of CR on metabolic and molecular adaptations in humans is also growing. In this review, we discuss findings from the NIH funded CALERIE randomized clinical trials and ongoing longitudinal studies on the effects to date of CR on human health. We also consider the importance of diet quality, and meal frequency and timing in mediating some of the beneficial effects of CR. Finally, we highlight the importance of human studies in the context of aging research and its potential to advance our understanding of interventions that could prevent the accumulation of molecular damage leading to multiple chronic diseases in humans.

## INSIGHTS FROM POPULATION STUDIES WITH CALORIE RESTRICTION IN HUMANS

### Calorie restriction without malnutrition in World War I and II

Involuntary episodes of CR are not uncommon in human history, but only few of these events were not accompanied by malnutrition, because the local governments wisely enforced food restriction with an adequate consumption of essential nutrient-dense foods. During World War 1 in 1917, Danish men and women were forced to reduce food consumption for 2 years, but with a well-planned and adequate consumption of whole grain cereals, vegetables, and milk. The result of this undesired experiment was an impressive

34% reduction in death rates (Hindhede, 1920). Similarly, in Norway during World War 2, the citizens of Oslo underwent a forced 20% CR without malnutrition (i.e. Norwegians were provided with adequate intake of fresh vegetables, potatoes, fish and whole cereals) for approximately 4 years (1941–45). In this forced experiment, mortality dropped by 30% compared to the pre-war level in both men and women (Strom and Jensen, 1951).

### **The Centenarians of Okinawa**

Another natural CR experiment took place in Okinawa, a beautiful island located 640 kilometers south of mainland Japan. On this island, an estimated 50 in every 100,000 people were 100 years of age or older; this is approximately 4–5 times higher than the number of centenarians residing in any other industrialized country (Japan Ministry of Health, 2005). All-cause mortality at age 60–64 for people living on Okinawa was half of that of other Japanese for the year 1995 (Willcox et al., 2006), and the mortality from ischemic heart disease and cancer (i.e. prostate, colon, breast, and lymphoma) was markedly lower than in the average mainland Japanese and US population (Kagawa, 1978). In the same year, both the average and maximum lifespan of people living on Okinawa (average 83.8 years, maximum: 104.9 years) was higher compared to Japanese living on mainland Japan (82.3 years, 101.1 years), and Americans residing in the USA (78.9 years, 101.3 years) (Willcox et al., 2007a).

Studies investigating the dietary intake of adults living on Okinawa suggest that Okinawans' consumed approximately 17% fewer calories than the average adult in Japan (Suzuki et al., 2001), and 40% less than the average adult in the United States (Bureau of Agricultural Economics, 1950). Importantly, the lower energy intake was not (exclusively) explained by overeating (energy intake exceeding energy requirements) in the latter populations. For Okinawans, a 10–15% deficit in energy intake was estimated according to the Harris-Benedict-equation of the energy requirements (Frankenfield et al., 1998; Willcox et al., 2007b). The Okinawan diet is also reported to be lower in protein (9% of calories) and rich in fresh vegetables, fruits, sweet potatoes, soy and fish (Willcox et al., 2006). Sadly, because of an increase in fast food chains brought by US soldiers in the 1960's, dietary habits of Okinawans' have become more westernized and as a result, body mass index has increased and so has mortality (Kagawa, 1978). In 2010, life expectancy for newborns on Okinawa is no longer different than in mainland Japan (girls, 87.0 vs. 86.4; boys, 79.4 vs. 79.5), whereas life expectancy for Okinawans, aged 65 and older, was still higher (women, 89.9 vs. 88.9; men, 84.5 vs. 83.8) (System of Social and Demographic Statistics, 2016).

## **INSIGHTS FROM RANDOMIZED CONTROLLED TRIALS OF CALORIE RESTRICTION**

### **Short duration CR clinical trials**

In three independent studies of short-term CR in non-obese humans, energy expenditure assessed as resting metabolic rate was reduced by  $-100 \pm 27$  kcal/day after 3 weeks of 20% CR (Heyman et al., 1992), by 12% after 6 weeks of 1000 kcal/d CR (Webb and Abrams, 1983) and by  $-255 \pm 151$  kcal/day after 10 weeks of 20% CR (Velthuis-te Wierik et al., 1995c). Changes in total daily energy expenditure assessed by doubly labeled water after 3

weeks ( $-296 \pm 170$  kcal/day) also were decreased, however did not reach statistical significance (Velthuis-te Wierik et al., 1995c). No change in sedentary energy expenditure with a 25% reduction in food intake has also been reported (Garby et al., 1988), however this may be due to poor compliance, since subjects were only asked to reduce food intake by 25% without any oversight or scientific control. Interestingly, after 20% CR, the resting metabolic rate per kg of FFM was reduced (Velthuis-te Wierik et al., 1995c), indicating that the energy expended per FFM is not fixed, and thus indicating metabolic adaptation ( $-7.4$  kg body mass,  $-1.3$  kg FFM). In this study, the reduction in energy expenditure after 10 weeks of 20% CR did not lead to a concomitant reduction of indicators of oxidative stress or insulin levels (Loft et al., 1995; Velthuis-te Wierik et al., 1995b). In the 10-week CR study of Velthuis *et al.* (Velthuis-te Wierik et al., 1994), blood pressure was significantly reduced (124 to 118 mmHg and 79 to 74 mmHg, for systolic and diastolic blood pressure, respectively). Impaired fibrinolytic activity has been shown to be predictive for developing CVD (Anand et al., 2003; Tofler et al., 2016) and the 10 week CR intervention reversed markers of these impairments and induced fibrinolytic activity, particularly in subjects that were more impaired at baseline (Loft et al., 1995; Velthuis-te Wierik et al., 1995a). Also in the study of Velthuis and Loft *et al.* (Loft et al., 1995), glucose concentrations were reduced significantly from 4.8 to 4.6 mmol/L in the CR-group (age, 42.9 years; BMI, 24.6 kg/m<sup>2</sup>). Of note, the subjects in this study, were likely still in active weight loss and had not reached new energy balance or weight maintenance on the CR diet. Thus, longer term studies of CR in humans were designed to elucidate the metabolic effect of prolonged CR in non-obese humans.

### **CALERIE randomized clinical trials**

The CALERIE (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) trials were initiated by the US National Institute of Aging to provide the first controlled clinical trials of CR with adequate nutrient provision in healthy, non-obese humans. In CALERIE-1, three pilot studies were performed to evaluate the feasibility and effects of CR on metabolic health after 6 months (Das et al., 2007a; Heilbronn et al., 2006) or 12 months (Racette et al., 2006a). In the pilot studies, CR was studied by achieving a negative energy balance through different modalities 1) reduced calorie intake (CR), 2) increased exercise energy expenditure, or 3) CR and exercise in combination. The volunteers randomized in the CALERIE-1 trials were mostly overweight and their body mass index dropped to the upper limit of normality at the end of the study. The results of these studies were collated and thereafter a phase 2 multi-center trial was conducted to investigate the efficacy and safety of 2 years 25% CR in leaner and younger men and women. The CALERIE 2 study enrolled 220 healthy, young and middle-aged (21–51 years old), healthy non-obese (body mass index between 22 to 27.8 kg/m<sup>2</sup>) men and women and tested the effectiveness of the CR intervention on energy metabolism, metabolic adaptations, immune function, chronic disease risk factors and quality of life (Rickman et al., 2011; Rochon et al., 2011).

### **CALERIE-1 at the Pennington Center in Baton Rouge**

At Pennington Biomedical Research Center (PBRC), a reduction of energy intake alone (25% CR) was compared to combined reduction in energy intake (12.5%) and a 12.5%

increase in energy expenditure through exercise (-12.5% energy intake +12.5% energy expenditure = 25% CR), a positive weight loss control group that through a very low-calorie diet achieved a 15 kg weight loss and a weight-maintenance control group (Heilbronn et al., 2006; Redman et al., 2007). In the 6 month study the level of CR was estimated (by doubly labeled water) to be 18% (Redman et al., 2007). The CALERIE phase 1 trial conducted at Pennington Biomedical in Louisiana arguably provides the most compelling evidence for CR-induced metabolic adaptation. Energy expenditure was assessed by various independent gold-standard measurement techniques: doubly-labeled water for 14 days assessing daily free-living energy expenditure (Redman et al., 2009), metabolic chamber for 23 hours assessing sedentary and sleeping energy expenditure (Heilbronn et al., 2006), and ventilated hood system for assessing resting metabolic rate (Martin et al., 2007b). In this 6-month study, all components of energy expenditure, that is, sleeping (Heilbronn et al., 2006), resting (Martin et al., 2007b), 24h sedentary (Heilbronn et al., 2006) and free-living (Redman et al., 2009) were reduced below baseline levels. The metabolic adaptation in sedentary energy expenditures (sleep/24h) was ~6% based on a smaller energy expenditure than predicted on the basis of the metabolic size of the individuals at the end of the study (Heilbronn et al., 2006). The 24h sedentary energy expenditure was also significantly lower than 865 individuals matched for age and BMI studied in the NIDDK Chambers in Phoenix (Heilbronn et al., 2006; Weyer et al., 1999). Interestingly it does not appear that the metabolic cost of activity is affected by CR, as the relation between energy expenditure to spontaneous physical activity (simultaneously assessed in metabolic chamber, spontaneous physical activity as % activity by radar motion detector) was unchanged (Martin et al., 2007b). Since the metabolic cost of spontaneous activity was unchanged and the energy expenditures of free-living activity were reduced, the findings from this study suggest that individuals undergoing CR also make behavioral adjustments (conscious or unconscious) to decrease physical activity during CR (Redman et al., 2009).

In this pilot study, core body temperature was assessed over 24 hours on a metabolic ward and was reduced by 0.2° C after 6 months of 25% CR (Heilbronn et al., 2006). Also, fasting insulin concentrations declined after CR in CALERIE 1 and more sophisticated assessments of glucose homeostasis suggest a more pronounced benefit of CR on carbohydrate metabolism and potential risk for development of T2DM. Insulin sensitivity, assessed by an intravenous glucose tolerance test (with delayed insulin-infusion) was improved by 40% (p=0.08) (Larson-Meyer et al., 2006). Moreover, the acute insulin-response to glucose-infusion was increased by 29% which suggests an improvement in  $\beta$ -cell function (Larson-Meyer et al., 2006). Similarly, CR (-1000 kcal/d) increased insulin sensitivity (glucose infusion rate during hyperinsulinemic euglycemic clamp) by 46% (p<0.0001) after 16 weeks in older, obese subjects (55±2 yrs, 35.2±1.3kg/m<sup>2</sup>) (Johnson et al., 2016).

T2DM and insulin resistance are related to increased adipocyte size and ectopic fat deposition including the liver and skeletal muscle (Fox et al., 2007; Perry et al., 2014; Weyer et al., 2000). In line with the improvements in glucose homeostasis, 25% CR in CALERIE 1 resulted in a significant reduction in subcutaneous adipocyte size and deposition of lipid in the liver (intrahepatic lipid) but not in skeletal muscle (Larson-Meyer et al., 2006; Redman et al., 2007).

Surprisingly, in this CALERIE I trial, various factors that are associated with CVD (blood pressure, LDL, HDL, fibrinogen, homocysteine, CRP, TNF $\alpha$ , and Factor VIIc) were not affected by CR (Lefevre et al., 2009; Tam et al., 2012). Also, flow-mediated dilatation, a measure of endothelial function, was not improved and factors that have been associated with CR and aging such as IGF-1, DHEA-s, GH secretion (both GH pulse amplitude and pulse mass) were also unchanged in overweight subjects studied in CALERIE 1 (Heilbronn et al., 2006; Lefevre et al., 2009; Redman et al., 2010). This could be related to the young age of the study participants and their impeccable health status even before beginning the CR intervention (age, 39.0 years; BMI, 27.8 kg/m<sup>2</sup>, fasting glucose, 4.9 mmol/l). Nevertheless, based on values for total and HDL cholesterol (expressed as their ratio), systolic blood pressure, age and gender (Anderson et al., 1991), it was estimated that the 25% CR diet for 6 months induced a 29% reduction in the ten-year risk for CVD (Lefevre et al., 2009).

For the first time in humans undergoing CR, a decline in markers of oxidative stress was observed (DNA-damage and SOD-activity, indicating oxidative stress defense activity) (Civitarese et al., 2007; Heilbronn et al., 2006). The reduction in DNA damage to red blood cells was however not correlated to any measure of energy expenditure (Heilbronn et al., 2006). In line with previous results of the short-term CR-intervention studies discussed earlier (Velthuis-te Wierik et al., 1995b; Walford et al., 2002), thyroid hormone (T3 and T4) concentrations were reduced in CALERIE 1 after 6 months (Heilbronn et al., 2006). More important than the absolute changes in thyroid hormones which might be solely related to the energy deficit/weight loss, is that changes in thyroid hormone and leptin concentrations were significantly related to metabolic adaptation (Heilbronn et al., 2006; Lecoultre et al., 2011). Since the major contributor of metabolic rate is fat-free mass, molecular studies in the skeletal muscle of CR people may provide insight into the mechanisms of CR-induced metabolic adaptation or metabolic efficiency. In skeletal muscle biopsies collected from the CR participants, mitochondrial DNA content increased by 35%, suggesting an increase in mitochondrial mass. In line with this observation, expression of genes encoding proteins involved in mitochondrial function such as PGC1 $\alpha$ , TFAM, eNOS, SIRT1, and PARL were also increased by CR (Civitarese et al., 2007). An increased number of mitochondria may enhance coupling due to a reduced mitochondrial membrane potential and contribute to reduce energy expenditure and oxidative stress. However, the activity of enzymes of the tricarboxylic acid cycle (citrate synthase),  $\beta$ -oxidation ( $\beta$ -hydroxyacyl-CoA dehydrogenase), and ETC (cytochrome C oxidase II) was unchanged. This lack of effect on mitochondrial enzyme activities questions whether skeletal muscle mitochondrial dynamics could affect whole-body energy expenditure.

### **CALERIE-1 at Washington University in St. Louis**

At Washington University, 48 overweight (BMI, 23.5–29.9 kg/m<sup>2</sup>) individuals, aged 50–60 years, were randomized for 1-year to 20% CR, or 20% increase in energy expenditure by means of endurance exercise or to a control group of healthy lifestyle (Racette et al., 2006a). At the end of the study, the CR group achieved only a 11.5 $\pm$ 2.1% reduction in calorie intake (measured with doubly-labeled water), BMI dropped from 27.3 $\pm$ 0.3 kg/m<sup>2</sup> to 24.4 $\pm$ 0.6 kg/m<sup>2</sup>, and visceral fat mass measured by MRI was reduced by 37% (Racette et al., 2006b).

As in experimental animals, CR-induced weight loss improved insulin sensitivity, increased adiponectin and reduced the serum concentrations of leptin, insulin, LDL-cholesterol, and C-reactive protein (Fontana et al., 2007b; Villareal et al., 2006; Weiss et al., 2006). Plasma triiodothyronine levels were decreased in the CR, but not in the exercise groups (Weiss et al., 2008). In this study, both CR and exercise induced weight loss resulted in a significant reduction in oxidative damage to DNA and RNA measured ex-vivo in white blood cells, and in improvements of left ventricular diastolic function (Hofer et al., 2008; Riordan et al., 2008). CR decreased bone mass, muscle size and strength, and maximal aerobic capacity in proportion to the reduction in body weight (Villareal et al., 2006; Weiss et al., 2007). In this pilot trial, unlike in CR rodents, serum concentration of IGF-1, estradiol, testosterone and cortisol did not change with CR (Fontana et al., 2008; Villareal et al., 2006; Weiss et al., 2006).

### **CALERIE-1 at Tufts University in Boston**

At Tufts University, 46 young (24–42 years old) overweight (BMI, 25–29.9 kg/m<sup>2</sup>) individuals were randomized to low *versus* high glycemic load during 30% CR (Das et al., 2007a). At 6 months, the individuals randomized to 30% CR experienced a significant reduction in body weight, and improvements in fasting insulin concentration, insulin sensitivity, first-phase acute insulin secretion, and lipid profile independent of the glycemic load of the diets (Das et al., 2007b; Pittas et al., 2006). Serum concentrations of CRP were reduced in the 30% low-glycemic CR group, but not in the 30% high-glycemic CR group (Pittas et al., 2006). Moreover, 30% CR significantly improved T-cell function (i.e. delayed-type hypersensitivity response and proliferative response of T cells to T-cell mitogens), and prostaglandin E<sub>2</sub> production (Ahmed et al., 2009).

### **CALERIE-2 multicenter trial**

The main goal of CALERIE-2 was to test the hypothesis that 2-years of sustained CR, involving a reduction in energy intake to 75% of baseline (25% CR) will result in the same adaptive changes that occur in rodents subjected to CR (Ravussin et al., 2015). One reason for 2-years of CR intervention is that the 6-mo and 1-yr in CALERIE-1 pilot trials were not sufficient to induce many of the metabolic and hormonal adaptations in humans that are thought to increase longevity in rodents. Overall, the intervention was safe and well-tolerated; only two participants were withdrawn from the study because of treatment-resistant anemia and one because of excessive bone mass loss ( 5% compared to baseline) (Romashkan et al., 2016). Unfortunately, in this large trial (CR: n=143, *ad libitum*: n=75), which required study subjects to self-select their own diets, compliance was not as good as expected, and average CR during the first 6-months was 19.5±0.8% and 9.1±0.7% over the next 18 months of the study.

In this study, food intake was monitored by self-reporting and estimated by the intake/balance method, which is described in Racette *et al.* (Racette et al., 2012). As imposed by the study design, the reduction in weight and body composition was achieved at 12 months; body weight at month 12 was reduced by 11.5% and was maintained throughout the study (9.9% at month 24 *vs.* baseline), FFM by 4.3% (4.2%) and FM by 23% (26%) (Ravussin et al., 2015; Villareal et al., 2016). Metabolic adaptation in resting metabolic rate was evident

in the CR group at both 12 months and 24 months. However, it was only significantly different from the group adhering to the *ad libitum*-diet after the first period of 12 months (Ravussin et al., 2015). A declining level CR throughout the study might explain this lack of significance at the 24 month-assessment. The measurement of indirect calorimetry in the metabolic chamber is considered the gold-standard because all components of daily energy expenditure are assessed and the individual can properly equilibrate to sedentary activities over the course of 24 hour measurements. While we await official publication from an ancillary study of CALERIE-2 where additional measurements of 24h energy expenditure were performed at one center ([ClinicalTrials.gov Identifier: NCT02695511](https://clinicaltrials.gov/ct2/show/study/NCT02695511)) and in a subset of people, the first reported results indicate confirmation of metabolic slowing with a CR diet (Redman et al., 2014). In the ancillary, both 24h and sleeping energy expenditure were reduced to a larger extent than predicted based on their body composition, indicating metabolic adaptation which was significant after both one and two years of intervention in the CR group. Additional insight on the effect of CR on skeletal muscle metabolism will be provided by skeletal muscle biopsies that have been collected in CALERIE-2, yet these data have not been published. More advanced measurement techniques of mitochondrial respiration and oxidative stress by *ex vivo* respirometry may provide a clearer picture of mitochondrial adaptations to CR. Also, the involvement of other tissues in the reduced energy expenditure observed with CR remains to be investigated since skeletal muscle only accounts for ~20% of resting energy expenditure and metabolic adaptations are likely to occur in other tissues as well (Heymsfield et al., 2007; Muller et al., 2015).

The results of this large trial are important because they demonstrate that mild CR improves cardiometabolic risk factors, well below the conventional thresholds used in the clinical practice, even when implemented in healthy lean or overweight young and middle-aged men and women. Total cholesterol, LDL-cholesterol, triglycerides, C-reactive protein, TNF $\alpha$  and blood pressure decreased significantly and HDL-cholesterol increased in CR group, even in people who had normal risk factor at baseline (Ravussin et al., 2015). HOMA-IR, which is a measure of insulin resistance, was also reduced from baseline to 24 months (1.2 to 0.9), (Ravussin et al., 2015). Importantly, the baseline values of the participants in all studies are already within the normative ranges (<6.1 mmol/L for glucose and <2.2 for HOMA-IR), thus it remains arguable whether the observed reductions of these fasting parameters are physiologically relevant with respect to the development of T2DM.

Finally, in CALERIE-2 CR caused a significant increase in serum IGFBP-1 and a reduction in leptin, and T3, but unlike in rodents did not change IGF-1 and IGF-1:IGFBP-3 ratio or cortisol levels (Fontana et al., 2016). Moreover, in this study CR like the CALERIE 1 trials had no effect on core body temperature, and serum concentrations of DHEA-s, PDGF-AB and TGF $\beta$ -1 (Fontana et al., 2016). Most likely, the lack of the typical CR-induced adaptations on several important hormonal and physiological factors is due to insufficient reduction in calorie intake achieved in the CALERIE trials. Thus, the findings of these studies may be more relevant to the effects of weight loss than of chronic severe CR.



## INSIGHTS FROM OBSERVATIONAL STUDIES OF SEVERE CALORIE RESTRICTION

### Biosphere 2 Study

Biosphere 2 is a 3.15-acre ecological enclosure that was designed as a laboratory for ecological investigations (e.g., biogeochemical cycles, food web systems, and the population genetics of speciation), and unintentionally provided an opportunity to study the effects of severe CR in non-obese metabolically healthy human beings (Walford et al., 1992). A very small group of 8 non-obese individuals between the ages of 25 and 67 entered Biosphere 2 for 24 months. Due to unforeseen agricultural problems with growing foods, the crew underwent a forced ~29% CR for 18 months. The diet composition was diverse, largely vegetarian, very low in fat (~10% energy from fat) and provided adequate protein and high fiber. The CR diet coupled with high levels of physical activity (70–80 hours of high intensity work per week) resulted in a ~15% weight loss (BMI decreased from 23.9 to 19.7 kg/m<sup>2</sup>). The 8 members of this crew experienced several of the same adaptations previously reported in CR rodents, including marked reductions in levels of insulin, cholesterol, triglycerides and white blood cell count, and an increase in cortisol concentration (Walford et al., 1992; Walford et al., 2002). Furthermore, glucose concentrations (5.1 to 4.1 mmol/L), HDL (62 to 38 mg/dl), systolic (109 to 89 mmHg) and diastolic blood pressures (74 to 58 mmHg) were significantly decreased below values that are considered healthy.

However, CR in these individuals did not result in reductions of serum concentration of IGF-1, testosterone and DHEA-s (Walford et al., 2002). Despite a significant reduction in serum T3 concentrations, this degree of CR produced only a subtle effect on core body temperature, but this may have been masked by technical limitations because the thermostats were not calibrated for temperatures <96 °F (Walford et al., 1999). Interestingly, 24-hr energy expenditure in the Biosphere inhabitants assessed one week and 6 months after exiting the Biosphere enclosure was lower when compared to non-CR subjects who were free-living and with appropriate statistical adjustment for age, sex, fat-free mass (FFM) and fat mass (FM) (Weyer et al., 1999). It should be noted that several years after the first publication, it was discovered that the 8 biospherians most likely endured chronic hypoxia (i.e. oxygen in the internal atmosphere recorded at only ~14%), which may have influenced the observed results and conclusions related to the potential role of the CR diet on some metabolic outcomes (Paglia and Walford, 2005). For example, chronic environmental (hypobaric) hypoxia has been reported to lead to weight loss and reduced appetite in obese men (Lippl et al., 2010). Furthermore, chronic hypoxia is also known to induce favorable effects on insulin sensitivity (Jain et al., 2016; Lecoultre et al., 2013).

### CRON Study

Currently, the only direct evidence that CR may influence the biology of aging in humans comes from data collected in members of the Calorie Restriction Society, who follow a regimen of self-imposed severe CR with optimal nutrition (CRON) in the belief that this dietary lifestyle will prolong their healthy lifespan. These individuals are very lean (BMI 19.7±1.8 kg/m<sup>2</sup>) men and women, who had been voluntary restricting their caloric intake (~1800 kcal/d) for an average of 15 years, and consume approximately 30% less energy as

compared to a group of individuals (matched for age, sex and socioeconomic status) consuming a regular Western diet. Importantly, their diet meets all dietary recommendations for essential nutrients, and is very high in vegetable fiber and low glycemic foods packed with a wide variety of phytochemicals, which may modulate metabolic health independently of caloric intake (Fontana et al., 2004).

While data on longevity and mortality are not yet available, data indicate that moderate/severe CR in humans results in the same metabolic and molecular adaptations typical of long-lived CR animals (Fig.1). Moreover, CR with optimal intake of nutrients in these individuals reduces metabolic and hormonal risk factors implicated in the pathogenesis of type 2 diabetes, cardiovascular disease, stroke, cancer and vascular dementia. All cardiometabolic risk factors in the members of the CR Society are remarkably low (Fontana et al., 2004). The cholesterol-HDL ratio was 2.6 with triglycerides in the 50 mg/dl range. Systolic and diastolic blood pressure, even in people in their late 70s, was 110/70 mmHg, and C-reactive protein almost undetectable (Fontana et al., 2004; Meyer et al., 2006). Serum TNF $\alpha$ , IL6, fasting glucose and insulin were also remarkably low, and insulin sensitivity based on HOMA-IR and the Matsuda and DeFronzo indexes were improved (Fontana et al., 2010). Historical data from their medical records prior to starting CR strongly supports a cause-effect diet-induced beneficial adaptation on cardiovascular risk (Holloszy and Fontana, 2007). As a consequence of these cardiometabolic improvements, the intima-media thickness of the common carotid arteries was significantly lower in these CR individuals than in the age-matched controls eating usual Western diets (Fontana et al., 2004). Additionally, the CRONies had significantly better echocardiographic markers of left ventricular diastolic function (i.e. lower chamber stiffness and augmented viscoelasticity), and improved autonomic function with a reduction in sympathetic and increased in parasympathetic modulation of heart rate variability (Fontana et al., 2007a; Meyer et al., 2006; Stein et al., 2012). Heart rate variability in the CR practitioners was comparable with published norms for healthy men and women 20 years younger (Stein et al., 2012).

Unlike in the CALERIE trials, the great majority of the hormonal adaptations that have been reported in long-lived CR rodents, and are also implicated in the pathogenesis of several common cancers (Longo and Fontana, 2010), occurred in these individuals practicing severe CR. Plasma triiodothyronine concentration, and as a consequence 24-hr core body temperature, were significantly lower in the CR society members (Fontana et al., 2006). Total and free testosterone and estradiol were also lower, and serum SHBG, adiponectin and cortisol concentration were higher in the CR group than in the age- and sex- matched control group. Consistent with the study of Biosphere 2 inhabitants, long-term CR in the CRONies did not affect DHEAS concentrations (Cangemi et al., 2010). However, unlike in rodents in which CR decreases circulating IGF-1 levels by ~20–40%, even in these individuals severe CR did not decrease total IGF-1 or IGF-1/IGFBP-3 ratio levels, unless protein intake was also reduced (Fontana et al., 2008; Sonntag et al., 1999).

At a molecular levels metabolic pathways that modulate the accumulation of molecular damage such as the PI3K/AKT and AMPK/SIRT pathway were changed by CR to an extent that resembles younger individuals, which again indicates the anti-aging potential of CR (Mercken et al., 2013). In particular, AKT mRNA expression and protein phosphorylation

were significantly reduced by CR in the skeletal muscle, and transcription factors downstream of AKT, such as FOXO-3A and FOXO-4, were up-regulated. FOXO activation resulted in up-regulation of several anti-aging genes, including the antioxidant enzyme SOD2, the DNA repair transcript DDB1, and the autophagy genes beclin-1 and LC3 (Mercken et al., 2013). Consistent with some of these gene expression changes, beclin-1 and LC3 protein levels were significantly higher in the skeletal muscle of the CR volunteers (Yang et al., 2016). Moreover, key stress-induced cytosolic chaperones transcript and protein levels, such as HSP70 and GRP78, were significantly higher in the CR skeletal muscle (Yang et al., 2016). These data strongly suggest that CR in humans is associated with an increase in key molecular chaperones and autophagic mediators involved in cellular protein quality control and removal of dysfunctional proteins and organelles. To our knowledge, this is the first set of data showing that long-term CR in humans up-regulates the HSF/HSP pathway and down-regulates the activity of the insulin/IGF pathway, which have been shown to play a key roles in promoting health and longevity in several experimental model organisms (Hsu et al., 2003; Kenyon et al., 1993; Yokoyama et al., 2002).

## INSIGHTS FROM STUDIES OF EXTREME CALORIE RESTRICTION

It is well established that energy restriction lacking sufficient protein and micronutrients is associated with, but not limited to, short stature, late reproductive maturation (Eveleth and Tanner, 1990), lower baseline gonadal steroid production in adults (Ellison, 1996a, b), suppressed ovarian function (Ellison et al., 1993), impaired lactation performance (Roberts et al., 1982), impaired fecundity (Lee, 1979), and impaired immune function (Martorell, 1980; Ulijaszek, 1990). However, the health effects of extreme CR without protein and vitamin malnutrition in humans are less understood. Data from patients with anorexia nervosa - a psychiatric disease characterized by disturbed body image, heightened desire to lose more weight, and pervasive fear of fatness – indicate that extreme CR (i.e. weight <75% of the expected weight) may be associated with increased mortality, hypokalemia, hypophosphatemia, symptomatic hypoglycemia, dehydration, orthostatic hypotension, hypothermia (temperature <36.1°C), impaired menstrual and reproductive function, anemia, osteoporotic bone fractures, and cardiac arrhythmias (Fairburn and Harrison, 2003). The effects of extreme CR in metabolically and psychologically healthy individuals have been studied in the Minnesota Semi-Starvation Study.

### The Minnesota Semi-Starvation Experiment

The pioneering ‘Minnesota Starvation Experiment’ by Ancel Keys (Kalm and Semba, 2005; Keys et al., 1950) is the only human study describing the physical and psychological effects of extreme CR in a clinical experiment. Designed to mimic dietary conditions during World War II, 32 lean and young 24 years old conscientious objectors were carefully studied during a 40% reduction in energy intake for 6 months. Participants were allowed to consume 1800 kcal/d, but were expected to walk 5 km/d and expend 3000 kcal/d. At the conclusion of the study, the men had lost ~25% body weight, of which 67% was fat mass (FM) and 17% fat-free mass. To properly reflect the diet during war, the quality of the diet was poor owing to the inadequate intake of protein (<0.8 g/kg body mass/d) as well as insufficient intake of fruits and vegetables. Therefore while this may be considered a study of extreme CR, the

study diet was not indicative of true CR diets, which meet intake recommendations for macro- and micronutrients. The malnourished CR diet led to chronic weakness, reduced aerobic capacity, and severe painful lower limb edema (Keys et al., 1950). Abnormal psychological behaviors which emerged by 6 weeks included severe emotional distress, confusion, apathy, depression, hysteria, hypochondriasis, suicidal thoughts, and loss of sex drive. This study was concluded when these young men reached the 25% target weight loss by 6 months at average body mass index of 16. With refeeding, mood, behavior, and muscle mass very slowly normalized (Kalm and Semba, 2005). Interestingly, 50% of these objectors who survived to age 80 lived at least 8 years longer than expected for men born in 1920 (Kalm and Semba, 2005).

### **Psychological and mental capacity**

In contrast to the Minnesota Semi-Starvation Experiment, well-controlled studies with a lesser degree of CR with adequate nutrition and interventions providing behavioral support to participants have reported no adverse effects of CR on psychological and mental capacity. For example, mood and mental performance were unchanged after 20% CR for 10 weeks (Velthuis-te Wierik et al., 1994) and cognitive performance, memory, attention and concentration were not affected by 6 months of 25% CR in CALERIE-1 (Martin et al., 2007a). Moreover, a 25% CR did not lead to the development of clinical signs or symptoms of eating disorders or binge eating (Williamson et al., 2008). Despite having higher dietary restraint scores the CR subjects also had reduced disinhibition regarding food and fewer concerns about body weight. Recent data from CALERIE-2 consistently showed, that mild CR for 2 years had no negative effects on health-related quality of life, based on assessments of vitality, mental health and bodily pain (SF-36) (Martin et al., 2016). Moreover, measures of mood were unaffected (anger, fatigue, confusion) or even improved (depression, less tension) in the CR-group as compared to the control group. Also, quality of sleep (assessed by PSQI) and sexual function (DISF-SR) were not affected or even improved (sexual drive and relationship). Nonetheless, more severe energy restriction such as in some members of the CR Society may result in reduction of libido, which seems to correlate with the reduction in circulating testosterone levels.

### **Bone mineral density and metabolism**

No untoward effect of CR was observed on bone mass after 6 months of CR, but turnover increased, which may relate to the altered hormonal environment caused by the reduced body mass (Redman et al., 2008). In the longer term CALERIE-2 trial, bone mass significantly decreased at clinically important sites of osteoporotic fractures such as the hip and femoral neck and the lumbar spine (Villareal et al., 2016). Changes in body composition, macronutrient intake, physical activity and plasma hormones (Vitamin A and D, cortisol, IGF-1, adiponectin and leptin) explained 31% of these changes in a multiple regression analysis. These findings clearly represent a potential limitation of the implementation of CR perhaps in some older persons where accelerated bone loss is a cause for concern. Longer term studies should assess trabecular bone architecture (a major determinant of bone strength and a good predictor of the risk of developing fragility fracture) to ensure that bone quality is preserved with CR, and whether the observed reductions in bone mass indeed contribute to increased fracture risk. However, data from

members of the CR Society have shown that trabecular bone microarchitecture of the distal radius (i.e. surface-to-curve ratio and erosion index) assessed by high resolution MRI was not significantly different between the CR and control groups, despite the CR-mediated low bone mineral density (Villareal et al., 2011). Serum CTX-1 and bone specific ALP, two well-accepted markers of bone resorption and formation, were also not significantly different between the two groups, which suggest that long-term CR with adequate micronutrient intake does not persistently increase the rate of bone turnover (Villareal et al., 2011). These results are consistent with the majority of studies published so far indicating that CR reduces bone mineral density in both rodents and monkeys, but improves bone quality and strength through a reduction of bone turnover and a prevention of secondary hyperparathyroidism (Kalu et al., 1984; Tatsumi et al., 2008). More studies are needed to understand the interactions between CR, dietary micronutrient content and resistance exercise in preserving bone health.

### Physical functioning

In short-term CR studies, in which nutritional requirements are met, a reduced physical functioning was indicated by a reduced cycling time until exhaustion and reduced maximal power (Velthuis-te Wierik et al., 1995b). These findings are in line with CALERIE-1, which reported declines in muscle mass, strength and aerobic fitness, which were assessed by MRI of both thighs, concentric isokinetic and isometric knee flexor and knee extensor strength by dynamometer and absolute maximal aerobic capacity ( $VO_{2max}$  and  $VO_{2peak}$ ) by a progressive treadmill test (Larson-Meyer et al., 2010; Weiss et al., 2007). However,  $VO_{2peak}/VO_{2max}$  expressed per kilogram of body mass were maintained or increased during CR, suggesting that the reduction in body weight may preserve or even beneficially affect physical functioning. A similar effect of CR on aerobic fitness has been observed in older and more obese subjects (>60 yrs, BMI 30kg/m<sup>2</sup>) with heart failure with preserved ejection fraction (>50%) (Kitzman et al., 2016). Despite decreased body mass and lean body mass (-7 and -2%), CR by 400 up to 1000 kcal/d significantly increased  $VO_{2peak}$  (expressed per kg body weight or per lean body mass, but not expressed without correction), 6-min walking distance and leg muscle quality (expressed in Watts per cm<sup>2</sup> thigh muscle area). In addition, quality of life improved based on data on increased Short Form 36 Health survey physical component scores, reduced MEADS depression scores, and physical functioning improved after 4, 6 or 24 months of ~25% CR in metabolically compromised (Kitzman et al., 2016) and healthy participants (Ravussin et al., 2015; Williamson et al., 2008).

## BEYOND SEVERE CALORIE RESTRICTION

Data from the CALERIE trials, the Biosphere-2 and CRON studies clearly indicate that moderate CR with adequate nutrition improves human health and drastically reduces multiple metabolic factors implicated in the pathogenesis of the most common chronic diseases typical of Western countries, including type 2 diabetes, heart and cerebrovascular disease, and cancer. Accumulating data suggest that more severe CR may slow, or even revert, the accumulation of molecular damage with age and preserve some key physiological functions (i.e. left ventricular diastolic function and heart rate variability) at a younger stage (Mercken et al., 2013; Meyer et al., 2006; Stein et al., 2012; Yang et al., 2016). However,

severe CR with optimal intake of vitamins and minerals is not feasible for most people because it is impractical and very difficult to sustain. Side effects such as extreme leanness, loss of sex drive, cold sensitivity and impaired menstrual cycles are also important deterrents to severe CR. Moreover, it is currently unknown which is the ideal caloric intake associated with optimal health. As studies in recombinant inbred mouse strains suggest (Harper et al., 2006; Liao et al., 2010), a definite degree of energy restriction that is ideal for some individuals might be excessive and detrimental in others. Interestingly, accumulating data suggest that severe CR possibly is not needed, other interventions such as intermittent fasting, restricted feeding, protein or selective amino acid restriction may recapture some of the beneficial effects of severe CR in modulating certain anti-aging pathways (Fontana and Partridge, 2015). The effects and mechanisms through which intermittent fasting, restricted feeding, protein or selective amino acid restriction modulates health and lifespan in model organisms will be discussed in more detail in two separate review articles of this special issue of Ageing Research Review (ref #3, ref #4).

## CONCLUSION

Data from epidemiological, experimental and clinical studies strongly indicate that maintaining a healthy body weight and preventing the accumulation of abdominal fat is essential for the prevention of multiple chronic diseases and the promotion of healthy aging (Fontana and Hu, 2014). Hundreds of preclinical studies have shown that dietary restriction, by inhibiting key nutrient-sensing and inflammatory pathways, activates multiple molecular pathways that promote proteostasis, genome stability, stress resistance and stem cell function (Fontana and Partridge, 2015). Data collected in non-human primates indicate that CR in combination with diet quality modifications markedly decrease the incidence of cardiovascular disease, cancer and diabetes, and attenuates age-related neurodegeneration, sarcopenia, and auditory loss. Finally, data from human studies show that CR remains the cornerstone in the prevention and treatment of obesity and its complications. Moderate CR achieved through intermittent fasting or restricting feeding in combination with regular physical activity most likely exerts additional beneficial health effects even in non-obese individuals. More studies are warranted to elucidate the role of specific amino acid restriction with and without CR, and the effects of nutritional modulation of gut microbiome in promoting health and longevity in humans.

## Acknowledgments

We apologize for the omission of relevant works due to space constraints. This work was funded in part by the National Institutes of Health in support of the CALERIE studies: U01-AG-020487, U01-AG-020480, U01-AG-022132 R01, AG029914 (Ravussin, E); U01 AG020478 (Ravussin, E). Dr. Fontana's research was also supported by grants from the Bakewell Foundation, the Longer Life Foundation (an RGA/Washington University Partnership), the National Center for Research Resources (UL1 RR024992), and the European Union's Seventh Framework Programme MOPACT (Mobilising the Potential of Active Ageing in Europe; FP7-SSH-2012-1 grant agreement No. 320333).

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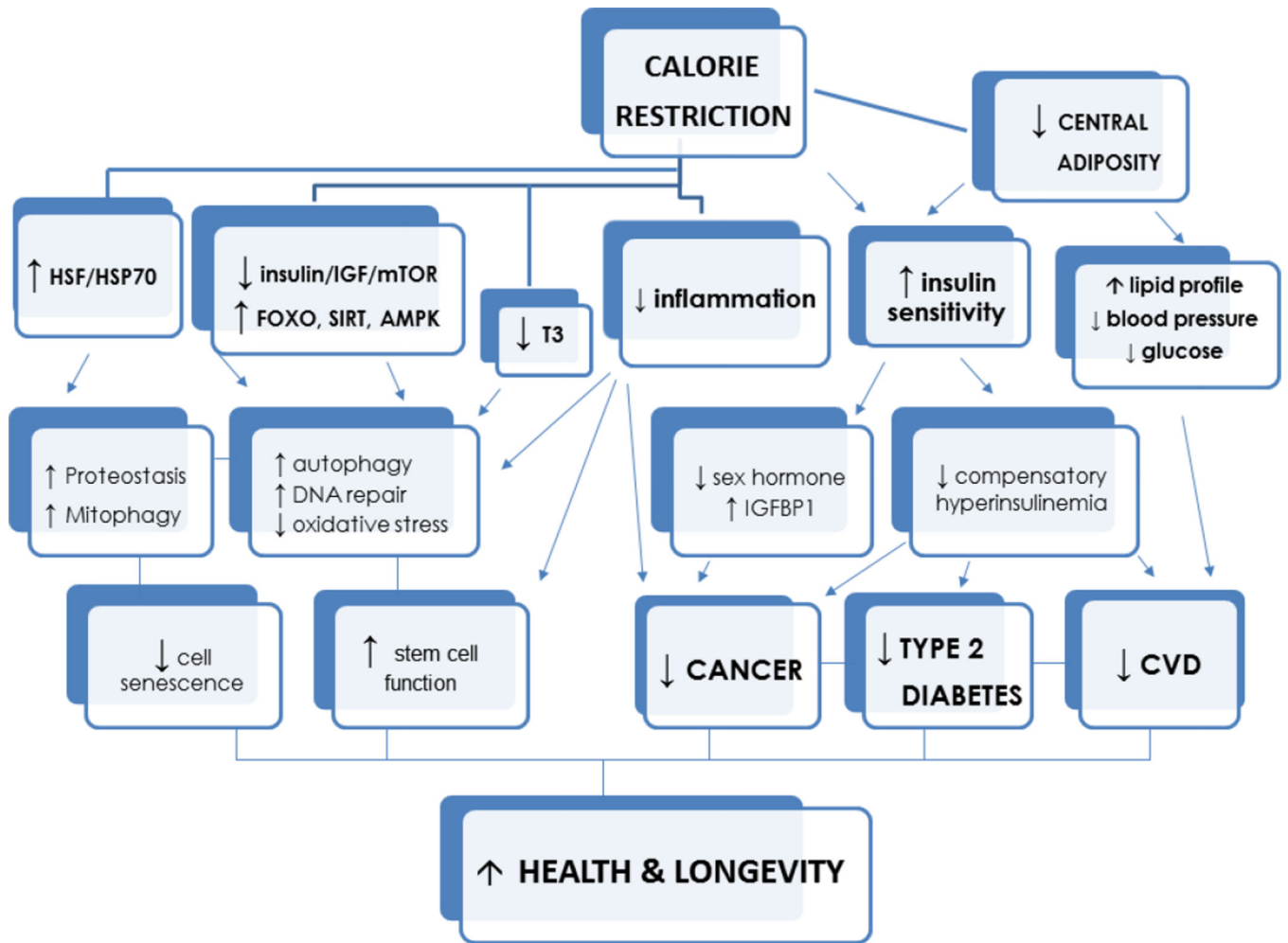
### Highlights

Well-nourished calorie restriction promotes metabolic and molecular health in non-obese humans

Calorie restriction reduces aging-associated biomarkers in humans

Calorie restriction induces metabolic adaptation and behavioral compensation in physical activity

Calorie restriction induces no adverse effects on quality of life or eating behavior



**Fig.1.**  
A proposed hierarchical model for the effects of calorie restriction on health and longevity