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Intermittent and Periodic Fasting, Aging and Disease

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Preface

Various forms of fasting, which have been part of the common and in some cases required practices by many religious groups for thousands of years, have recently become some of the most adopted interventions to promote health. However, fasting, like eating, can have positive, negative, and neutral effects on health and longevity depending on its length, type, and frequency but also on a wide range of characteristics of the person who is fasting. For example, 12–13 h of daily fasting is associated with health and sleep benefits but when the fasting period surpasses 14–16 h it is associated with an increased risk of hospitalization with gallstone disease (Sichieri et al. 1991) and even higher cardiovascular and overall mortality if the prolonged fasting involves skipping breakfast (Rong et al. 2019).

Thus, to allow fasting to become part of the toolkit of healthcare professionals for disease prevention and treatment it will be important to standardize and test in randomized clinical trials the fasting method utilized while explaining the exact method by which it was carried out. Whereas only some fasting-based interventions may seek FDA approval, a standard similar to the one leading to drug approval should be used to determine whether a particular type of fasting is, in fact, able to prevent or treat a disease or if it may affect biological age.

This book will first focus on calorie and protein restriction research, which has generated an enormous number of valuable publications in the past 100 years, most pointing to their anti-aging properties, but many also pointing to side effects or their efficacy only in certain genetic backgrounds or in specific age ranges. Following the chapters on these everyday restrictions, it will focus on time-restricted eating (TRE) and therefore on the length of the daily periods in which food is consumed or not. These TRE chapters will address its benefits on markers for aging and diseases but also on the quality and length of sleep, as well as its origins in circadian biology. Several chapters will also focus on alternate day fasting (ADF) and therefore the alternation of days of normal or high calorie consumption with days of no or low calorie intake and its effect on aging and disease markers, with one chapter emphasizing fasting responses in the brain. From these forms of intermittent fasting (IF), the subsequent chapters will shift to periodic fasting (PF) or the studies of longer fasting periods usually lasting 3 days or longer but applied once a month or less, in

most cases. These chapters will cover water only or similar very low calorie consumption fasting, or the use of fasting mimicking diets (FMDs) developed to allow a much higher calorie consumption while achieving the fasting response. First the focus will be on periodic fasting and FMDs lasting 3–7 days and in many cases applied once a month and then on periods of water only or similar fasting lasting up to several weeks or longer but in most cases carried out only once a year or less frequently.

In general, there is no doubt that the various fasting methods can have remarkable effects on aging, disease risk factors, but can also reverse or at least ameliorate a number of diseases. However, the near future will require many additional randomized clinical trials, to allow healthcare professionals to adopt only the fasting methods demonstrated to be effective against aging and/or disease prevention or treatment. It will be important to assess their overall effect not only on the disease being treated but also on both the short- and long-term effects, so that lifespan and health span are both optimized. For example, achieving weight loss or diabetes reversal by fasting methods may not only be beneficial but it may also be detrimental if the patient regains the weight or if the insulin resistance and diabetes return.

Taking into account multiple pillars including clinical, basic, and epidemiological studies but especially those examining health span will be of central importance for these dietary interventions to be considered to complement or replace pharmacological interventions.

Los Angeles, CA, USA   Valter Longo

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About the Editors

Krista Varady, PhD, is a Professor of Nutrition at the University of Illinois, Chicago. Her research focuses on the efficacy of intermittent fasting for weight management and metabolic disease risk reduction in adults with obesity. She has been studying fasting for almost 20 years and is one of the top researchers in this field. Her work is funded by the NIH, American Heart Association, and the University of Illinois. She has published over 100 publications on this topic and is also the author of two books for the general public, entitled the "Every Other Day Diet" and "The Fastest Diet."

Emily N. C. Manoogian, PhD, is a staff scientist and the head of clinical research in Dr. Satchin Panda's lab at the Salk Institute for Biological Studies. She has studied circadian rhythms for the past 15 years at the mechanistic and clinical levels. Currently, her research focuses on how lifestyle interventions that optimize circadian health, such as time-restricted eating, can improve a variety of age-related diseases including pre-diabetes, diabetes, cardiovascular disease, cancer, mental health, and cognitive decline.

Valter Longo, PhD, is the Edna Jones Professor in Gerontology, the Director of the USC Longevity Institute and group leader at the IFOM Cancer Research Institute in Milan, Italy. His laboratories study the fundamental mechanisms of aging in yeast, rodents, and humans by using genetics and biochemistry techniques. The focus is on the nutrient-response signal transduction pathways that regulate disease and longevity. This work led to the discovery of the effects of periodic fasting and fasting mimicking diets on multi-system stem cell activation and regeneration in mice and to clinical trials on a range of age-related diseases.

Part I
Calorie Restriction

Chapter 1
Caloric Restriction and Biomarkers of Aging

Susan B. Racette and Sai Krupa Das

Abstract Calorie restriction (CR) is characterized by a reduction in calorie intake without malnutrition. In the context of geroscience, CR is a promising nutritional strategy that targets the biology of aging and therefore has the potential to delay the onset or slow the progression of age-related diseases. Life span extension by CR has been demonstrated in a variety of species, including yeast, drosophila, worms, rodents, and dogs, providing compelling evidence for the geroprotective potential of CR. In humans, optimizing health span is arguably a more meaningful goal of CR interventions and CR lifestyles. Numerous physiological effects of CR on biomarkers of human aging and health span have been explored, both in observational studies and in well-controlled intervention trials, providing an abundance of rich data that overwhelmingly support CR as a promising strategy for attenuating biological aging. The development of novel biomarkers of aging has advanced the field of geroscience and provided new opportunities for exploring the impact of CR on biological aging. In this chapter, we discuss the influence of CR on various biomarkers of aging and health span, with the biomarkers organized in three broad categories: cellular aging, phenotypic aging, and functional aging. We present results of CR studies in humans that demonstrate typical improvements in cardiometabolic indices, as well as effects on novel epigenetic biomarkers of cellular aging and the pace of biological aging that are based on DNA methylation. Finally, the chapter closes with highlights of ongoing CR initiatives funded by the National Institute on Aging and future directions for exploring numerous unanswered but important questions about the role of CR in enhancing health span.

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1.1   Introduction

Biological aging is the gradual deterioration in the integrity of bodily systems over time, during which the loss of molecular fidelity in tissues exceeds repair capacity (Hayflick 2007). Chronological aging increases at the same pace for everyone, whereas biological aging can be accelerated or decelerated as a consequence of lifestyle behaviors (Hayflick 2007; Ferrucci et al. 2018). Biological aging can be described in terms of primary aging and secondary aging (Holloszy 2000). Primary aging is the progressive deterioration in tissue structure and function that occurs with advancing age and is proposed to occur independently of disease, lifestyle behaviors, and environmental factors. Secondary aging, in contrast, reflects the deterioration in tissue structure and function that occurs due to disease processes, adverse lifestyle behaviors, and harmful environmental exposures. Various biomarkers have been identified to reflect biological aging, which is important for evaluating the merits of interventions that are designed to be geroprotective, such as calorie restriction (CR).

1.2   Biological Aging Is Associated with Functional Decline and Chronic Disease Risk

The physiological process of aging is characterized by molecular, cellular, metabolic, hormonal, immune, and neurocognitive changes that independently and collectively contribute to decrements in physical function and increases in chronic disease risk (Hayflick 2007; Lopez-Otin et al. 2013). Recent scientific advances have led to the transformative hypothesis that interventions targeting the fundamental biology of human aging have the potential to delay, if not prevent, the onset of aging-associated conditions, thereby extending the length of time a person is healthy—referred to as health span. The unprecedented growth of the aging population and increasing prevalence of chronic diseases have created an urgent need for interventions that promote the maintenance of health into old age.

1.3   Calorie Restriction as a Geroprotective Strategy

CR is characterized by a reduction in calorie intake without malnutrition. In the context of geroscience, CR is a strategy that is intended to impact primary aging favorably; this is distinct from obesity treatment approaches that are designed to promote weight loss and ameliorate the metabolic consequences associated with obesity. CR has the distinction of being one of the few non-pharmacological, non-genetic interventions that directly target the biology of aging. Other promising dietary approaches include intermittent fasting regimens (de Cabo and Mattson

2019) and methionine restriction (Kitada et al. 2021; Zhang et al. 2022). By targeting biological aging, CR has been shown to delay the onset or slow the progression of age-related diseases in various organisms (Fontana et al. 2010a; Colman et al. 2009, 2014; Mattison et al. 2017). In rodents, CR has potent anticancer effects, reduces adiposity, and protects against atherosclerosis, cardiomyopathy, and neuro-degeneration as well as autoimmune, renal, and respiratory diseases (Lane et al. 1996, 1997; Fontana and Partridge 2015; Ingram and de Cabo 2017). In rhesus monkeys, which are genetically and physiologically similar to humans, long-term CR conferred profound benefits in protecting against conditions and diseases that are common in human geriatric populations, such as sarcopenia, osteoporosis, arthritis, cancer, type 2 diabetes, and cardiovascular disease, which lowered the risk of age-related morbidity more than twofold (Colman et al. 2009, 2014; Mattison et al. 2017; Lane et al. 1999). Collectively, the geroprotective effects of CR interventions in yeast, worms, flies, rodents, and non-human primates raised the possibility that CR acts through mechanisms that are conserved across species, providing strong rationale for studying CR in humans.

Effects of CR on Longevity Equally impressive are the effects of CR on increasing longevity, which may be considered the most definitive evidence of geroprotection. Longer life span with CR has been demonstrated convincingly in yeast, drosophila (Mair et al. 2003), worms, rodents (McCay et al. 1935; Holloszy and Schechtman 1991; Holloszy 1997), and dogs (Kealy et al. 2002; Lawler et al. 2008). The earliest experimental evidence for CR's benefits date back to the 1930s, when Dr. Clive McCay et al. discovered that retarding the growth rate of young rats extended their life span (McCay et al. 1935; McCay and Crowell 1934). Elegant CR intervention studies in rats conducted several decades later in Dr. John Holloszy's lab revealed that CR, whether implemented as the sole intervention or combined with exercise, increased both mean and maximal life span (Holloszy 1997). This benefit is distinct from that of exercise alone, which promoted a smaller increase in mean life span compared to CR and did not extend maximal life span (Holloszy 1988). A very intriguing study that demonstrated the life-extending effects of CR in drosophila addressed the important question of whether CR must be initiated in early life to exert longevity benefits (Mair et al. 2003). The results were both encouraging and sobering: CR initiated in midlife had robust, rapid, and comparable benefits on longevity as CR initiated in early life; however, switching from a CR diet in early life to a fully fed condition in midlife quickly reversed the benefits of CR and caused even higher mortality compared to flies that were fully fed throughout life.

The effects of long-term, controlled CR interventions on life span in rhesus monkeys were variable, showing benefits in one of the two colonies (Colman et al. 2009; Mattison et al. 2017), but not in the other (Mattison et al. 2012). This discrepancy is believed to be attributable to diet quality and other differences in experimental designs between the two studies (Maxmen 2012). In humans, there is very intriguing observational evidence from Okinawa, Japan, that a dietary pattern characterized by approximately 11% CR initiated during youth and continued into mid-adulthood was associated with greater mean and maximal life span and likely

contributed to the Okinawans' distinction as the longest-lived human population (Willcox et al. 2006).

CR Studies in Humans While longer life span is a strong indicator of the gero-protective effects of CR, greater health span is perhaps a more meaningful goal of CR interventions and CR lifestyles. Numerous physiological effects of CR on biomarkers of human aging have been explored both in observational studies and in well-controlled intervention trials. Figure 1.1 provides an outline (for reference throughout this chapter) of human studies in which CR was implemented for at least 6 months' duration in adults without obesity and in which aging biomarkers were quantified.

Observational Studies of CR in Humans Although randomized controlled trials are considered the gold standard for many research studies, natural experiments and observational studies provide unique and unmatched opportunities to examine the influence of specific dietary patterns followed for long durations. Such lengthy interventions and follow-up periods are beyond the time frame that is feasible in most human intervention studies. A noteworthy example was Biosphere 2, an ecological mini-world located near Tucson, Arizona, in which Dr. Roy Walford, one of the pioneers of CR, together with seven other healthy adults without obesity, lived for a 2-year period (1991–1993). An unexpected problem growing crops resulted in a calorically restricted diet (~29% CR) during most of the 2-year experiment. The silver lining for geroscience was the rich biomarker data that were obtained from the eight inhabitants (Walford et al. 1992, 2002). Another unique opportunity to evaluate the effects of long-term CR was provided by "CRON" individuals who voluntarily and independently had been following a CR

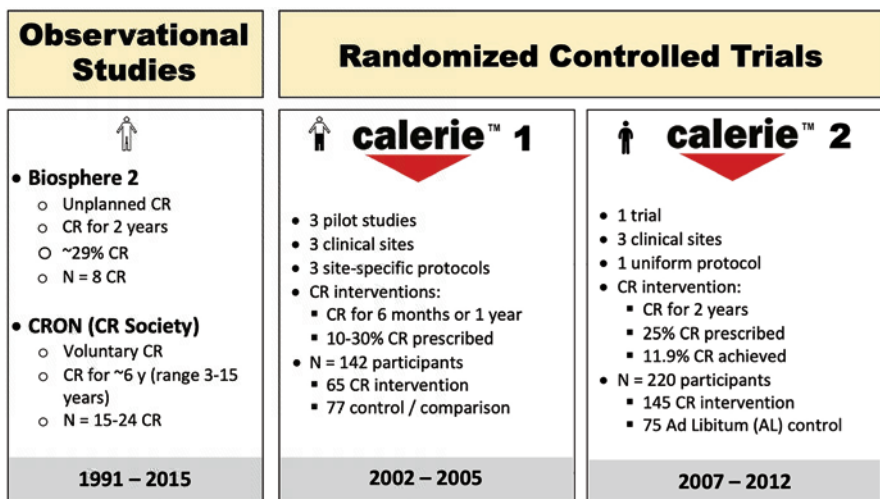| Observational Studies	Randomized Controlled Trials	
☖	☖ **calerie™ 1** ▼	☖ **calerie™ 2** ▼
• **Biosphere 2** ○ Unplanned CR ○ CR for 2 years ○ ~29% CR ○ N = 8 CR • **CRON (CR Society)** ○ Voluntary CR ○ CR for ~6 y (range 3-15 years) ○ N = 15-24 CR	• 3 pilot studies • 3 clinical sites • 3 site-specific protocols • CR interventions: ▪ CR for 6 months or 1 year ▪ 10-30% CR prescribed • N = 142 participants ▪ 65 CR intervention ▪ 77 control / comparison	• 1 trial • 3 clinical sites • 1 uniform protocol • CR intervention: ▪ CR for 2 years ▪ 25% CR prescribed ▪ 11.9% CR achieved • N = 220 participants ▪ 145 CR intervention ▪ 75 Ad Libitum (AL) control
1991 – 2015	2002 – 2005	2007 – 2012

Fig. 1.1 Studies of calorie restriction in humans

diet for an average of 6 years (range 3–15 years) when Dr. Luigi Fontana began assessing their cardiometabolic health (Fontana et al. 2004). The goal of their dietary approach was to achieve caloric restriction with optimal nutrition (CRON); this is the lifestyle adopted by the CR Society. The physiological and metabolic profiles of the CRON individuals were impressive and supported the geroprotective effects of CR observed in other species.

Randomized Controlled Trials of CR in Humans The first randomized controlled trial of CR in humans without obesity was the *C*omprehensive *A*ssessment of *L*ong-term *E*ffects of *R*educing *I*ntake of *E*nergy (CALERIE) trial. Funded by the National Institute on Aging (NIA), CALERIE™ was conducted in two phases at three clinical centers: Pennington Biomedical Research Center in Baton Rouge, Louisiana; the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts; and Washington University School of Medicine in St. Louis, Missouri. CALERIE™ Phase 1 was conducted between 2002 and 2005 and involved site-specific pilot studies to test approaches to achieve and maintain CR (Das et al. 2007; Heilbronn et al. 2006; Racette et al. 2006). The CALERIE™ Phase 2 trial, conducted from 2007 to 2012, was a multi-site, single-protocol study designed to test the hypothesis that 2 years of 25% CR would improve biomarkers of aging and biomarkers of age-related chronic disease (Rochon et al. 2011; Ravussin et al. 2015). CALERIE™ 2 participants were 220 healthy adults aged 21–50 years at baseline (upper age limit was 47 years for women to avoid the metabolic effects of menopause) who were randomized in a 2:1 allocation ratio to a CR intervention ("CR" group) or an ad libitum intake control condition ("AL" group). All participants were healthy and not taking medications other than oral contraceptives and had no evidence of disease or disease risk factors based on extensive blood, urine, physical, and psychological testing for eligibility.

The CR prescription was 25% for 2 years, reflecting a daily energy intake level that was 25% below baseline, weight-maintenance energy needs. Each participant in the CR group received an individualized daily energy intake prescription (kcal/day) that was 25% lower than their baseline daily energy expenditure; baseline energy expenditure was measured for 4 consecutive weeks using doubly labeled water. This method provides the most accurate estimate of free-living energy expenditure and energy intake while people lead their usual lives. The CALERIE™ 2 trial was characterized by careful design, rigor, and high commitment of the research team personnel and study participants. An important consideration when reviewing the results of the CALERIE™ 2 trial is that the average level of CR achieved throughout the 2-year intervention was $11.9 \pm 0.7\%$ (mean $\pm$ SE), with a range of 0.9%–31.2% among CR participants. These results highlight the difficulty of sustaining a CR diet for 2 years and the inter-individual variability in adherence. Despite achieving a more moderate level of CR, on average, than the prescribed level of 25%, it is encouraging that this modest level of CR yielded improvements in several biomarkers of aging, with relatively few effects that were deemed potentially adverse.

1.4 Biomarkers of Aging Relevant to Calorie Restriction

Biomarkers are intended to reflect important biological indices of health. Biomarkers of aging can be categorized as cellular, phenotypic, and functional to reflect the physiological mechanisms involved and the clinical manifestations that are associated with aging. Several novel and traditional biomarkers have been proposed to reflect human aging and health span. The focus of this chapter is the various biomarkers that reflect health span and the potential influence of CR on biological aging indices in humans and other species.

Table 1.1 provides an overview of the favorable, neutral, and negative effects of CR on cellular, phenotypic, and functional biomarkers that have been observed in rodent intervention studies 🐭, rhesus monkey intervention studies 🐒, human observational studies 🧍, human intervention studies of 6 months to 1 year in duration 🧍, and human intervention studies of 2 years in duration 🧍. We intentionally included only studies that assessed age-associated biomarkers among rodents (mice and rats), monkeys (rhesus macaques), or humans without obesity. Human studies were restricted to those in which the duration of CR was at least 6 months. We acknowledge that this does not represent an all-inclusive compilation of CR studies.

1.4.1 Calorie Restriction and Cellular Aging Biomarkers

TAME Biomarkers Novel surrogates for cellular aging include blood-based biomarkers proposed by the Targeting Aging with MEtformin (TAME) Biomarkers Workgroup (Justice et al. 2018). TAME encompasses the following multi-system metabolic markers that reflect the health status of various tissues, organs, and metabolic processes: interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP), and tumor necrosis factor α receptor II (TNFRII) as markers of inflammation and intercellular signaling; growth differentiation factor 15 (GDF15) as a marker of a stress response and mitochondrial dysfunction; insulin-like growth factor 1 (IGF-1) and insulin as markers of nutrient signaling; cystatin C as a marker of renal aging; N-terminal B-type natriuretic peptide (NT-proBNP) as a marker of heart failure and cardiovascular health; glycated hemoglobin (HbA1c) as a marker of glucose regulation and metabolic aging; and molecular signatures that reflect epigenetic, transcriptomic, and proteomic processes that reflect aging.

Inflammation Reduced inflammation is considered an important mechanism that mediates the beneficial effects of CR on aging (Heilbronn and Ravussin 2003). In the CRON population, long-term CR was associated with lower concentrations of the inflammatory protein hsCRP (Fontana et al. 2004), which may be mediated, in part, by an increase in serum cortisol (Yang et al. 2016). In the CALERIE™ 2 trial, 2 years of CR resulted in 40% to 50% reductions in circulating concentrations of the pro-inflammatory cytokines TNFα and hsCRP (Ravussin et al. 2015), as well as

Table 1.1 Effects of calorie restriction on biomarkers of aging and health span

	Favorable Effects	Neutral Effects	Negative Effects	References
Biomarkers of cellular aging				
Inflammatory markers: IL-6, hsCRP, TNFα				Lane et al. (1997), Meyer et al. (2006), Weiss et al. (2006), Fontana et al. (2007), Yang et al. (2016), Meydani et al. (2016), Dorling et al. (2021), Spadaro et al. (2022)
Nutrient signaling: IGF-1, insulin				Fontana et al. (2004), Das et al. (2007), Heilbronn et al. (2006), Weiss et al. (2006), Dorling et al. (2021), Mercken et al. (2013a), Ramsey et al. (2000)
Dehydroepiandrosterone sulfate (DHEAS)				Willcox et al. (2006), Heilbronn et al. (2006)
Oxidative stress or oxidative damage				Ning et al. (2013), Zainal et al. (2000), Meydani et al. (2011), Il'yasova et al. (2018), Redman et al. (2018)
Cellular senescence				Ning et al. (2013), Cohen et al. (2004), Fontana et al. (2018a)
Autophagy				Yang et al. (2016), Ning et al. (2013)
Mitochondrial function or mitochondrial damage				Mercken et al. (2013a), Ning et al. (2013), Civitarese et al. (2007), Sparks et al. (2017)
DNA methylation, gene expression, RNA processing, transcription				Spadaro et al. (2022), Maegawa et al. (2017), Rhoads et al. (2018), Mercken et al. (2013b)
Biological age—Epigenetic clocks (DNA methylation-based): GrimAge, DNAm PhenoAge				Waziry et al. (2023)
Pace of biological aging (DNA methylation-based): DunedinPoAm, DunedinPACE				Waziry et al. (2023)
Biomarkers of phenotypic aging				
Blood pressure				Walford et al. (1992), Meyer et al. (2006)

Table 1.1 (continued)

	Favorable Effects	Neutral Effects	Negative Effects	References
Cardiovascular health: diastolic function, carotid intima-media thickness, heart rate variability				Meyer et al. (2006), Riordan et al. (2008), Stein et al. (2012)
Metabolic syndrome, insulin resistance, glucose regulation				Ravussin et al. (2015), Weiss et al. (2006), Dorling et al. (2021), Ramsey et al. (2000), Kalant et al. (1988), Kemnitz et al. (1994), Gresl et al. (2001), Fontana et al. (2010b), Larson-Meyer et al. (2006), Fontana and Klein (2007), Huffman et al. (2022)
Anthropometrics: weight, waist and hip circumferences				Fontana et al. (2004), Das et al. (2007), Racette et al. (2006), Colman et al. (1998), Redman et al. (2007), Das et al. (2017)
Body composition: whole-body and abdominal adiposity				Fontana et al. (2004), Das et al. (2007), Racette et al. (2006), Colman et al. (1998), Redman et al. (2007), Das et al. (2017), Phillips et al. (2022)
Bone mineral density, bone mineral content, bone architecture, bone strength, bone turnover				Ingram and de Cabo (2017), Colman et al. (2007, 2008), Redman et al. (2008), Villareal et al. (2006, 2016)
Core body temperature				Lane et al. (1996), Heilbronn et al. (2006), Ravussin et al. (2015), Soare et al. (2011)
Energy expenditure or metabolic rate (basal, resting, sleeping, daytime, or 24-h metabolic rate)				Lane et al. (1996), Das et al. (2007), Heilbronn et al. (2006), Raman et al. (2007), Weyer et al. (2000), Ravussin et al. (2015), Redman et al. (2018)
Biological age (physiology-based): Klemera–Doubal method (KDM), homeostatic dysregulation index (HDI), PhenoAge				Belsky et al. (2017)

	Favorable Effects	Neutral Effects	Negative Effects	References
Biomarkers of functional aging				
Cardiorespiratory fitness: VO_{2max} (ml·kg^{-1}·min^{-1})	🐵	🐵		Racette et al. (2017), Weiss et al. (2007)
Muscle strength	🐵	🐵	🧍	Racette et al. (2017), Weiss et al. (2007)
Grip strength	🐭	🧍🐭		Villareal et al. (2016), Peters et al. (2022)
Cognition	🧍	🧍		Leclerc et al. (2020), Silver et al. (2023)
Sleep	🧍	🧍		Martin et al. (2016)
Sexual health	🧍	🧍		Martin et al. (2016)

Key: 🐭 rodents, 🐵 rhesus monkeys, 🧍 human observational studies, 🧍 human interventions of 6 months to 1 year, 🧍 human intervention of 2 years

their downstream target intercellular adhesion molecule-1 (Meydani et al. 2016). The anti-inflammatory effects of CR were further supported by reductions in total white blood cell, lymphocyte, and monocyte counts (Meydani et al. 2016). Serum cortisol, however, was increased to a small but statistically significant extent only after the first year of CR in the CALERIE™ 2 trial; the increase was not sustained at the 2-year time point (Fontana et al. 2016).

Oxidative Stress Oxidative stress and the accumulation of oxidative damage are implicated strongly in the pathogenesis of multiple age-associated chronic diseases, including heart disease, type 2 diabetes, cancer, and neurodegeneration (Valko et al. 2016). A reduction in oxidative stress is one mechanism by which CR is hypothesized to slow aging (Fontana et al. 2010a; de Cabo et al. 2014). Urinary F2-isoprostanes are considered reliable markers of non-enzymatic lipid peroxidation and serve as indicators of tissue oxidative damage and systemic oxidative stress (Il'yasova et al. 2010). In the CALERIE™ 2 trial, reductions of 13% and 27% in 2,3-dinor-iPF(2α)-III and iPF(2α)-II, respectively, were observed after 2 years of CR (Il'yasova et al. 2018). Furthermore, a sub-study of participants at the Pennington site found that change in 2,3-dinor-iPF(2α)-III was associated with metabolic adaptation in 24-h energy expenditure, suggesting that CR in humans may slow aging via a biological link between reductions in energy expenditure and oxidative stress (Redman et al. 2018).

SASP, Sirtuins, DNA Repair, and Autophagy Other important biomarkers of cellular aging include senescence-associated secretory phenotype (SASP) proteins, which are cellular senescence biomarkers (Fontana et al. 2018b); silent information regulators (sirtuins) (Cohen et al. 2004; Cantó and Auwerx 2009), which are proteins that regulate important biological pathways, such as ribosomal DNA recombination, gene silencing, and DNA repair; mitochondrial function; and autophagy, which is a favorable process through which damaged or dysfunctional proteins are degraded and recycled (Chung and Chung 2019). Long-term, voluntary CR among CRON practitioners in the CR Society contributed to favorable effects on cellular quality control processes in vastus lateralis muscle (Yang et al. 2016). Specifically, molecular chaperones and mediators of autophagy were higher among 15 men and women who had been following a CRON diet for 3–15 years compared to 10 age-matched control participants who were not practicing CR. Six months of ~25% CR in the CALERIE™ 1 pilot study was shown to increase mitochondrial DNA content, increase the expression of genes that encode mitochondrial proteins, and reduce DNA damage, but did not alter the activity of several key mitochondrial enzymes (Civitarese et al. 2007). These predominantly promising findings were not replicated in the CALERIE™ 2 trial, in which an investigation of potential mitochondrial effects after the first year of CR revealed no significant alterations in pathways involved in mitochondrial biogenesis, maximal muscle ATP synthesis rate, or other indices of mitochondrial function (Sparks et al. 2017). The effect of CR on autophagy in mice was dependent on the age at which CR was initiated (Sheng et al. 2017). Interestingly, favorable

increases in autophagy occurred in middle-aged and older mice, whereas suppression of autophagy occurred in young mice.

Biological Aging Biomarkers   Novel epigenetic biomarkers of cellular aging that were developed to reflect biological age and the pace of biological aging are based on DNA methylation (DNAm) as an indicator of epigenetic modifications. Epigenetic clocks (Horvath and Raj 2018) provide estimates of biological age and age advancement, relative to chronological age. Horvath's epigenetic clock (Horvath 2013) predicts the DNA methylation age of various human tissues and cells based on the methylation state of 353 CpG dinucleotides. Hannum's clock (Hannum et al. 2013) represents a quantitative model of the rate at which a person's methylome ages and was developed using more than 450,000 CpG sites from 656 adults aged 19–101 years. Levine's DNAm PhenoAge biomarker (Levine et al. 2018) was developed using 513 CpG sites selected from 20,169 CpGs tested, 9 blood biomarkers (selected from 42 clinical markers), chronological age, biomarker and mortality data from 9926 adults aged 18 years and older in the National Health and Nutrition Examination Survey (NHANES) III, and DNA methylation and blood chemistry data obtained at two time points from 456 adults aged 21–100 years in the Invecchiare in Chianti (InCHIANTI) Study. Lu et al.'s DNAm GrimAge biomarker (Lu et al. 2019) was designed to predict life span and health span using DNAm-based surrogate biomarkers for seven plasma proteins (adrenomedullin, beta-2 microglobulin, cystatin C, GDF-15, leptin, plasminogen activation inhibitor 1, and tissue inhibitor metalloproteinase 1), a DNAm-based biomarker for smoking pack years, as well as chronological age and biological sex of 2356 adults in the Framingham Heart Study Offspring Cohort. Another novel metric, AgeAccelGrim, was developed from DNAm GrimAge to estimate epigenetic age acceleration (Lu et al. 2019). An enhanced version of DNAm GrimAge, named GrimAge2 (Lu et al. 2022), is proposed to be a stronger epigenetic biomarker of mortality and morbidity risk by incorporating two additional DNAm-based surrogates of the plasma proteins hsCRP and HbA1c.

In the CALERIE™ 2 trial, 2 years of CR did not significantly alter either the GrimAge clock or the DNAm PhenoAge clock relative to the AL control condition (Waziry et al. 2023). A subsequent exploration of a dose-response relationship was conducted to determine whether achieving greater than 10% CR during the 2-year intervention was associated with favorable changes in biological clock ages. Neither the GrimAge clock nor the PhenoAge clock differed based on this criterion of achieving greater than 10% CR (vs. achieving 10% CR or less).

DNA Methylation Pace of Aging   Whereas the biological aging clocks are regarded as static metrics that reflect mortality risk at a single point in time, the pace of biological aging indices represents the rate at which an individual is aging relative to each calendar year increase in chronological age. DunedinPoAm, which stands for Dunedin Study *P*ace *o*f *A*ging from *m*ethylation, was developed by Dr. Daniel Belsky et al. (Belsky et al. 2020) to quantify the rate of biological aging based on changes in several age-related blood-based biomarkers that reflect the integrity of

the cardiovascular, metabolic, renal, hepatic, immune, periodontal, and pulmonary systems. An enhanced pace of aging metric, DunedinPACE (Belsky et al. 2022), was later developed by the same investigators; PACE stands for *P*ace of *A*ging *C*alculated from the *E*pigenome. Unique and important aspects of DunedinPACE are that it was developed using data from the Dunedin study cohort, which consisted of 1037 individuals who were born in the same city (Dunedin, New Zealand) as part of a single birth cohort (born 1972–1973) and assessed at the same four time points (baseline and 6, 12, and 20 years thereafter) at the same ages (26, 32, 38, and 45 years of age). DunedinPACE is comprised of the following 19 biomarkers: body mass index (BMI), waist-to-hip ratio, glycated hemoglobin, leptin, mean arterial blood pressure, VO_{2max} as a measure of cardiorespiratory fitness, forced vital capacity ratio (FEV1/FVC), forced expiratory volume in one second (FEV1), total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, lipoprotein(a), apolipoprotein B100/A1 ratio, estimated glomerular filtration rate (eGFR), blood urea nitrogen (BUN), high-sensitivity C-reactive protein (hs-CRP), white blood cell count, mean periodontal attachment loss, and the number of tooth surfaces affected by dental caries (as a reflection of tooth decay). The advantages of DunedinPACE over DunedinPoAm (as stated by the investigators who developed these metrics) are that it includes four time points (versus three time points) over a 20-year follow-up period (versus 12 years of follow-up) and includes only highly reliable DNA methylation probes that were demonstrated to have low variability across replicate measurements (vs. including all CpG sites without restriction).

In the CALERIE™ 2 trial, the pace of aging based on DunedinPACE was slower in the CR group than in the AL group (Waziry et al. 2023; Rubin 2023). Reductions in DunedinPACE after 1 year of CR (Cohen's $d = -0.29$; 95% confidence interval -0.45 to -0.13) were maintained at the 2-year time point (Cohen's $d = -0.25$; 95% confidence interval -0.41 to -0.09; $P < 0.003$ for both time points). These results correspond to a favorable average reduction of 2–3% in the pace of aging among CR participants (Waziry et al. 2023), with larger than 3% improvements among some participants, but worsening observed in other participants. Interestingly, a dose-response relationship was observed: the CR treatment effect of achieving >10% CR was $d = -0.33$ at 1 year and $d = -0.33$ at 2 years, whereas the effects were less in the group that achieved <10% CR ($d = -0.19$ at 1 year; $d = -0.14$ at 2 years. An instrumental variable analysis that modeled the effect of 20% CR revealed that the treatment effect would be $d = -0.43$ (95% confidence interval -0.67 to -0.19) at 1 year and $d = -0.40$ (95% confidence interval -0.67 to -0.12) at 2 years ($P < 0.005$ for both).

1.4.2 Calorie Restriction and Phenotypic Aging Biomarkers

Cardiovascular and Cardiometabolic Disease Risk Phenotypic aging biomarkers are broad and reflect numerous risk factors for cardiovascular and cardiometabolic diseases, such as heart disease, stroke, atherosclerosis, and type 2 diabetes.

Decreasing risks for these diseases reduces lifetime coronary events and morbidity (Hwang et al. 2018). Cardiovascular and cardiometabolic benefits of long-term CR in the CRON population included lower blood pressure, lower glucose and insulin concentrations, and a more favorable blood lipid profile (i.e., lower total and LDL-cholesterol, higher HDL-cholesterol, lower triglycerides, and lower ratio of total/HDL cholesterol). Importantly, several CRON participants provided medical records that preceded their initiation of a CR diet, providing evidence that their baseline values for the various health metrics were comparable to the age-matched referent (non-CR) group and that statistically significant and clinically meaningful improvements in these parameters occurred after following a CR diet (Holloszy and Fontana 2007).

In the CALERIE™ 1 CR intervention studies, 6 months and 1 year of CR led to significant improvements in blood pressure, insulin sensitivity, serum lipid and lipoprotein concentrations, and left ventricular diastolic function (Riordan et al. 2008). In the CALERIE™ 2 trial, 2 years of CR profoundly improved glucose regulation and insulin sensitivity, based on the homeostasis model assessment of insulin resistance (HOMA-IR), and improved multiple risk factors to levels well below the conventional risk thresholds used in clinical practice (Ravussin et al. 2015; Il'yasova et al. 2018; Kraus et al. 2019). Importantly, the baseline values already were in the normal range and the majority of vascular and metabolic indices improved significantly in the CR vs. AL group at 1 year, with maintenance of the improvements at 2 years.

Anthropometrics and Body Composition Midlife adiposity has been associated with earlier onset of Alzheimer's dementia, neuropathology, pre-symptomatic cerebral amyloid accumulation (Chuang et al. 2016), and frailty in old age (Stenholm et al. 2014). Anthropometric and body composition measures reflect whole-body fat mass as an index of adipose tissue-related health risk, regional adiposity as a reflection of metabolic risks associated with visceral and subcutaneous abdominal adiposity, lean body mass as a metric of sarcopenia risk, and bone mineral density as a metric of osteoporosis risk. CR has consistently been shown to improve adiposity-related anthropometric measures (i.e., reductions in body weight, body mass index [BMI], and waist circumference) and reduce adiposity (whole-body and regional) in animal models (Mattison et al. 2003) and in humans (Fontana et al. 2004). In the CALERIE™ 1 pilot trials of 6 months or 1 year of CR, significant reductions were observed for weight, BMI, waist circumference, whole-body fat mass (Das et al. 2007; Racette et al. 2006; Redman et al. 2007), and visceral and subcutaneous abdominal adipose tissue by computed tomography and magnetic resonance imaging (Racette et al. 2006). Results of the CALERIE™ 2 trial were consistent; CR induced significant reductions in whole-body fat mass (−5.4 kg) and central adiposity (6.1 cm decrease in waist circumference and 2.8 kg decrease in trunk fat mass determined by dual-energy x-ray absorptiometry) (Das et al. 2017). Additionally, 1 year of CR attenuated the increase in extramyocellular lipid content of the tibialis muscles of CR participants relative to AL controls (Sparks et al. 2017).

In contrast to the predominantly favorable changes in adiposity that occur with CR, the reduction in fat-free mass was not trivial (−2.0 kg) (Das et al. 2017). This

result is consistent with the composition of weight loss observed in many other dietary intervention studies that did not include an exercise component. A portion of the fat-free mass loss is attributable to bone loss, a potentially adverse consequence of long-term CR diets (Liu and Rosen 2023). In fact, change in bone mineral density was used as a safety metric during the CALERIE™ 2 intervention (Rochon et al. 2011). Three CR participants had decreases in bone mineral density of 5% or more from baseline, necessitating temporary discontinuation of CR for two participants and permanent discontinuation for one participant (Ravussin et al. 2015). In the overall CALERIE™ 2 sample, bone mineral density decreased significantly in the lumbar spine, total hip, femoral neck, and other regions of the hip at both 1 year and 2 years in the CR group relative to the control group (Villareal et al. 2016). Bone turnover, another metric of bone health, also showed adverse changes in response to CR, with bone resorption exceeding bone formation (Villareal et al. 2016). The influence of CR on bone architecture and quality has yet to be explored.

Core Body Temperature and Energy Metabolism   Lower core body temperature and reductions in core body temperature in response to CR are considered favorable adaptations that may contribute to slower aging. This has been demonstrated in rodents, monkeys (Lane et al. 1996), and humans after 6 months of CR (Heilbronn et al. 2006), but not after 2 years of modest CR in the CALERIE™ 2 trial (Ravussin et al. 2015). Energy metabolism metrics that are used as indicators of energy conservation and potentially slowed aging include resting metabolic rate (RMR), RMR adjusted for changes in body composition (RMR residual), sleeping metabolic rate, and 24-hour sedentary energy expenditure. While acute weight loss is known to lower resting metabolism, the effects of longer-term CR have been variable in rhesus monkeys. In the CALERIE™ 1 pilot studies, 6 months of CR led to reductions in RMR (Das et al. 2007), sleeping energy expenditure (Heilbronn et al. 2006), and 24-hour sedentary energy expenditure in a metabolic chamber (Heilbronn et al. 2006). In the CALERIE™ 2 trial, the effect of the CR intervention on RMR residual was significant only at 1 year (Ravussin et al. 2015); the lack of difference between the CR and AL groups at 2 years may be attributable to the lower level of CR achieved during year 2 (~8.3% CR) than during year 1 (~15.2% CR) (Dorling et al. 2020).

Biological Age   Biological aging algorithms based on phenotypic biomarkers (Hastings et al. 2019; Kwon and Belsky 2021) incorporate various indices, such as cholesterol, hemoglobin A1c, systolic blood pressure, white blood cell count, uric acid, and high-sensitivity C-reactive protein (Belsky et al. 2015). Phenotypic aging algorithms include the Klemera–Doubal Method (KDM) Bioage (Klemera and Doubal 2006), Homeostatic Dysregulation Index (HDI) (Cohen et al. 2013), and Levine phenotypic age (Levine 2013). KDM and HDI were assessed in the CALERIE™ 2 trial to address the question of whether long-term modest CR influences biological age. Favorable effects were observed: the CR group had a younger KDM biological age than the AL control group at both the 1-year and 2-year time points (treatment by time interaction: $\beta = 0.60$; 95% confidence interval -0.99 to 0.21; $P = 0.003$) (Belsky et al. 2017). In addition, KDM biological age advanced more

slowly during the 2-year CR intervention (0.11 years per calendar year, 95% confidence interval: −0.13 to 0.36) than during 2 years of the AL control condition (0.71 years per calendar year, 95% confidence interval: 0.41 to 1.01). Interestingly and not surprisingly, there was a trend for an inverse dose-response relationship between %CR and the rate of biological aging: CR participants who achieved a higher %CR exhibited a slower rate of biological aging compared to participants who were less adherent to the CR diet (Belsky et al. 2017). Consistent with the KDM Bioage result, HDI was suppressed significantly in CR but not AL ($P < 0.001$). An important observation, based on sensitivity analyses, was that the beneficial effects of CR on biological age were independent of weight loss, challenging the view that weight loss is a prerequisite for CR-related improvements in biomarkers of aging and longevity.

1.4.3 Calorie Restriction and Functional Aging Biomarkers

Cardiorespiratory Fitness Arguably, the most relevant biomarker of functional aging is cardiorespiratory fitness, which has been deemed a vital sign due to its very strong association with longevity in numerous large-scale epidemiologic studies in the United States and worldwide (Hanscombe et al. 2021; Davidson et al. 2018). The Henry Ford Exercise Testing Project of more than 57,000 adults revealed that cardiorespiratory fitness was a strong predictor of survival during more than 10 years of follow-up (Blaha et al. 2016). Similarly, the Cooper Center Longitudinal Study of 16,533 adults indicated that lower cardiorespiratory fitness was associated with a higher risk of cardiovascular death during 28 years of follow-up (Wickramasinghe et al. 2014). The largest study, which included 498,135 participants in the UK Biobank, demonstrated that lower cardiorespiratory fitness was associated with higher mortality during 4.9 years of follow-up (Celis-Morales et al. 2017).

The gold standard measure of cardiorespiratory fitness is maximal oxygen consumption (VO_{2max}), determined during a graded exercise test with respiratory gas exchange analysis. In the CALERIE™ 2 trial of 2 years of CR, VO_{2max} was quantified using the Cornell treadmill protocol (Racette et al. 2017). VO_{2max} increased when expressed relative to body mass (ml·kg^{-1}·min^{-1}), which is the most common metric used when classifying an individual's fitness level based on sex- and age-specific reference tables (Kaminsky et al. 2015) or assessing changes in fitness over time. In CALERIE™ 2, relative VO_{2max} increased 5% from baseline to 2 years, whereas a decrease of 3% was observed in the AL control group (Racette et al. 2017). Exercise time during the VO2max treadmill test also increased to a greater extent in the CR group (+2.9 min) than in the AL group (+1.8 min), suggesting that aerobic capacity and endurance were enhanced by CR. In contrast, absolute VO_{2max}, expressed as L/min, decreased in both the CALERIE™ 1 trial (Weiss et al. 2007) and the CALERIE™ 2 trial (Racette et al. 2017), likely due to smaller body size and smaller muscle mass after the CR interventions.

Functional Aging Biomarkers Other biomarkers of functional aging include gait speed, energy efficiency, muscle strength, grip strength, frailty, fatigue, cognition, and

sleep quality. Consistent with the reductions in lean body mass that accompany long-term CR, muscle strength of the knee extensors and knee flexors decreased in absolute terms in CALERIE™ 1 (Weiss et al. 2007) and CALERIE™ 2 (Racette et al. 2017). However, when expressed relative to body mass, muscle strength was either unchanged (Weiss et al. 2007) or increased (Racette et al. 2017) after 1 or 2 years of CR, respectively. The clinical implications of these findings with aging are uncertain. Hand grip strength did not change significantly in CALERIE™ 2 (Villareal et al. 2016). Male C57BL/6NCrl mice that followed a 15% CR regimen initiated at 4 months of age and continued throughout life demonstrated greater limb grip strength at ages 10 and 18 months, but not at ages 26 or 28 months, relative to controls (Peters et al. 2022).

Cognition, Sleep, and Hunger CR is proposed to have benefits on cognitive health for individuals with or at risk for various neurodegenerative diseases (Dias et al. 2020). In the CALERIE™ 2 trial, spatial working memory improved on the Cambridge Neuropsychological Test Automated Battery (CANTAB), whereas other indices of cognitive function remained unchanged (Leclerc et al. 2020). The change in spatial working memory was not associated with diet quality, assessed using either the Dietary Inflammatory Index or the Healthy Eating Index (Silver et al. 2023). While there is intriguing data that CR may improve cognition in animal models, depending on the level of CR achieved and the time of life that it is initiated (Dias et al. 2020), human trials of adults with normal cognition at baseline have not shown impressive effects of medium-term or long-term CR interventions thus far. It is likely that much longer-term CR and follow-up periods or older adult populations are required to determine the impact of CR on cognitive health. Sleep quality often deteriorates with aging, and there is an extensive array of adverse health consequences associated with sleep deprivation and poor sleep quality. Sleep metrics did not change after 2 years of CR in the CALERIE™ 2 trial; the only benefit observed was on sleep duration at the 1-year time point (Martin et al. 2016). Perceived hunger assessed by the Eating Inventory did not change in the CR group at 1 year or 2 years in the CALERIE™ 2 trial (Ravussin et al. 2015), whereas hunger assessed using a visual analog scale increased to a small but statistically significant extent (~3 mm on a 100 mm scale) in the CR group during the 2-year intervention (Dorling et al. 2020).

1.5 Other Considerations of Calorie Restriction on Biomarkers of Aging

As is evident from studies of CR in humans, non-human primates, and other organisms, the influence of CR on biomarkers of aging is variable and dependent on numerous factors. The level of CR, duration of CR, quality of the CR diet, and age at which CR is initiated appear to be of major importance for many biomarkers. Additional factors that would be expected to impact one or more aging biomarkers include specific dietary components, physical activity and exercise patterns, cardiorespiratory