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Paul C. Guest *Editor*

Reviews on Biomarker Studies in Aging and Anti-Aging Research

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Reviews on Biomarker Studies in Aging and Anti-Aging Research

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Preface

The lifespan of every organism is limited by the aging process, which involves physical decline, an increase in chronic diseases, and ultimately death. It has been an ongoing quest of mankind to understand the aging process and use this information to develop ways of extending both the health and lifespan of individuals. Through these efforts, researchers have gained new insights into the physiological and molecular aspects of aging using both epidemiological and model organism approaches, and this has led to significant advancements in potential antiaging strategies. This is important as most chronic diseases in the world are intertwined with the aging process and occur more frequently in the aged population. According to the World Health Organization, non-communicable diseases affect mainly adults and elderly individuals, and this imposes the greatest burden on global health with staggering costs to the healthcare services. This book presents a series of reviews in various aspects of aging and age-related disease research along with several methods which have shown progress as potential antiaging approaches.

Chapter 1 covers studies which have focused on long-lived mutant and naturally occurring animal species. Chapter 2 describes the association of glycolytic dysfunction with the accelerated aging of neuronal cells in schizophrenia patients. Chapter 3 covers studies on the screening of antiaging drugs and gives clues into further research of aging biomarkers and antiaging targets. Chapter 4 describes the effects of sex differences in the aging process and the role of sex hormones in this process. Chapter 5 covers the efficacy of the ketogenic diet in a variety of neurodegenerative, neurodevelopmental, and metabolic conditions throughout different stages of life. Chapter 6 reviews the potential role of CoQ10 supplementation in the treatment of tissue fibrosis, implicated in the age-related loss of function of various organs including the heart. Chapter 7 reviews the evidence that associates dietary restriction, cardiovascular aging, and age-related cardiovascular diseases, and related strategies to prevent or retard age-related cardiovascular diseases in the elderly. Chapter 8 focuses on the possible use of fecal microbiota-related parameters and microbiota-derived metabolites as biomarkers of cognitive performance and dementia, with a spotlight on the most promising areas of future research. Chapter 9 looks at the potential impact of herbal products on the prevention, regeneration, and delayed

aging of skin. Chapter 10 explores the influence of epigenetics on aging and the potential of restoring age-related changes to a "younger" state. Chapter 11 describes the basics of adipose tissue biology, growth hormone secretion, and action, and how the interactions of these may play a critical role in determining lifespan and healthspan. Chapter 12 provides a brief overview of cytoskeletal structure and function, and discusses the evidence which links cytoskeletal function and dynamics with aging and neurodegeneration. Finally, Chapter 13 details how the most promising avenues to halt the aging process have come from studies of the molecular pathways involved with caloric restriction, insulin/insulin-like growth factor signaling, and mitochondrial ROS production, in nematode, fly, and rodent models.

As a way of highlighting the growing interest in this topic throughout the world, the authors in this series come from all six of the world's habitable continents. This includes the countries Australia, Brazil, China, Indonesia, Iran, Italy, Greece, Russia, the United Kingdom, and the United States of America. As each review describes the cutting edge of research in this important field in a functional blend of scientific and layperson's language, this volume will be of interest to scientists, medical practitioners, members of health organizations, and pharmaceutical company employees, as well as those without professional or specialized knowledge.

Campinas, Brazil Paul C. Guest

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Chapter 1
Of Mice, Whales, Jellyfish and Men: In Pursuit of Increased Longevity

Paul C. Guest

1 Introduction

On August 4, 1997, a French woman from Arles named Jeanne Louise Calment died at the age of 122 years and 164 days [1]. She was widely reported to have been the oldest human and there were documents to confirm this. Her incredible longevity attracted significant attention from the media worldwide and the secrets of how she achieved this long healthy life sparked considerable medical research and speculation. According to Dr. Jean Marie Robine, a public health researcher and biographer, Madame Calment ate pounds of chocolate, smoked cigarettes and drank red wine every day, and she rode a bicycle up to the age of 100 [2]. Dr. Robine stated, "I think she was someone who constitutionally and biologically speaking, was immune to stress." Madame Calment herself was fond of saying, "If you can't do anything about it, don't worry about it." The book speculates that this way of handling stress helped to strengthen Madame Calment's immune system, which normally declines with age [2].

It is a well known fact that women live longer than men, making sex one of the strongest predictors of human lifespan [3]. This is borne out by the current list of the oldest verified people (Tables 1.1 and 1.2). This shows that the top 10 females lived and average of 3% longer than the top 10 males. This is also borne out by World Health Organization (WHO) statistics for the year 2015 across the world, in terms of life expectancy at birth [4]. The WHO estimated life expectancies were 74.2 years for females and 69.8 years for males (a difference of 6% in favour of females). These statistics differ across the world and countries such as Japan having the

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Table 1.1 List of the top 10 oldest verified females as of May 8, 2019

Name	Birth date	Death date	Age at death	Country
Jeanne Calment	Feb 21, 1875	Aug 4, 1997	122 years, 164 days	France
Sarah Knauss	Sep 24, 1880	Dec 30, 1999	119 years, 97 days	USA
Nabi Tajima	Aug 4, 1900	Apr 21, 2018	117 years, 260 days	Japan
Lucy Hannah	Jul 16, 1875	Mar 21,1993	117 years, 248 days	USA
Marie-Louise Meilleur	29 Aug, 1880	Apr 16, 1998	117 years, 230 days	Canada
Violet Brown	Mar 10, 1900	Sep 15, 2017	117 years, 189 days	Jamaica
Emma Morano	Nov 29, 1899	Apr 15, 2017	117 years, 137 days	Italy
Chiyo Miyako	May 2, 1901	Jul 22, 2018	117 years, 81 days	Japan
Misao Okawa	Mar 5, 1898	Apr 1, 2015	117 years, 27 days	Japan
María Capovilla	Sep 14, 1889	Aug 27, 2006	116 years, 347 days	Ecuador

Table 1.2 List of the top 10 oldest verified males as of May 8, 2019

Name	Birth date	Death date	Age at death	Country
Jiroemon Kimura	Apr 19, 1897	Jun 12, 2013	116 years, 54 days	Japan
Christian Mortensen	Aug 16, 1882	Apr 25, 1998	115 years, 252 days	USA
Emiliano Mercado del Toro	Aug 21, 1891	Jan 24, 2007	115 years, 156 days	Puerto Rico
Mathew Beard	Jul 91,870	Feb 16, 1985	114 years, 222 days	USA
Walter Breuning	Sep 21, 1896	Apr 14, 2011	114 years, 205 days	USA
Yukichi Chuganji	Mar 23, 1889	Sep 28, 2003	114 years, 189 days	Japan
Joan Riudavets	Dec 15, 1889	Mar 5, 2004	114 years, 81 days	Spain
Fred H. Hale, Sr.	Dec 1, 1890	Nov 19, 2004	113 years, 354 days	USA
Yisrael Kristal	Sep 15, 1903	Aug 11, 2017	113 years, 330 days	Israel
Johnson Parks	Oct 15, 1884	Jul 17, 1998	113 years, 275 days	USA

highest life expectancy with 86.8 years for females and 80.5 years for males, and Sierra Leone having the lowest at 50.8 years for females and 49.3 years for males (Fig. 1.1). One factor which might account for the lower life expectancy in Sierra Leone and other African countries may be related to healthcare expenditures. A study published in 2017 found a positive relationship between healthcare expenditures and life expectancy and a negative correlation with the number of neonatal, infant, and under-five deaths [5]. From these data, it is clear that environmental factors can also have an impact on life expectancy. A study of 28 European Union countries published in 2014 explored potential determinants of life expectancy at birth, namely gross domestic product (GDP) growth rate, population growth rate, level of education attained, education enrolment, GDP per capita and life expectancy in the period from 2001 to 2011 [6]. The results of this study found that GDP per capita and attained education level explained 72.6–82.6% of the differences, depending on the year of observation.

Non-communicable diseases account for 63% of all deaths worldwide and approximately three quarters of these were incurred by individuals over 60 years old [7]. In 2016, ischemic heart disease and stroke were among the highest causes of

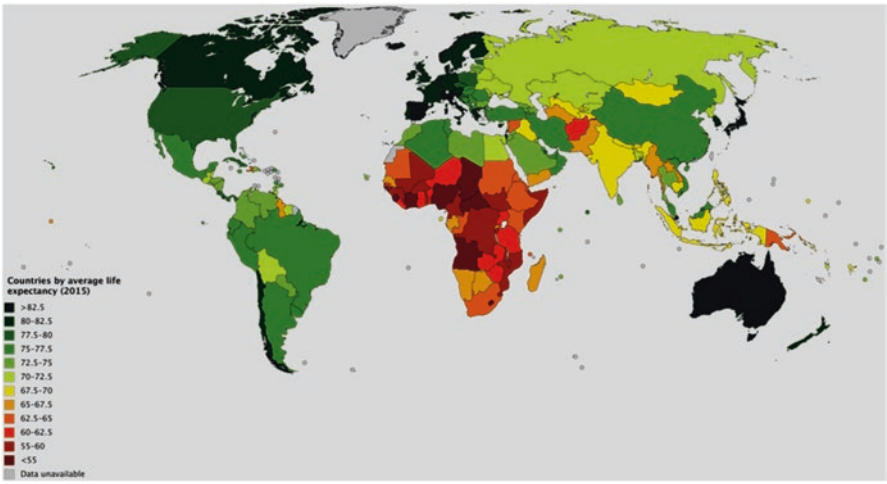

Fig. 1.1 Diagram of the world showing differing life expectancies of countries (according to the WHO, 2015). (Figure by JackintheBox - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=69178739)

death in the world with a combined 15.2 million deaths out of 56.9 million total deaths and chronic obstructive pulmonary disease, Alzheimer's disease and other dementias, lung and tracheal cancers and diabetes were responsible for a further 8.3 million deaths [8]. In addition, these age-related diseases are associated with prolonged disability and reduced productivity, and of course they also have a negative financial impact within families and heavy demands on healthcare systems [9].

Improvements in human longevity and healthy aging are among the most difficult challenges facing modern medicine. Life expectancy has more than doubled in the last hundred years and people aged over 85 (the "very old") constitute the fastest growing across all age groups due to increases in life expectancy and advances in medical support [10]. Several studies on the very old have found that the appearance or progression of non-communicable diseases is delayed in this group compared to other age groups, suggesting that healthspan and longevity are intimately linked [11–14]. These characteristics suggest that other factors beyond environmental conditions play an important role in regulation of lifespan.

The molecular mechanisms by which longevity has evolved are still mostly unexplained. However, the comparative analyses of genomes, transcriptomes, proteomes and metabolomes of long-lived and short-lived species, along with those of model organisms, has allowed us to increase our understanding of this process [15–23]. This review presents an overview of these studies with a focus on long-lived mutant mice and worms, and the longest living natural species from giant tortoises to whales, sharks, clams and the immortal jellyfish (Fig. 1.2). The main aim is to identify conserved genes and physiological networks involved in the aging process and longevity.

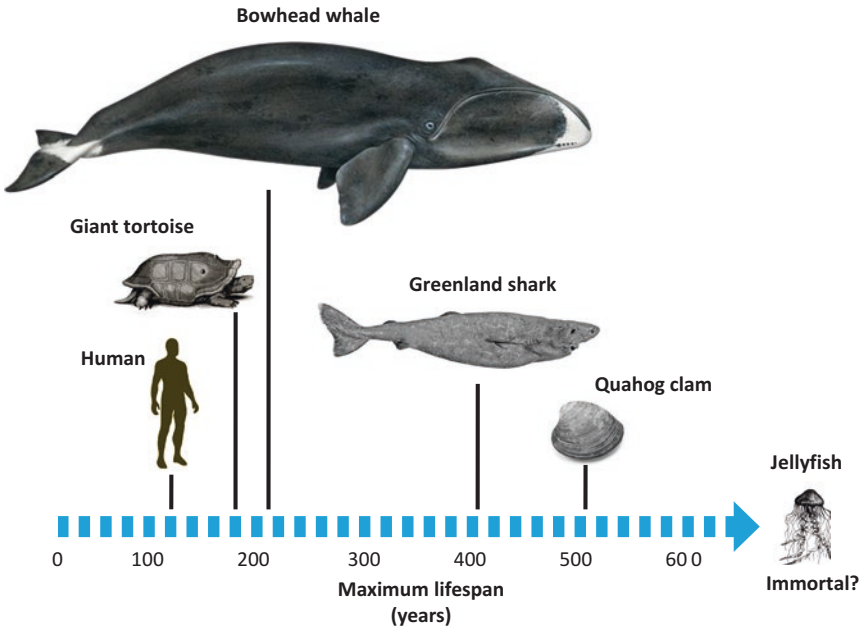

Fig. 1.2 Timeline showing the maximum lifespan recorded for some of the longest-lived species

2 What Have We Learned from Studying the Very Old: The Human Perspective?

There has been an increase in research since the death of Jeanne Calment to identify the genetic, environmental and lifestyle components associated with extreme longevity. One approach has been to study population groups sometimes called the oldest-old, such as centenarians who lived long lives essentially free of disease. With this in mind, a number of genome-wide association studies (GWAS) of various human populations have been performed to identify genes associated with living a long time [12, 24–28]. Such studies have shown associations of *APOE* and *FOXO3A* variants with longevity and these findings have been confirmed in other population studies [29–31]. Not surprisingly, maintenance and repair of the genome have also been identified as important factors for supporting a long life. This has been shown by studies on centenarian populations which found that such individuals appear to have an enhanced DNA repair capacity with significantly lower genomic and cellular damage [32–35].

In an interesting approach, a genetic study of long-lived smokers identified an interaction network of 215 single nucleotide polymorphisms (SNPs) that was significantly associated with increased survival and low cancer rates [36]. The strategy was to potentially identify genes involved in enhanced maintenance and survival by

studying individuals who had lived long healthy lives despite the fact that they were smokers. The identified network was enriched in platelet-derived growth factor (*PDGF*), *RAS* and *RAP1* and pathways which have implications for cancer pathogenesis [37–39]. In addition, the results showed that pathways potentially linked with aging were enriched and of these, PI3K/AKT signalling showed the highest enrichment score. This pathway comprised genes related to stress resistance, DNA repair, cell death, protein turnover and antioxidant function [40]. The PI3K/AKT pathway is known to be activated by insulin and insulin-like growth factor (IGF) signalling, which have also been implicating in the aging process and longevity [41–43]. Another SNP in the forkhead transcription factor *FOXO3A* gene was identified consistent with a previous study which found that two SNPs in *FOXO3* were associated with extreme longevity in two different groups comprised of individuals with German [29] or Japanese [44] ancestry.

In addition to looking at gene polymorphisms, another study compared the circulating levels of specific proteins, lipids and glucose as potential biomarkers in long-lived and short-lived families as a means of identifying protective genes linked to healthy aging [45]. This showed that the oldest-old individuals had higher serum levels of apidonectin. This is interesting as adiponectin is produced by adipose tissue and has anti-atherogenic and insulin-sensitizing activities, and low levels of adiponectin have been used as a biomarker of metabolic syndrome [46, 47]. This is consistent with a recent study that used genetic analyses as a way of identifying circulating factors as potential biomarkers of aging, longevity and age-related diseases [48]. As part of this study, a panel of hormones was identified, consisting of adiponectin, IGF-1, resistin, ghrelin and growth hormone (Table 1.3).

An earlier database study identified 20 circulating biomarkers associated with mortality risk through examination of 11,555 different publications [49]. This most significant associations were found for brain natriuretic peptide, cholesterol fractions, C-reactive protein, erythrocyte sedimentation rate, fibrinogen, granulocytes, homocysteine, intercellular adhesion molecule-1, N terminal-pro brain natriuretic peptide, white blood cell count, granulocytes, homocysteine, intercellular adhesion molecule-1, neutrophils, osteoprotegerin, procollagen type III aminoterminal peptide, uric acid, soluble urokinase plasminogen activator receptor, tissue inhibitor of metalloproteinases 1 and tumour necrosis factor receptor II. The fact that only one biomarker, intracellular adhesion molecule 1, overlapped across both studies suggests that considerable further research is necessary.

In terms of microRNAs, miR-17-5p has been identified as potentially being involved in longevity and cancer through its role in cell cycle regulation, proliferation and apoptosis [50]. This microRNA is expressed highly in embryonic stem cells and a transgenic miR-17-5p mouse model showed an extended lifespan [51]. However, this microRNA may also be involved in cancer regulation and so further studies are again merited to determine its use as a potential biomarker or therapeutic target.

In the cases of hormones, most of the research has been conducted on insulin, IGF-1 and growth hormone. It is well known that serum levels of both of these hormones are altered with advancing age. One study of 49 centenarians found that

Table 1.3 Panels of potential circulating biomarkers associated with aging, longevity and age-related diseases identified through genetic analyses. The panels are divided into seven major pathways [48]

Pathway	Potential biomarker panel component
Calcium homeostasis	Calreticulin
	Regucalcin
	S100 calcium binding protein B
Cytoskeleton and hormones	Alpha-klotho
	Adiponectin
	Fibroblast growth factor 21
	Fibroblast growth factor 23
	Ghrelin
	Growth hormone
	Insulin-like growth factor 1
	Leptin
	Resistin
Fibrosis	Angiotensinogen
	Plasminogen activator
	Plasminogen activator inhibitor
	Transforming growth factor beta
Inflammation	Alpha-defensin
	C-X3-C motif chemokine ligand 1
	C-X-C motif chemokine ligand 10
	Interleukin 6
	Pentraxin
	Intercellular adhesion molecule-1
Mitochondria and apoptosis	Amyloid beta precursor protein
	Fibronectin type III domain containing 5
	Growth differentiation factor 15
	Lactate dehydrogenase
	Vimentin
Neuromuscular junction	Agrin
	Brain derived neurotrophic factor
	Complement factor 3
	High mobility group box 1
	Interleukin 1 receptor like 1
	Progranulin
	Soluble receptor for advanced glycosylation end products
Other principles	Adenosylhomocysteinase
	Glycoprotein nonmetastatic melanoma protein B
	Keratin 18
	Lactoferrin
	Micro ribonucleic acid panel (to be defined)

serum IGF-1 levels appeared to be correlated to short-term nutritional status and low levels of this hormone may be involved in progression of dementia [52]. A randomized control trial of overweight, sedentary individuals placed on a calorie-restricted diet for 6 months found that fasting insulin levels and body temperature were both reduced [53] and survival studies of overweight and obese people have led to the suggestion that long-term calorie restriction could prevent weight gain and add 3–13 years to life expectancy [54]. In line with this, a recent study found that changes in the levels of insulin, IGF-1 and IGF binding proteins 1 and 2 could be used as biomarkers to monitor the effects of dietary restriction [55]. Another study which assessed various hormones and cytokines in centenarians found that low levels of leptin, IGF-1 and IGF binding protein 3, and high levels of tumor necrosis factor-alpha were associated with increased mortality [56]. An investigation of a healthy cohort of 476 individuals aged 16–104 years, living in the West Coast of Southern Italy, found that both blood levels of IGF-1 and leukocyte telomere length decreased significantly with age, consistent with the cellular senescence hypothesis of aging [57]. In addition, the role of eroded telomeres in cellular aging has been well established and has been reviewed elsewhere [58–60].

In possible contrast to these studies, the Baltimore Longitudinal Study of Aging, which compared the levels of various circulating biomarkers between participants who survived to at least 90 years of age and those who died between 72–76 years of age, found no differences in the circulating levels of ghrelin, insulin, leptin, interleukin 6, adiponectin and testosterone [61]. Taken together, these findings indicate that there is still a considerable amount of research warranted concerning the levels of circulating biomarkers of aging and longevity.

Another factor which should be taken into account in human aging and longevity studies is the effect of potential biomarkers related to oxidative damage. One study found a lipid profile characterized by a higher ratio of monounsaturated compared to polyunsaturated lipids, indicative of lower oxidative stress [62]. This was consistent with a combined nuclear magnetic resonance imaging metabonomic and shot-gun lipidomic analysis of human serum which found differences in the levels of 41 lipids associated with greater antioxidant capacity in centenarians compared to younger controls [63]. Another study tested the potential of administering a dietary antioxidant in the form of pomegranate juice in preventing memory loss in old subjects, using memory testing and functional magnetic brain imaging as the outcome measures [64]. This demonstrated an increase in visual memory task scores and functional brain activity in the group receiving the pomegranate juice. One inconsistent study of older community-dwelling adults found that urinary levels of the antioxidant resveratrol was not associated with inflammatory markers, cardiovascular disease or cancer, and was not predictive of all-cause mortality [65].

3 The Lessons from Model Species

3.1 Mice

The main parameters seen in association with increased longevity in mouse models are: (1) suppression of the growth hormone/IGF-1axis; (2) decreased metabolism; (3) increased resistance to oxidative stress; (4) reduced insulin secretion; (5) enhanced insulin sensitivity, (6) delayed maturation; and (7) reduced fecundity [66]. Studies of growth hormone-deficient mutant mice show that these animals live 50% longer and show differences in energy metabolism regulation, compared to wild-type mice [67]. In line with the above parameters, reduced growth hormone signalling is thought to increase longevity by leading to increased stress resistance, reduced growth, altered cytokine production by adipose tissue, increased insulin sensitivity and increased oxygen consumption [68]. Mice with an extended lifespan have also been produced by gene deletions or other manipulations leading to a deficiency in the levels of IGF-1 [66, 67, 69, 70], growth hormone [66–69], insulin [43, 70–72] and insulin receptor substrates (IRS) 1 [73] and 2 [74], and in the p70 ribosomal protein S6 kinase 1 (S6K1) [75]. S6K1 is involved in regulation of cell growth via the mammalian target of rapamycin (mTOR), a pathway known to be involved in aging through studies showing that the mTOR inhibitor rapamycin increases mouse longevity [76–78]. Furthermore, insulin is known to regulate nutrient and metabolic homeostasis via the IRS/PI3K/AKT signalling pathway that converges on regulation of FOXO1 and mTOR [79].

There is also an oxidative stress theory of aging which proposes that the rate of aging is regulated by accumulation of oxidative damage [80]. According to this hypothesis, damage occurs through accumulation of reactive oxygen species (ROS) which induces tissue degeneration. The oxidative stress theory of aging was later expanded to include the mitochondrial theory of aging, which proposes that ROS resulting from the mitochondrial respiratory chain damages macromolecules such as lipids, proteins and mitochondrial DNA [81]. This leads to a viscous cycle of defective mitochondrial function, in turn causing further ROS generation and oxidative damage. Both theories are supported by the fact that animals accumulate oxidative damage as they age [82]. Early on, this theory gained a lot of support although the results of recent studies suggest that oxidative stress or damage does not affect the aging process, measured as the mean or maximum lifespan [83]. However, a number of studies have demonstrated that reduced expression of antioxidants is associated with accelerated age-related diseases. For example, heterozygous knockout mice with reduced manganese superoxide dismutase ($SOD2^{+/-}$) show increased incidence of neoplasia with age, compared to wildtype control mice ($SOD2^{+/+}$) [84]. The $SOD2^{+/-}$ mice also have increased incidences of cardiac hypertrophy [85] and deficits in glucose metabolism [86]. Conversely, overexpression of *SOD1* has been found to have beneficial effects in mouse models of atherosclerosis [87], metabolic dysfunction [88], Alzheimer's disease [89] and Parkinson's disease [90]. Similarly,

overexpression of *SOD2* has been found to prevent cardiac myopathy [91], age-related defects in glucose metabolism [88] and Alzheimer's disease [92].

Caloric restriction has been shown to decrease age-related chronic diseases and extend lifespan in many species, including mice and rats [93, 94]. In rodents and primates, caloric restriction has been found to lower circulating glucose, insulin, cholesterol, triglycerides and IGF-1 levels [95]. Many of the effects of calorie restriction are also seen in dwarf mice. The lifespan of the Ames and Snell dwarf mice is significantly longer than that of their phenotypically normal control siblings [96, 97]. The mean age at death of dwarf mice exceeds the corresponding value in the normal controls by approximately 40% and is thought to be due to delayed aging of the dwarf mice. Consistent with the theories on aging and longevity, the phenotype of these dwarf mice is also characterised by increased levels of the antioxidant enzymes catalase and SOD [98, 99], a reduced metabolic rate and core temperature [100], lower plasma glucose levels and increased insulin sensitivity [101], and reduced growth hormone and IGF-1 levels [102].

Finally, the *SIR2* gene is linked to longevity in yeast, Caenorhabditis elegans and Drosophila [103]. *SIR2* and the mammalian orthologue *SIRT1* are NAD-dependent deacetylases, and both are thought to link metabolism with lifespan [104]. This is consistent with the finding that some of the effects of moderate calorie restriction include changes in expression of *SIRT1*-related genes in mice and other species [105]. SIRT1 inhibits adipogenesis in white adipose cells via repression of peroxisome proliferator-activated receptor γ (PPARγ), it regulates insulin secretion by pancreatic β cells and it deacetylates and activates expression of genes regulating gluconeogenesis in the liver [103].

To summarize this section, insulin signalling regulates nutrient and metabolic homeostasis via the IRS/PI3K/AKT signalling pathway that targets FOXO1 and mTOR. Insulin signalling also regulates the mitochondrial electron transport chain by suppression of FOXO1 to maintain the NAD+/NADH ratio, which in turn regulates the SIRT1 pathway required in mitochondrial function. Conversely, mitochondrial dysfunction can lead to high ROS levels that could further damage mitochondria and other cellular components leading to insulin resistance.

3.2 Worms

FOXOs are transcription factors, featuring a conserved Forkhead box, involved in regulation of important cellular processes such as cell cycle arrest, apoptosis, metabolism, stress resistance, aging and longevity [106]. Invertebrates such as Caenorhabditis elegans (C. elegans) have only one FOXO gene, named *DAF-16*, named after the dauer phenotype of a developmentally arrested larval form of greater durability that is induced by crowding or starvation [107]. More recent investigations have found that *DAF-2*, *DAF-23* and *DAF-16* are all part of the same branch of the dauer regulatory pathway involved in transduction of a pheromone signal for dauer formation [108]. An elegant study carried out by Kenyon et al.,

published in 1993 showed that *DAF-2* C. elegans mutants have a lifespan that is approximately doubled and this effect is suppressed by a mutation in *DAF-16* [109]. These findings suggested that *DAF-2* mutations can extend lifespan through activation of *DAF-l6*.

Several other gene mutations have been identified in C. elegans that lead to increased longevity and these can be assigned to pathways such as insulin/insulin-like signalling, the dietary restriction pathway and translation control [110–114]. A recent study showed that administration of the traditional Chinese herbal medicine Ganoderma lucidum promoted C. elegans to resist oxidative stresses induced by paraquat and the heavy metal Cr^{6+}, and it prolonged lifespan [115]. This protection occurred through the same pathways seen though dietary restriction and mTOR/S6K signalling, respectively. Another traditional Chinese medicine was found to extend the lifespan of C. elegans though the insulin/IGF-1 signalling pathway [116]. Another study suggested that administration of the pro-antioxidant compound selenocysteine can mimic the effect of dietary restriction on lifespan in C. elegans [117]. This was consistent with previous studies of C. elegans showing that administration of selenium inhibited Mn-induced toxicity and led to increased expression of SOD-3 and nuclear localization of DAF-16 [118]. Furthermore, dietary supplementation with selenocysteine increased resistance to oxidative stress, extended lifespan without reduction of fertility, and delayed age-related decline of motility [119].

Another recent study showed that the levels of the miR-58 family of miRNAs, which are effectors of insulin signalling, are increased in long-lived C. elegans mutants, again providing a link to dietary restriction effects [120]. This was consistent another miRNA study which identified a number of factors involved in regulation of lifespan, as reviewed by Smith-Vikos and Slack in 2012 [121] and summarized in Table 1.4. The pathways involved were related to either insulin/IGF-1 signalling or cell cycle control. Another investigation into the role of miRNAs in regulation of C. elegans lifespan found that 6 miRNAs were either up- or down-regulated during aging and in *DAF-2* long-lived mutants and five of these showed an opposite regulation (Table 1.5) [122]. Specifically, miR-237, miR-62 and miR-252 were found to be decreased during aging and increased in the *DAF-2* mutants. Conversely, miR-239b and miR-246 were increased during aging and decreased in mutants. Thus, these miRNAs are potentially involved in regulation of genes that might represent good targets for regulation of lifespan.

Taken together, these studies of rodents and worms indicate the conservation of insulin/IGF signalling pathways involved in the aging process and longevity

Table 1.4 miRNAs involved in the aging process identified through loss of function studies in C. elegans

miRNA	Loss of function mutant	Pathway
Lin-4	Shortened lifespan - aging phenotype	DAF-2 activation, DAF-16 repression
miR-71	Shortened lifespan - aging phenotype	Represses AGE-1/PDK-1/CDC-25.1/CHK-1
miR-239	Longer lifespan	Activates AGE-1 and PDK-1

Table 1.5 miRNAs up- or down-regulated >two-fold during normal aging (day 0–10) or in DAF-2 C. elegans mutants at day 0 or day 10 [122]

miRNA	Regulated during aging	Regulated in DAF-2 mutants
miR-237	↓	↑
miR-253	↑	↑
miR-62	↓	↑
miR-252	↓	↑
miR-239b	↑	↓
miR-246	↑	↓

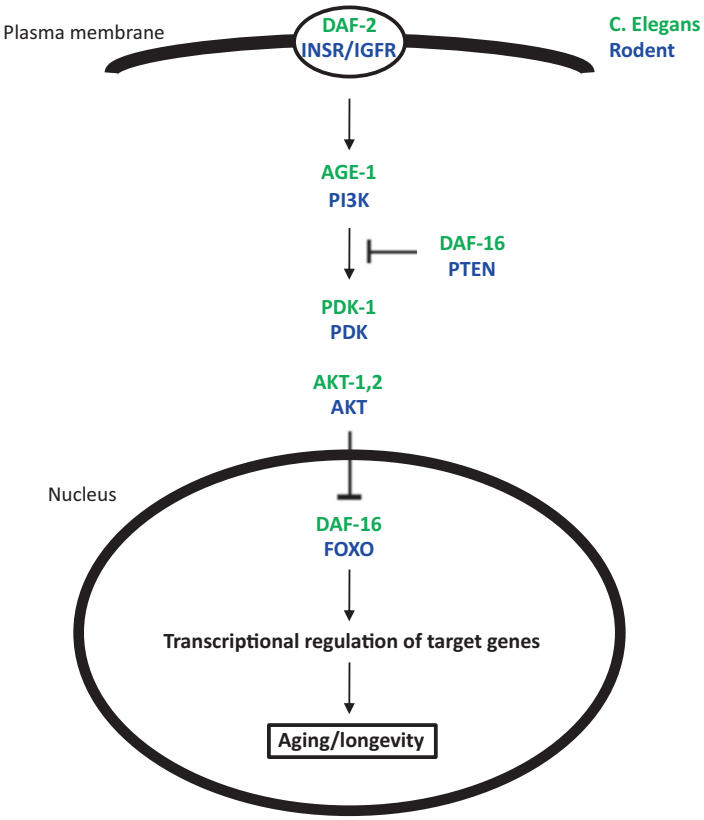

Fig. 1.3 Corresponding insulin/IGF signalling pathways form C. elegans and rodent studies indicating corresponding proteins or classes of proteins

(Fig. 1.3). Reduced insulin-like signalling through a cascade orthologous to the rodent and other mammalian insulin receptor/PI3K/AKT pathway, leads to an extended lifespan [123, 124]. Binding of the DAF-16 transcription factor to various genes leads to the lifespan extension phenotype.

4 Giant Tortoise

Little has been published on the giant tortoise regarding lifespan research although this should be an excellent point of focus in this field. The Galapagos giant tortoise (*Chelonoidis nigra*) has one of the longest life spans among vertebrate species [125]. A female named "Harriet" was thought to have been collected from the Galapagos Islands by Charles Darwin and estimated to be 176 years old when she died in 2006 at the Famous Australia Zoo on the Sunshine Coast in Queensland, Australia [125–127]. I had the opportunity to visit the Galapagos Islands in 1987 and saw many of these marvellous creatures on various islands, some of which could have been alive at the time Charles Darwin visited these islands during the second voyage of the HMS Beagle in the latter half of 1835 [128].

The only molecular study carried out thus far on this species was just published in 2019 [129]. This was a global comparison analysis of the genomes of Lonesome George (the last known member of Chelonoidis abingdonii) and the Aldabra giant tortoise (Aldabrachelys gigantea) using both unsupervised and supervised analyses. This analysis resulted in identification of gene variants associated with DNA repair, mitochondrial function, inflammation and cancer development, which may be associated with some of the characteristics of these animals, including their gigantism and longevity.

5 Bowhead Whales

The lifespan of some whale species is approximately double than that of humans [130]. The bowhead whale (Balaena mysticetus), a species of right whale (Balaenidaem) that lives in Arctic and sub-Arctic waters, and subsists on a lipid-rich diet of krill and other small marine animals, has been estimated to live over 200 years [131]. This has been determined by chemical analyses of eye lenses of bowhead whales captured by native Alaskan hunters in the Arctic [132]. In addition, stone and ivory weapon fragments have been recovered from bowhead whales hunted near Alaska, USA, in the 1980s and 1990s although the dates of manufacture are not known. More recently bomb lance fragments that were likely to have been manufactured between 1879 and 1885 were recovered in 2007 from four bowhead whales [133]. From this information, the hunters estimated that the oldest of these whales was approximately 211 years old and the younger specimens were between 135 and 172 years old [134]. In addition to a longer lifespan, the bowhead whale has a lower incidence of disease in old age compared to humans [135]. Consistent with evolutionary theory, the longevity of whales has been associated with low fertility and extended developmental times, as seen for humans [136]. The longevity of whales is also consistent with theories that larger animal species tend to live longer [137, 138].

The molecular mechanisms underlying the increased longevity in the bowhead whale have not been fully elucidated, although cross species comparisons have allowed us to gain some insights. In genomic studies, the identification of loci most undergoing adaptation allows us to gain insights into evolutionary biology. This is achieved by comparing the codon substitution at presumably silent sites with those experiencing the most selection [139]. A higher rate of substitution occurs if natural selection favours changes in the protein sequence. Thus by comparing the genomes of long- versus short-lived species, we may be able to highlight those genes which are most associated with the aging process and longevity [24]. The publication of the genomes of the shorter-lived minke [140] and longer-lived bowhead [22] whales has made this possible. To determine selective pressure variation in bowhead whale, a protein-coding database was constructed for the bowhead, minke and orca whales, in a comparison with several terrestrial mammals of varying lifespans (chimpanzee, cow, dog, elephant, gibbon, gorilla, guinea pig, horse, human, macaque, marmoset, microbat, mouse, opossum, orangutan, platypus, rabbit and rat). In addition, the authors aligned each bowhead whale protein with orthologues from 9 other mammals (cow, dog, dolphin, elephant, horse, human, minke whale, mouse and rat) to identify the unique bowhead amino acid residues. Next gene expansion analysis was performed by construction of gene trees assembled from 21,069 (human), 22,275 (mouse), 19,292 (dog), 19,988 (cow), 15,769 (dolphin), 17,936 (platypus), 20,496 (minke whale) and 22,733 (bowhead whale) genes to identify duplications in the bowhead whale genome.

Their findings identified several genes under positive selection and mutations in genes specific in the bowhead whale associated with cancer and aging. They also indentified both gains and losses involving genes linked with DNA repair, cell-cycle regulation, cancer and aging. In addition, they found changes in genes related to thermoregulation, sensory perception, dietary adaptations and immune response. Several genes related to aging and cancer showed high selective pressure in whales, including suppressor of cytokine signaling 2 (*SOCS2*), aprataxin (*APTX*), noggin (*NOG*) and leptin (*LEP*). The highest selective pressure was seen for the genes *FOXO3*, *ERCC3* (DNA repair), *FGFR1* (fibroblast growth factor receptor) in the bowhead whale and amino acid changes unique to the bowhead whale were found in *ERCC1* (DNA repair), *HDAC1* and *HDAC2* (histone deacetylases). In addition, gene duplications and unique amino acid changes were seen for PCNA (proliferating cell nuclear antigen; involved in DNA replication) and the amino acid sensor and mTORC1 activator *LAMTOR1*, which is associated with aging and cancer [141]. Finally, the *UCP1* gene (uncoupling protein 1; involved in thermoregulation) has a premature stop codon in the bowhead whale, which is likely to change its function. This is interesting as bowhead whales need to maintain stable body temperatures in the cold water and this stop codon distinguishes them from smaller mammalian species.

In 2014, a genome-wide gene expression analysis of the bowhead whale was published based on *de novo* assembly of its mRNA transcriptome using the Illumina RNAseq technology [21]. To identify transcripts important in longevity, a comparison was carried out of the bowhead whale liver, kidney and heart transcriptomes

with those of the minke whale, cow, yak, Brandt's bat, Chinese tree shrew, naked mole rat, rhesus macaque, mouse and rat. A total of 45 transcripts were found to be differentially expressed in the bowhead whale liver compared to the other mammals and the greatest difference was a reduction in the levels of growth factor receptor-bound protein 14 (*GRB14*). The encoded protein (GRB14) binds to the insulin and IGF-1 receptors, again implicating this signalling pathway in longevity and aging. Changes in proteins related to apoptosis and cell survival were also found, including Cbp/P300 interacting transactivator (CITED2) and TP53 apoptosis effector (PERP). In addition, altered levels of EPH receptor A2 (EPHA2) and replication protein A2 (RPA2) were detected, which promote vascular tone/proliferation and DNA repair, respectively. In the kidney, 53 transcripts were differentially expressed and many of these were associated with DNA repair and tumour suppression, and only 4 transcripts were altered in the heart transcriptome and one of these [argininosuccinate lyase (ASL)] is required in the production of nitrous oxide (NO), which is known to protect the heart during hypoxic events [142]. In the case of the whale, this could be related to its deep diving activities.

6 Greenland Shark

The Greenland shark (Somniosus microcephalus) is a resident of the seas of North Atlantic and Arctic regions [143, 144]. This shark is known to mature slowly, reach sexual maturity after approximately 150 years and grow to a length of approximately 5 m [145]. A radiocarbon dating study of eye lens from 28 female Greenland sharks (0.81–5.02 m in total length) revealed a life span of greater than 272 years [145]. The age of the largest shark was approximately 392 years. Another investigation studied several metrics of oxidative status measured in skeletal muscle and red blood cells of the Greenland shark but found no correlation with the species lifespan [146]. Instead the authors proposed that these matrices were linked to ecological features, such as adaptation to the cold water environment and the extreme depth that sharks of this species dive to. Although further studies are required to understand the mechanisms of how these sharks reach this great age, the results indicated above place the Greenland shark as the longest-lived vertebrate on Earth [147].

7 Ocean Quahog

The burrowing ocean quahog clam (Arctica islandica) is the longest lived of all non-colonial animal species on earth with a reported maximum species lifespan of more than 400 years [148, 149]. One study sampled the bivalve shell from a quahog clam specimen collected alive in 2006 from the north Icelandic shelf for radiocarbon analysis and annual band counting and found that this clam had lived for more than 405 years [150] and a more recent study found that one specimen had lived for

507 years [151]. Another study found that the extreme longevity of this species of clam might be due to a higher antioxidant capacity compared to shorter-lived clams [152]. These authors examined gill and mantle tissues of 4–192 year old quahogs and found that catalase and citrate synthase activities and glutathione concentrations declined rapidly within the first 25 years of life but thereafter remained stable for more than 150 years, and superoxide dismutase activity remained high over the entire life time. Another study tested this hypothesis by comparing reactive oxygen species production, resistance to oxidative stress and antioxidant defences in long-lived ocean quahogs with the shorter-lived hard clam (Mercenaria mercenaria) [153]. These analyses revealed lower protein carbonyl levels in the ocean quahog compared with the hard clam, consistent with the possibility of reduced ROS production resulting in lower macromolecular damage. In addition, exposure to the oxidative stressor tert-butyl hydroperoxide (TBHP) revealed a greater resistance to oxidative stress-induced mortality in the quahog. However, the authors found that the quahog and hard clams had similar antioxidant capacities and homeostatic antioxidant responses following TBHP exposure. Therefore, variations in antioxidant capacities do not account for the difference in the longevities of these two clam species. Nevertheless, more recent studies found that the ocean quahog have a lower degree of lipid peroxidation [154], more resistance to genotoxic stressors [155] and greater maintenance of protein homeostasis [156], compared to other short-lived clams species. Taken together, these findings suggest that increased antioxidant capacity and resistance to stressors may be linked with the long lifespan of the ocean quahog, although other potential mechanisms should also be explored.

8 Immortal Jellyfish

The immortal jellyfish (Turritopsis dohrnii) is thought to be the only animal capable of immortality [157, 158]. However, there is a trick to this. It's not because this jellyfish lives a long time. It's because it can effectively regenerate itself.

The adult form of the immortal jellyfish is approximately 0.5 cm wide. Once it has reproduced it can transform itself back into a juvenile polyp state. This involves retraction of the tentacles and shrinkage of the body using tissue from the circulatory canal and bell surface, in a process called transdifferentiation [159]. After or during this process, the jellyfish sinks to the seabed. The rejuvenated creature will then regain its sexual maturity in less than 30 days if the water temperature is at least 20 °C. This process has been observed to repeat itself indefinitely and so this jellyfish does not experience aging in the true sense [160]. Obviously, this incredible attribute of this creature has sparked considerable interest in the area of aging, stem cell and cancer research.

Although the transdifferentiation process has been documented, the underlying biological mechanisms of this process have not been fully elucidated. It is known that some jellyfish and other basal metazoans possess high levels of pluripotent stem cells capable of differentiating into all cell types [161]. However, during

evolution of more complex organisms, these capacities were thought to have been reduced or lost. One study carried out a phylogenetic analysis based on available 16 s rRNA gene and protein sequences of cytochrome oxidase subunit-I (COI or COX1) of the immortal jellyfish in comparison to similar species in order to identify its closest relatives [162]. This led to identification of 6 closely related species for further research. A more recent study sequenced the complete mitochondrial genome sequence of the immortal jellyfish, which contains genes encoding 13 proteins involved in oxidative phosphorylation and respiration, 2 transfer RNAs and 2 ribosomal RNAs [163]. Although this provided an initial characterization, thus far it has not allowed any insights into how this species can regenerate itself. This will require complete sequencing of the genome and appropriate cross comparisons with other species.

9 Conclusions and Future Perspectives

Aging of differentiated adult cells occurs via several mechanisms. These include: (1) damage to DNA molecules; (2) perturbations in proteostasis; (3) mitochondrial dysfunction; (4) inappropriate insulin/IGF-1 signalling; and (5) damage resulting from oxidative stress. Most of the species discussed in this chapter characterized by long lifespans appear to have the capacity to reduce the effects of one or more of these pathways through evolution of specific counter measures. The study of model organisms such as long-lived rodent and worm mutants has allowed us to investigate these pathways in detail and identify potential modulatory agents. Furthermore, the investigation of differences in these pathways in short- and long-lived animal species has provided additional insights into how exceptional longevity can be achieved. Together, these approaches have improved our understanding of longevity regulation and provide a starting point for the development of interventions that can potentially maximise the human healthspan.

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Chapter 2
Effects on Glial Cell Glycolysis in Schizophrenia: An Advanced Aging Phenotype?

Giuliana S. Zuccoli, Paul C. Guest, and Daniel Martins-de-Souza

1 Introduction

Schizophrenia is a psychiatric disorder known to affect approximately 1% of the world population. It is manifested through a wide range of severe symptoms and onset occurs typically in late adolescence or early adulthood. However, the origins of schizophrenia are likely to be found early in development, long before the onset of symptoms [1, 2]. Over the years, many studies have been conducted in attempts to understand this disorder and it has been proposed that the disease occurs due to small flaws in several brain areas rather than more global damage in particular brain regions. Therefore, the disorder could arise from defective connections between components of the neural system [3].

Imaging, proteomic and genetic studies have found evidences of alterations in metabolic processes related to energy pathways in patients suffering from this disorder and, given the importance of proper functioning of energy handling in the brain (Fig. 2.1), this has been accepted as one important feature of schizophrenia [4–6]. In fact, the findings of proteomic profiling studies have suggested that, in the context of energy metabolism, glycolysis is the predominantly affected pathway in schizophrenia (Fig.2.2) [7].

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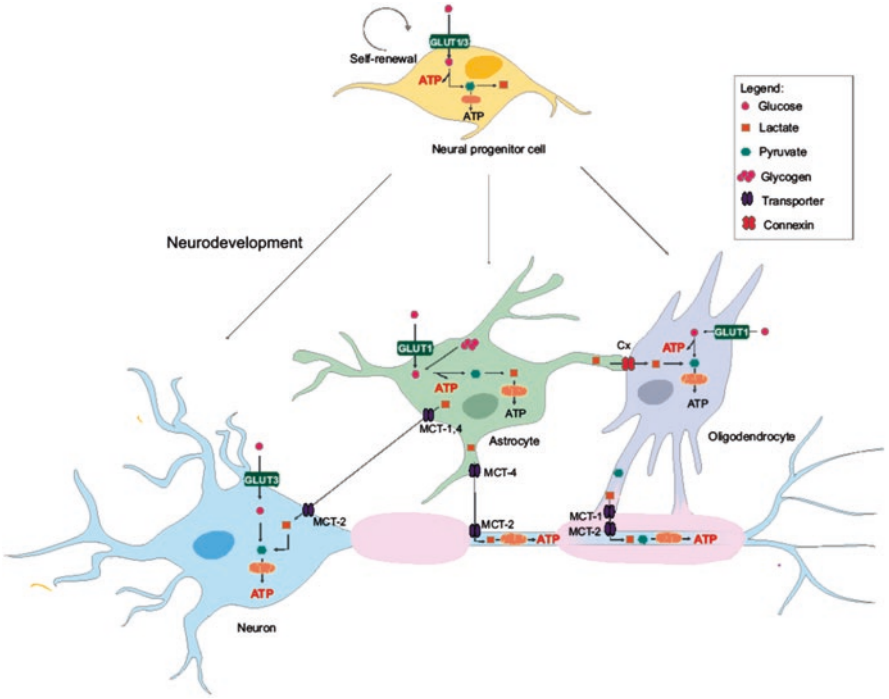

Fig. 2.1 Schematic representation of glucose handling during neurodevelopment and in the adult brain, showing the importance of cooperation between neurons, astrocytes and oligodendrocytes in the context of energy metabolism. ATP molecules marked in red represents the pathway that is the major source of energy: Glycolysis for neural progenitor cells, astrocytes and oligodendrocytes; Oxidative phosphorylation for neurons (cell body and axons)

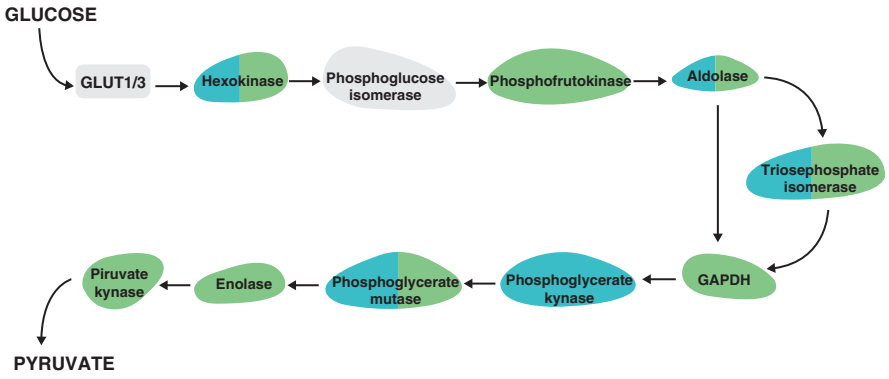

Fig. 2.2 Evidence from proteomic studies showing compromised glycolysis in schizophrenia. Proteins in green represent those found to be altered in postmortem brain tissue from schizophrenia patients, whereas those in blue represent proteins found to be altered in cell-culture models of the disorder

Glucose is the obligatory energy substrate of the brain and this organ accounts for approximately one quarter of the whole-body glucose utilization despite the fact that it only accounts for 2% of the total body weight, [8]. In addition, glucose is an essential component of macromolecules such as glycolipids and glycoproteins in neural cells, and it enters the metabolic pathways that result in the synthesis of three key neurotransmitters of the brain, glutamate, GABA and acetylcholine [9].

Glucose is sequentially processed by glycolysis in the cell cytosol and processing through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation takes place in the mitochondria [10]. The main energy-consuming process of the brain is the maintenance of ionic gradients across the plasma membrane, a condition that is crucial for excitability. Maintenance of these gradients is achieved predominantly through the activity of ionic pumps fueled by ATP [11]. Neurons represent approximately 80% the brain energy requirements whereas glia account for around 10% [12]. Axonal transport of molecules synthesized in the cell body along the axon is another process fueled by cellular energy metabolism [10].

Given the cellular heterogeneity of the brain, which is composed of neurons and glial cells, is important to note that different cell types have distinct metabolic profiles. In fact, neurons are predominantly oxidative whereas glycolysis predominates in astrocytes and oligodendrocytes [13, 14]. Hence, astrocytes and oligodendrocytes are responsible for providing lactate to the neuronal cell body and axon, respectively, where it can be further metabolized and provide ATP to insure the maintenance of neuronal function [15].

Given the evidences of compromised glycolysis in schizophrenia, is important to understand energy metabolism from a glial perspective, as opposed to a neuronal-centric view. In addition, metabolism in neuronal cells is altered in the aging process and various regions of the brain are thought to undergo accelerated aging in individuals with schizophrenia compred with healthy controls. Therefore, we present the role of glial cells in energy metabolism of the brain and consider the evidence for the advanced aging theory of schizophrenia in these cells. Focussed efforts in this area could lead to identification of novel biomarkers and potential drug targets for improved treatment of individuals with schizophrenia and other psychiatric or neurodegenerative disorders.

2 The Role of Glia Cells in Brain Energy Metabolism

2.1 Astrocytes

Astrocytes are the most abundant glial cells in the brain, exceeding the number of neurons by five fold. Astrocytes are involved in many brain functions, such as the formation of the blood-brain barrier (BBB), taking up neurotransmitters at glutamatergic synapses, immune function support, composing tripartite synapses with pre and post-synaptic neurons and providing energy support to neurons [16, 17].

Astrocytes are essentially the only cells containing glycogen in the adult central nervous system (CNS), and glycogen metabolism followed by glycolysis provides a source of lactate to other cells [18]. The most accepted hypothesis to explain this supply of lactate is the astrocyte-neuron lactate shuttle (ANLS) [19]. This hypothesis postulates that upon glutamate release from the presynaptic terminal of a neuron, the neurotransmitter activates the astrocyte membrane transporters and/or receptors leading to the transport of glutamate into astrocytes. This uptake of glutamate leads to the activation of glucose utilization in astrocytes and glucose uptake from the circulation through the glucose transporter GLUT1 [15]. Upon the synthesis of astrocyte-derived lactate via glycolysis, it diffuses out via the astrocyte monocarboxylate transporter types 1 and 4 (MCT-1 and has a high affinity for lactate [20]. Once inside the neuron cell body, lactate undergoes subsequent metabolic reactions in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation to produce more ATP and other substances required for neuronal functioning (Fig. 2.1) [21].

In addition, studies of both astrocytes and oligodendrocytes have demonstrated that astrocytes also transfer energy metabolites directly to those cells. Connections between astrocytes and myelinating cells occur via gap junctions formed by connexins in the plasma membranes of both adjacent cells [22]. Those connexins form channels that promote the exchange of molecules, including lactate, between connected cells [23, 24]. Also, the formation of gap junctions between astrocytes and oligodendrocytes have an important role in K+ release during neuronal activity and in buffering extracellular glutamate [25].

Studies using a coculture model of astrocytes and neurons have shown that the enhancement of the astrocyte-neuron lactate shuttle process through over-expressing GLUT1 in astrocytes and MCT2 in neurons leads to neuroprotection against excitotoxic insults [26]. In contrast, the impairment of lactate delivery to neurons leads to neuronal damage. Therefore, lactate transfer from astrocytes to neurons seems to exert an important neuroprotective role and possibly indicates a therapeutic strategy under glucose-deprivation conditions and against neuronal insults associated with intracellular energy deficiency such as excitotoxicity [15].

2.2 Oligodendrocytes

Oligodendrocytes are the most abundant cell type in white matter and are responsible for providing protection and electrical insulation of axons, through myelination. Neurons may have axonal processes that exceed the length of the neuronal body and this represents a challenge when considering electrical impulse conduction, transport of molecules to the axon end and metabolic maintenance [27]. In this way, oligodendrocytes are crucial to circumvent those possible limiting factors, given that the myelination of axons allows a saltatory nerve conduction, in which neuronal action potentials are restricted to the nodes of Ranvier, increasing conduction speeds by up to 100-fold [28].

As mentioned above, there must be a metabolic support for axons of energy substrates in order to assure axonal propagation of action potentials and rapid transport along axons. This important task is fulfilled by oligodendrocytes since the myelin sheath can be considered metabolically active, allowing the movement of molecules to the subjacent axon [29–31]. The metabolic coupling between oligodendrocytes and axons begins with the transfer of lactate or pyruvate from oligodendrocytes to the peri axonal space via MCT-1. Following this, lactate or pyruvate can enter axons through MCT-2 and further metabolized to then be processed in the axonal mitochondria for ATP generation (Fig. 2.1) [32, 33]. This metabolic coupling assures axonal and neuronal health and survival, as studies showed that in both cell-culture and animal models, the disruption of MCT-1 expression in oligodendrocytes resulted in impairments in the delivery of lactate, leading to axonal damage and neuronal loss [22, 32, 33].

One important aspect to consider is that the constant over-production of lactate would result in lactic acidosis, which could be detrimental for white matter function [34]. Following the discovery that oligodendrocytes have functional NMDA receptors [35, 36], there was much interest in understanding the function of these receptors. NMDA receptor activation in oligodendrocytes promotes the increased incorporation of GLUT1 into the membranes of oligodendrocytes and myelin and enhances glucose import into those cells. Therefore, it has been hypothesized that a mechanism to prevent exacerbated production of lactate could be that oligodendrocytes adapt their energy metabolism based on activity-dependent glutamate release [37].

3 Astrocytes and Schizophrenia

Astrocytes have been associated with neuroinflammation in some brain areas [38] and gene sets from these cells have been associated with increased risk for schizophrenia [39]. In addition, gene expression analysis of postmortem brain tissue from schizophrenia patients has shown alterations in the expression levels of glial fibrillary acid protein (GFAP), which is an astrocyte marker [40, 41]. S100B is an astrocytic protein that has been implicated in the regulation of several intracellular activities and is used as a marker of astrocyte function [42]. Increased levels of this protein have been observed in serum [43] and cerebrospinal fluid [44] of schizophrenia patients and high amounts of S100B have been hypothesized to be predictive for negative symptoms [45].

The administration of MK-801 in animals has been proposed as one possible model of schizophrenia and reduced levels of glutamine and glutamate derived from acetate have been observed, indicating changes in astrocytes metabolism upon treatment with this drug [46]. One study that analyzed the effects of acute and long-term treatment of MK-801 on an astrocyte cell line revealed that the levels of aldolase C were affected under acute conditions and aldolase C and hexokinase 1 levels were altered following long-term treatment, both being enzymes involved in glycolysis (Fig. 2.2) [47].

The protein disrupted in schizophrenia-1 (DISC-1) is expressed in astrocytes and a study showed that a mutated DISC-1 protein was associated with decreased levels of d-serine, which is a co-ligand of NMDA receptors [48]. The co-culture of DISC-1 mutated astrocytes with neurons promoted deficits in synaptic and dendritic maturation, and this phenotype was reversed after d-serine treatment [49]. In addition, one study observed that treatment of a primary astrocyte culture with the antipsychotic drug clozapine increased d-serine levels released by these cells, which subsequently enhanced the glutamate release by neurons [50]. These results indicate that astrocytes could be a target cell in the treatment of schizophrenia. In a recent study, the knockdown of endogenous DISC-1 in primary astrocytes resulted in decreased levels of GLUT4, reduced glucose uptake and diminished production of lactate by astrocytes [51]. In addition, the same study showed that the expression of a truncated version of the DISC-1 protein in mice resulted in metabolic perturbations associated with altered behavior and memory deficits, and this phenotype was reversed upon systemic treatment with lactate. Hence, there is the indication that alterations in astrocytes could lead to abnormalities in energy supply and may be associated with some aspects of schizophrenia.

In the context of neurodevelopment, astrogenesis is a major feature during brain development and one recurrent finding using mouse models of early stress has been deficits in glial density [52]. Astrocytes are derived from neural stem cells and play an important role in regulating neural development by coordinating synapse formation and function, neuronal survival and axonal guidance [53]. Given the important role of these cells, the high energetic demand during neurodevelopment (Fig. 2.1) and evidences of compromised glycolysis in schizophrenia, studies using iPSC derived astrocytes could bring insights into the underlying mechanisms of astrogenesis during neurodevelopment in the context of schizophrenia.

4 Oligodendrocytes and Schizophrenia

Several studies have found abnormalities related to functional and structural dysconnectivity in schizophrenia [54–56] and it has been hypothesized that oligodendrocyte dysfunction with subsequent abnormalities in myelin maintenance and repair could lead to the disrupted connectivity related to the disorder [57, 58]. Imaging investigations have shown decreased white matter in schizophrenia patients [59, 60] and postmortem studies have found alterations related to morphology and density of oligodendrocytes from schizophrenia patients, which suggests disturbances in energy metabolism in these cells and possible effects on axonal integrity [61–64]. Microarray analysis of postmortem dorsolateral prefrontal cortex from schizophrenia patients has revealed that oligodendrocyte gene transcripts are specifically reduced in the disorder [65]. Some of these are involved in myelination, such as myelin-associated glycoprotein (MAG), transferrin and myelin and lymphocyte protein (MAL). In addition to the altered myelinating genes, CNP1 was also found to be dysregulated and this gene encodes an oligodendrocyte-specific

protein located in the myelin sheath that is involved in oligodendrocyte differentiation and in maintaining axon function and survival [66].

Upon treatment of different cell lines with MK-801, which acts on the glutamatergic system and has been proposed as a pharmacological means of modeling schizophrenia, it was found that this glutamatergic manipulation resulted in significant alterations in proteins related to glycolysis in oligodendrocyte cell lines. Alterations in the levels of hexokinase 1, enolase 2, phosphoglycerate kinase, phosphoglycerate mutase 1 and triosephosphate isomerase have been observed following acute and long-term treatment (Fig. 2.2) [47, 67]. In addition, the subsequent treatment of these MK-801-treated oligodendrocytes with the antipsychotic drug clozapine reversed some of these glycolytic alterations, which leads to the possibility that certain antipsychotics may act as modulators of glycolysis in oligodendrocytes and thereby improve neuronal connectivity [68].

Given the neurodevelopmental origin of schizophrenia hypothesis, it has been hypothesized that early insults to the developing brain could promote an inflammatory environment, cause injuries to oligodendroglial progenitor cells and lead to the white matter abnormalities found in the disorder [69, 70]. Progenitor cells have a high division rate and, therefore, present an elevated energy demand, which is achieved by glycolysis (Fig. 2.1) [71, 72]. They present a complex program of differentiation until the formation of myelinating mature cells with several particularities related to their metabolism and physiology [73]. Therefore, alterations in energy metabolism in progenitor cells could have detrimental effects on oligodendrogenesis not only in the developing brain but in the adult brain as well, as they can differentiate into mature oligodendrocytes and promote re-myelination. In this way, the possibility to use human induced pluripotent stem cell (hiPSC) technology combined with the generation of oligodendrocyte progenitor cells to study the metabolic profile of progenitor cells in the context of schizophrenia could help to elucidate the role of those cells in the development of the disease [74, 75].

5 Effect of Aging on Oligodendrocyte and Astrocyte Metabolism

It is well known that the aging brain shows deficits in metabolic pathways such as those linked to glycolysis [76]. Aging appears to be associated with deteriorating systemic regulation of glucose metabolism which is linked in turn to declining brain glucose metabolism (Fig. 2.3). In addition, the progressive brain loss in schizophrenia appears to reflect an accelerated aging phenotype in various regions of the brain [77]. This has been seen through identification of a number of aging-related biomarkers in patients such as shorter telomere length in some brain areas and in various neuronal cell types [78]. Studies have also shown that telomere shortening occurs in peripheral blood cells in chronic stress [79], mood disorders [80] and schizophrenia [81], thus providing a potential peripheral biomarker of these diseases.

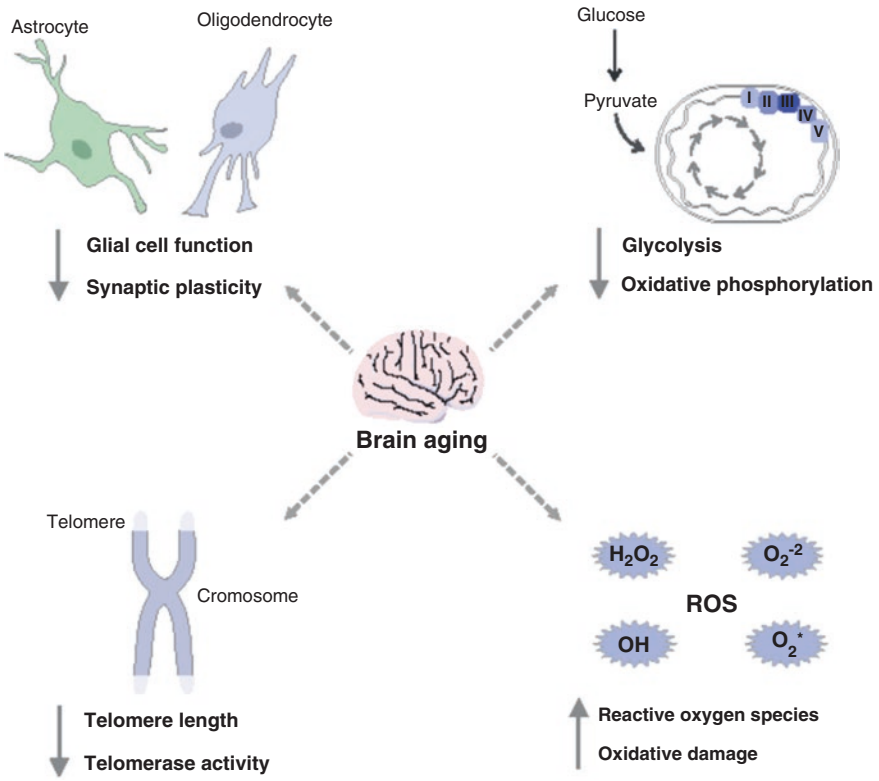

Fig. 2.3 The advanced aging hypothesis of schizophrenia: effects of disrupted glycolysis, oxidative damage and accelerated telomere shortening in glial cells of the brain

Telomeres are comprised of DNA and proteins at the end of each chromosome and these structures prevent the loss of genetic material during each cycle of cell replication. In differentiated cells, the telomeres shorten with each replicative division. This eventually induces DNA damage, leading to growth arrest and, ultimately, replicative senescence [82–84]. In undifferentiated stem cells, germ cells and progenitor cells, the loss of telomeres is protected by the enzyme telomerase, which adds TTAGGG repeats onto the ends of the chromosomes [85–89]. Experiments in telomerase-deficient mice provided the first proof that telomere erosion is a determinant of longevity and the occurrence of age-related pathologies [90, 91].

Studies have shown that schizophrenia patients exhibit an initial loss of gray matter followed by a deficit in white matter that progressively worsens with age [92]. Thus, the losses in white matter are likely to be linked more to aging and the effects on gay matter occur during the early stages of the disease in younger patients. Aging can cause a loss of normal astrocyte or oligodendrocyte function which reduces the ability of these cells to maintain a healthy environment in the brain [93, 94]. Culturing the oligodendrocyte cell line OLN-93 in the presence of glucose was

found to induce an aging phenotype [95]. This was seen as an elongation and thickening of cell processes, shortening of telomeres and increased expression of netrin. Consistent with our understanding of telomere shortening, this reflected a more mature state of oligodendrocyte development or differentiation. Furthermore, a laser capture micro-dissection study found an attenuated oxidative stress defence and deficient telomerase activity, contributing to telomere shortening in oligodendrocytes in major depressive disorder patients [96]. This suggests an aetiological link between telomere shortening and white matter abnormalities in schizophrenia, which may also occur in other psychiatric disorders. This is consistent with earlier studies which showed that differentiation of cultured oligodendrocyte precursor cells into mature oligodendrocytes is associated with lower activities of the telomerase enzyme [97]. These findings indicate that the population of neural stem cells that give rise to oligodendrocytes and astrocytes may be lower in patients with certain psychiatric or neurodegenerative disorders.

6 Conclusions

Many studies have suggested that energy metabolism dysfunction is an important feature of psychiatric disorders and the glycolysis pathway is believed to be the most affected in schizophrenia. In brain energy metabolism, glial cells are predominantly glycolytic and provide energy to the neuronal cell body and axon, respectively. Moreover, glial cells have important roles in neural development and in the adult nervous system and have surpassed their original definition as supportive cells. Thus, biomarkers associated with these cell types such as components of glycolysis and energy metabolism pathways may be useful in disease modelling and drug development studies. Finally, the hypothesis that schizophrenia may be seen as an advanced aging phenotype in some regions of the brain and neuronal cells such as glial cells is supported by the finding of accelerated telomere erosion in astrocytes and oligodendrocytes from schizophrenia patients. This may also be linked to advanced oxidative damage in these cells, consistent with the findings of studies on other diseases marked by accelerated telomere erosion [98]. Thus, studies of other biomarkers of the advanced aging phenotype, oxidative damage and disrupted glycolysis and energy production pathways may provide further useful information in studies of schizophrenia in both the acute and chronic stages (Fig. 2.3). In conclusion, evidence of glycolytic and aging-related alterations in astrocytes and oligodendrocytes in schizophrenia indicate the importance of studying this disorder from a glial-neuronal cell perspective.

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Chapter 3
Aging Biomarkers and Novel Targets for Anti-Aging Interventions

Kang Xu, Yannan Guo, Zhongchi Li, and Zhao Wang

1 Introduction

1.1 Aging of the Population

In 2017, the segment of the world population aged 60 and over reached 962 million, accounting for 13% of the world population, and the aged population is growing at a rate of 3% per year. At present, Europe has the highest proportion of the elderly, with a population 60 years and older accounting for 25% of the total population. By 2050, this proportion of the world population (except Africa) will reach 25% or even higher. At the same time, human life expectancy constantly rises, from 67 years in 2000 to 71 years in 2015, increasing by 4 years in a 5 year period [1]. Aging is unavoidable for mankind and the problem of population aging is a serious challenge faced by all countries. Aging has become a strategic issue affecting the long-term development of a country's economy. With the development of society and the gradual improvement of the public health system, people have begun to pay more and more attention to aging and age-related diseases. Aging is the strongest risk factor for diseases and dysfunction, ranging from neurodegeneration to cancer. This relationship likely arises from accumulation of genetic errors and effects of environmental factors [2]. In general, it is desirable to promote healthy aging.

Most species in nature obey the law of increasing mortality with age [3]. Aging and longevity are among the most complex topics in current biological and clinical research. Aging is the result of multifactorial effects, including genetic factors and genetic changes, external environmental stimuli and lifestyle factors. In aging research, we must be aware of the delicate balance between life extension and

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tumorigenesis. As reported in the past, the naked mole rat, the rodent species with the longest lifespan, has a maximum lifespan of over 30 years, surprisingly, without the extra incidence of cancer. Tian and colleagues have discovered that abundant high-molecular-mass hyaluronan (HA) in the skin and other organs/tissues plays a dominant role in longevity and cancer resistance of the naked mole rat [4]. We may obtain clues from such pioneering research to seek ways to slow down aging.

1.2 Biological and Chronological Age

Some scientists believe that chronological age is an essential risk factor for functional impairment and biological age may be a more accurate metric for prediction of aging [5]. Chronological age represents the actual age of a person, that is the time he or she has lived on this earth since birth. Chronological age insufficiently reflects an individual's general health status and susceptibility to diseases and disabilities associated with the process of aging. Biological age represents the physiological age of a person. Mostly, we have to take diet, exercise habits and other lifestyle factors and living environments into consideration. Patrick and colleagues have published a Health Aging and Body Composition Study and found that biological age is a better predictive factor for late-life depressive symptoms than chronological age [6]. Kim et al. has reported that biological age measurement can facilitate the assessment of colorectal adenoma risk associated with screening colonoscopy [7]. A cross-sectional study that has been conducted by Zhang and colleagues put forward a biological age-based formula to effectively monitor the degree and speed of aging in the Chinese population [8]. Biological age is better able to reflect a person's health state and indicates an individual's active-life expectancy, which is based on the studies above [9].

1.3 Aging Biomarkers and Breaking News

With the increasing worldwide life expectancy, the risk of aging-related diseases rises. Characterization of biomarkers associated with aging may open the door to aging predictions and antiaging-strategy discovery [10]. Over the years, numerous scientists have made important contributions to the search for accurate and adaptable aging biomarkers. Understanding the triggers for the aging process and the association between aging and diseases is important for the search and validation of aging biomarkers or a system that will boost basic gerontology and clinical translational research.

However, we have to deal with the complexity of the aging process, varying aging patterns and the diversity of aging causes. There are still no precise independent aging biomarkers that accurately reflect a person's aging status or predict aging speed and life expectancy.

Aging research has long been considered one of the most costly types of biological studies because it is time-consuming and expensive to use mammals as research subjects. Janssens et al. from the Karolinska Institute in Sweden has developed an innovative and age-stratified human-tissue transcriptomics-based cell culture system for geroprotector screening [11]. They found that monorden and tanespimycin, which are Hsp90 inhibitors, can induce a youthful transcriptional state in human cells, prolong the lifespan and help to maintain a healthy state in Caenorhabditis elegans. Nonetheless, the protective effect of these two compounds on humans remains to be investigated. From their article, maybe we can glean additional information related to aging and disease states of the cell from the transcriptome in specific tissues and at specific time points. Similarly, machine learning and artificial intelligence will greatly contribute to the efficiency of aging research.

This review collates current hot research topics and proposes promising aging biomarkers at the molecular, cellular and organismal levels, and we attempt to shed light on the relationship between various relevant factors. We provide a broader definition of aging biomarkers, including telomere length, protein expression levels and function, activation or inhibition of key signaling pathways, the gut microbiota, and metabolic patterns. We believe that combining different levels of aging biomarkers can better characterize aging and improve screening of aging interventions for targeted drugs.

2 Aging Biomarkers and Novel Targets for Interventions

2.1 The Molecular Level: The Telomere

Telomeres, the terminal nucleoprotein complexes of chromosomes in eukaryotes, play an important role in chromosomal DNA protection, gene expression, and regulation of stress-related signaling pathways by controlling cell senescence and organismal aging [12, 13]. Telomere length, reflecting a delicate balance between shortening and elongation of telomeres, is mainly controlled by telomerase and telomeric repeat-containing RNA (TERRA) [14]. A systemic knockout of a telomerase subunit in mice results in a decreased telomere length, accelerated organ dysfunction and a shortened lifespan [10, 15]. Reintroduction of telomerase into the $G4^{TERT\text{-}ER}$ mouse model can reverse the tissue degeneration and accelerated-aging phenotype, without an increased tumorigenesis risk. As a result, telomerase reintroduction has an excellent potential for organ or tissue regeneration and aging reversal [16]. There are also some chemical telomerase activators that are expected to be applied to clinical practice for aging research and treatment of degenerative disorders [10].

Telomere attrition may contribute to increased morbidity and mortality of aging-related diseases [17]. As Wang and colleagues found, shorter telomeres correlate with a higher risk of all-cause mortality. Average telomere length varies and there

are sex differences with women have a longer telomere length and life expectancy compared to men, perhaps owing to the hormonal differences, e.g., in estrogen levels, as well as the effect of the X chromosome [18, 19]. Decreasing immune surveillance and increasing inflammation are related to age, and both are linked to telomere shortening and lower telomerase activity [20, 21]. It has been proven that shorter telomere length is associated with oxidative-stress-induced diabetes, Alzheimer's disease, cardiovascular diseases and high levels of the proinflammatory cytokine tumor necrosis factor α [22–24]. Accumulation of reactive oxygen species (ROS) may be a contributing factor of telomere-dependent senescence [25]. It is worth noting that mitochondria-derived oxidative stress plays a key role here because mitochondrial depolarization induced by carbonyl cyanide–4(trifluoromethoxy) phenylhydrazone, an uncoupler of oxidative phosphorylation in mitochondria, can bring about mitochondrial dysfunction, increased ROS production and subsequent telomere attrition and genomic instability [26]. Health-promoting daily activities and habits also contribute to the maintenance of telomere length, e.g., low alcohol consumption and cigarette smoking and following a healthy diet and a physical-activity regimen [27].

Due to the ability to maintain telomere length, cancer cells can proliferate indefinitely [28]. However, the relevance of telomere length to cancer susceptibility is not clear because of the cancer type-specific characteristics [29].

Southern blotting, polymerase chain reaction (PCR)-based methods, single telomere length analysis (STELA) and fluorescence in situ hybridization (FISH) are relatively new technologies for measuring telomere length. Quantitative PCR and FISH are frequently applied in clinical and epidemiological studies [30].

The future research direction dealing with telomeres addresses various relevant questions including how to improve the accuracy of the methods measuring telomere length, telomerase activity, and telomeric repeat-containing RNA and whether or not telomere length and telomerase activity can be straightforward markers of aging and age-related diseases in clinical tests. Moreover, further investigations are still needed to look for specific pharmaceutical or nutraceutical approaches to maintain the telomere length and telomerase activity without increasing the risk of tumorigenesis and other adverse effects to achieve systemic healthy aging (Fig. 3.1) [30].

2.2 The Cellular Level

2.2.1 Longevity Proteins

These proteins are needed to protect the cell/organism from future damage. Impairment of the function of longevity proteins raises the risk of diseases associated with aging (Fig. 3.2) [31].

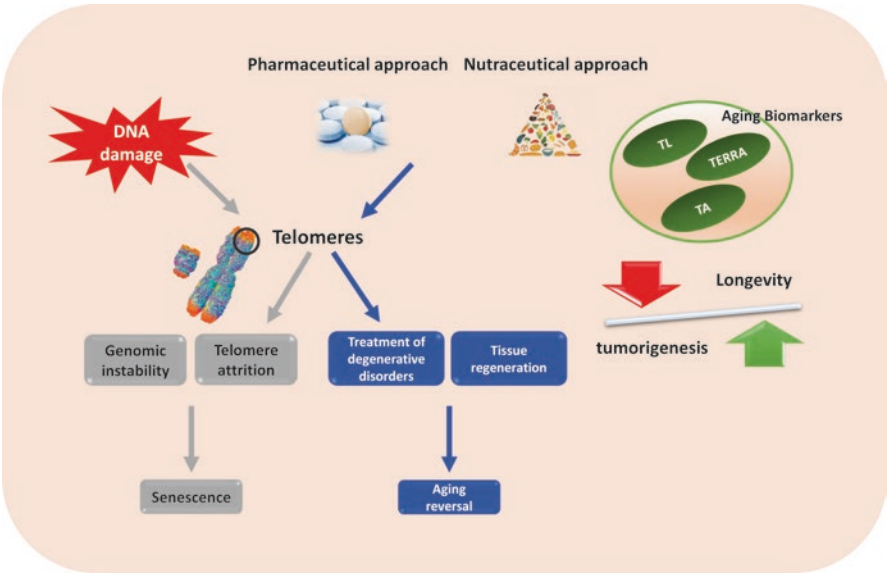

Fig. 3.1 The relationship of telomeres with cell senescence and aging

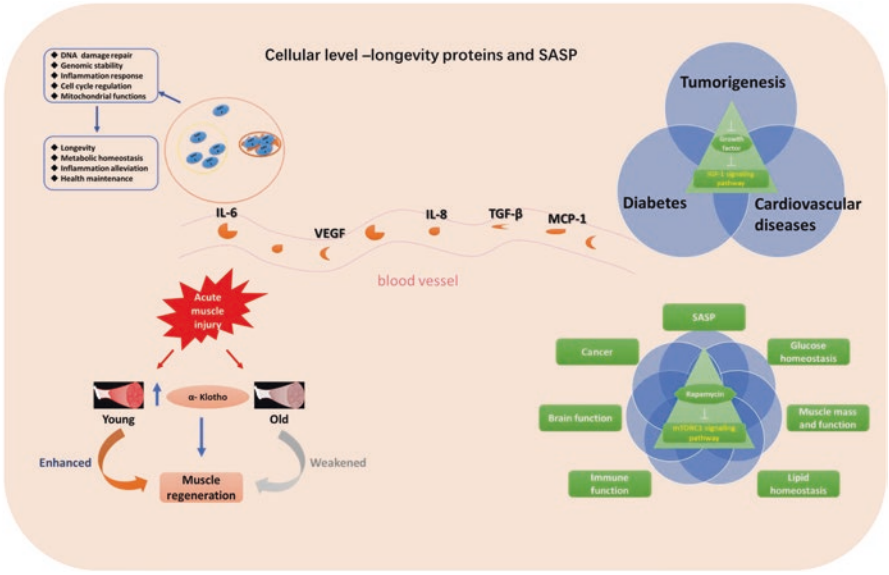

Fig. 3.2 Longevity proteins, the IGF-1 signaling pathway and mTORC1 signaling pathway affect the magnitude of a SASP, resulting in regulation of the aging process

Sirtuins Sir2 was discovered in yeast because of the significant correlation between the activity of Sirtuin family proteins and the rate of replicative aging [32, 33]. There are seven sirtuins in mammals which have different tissue expression patterns (and specificities for substrates) [34]. These are SIRT1, SIRT6 and SIRT7, which function in the nucleus, SIRT3, SIRT4 and SIRT5, which function in mitochondria, and SIRT2 which is active in the nucleus and cytoplasm. Sirtuins take part in various biological processes individually or cooperatively, including DNA damage repair, inflammation response, cell cycle regulation, and mitochondrial functions, and thus affect genomic stability, longevity, metabolic homeostasis, inflammation alleviation and health maintenance [35, 36].

SIRT1 and SIRT6 are two of the most promising regulators of longevity. Caloric restriction as a practical dietary regimen to protect from aging-related changes and to extend the lifespan for humans, can increase the expression and activity of SIRT1 and SIRT6 [37]. SIRT1 overexpression can attenuate many age-related disorders, including glucose resistance, neurodegenerative diseases and tumorigenesis but without the extension of the lifespan in a mouse model [38]. SIRT1 can also enhance DNA methylation, which is beneficial for genomic stability and longevity [36]. SIRT6 mainly functions through deacetylation and ADP ribosylation [39]. A systemic SIRT6 knockout in mice causes premature aging and death at 4 weeks of age [40]. SIRT6 overexpression extends the lifespan of male mice but does not affect female mice [41]. The latest studies have proved the new mechanism of SIRT6 action, including heterochromatin silencing, genome maintenance, epigenetic regulation of stem cell homeostasis and differentiation, tumor suppression, and regulation of glucose and lipid metabolism, which are closely linked to aging and age-related diseases [39].

α-Klotho (muscle regeneration) *Klotho*, a powerful aging suppressor gene, encodes a membrane-bound and circulating hormonal protein in mammals [42]. Mice with a systemic Klotho knockout present with a phenotype of accelerated aging, e.g., cognitive dysfunction and sarcopenia [43, 44]. After an acute muscle injury, there is increased expression of α-Klotho in young skeletal muscle, resulting in impaired tissue regeneration. However, there are no significant changes in old muscles [45].

The insulin/insulin-like growth factor (IGF) pathway The IGF-1/insulin signaling pathway is one of the most conserved pathways involved in longevity and lifespan regulation [46]. The production of IGF-1 is induced by growth hormone mostly in the liver and partially in the heart, kidneys and cartilage. Serum IGF-1 concentrations could reflect the secretion and activity of growth hormone. The IGF-1 signaling pathway also participates in the control of tumorigenesis, diabetes, and cardiovascular diseases, which are common diseases associated with aging [47]. It has been proven that inhibition of IGF-1/insulin signaling can extend the lifespan of C. elegans [48]. In mice, growth hormone deficiency or resistance can extend the lifespan, with a decreased IGF-1 level [49]. Some epidemiological studies indicate

that there is a relationship between longevity, human healthy aging and mutation of the growth hormone and IGF-1 receptors [50, 51].

Mammalian target of rapamycin (mTOR) Kinase mTOR is a serine/threonine protein kinase, including mTORC1, a metabolic sensor for nutrients, growth factors, energy metabolism and stressful stimuli, and mTORC2 an essential regulator of glucose metabolism. mTOR could be regarded as a central regulatory element of glucose homeostasis, muscle mass and function, lipid homeostasis, immune function, brain function, cancer and a senescence-associated secretory phenotype (SASP), which are closely associated with longevity and aging [52, 53]. Rapamycin, an inhibitor of mTOR, can extend the lifespan of mice when fed late in life but with the adverse effect of increased glucose tolerance because of inhibition of mTORC2 [54]. These findings indicate that future work should focus on the discovery of a specific mTORC1 inhibitor.

2.2.2 The SASP

Approximately 10 years ago, a SASP was first proposed by Coppé and colleagues [55]. This means that a senescent cell can produce and secrete some cytokines to affect surrounding cells and the microenvironment positively or negatively. Secreted factors include immunomodulators, inflammatory factors (and other interleukins and chemokines) and growth factors [56]. Senescence could occur in response to damage and danger signals, a metabolic abnormality, activated oncogenes or oncogenic mutations [57]. Due to differences in cell types and aging stimuli (including intensity and duration), the secretion associated with a SASP also varies. Some investigators have discovered that severe DNA damage causes persistent DNA damage response signals and initiation of a SASP [58]. In the initial stage, a SASP is implemented to enhance the senescence-related cell growth arrest, which is beneficial for clearance of senescent cells. However, with the rapid increase in the number and slowing clearance of senescent cells, chronic inflammation, as a hallmark of aging and a major contributor to age-related dysfunctions [58], and tumorigenesis are exacerbated [59, 60].

The classic SASP: cytokines IL-1α, as an important participant in a SASP, can also regulate the production and secretion processes of a SASP, especially those of IL-6, IL-8, vascular endothelial growth factors (VEGFs) and transforming growth factor β (TGF-β) [61, 62]. It is also a senescence marker of endothelial cells and of vascular aging [63]. The NF-κB signaling pathway, called the "master regulator of a SASP," plays a major role in the regulation of IL-6 and IL-8 expression [62]. GATA4 (which is degraded by p62-mediated autophagy [60]), upregulation of p38 MAPK [64], and activation of mTOR can increase NF-κB activity [65], resulting in a potent SASP. Many other SASP factors are influenced by the Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway, which is NF-κB independent [66].

Statins, such as simvastatin, can inhibit a SASP, including expression of IL-6, IL-8, and MCP-1, and this effect slows down cancer cell proliferation [67]. Metformin, rapamycin, and JAK1/2 inhibitors have been reported to act as SASP inhibitors, which can also improve an aging state and alleviate age-related diseases in mammals at a different level [68]. The clinical application of SASP inhibitors requires more research and validation because of their potential adverse effects [69].

Other factors Extracellular vesicles (EVs) is a general name of a variety of small membranous vesicles that are released into the extracellular environment by most cell types. It has been reported that the EVs secreted by older cells are markedly different from those from younger cells. As a result, EVs can be regarded as one of the special participants of a SASP. Senescent cells secrete more EVs when considering the cell type and stimulation [70–72]. Cell senescence also alters the contents of EVs, including an increase in the levels of IL-6 and IL-12 mRNAs [73]. Naturally, the composition of EVs, such as microRNA content, also regulates cellular senescence [74].

Gan and colleague published some research results showing that the level of urinary 8-oxo-7,8-dihydroguanosine (8-oxoGsn) increases with age, in samples from 1228 healthy Chinese residents 2–90 years of age [75]. Because urine sampling and the detection methods of 8-oxoGsn are convenient and operational, urinary 8-oxoGsn could be regarded as a promising aging biomarker, especially in clinical situations.

Detection of a SASP For SASP discovery, mRNA profiling, proteomics, antibody arrays, and other multiplex assays are widely used. However, due to their high cost and low sensitivity, the highly sensitive and customizable sandwich enzyme-linked immunosorbent assay (ELISA), a variation of the ELISA, is recommended for laboratory research [76].

2.3 The Organismal Level

2.3.1 The Gut Microbiota and Aging

People that grow older will experience a series of changes associated with the gut microbiota. With the development of large-scale bacterial DNA sequencing, gut microbiology research has made a breakthrough in recent years [77]. The gut microbiota performs important functions in the processes of development, maturation and aging, and in the incidence of diseases. The gut microbiota could provide various vitamins, short-chain fatty acids, essential amino acids, peptides and other organic compounds that are essential for biological processes. At the same time, these microbes directly or indirectly participate in the regulation of digestion, nutrient absorption, and metabolism, which are closely related to inflammation and immunity [78, 79] (Fig. 3.3).

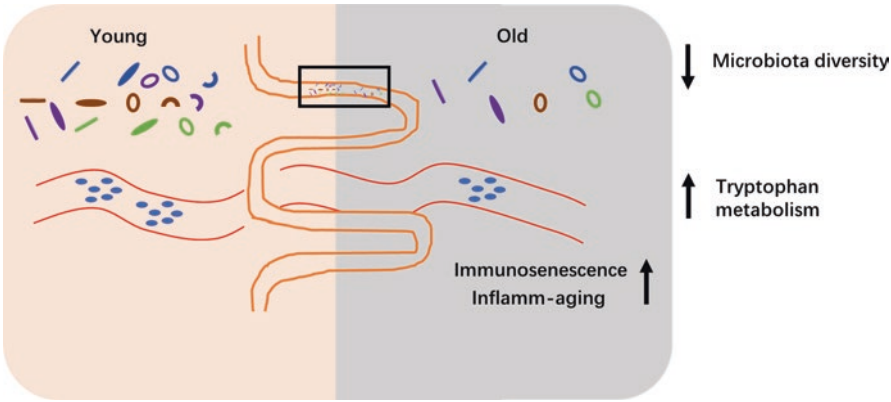

Fig. 3.3 Decreased microbiota diversity, increased tryptophan metabolism and elevated immunosenescence and inflamm-aging occur during the aging process, accelerate the aging rate and aggravate aging-related diseases

Immuno-senescence and inflamm-aging Long-term stimulation of the immune system can cause a decline of immune-system functioning, which is called immunosenescence. Low-grade chronic inflammation, along with many age-related diseases, metabolic syndrome and neurodegeneration, has been referred to as inflamm-aging [80]. Normal bacteria can act as antigens to stimulate the body's immune response. Immune aging is usually accompanied by up-regulation of the inflammatory response. During aging, a continuous gut microbiota imbalance leads to inflammatory responses to the intestinal mucosa. As a result, chronic inflammation develops throughout the body [81].

Microbiota diversity Changes in microbiota diversity with age are significant. Moreover, the diversity of the microbiota in elderly people is lower than that in younger people [82], with a reduction in relative abundance of *Bifidobacteria*, and increasing abundance of *Bacteroides* and *Enterobacteriaceae* [80]. The ratio of *Firmicutes* to *Bacteroides* can be a criterion of metabolic health and a decrease in this ratio is observed with aging [83].

Bifidobacterium species that are suggested to function in the maintenance of human health [84], as an important member of the gut microflora (especially probiotic species), show a decrease in relative abundance in elderly people as compared with younger adults [82]. It has been reported that long-term *Lactobacillus* and *Bifidobacterium* dietary supplementation can enhance memory and change metabolism of the brain in aged rat [85]. Compositional changes in *Bifidobacterium* take place with age. For example, there is a decrease in the abundance of *B. breve* in people who are over 50 years of age and a increase of *B. dentium* abundance in subjects over 60 years of age [84].

Tryptophan metabolism Tryptophan, as an intestinal microflora metabolite, performs a crucial function in the balance between gut immune tolerance and gut microbiota maintenance [86]. An enhancement of tryptophan metabolism is positively correlated with age, in agreement with the finding that elderly people have a lower serum tryptophan level [87]. There is another study which indicates that patients with senile dementia also have a decreased serum tryptophan level [88].

There are still many controversies regarding the notion that the gut microbiota is a specific and an unquestionable signature for evaluation of the aging status. However, there is reasonable evidence showing the importance of gut homeostasis for antiaging and microbiome-targeted interventions in antiaging medicine.

2.3.2 The Metabolic Pattern and Aging

Elderly people have a different body composition, energy intake, energy expenditure, physical activity, fuel utilization and the ability to regulate the energy balance, in comparison with young people [89].

Nicotinamide adenine dinucleotide (NAD^+) NAD^+, as a pivotal cellular factor participating in different metabolic pathways, functions as both a co-substrate and co-enzyme in different cellular compartments. Under aging and high-fat diet, the NAD+ level falls. Simultaneously, a decreased NAD^+ concentration can accelerate the aging process. However, calorie restriction (CR), fasting, glucose deprivation and other lower-energy load conditions can efficiently raise the concentration of NAD^+, resulting in an increasing lifespan and a better state of health in old age. It has been shown that a NAD^+ precursor, nicotinamide riboside, or nicotinamide mononucleotide dietary supplementation can contribute to health maintenance and increase longevity in mice [90, 91]. NAD^+ precursors could serve as therapeutic interventions for metabolic diseases, such as type 2 diabetes, fatty liver disease, glucose intolerance, and neurodegenerative disorders [90]. A reduced number and function of adult stem cells may induce injury and dysfunction of the tissue when it encounters external stimuli, including effects of the aging process. Nicotinamide riboside supplementation has been reported to induce the rejuvenation of muscle stem cells and intestinal stem cells in aged mice [92, 93].

Some researchers remain sceptical about the powers of NAD^+ because of some recent studies [94]. Nacarelli and colleagues have demonstrated that NAD^+ metabolism can promote an inflammatory environment and production and secretion of proinflammatory factors (i.e., SASP), which will facilitate tumorigenesis [94]. These results serve as a warning that the antiaging effect and potential tumorigenesis risk have to be balanced when oral NAD^+ is considered for clinical development as a nutraceutical (Fig. 3.4).

CR and a ketogenic diet (KD) The process of aging has been repeatedly proven to be closely related to metabolism and diet is the most direct factor affecting metabolism. How to regulate the aging process through dietary changes is a new

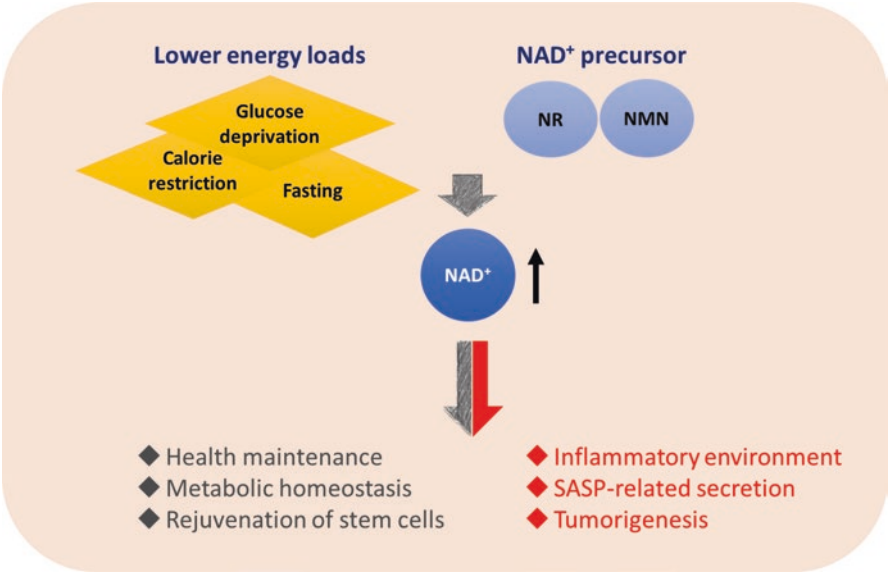

Fig. 3.4 Lower energy loads and NAD^+ precursor supplementation could increase the NAD^+ level. Nevertheless, the effect of a high level of NAD^+ is controversial, including health maintenance, anabatic inflammatory responses and tumorigenesis. NMN nicotinamide mononucleotide, NR nicotinamide riboside

topic in aging intervention research. Dietary interventions, as the most common and direct method for combating aging and diseases, are gradually attracting more attention. CR and KDs have been studied and discussed for many decades. CR can convert glycolysis to β-oxidation, which means a shift from carbohydrate to fat metabolism in cells and in the organism. Balasubramanian et al. have concluded that the beneficial effect of CR is related to human aging and CR could protect the organism from arthritis, cardiovascular diseases, age-related diabetes, colon lesions and delay osteoporosis, sarcopenia, brain atrophy and cognitive decline, as well as decrease cancer incidence and progression [95]. Just as CR, the KD is associated with changes in metabolic patterns, from carbohydrates toward fatty acid oxidation and induces an increase in the levels of ketone bodies in mice [96]. Roberts et al. have shown that a KD could promote longevity and increase the health span in adult mice, with increased motor function and memory and a decreased tumor incidence. In their research, the KD was found to function by increasing protein acetylation and regulating mTOR signaling, and this was tissue specific [97]. Almost simultaneously, Newman and Verdin reported that a KD can reduce midlife mortality and improve memory in aging mice, in line with the conclusion of Roberts [98]. In our own research, we found that both a high-fat diet and a KD could reverse shortening of lifespan and attenuate the premature aging phenotype in a mouse model of progeria. We attribute this effect to a shift in metabolic patterns (unpublished observations). In 2014, a paper presented the idea that a high-fat diet is beneficial for

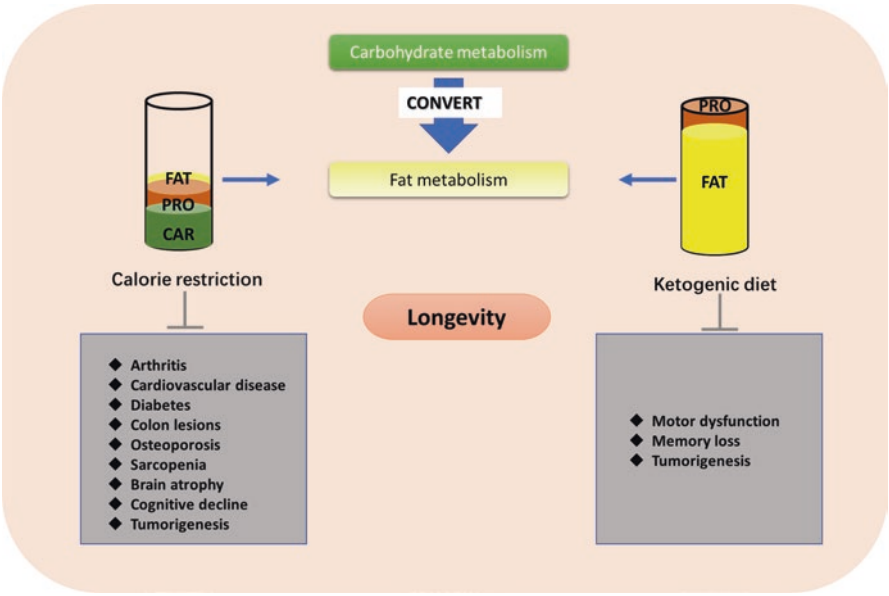

Fig. 3.5 CD and KD can convert carbohydrate metabolism to fat metabolism, which contributes to longevity

preventing premature aging in Cockayne syndrome, which has features that are similar to late-stage human aging [99]. All of these studies seemingly support this opinion that fat metabolism is a better choice for kerbing the aging process (Fig. 3.5).

It is also noteworthy that a KD shows promise for cancer therapy if tumor types and genetic changes are taken into account. In preclinical trials, there is evidence that some patients with glioblastoma, neuroblastoma, pancreatic cancer or lung cancer show better recovery when given an adjuvant KD therapy. The KD also has antitumor effects in stomach or liver cancer patients [100].

3 Conclusion and Perspectives

Focusing on the research on healthy aging and the rate of aging is more important than solely extending the lifespan. Various scientists have been contributing to the study of aging biomarkers and novel targets for anti-aging interventions and have made significant progress in these objectives. The biomarkers and targets mentioned in this review were hotspot issues in recent years and gained much recognition in the field of aging biology and geriatrics. What deserves our attention is that the effects of an individual's aging biomarkers vary at the cellular and systemic levels. For the exploration of aging biomarkers and anti-aging targets, we should pay more

attention to the overall effect. Of course, large-scale screening at the cellular level has dramatically improved the efficiency of interventions and success rates.

There are some limitations in this review. For example, it does not cover some other phenomena that play an important role in the aging process, e.g., autophagy. Recently, the role of autophagy in the process of aging gradually attracted the attention of scientists. Autophagy is activated under stressful conditions, such as oxidative stress, starvation or hypoxia, to ensure cellular homeostasis. To a certain degree, activated autophagy, which is induced by overexpression of Atg5 and enhancement of lysosomal receptor of chaperone-mediated autophagy (CMA), may significantly attenuate age-related organismal damage and functional decline in aged mice [101, 102]. Notably, reduced autophagic activity is observed in aged muscle, thus resulting in lower myofiber function and muscle strength, and this effect is conserved between mice and humans [103]. In addition, the correlation between metabolic rate and aging is controversial. Thus, considerable more work and information is required to shed light on this topic.

The benefits of the advances in computer sciences, including meta-analyses and artificial intelligence, will improve the speed and efficiency of aging biomarker research [95]. We need more research and validation for creation and application of an aging biomarker assessment system in clinical practice.

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Chapter 4
Sex Differences in Aging and Associated Biomarkers

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1 Introduction

Aging is a natural process defined by the gradual, time-dependent decline of physiological and behavioural function, and individuals of the same chronological age can show great variability in these parameters [1]. The capacity of biological systems to continuously adjust for optimal functioning despite ever changing environments is essential for healthy aging, and variability in these adaptive homeostatic mechanisms may reflect the heterogeneity observed [2].

Adaptive homeostasis refers to "The transient expansion or contraction of the homeostatic range in response to exposure to sub-toxic, non-damaging, signalling molecules or events, or the removal or cessation of such molecules or events" [3], and represents the framework in which the body dynamically maintains biological and behavioural processes required for life. The endocrine, metabolic and immune systems are well established in the literature as homeostatic mechanisms [3]. These systems are complex and integrated, involving feedback-dependent mechanisms and cross-talk across networks, thereby contributing to overall system regulation [4]. These integrated systems are ultimately governed by the complex interaction between genetic and environmental factors, including lifestyle factors, which may affect homeostatic mechanisms and ultimately change the progression of aging. Growing evidence suggests that the adaptability of these homeostatic systems decline with age, with many linked to age-related morbidities suggesting a shared pathophysiology of disrupted cellular homeostasis [2]. This change or decline in key homeostatic systems may therefore reflect the inability to activate or modulate several adaptive responses.

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With an ever-increasing aging population, interest in biomarkers of healthy or accelerated aging is growing. Although there is no universally accepted definition of 'aging' biomarkers, attempts to identify objective biomarkers often point to mediators of homeostatic systems including physiological functions, cognitive processes, and endocrine and immune functions [5]. Importantly, sex differences are often reported in many of these adaptive systems, and this may reflect, to some extent, sex differences observed in aging and age-related disease states. Therefore, it is imperative to consider sex and gender differences in aging and age-related diseases.

Known sex differences in key homeostatic mediators may influence the differential susceptibility and resilience to aging outcomes between males and females. Therefore, within the context of biomarkers in aging, it is important to consider biological sex differences and the role of gender, which includes a person's psychosocial and cultural self-identification [6], and how these may influence analytical measurement. From a physiological standpoint, biological sex may play an important factor in the variability of biomarkers due to the capacity of sex chromosomes and sex hormones (i.e., estrogen, testosterone), to alter biomarkers present in blood, saliva, cerebral spinal fluid, or tissue samples [4]. From a behavioural standpoint, biological sex hormones, sex chromosomes, and gender may impart differential adaptive processes seen in the cognitive domain [7].

This chapter aims to outline sex differences in key homeostatic domains thought to be associated with the pathophysiology of aging, and which are often proposed as biomarkers of aging and age-related disease states. This includes summarising the available literature of sex-based differences and hormonal status with regards to the gonadal and adrenal endocrine systems and immune function.

2 Sex Differences in Age-Related Diseases

Sex differences in longevity, mortality and age-related diseases are well established in the literature. In developed nations, females tend to live longer than males and have notably lower death rates than men at all ages. However, it is also known that females suffer from higher levels of morbidity than men [8]. Salient gender-related differences have been associated with risk, clinical expression, treatment response and course of several age-related neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease [9].

Alzheimer's disease, the most common cause of dementia, disproportionately affects females, with a higher incidence compared to males [10]. Females have a faster rate of cognitive and functional decline following diagnosis and appear to suffer significantly greater cognitive impairments after accounting for sex differences in age, education and dementia severity [11]. Sexual dimorphisms in Alzheimer's disease pathologies have been reported in several mouse models, with females expressing greater pathology [12]. Sex has also been demonstrated to modify genetic risk factors for Alzheimer's disease. For example, the apolipoprotein E gene ε4 allele confers a greater Alzheimer's disease risk in females, as compared to

males [13]. In contrast, males have at least a two-fold greater risk and prevalence of Parkinson's disease [14]. In comparison to females, males have a later onset of Parkinson's disease. Clinical and cognitive profiles also differ between males and females with Parkinson's disease. Males are more likely to present with rigidity, rapid eye movement behaviour disorder and sleep disturbances. The cognitive profile in males is more likely to include verbal fluency deficits and a lack of facial emotions. Females are more likely to present with a tremor dominant form of Parkinson's disease. A reduction in visuospatial cognition is more frequent in females [9, 15, 16]. Possible reasons for these sex differences in age-related, neurodegenerative disorders and longevity are thought to be due to intrinsic differences based on genes, sex hormones, and reproductive physiology. These can confer differential risks of morbidity. In addition, extrinsic factors such as lifestyle, health habits, exercise, nutrition, and others may also have a connection with sex difference in biological vigor as potential moderators.

3 Endocrine Markers of Aging

The endocrine system represents a key facilitator of homeostatic function, maintaining critical functions including development, growth, metabolism, stress responses, reproduction, and sleep patterns. As such, it is one of the most important regulators of physiology over the lifespan. The hypothalamus can be considered as the link between the nervous system and endocrine system, continually adjusting according to internal and external environments using feedback mechanisms, and subsequently regulating all hormone-related physiology and behaviour. With advancing age, alterations in hormonal networks, with concomitant excesses or deficits in steroidal hormones occur [17]. This is further exacerbated by poor sensitivity of tissues to their action, and a decreased sensitivity toward feedback control [18]. These alterations are thought to be translated clinically to losses of function seen in aging of the reproduction system including menopause, developmental and maintenance growth axes, and the adrenal endocrine axis [18].

4 Aging of the Female and Male Reproductive System and the Aging Gonadotropic Axis

Sex steroid hormones, including estrogens and androgens (testosterones) are regulated by the hypothalamus pituitary gonadal (HPG) axis. While the endocrine feedback mechanisms are complex, a simplified account of the HPG axis suggests that the hypothalamus releases gonadotropin-releasing hormone (GnRH), stimulating the anterior pituitary to produce and secrete gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The ovary, comprised of two cellular

components, is synergistically stimulated by FSH and LH, leading to the release of sex steroids [19]. FSH and LH then complete the endocrine feedback loop by posing both positive and negative feedback effects on the axes, depending on timing and context [20].

A clear change observed throughout aging is the decline in sex hormone production. As women age, the natural transition from reproductive years to menopause is associated with fluctuating and eventual decreasing levels of ovarian sex steroids. Suggested to be a predictor of reduced ovarian reserve, gonadotropin FSH increases pre-menopausally [21]. Subsequent to the menopausal transition, eventual decreases in FSH and LH occur [18]. Again, alteration at the level of the hypothalamus-pituitary-gonadal axis is thought to principally contribute to this progressive decline. This appears to be moderated by increased levels of GnRH which increase with age with hypothalamic adaptations during aging, favouring its release [22, 23]. Moreover, the feedback effect of estrogen on FSH responsiveness is greater than on LH and is attenuated with aging [24].

This natural consequence of aging of the reproductive axis and sex-steroid privation leads to the loss of reproductive capability, in addition to a sequelae of clinical symptomatologies including loss of muscle and bone mass, visceral tissue accumulation, insulin resistance, and negative impacts on mood, cognition, and importantly, quality of life [25]. A recent meta-analysis of 310,329 women demonstrated that younger age at menopause (occurring prior to 45 years old) was associated with a higher risk of coronary heart disease, stroke, cardiovascular mortality, and of all-cause mortality [26]. In addition, the occurrence of age-related morbidities including Alzheimer's disease and Parkinson's disease has led to the suggestion that the loss of sex steroid hormones, in both female and males, is a contributor to the aetiology [27, 28].

Although not as dramatic as seen during the female menopausal transition, a modest, progressive decline in anabolic androgen is observed in males throughout the aging process [29]. No specific age can be defined as to when this process begins, although several cross-sectional and longitudinal studies have confirmed that testosterone (free and bound) decline in aging, while LH increases with age [30–34]. Recently, weight and lifestyle factors have been shown to moderate these hormonal changes [30]. In addition, the levels of sex hormone binding globulin (SHBG) increase during aging which binds testosterone, resulting in relatively less free biologically-available testosterone [35]. Clinically, low testosterone levels have been associated with increased mortality in men, showing an approximate doubling in mortality risk compared to men with normal levels [36]. Associations between low testosterone and adverse outcomes have also been reported, including diabetes mellitus, osteoporosis, decreased libido, and cardiovascular disease [37–39]. In a recent biomarker analysis that included sex hormones in an aging 'panel', it was reported that low levels of testosterone are correlated with high-sensitivity cardiovascular risk factors, consistent with a potential increased risk of cardiovascular disease [40].

Fluctuating hormone levels throughout the menstrual cycle of reproductive aged women and in perimenopausal women should be also be considered, as female hormonal status has been shown to modulate many age-related biomarkers. As an

example, stress-induced cortisol levels are associated with impaired emotional retrieval [41] and affect serum biomarkers in clinical tests only in some menstrual phases [42]. In addition, hormonal contraceptives used by many women of reproductive age have shown to vary clinical measurement of serum biomarkers [42], and this is rarely considered within the context of system dynamics and levels of molecular outputs. Although the contributions and mechanisms are yet to be fully elucidated, sex steroid hormones exhibit clear functional activity on age-related processes and may govern, in large part, the sexual dimorphisms observed in many age-related morbidities.

5 Dehydroepiandrosterone in Aging

Adrenal secretion of dehydroepiandrosterone (DHEA) also profoundly fluctuates across the lifespan and represents one of the largest endocrine changes observed in human aging. Although the physiological role of DHEA is not yet completely understood, its apparent influence in age and age-related disease states has yielded much interest.

DHEA is predominately synthesised within the adrenal zona reticularis of the adrenal cortex in response to adrenocorticotropic hormone (ACTH) and subsequently released into the bloodstream [43]. The majority of circulating DHEA is converted to its sulphated derivative DHEA-S via the enzyme steroid sulphotransferase, which is mostly bound to the carrier protein, albumin. As the precursor to the sex steroid hormones, it is also synthesised to a smaller extent in human gonads [44]. DHEA-S serves as a precursor to approximately 50% of androgens in adult men, 75% of active estrogens in premenopausal and nearly 100% in postmenopausal women [45]. More recently, reports pertaining to its de novo synthesis in human brain highlights the important neurosteroidal properties that DHEA may have, posited to have direct activity at estradiol receptors [46] and NMDA receptors [47], possess neuroprotective properties, and participate in neurogenesis, catecholamine synthesis and secretion, in addition to anti-inflammatory effects [44]. Indeed, it has been reported that concentrations of DHEA/DHEA-S are considerably higher in the brain than in any other organ.

At birth, DHEA-S levels are markedly high until involution of the adrenal fetal zone, which leads to a cessation in DHEA production and it remains dormant until the childhood period, when production once again commences. Peak levels for both females and males are observed when they are in their thirties, after which point DHEA levels fall by approximately 2–3% each year [48], declining to levels that are approximately 20% of peak values in men and 30% of those in women [43, 49].

Several studies have reported the association between low levels of DHEA-S and DHEA and frailty in older populations [50–52]. In addition, several cross-sectional studies, including investigations of serum and cerebrospinal fluid (CSF), have implicated DHEA levels in a number of age-related pathologies including type 2 diabetes mellitus, psychiatric disease states, and neurocognitive disorders, including

Alzheimer's disease [29, 43]. Although many studies have provided support for a positive correlation between higher levels of DHEA and improved health outcomes, including muscle strength, higher bone density and increased longevity in males, other studies have demonstrated that DHEA is associated with negative health outcomes. For example, lower DHEA-S levels have been associated with greater risk of ischemic stroke, having been adjusted for appropriate potential confounders [53].

Due to such accumulating literature, DHEA has drawn much attention as a putative 'anti-aging' supplement, although little is known about the underlying mechanism with regards to its progressive decline and associated pathologies. Hypotheses pertaining to plausible biological mechanisms include the ability of DHEA/DHEA-S to modulate energy metabolism, systemic inflammation, and that it may counteract or modulate the stress endocrine hormone, cortisol [54–56]. Given the critical role of sex steroids and age-related associations, the function of DHEA as an alternative source of androgens and estrogens to local tissues may be a plausible way in which DHEA/DHEA-S protects against aging and age-related pathologies [57]. Although there is some evidence to suggest that DHEA supplementation may improve skin status and bone turnover in older females, there are few clinical studies powered enough to conclude the effects of DHEA on aging and age-related diseases. In addition, data on the safety profile of long-term DHEA supplementation are still lacking [58].

5.1 Sex Differences in DHEA

Differential trajectories of DHEA levels between the sexes begin following the onset of adrenarche, and differences in levels can again be observed during the perimenopausal period in women, and in older people [59]. Peak levels have also been reported to occur earlier for females [60]. The biological reason for this difference has yet to be elucidated but it has been suggested that higher levels observed in males may reflect chromosomal differences [61]. The gene for steroid sulfatase, which degrades androgens, is located on the X chromosome. Therefore, as males only have one copy of the gene, they consequently have less degradation and higher DHEA concentrations [57]. Although the literature reports somewhat inconsistent results, a recent review concluded that enough evidence supports the association between DHEA and aging in males, but in females this may not exist, or displays a non-linear, U-shaped association. Larger studies, with a prior stratification of sex, are required to confirm these findings [62].

6 Analyses of Steroidal Hormones

The complexity of sex steroid measurement should not be underestimated, and there is current debate over the best technologies to use (see [63] for overview). These steroids exhibit only modest differences in structure, but which amount to profound

functional differences. Owing to such subtle differences in structure and small size, steroids are not easy to measure.

Although immunoassays have remained the mainstay of steroid analysis, the reliability of the specificity and sensitivity of these assays have come into questions, particularly commonly used commercially derived assays [64]. Unless preanalytical extraction methods or chromatographic steps are introduced to purify samples prior to the immune-assay, these assays are prone to inaccuracy due to their non-specificity [63]. Mass spectrometry has introduced far greater specificity of steroidal hormones and allows for specific analysis of different steroids (i.e., estrogen, and testosterone). Currently this technique provides the highest specificity due to mass spectrometry providing the exact structural information of the analyte, allowing for specificity between steroid analytes and their precursors. However, it is noted that costs and expertise required for such analyses may prevent many researchers from conducting such analyses. As technology advances and becomes cheaper, this gold standard technology will grow in use.

Relevant to this chapter, difficulty in the measurement of steroids has been proposed for the reason for the inconsistent findings seen in the DHEA aging literature. The low specificity and precision of immunoassays may have yielded incorrect measurements in subjects low in DHEA/ DHEA-S [62].

7 Aging of the Adrenal Endocrine System: The Hypothalamus-Pituitary-Adrenal Axis

Activation of the hypothalamus-pituitary- adrenal (HPA) endocrine axis and the subsequent stress response is a fundamental homeostatic mechanism that enables a wide array of physiological and behavioural responses to actual or perceived threat. Initiated at the level of the hypothalamus via corticotrophin releasing hormone (CRF), a signalling cascade then ensues with adrenocorticotropin hormone (ACTH) released from the anterior pituitary, followed by glucocorticoids being synthesised from cholesterol in the adrenal cortex, and released directly into the bloodstream. Glucocorticoids are lipophilic and largely transported bound to cortisol binding globulin (CBG) leaving only a small fraction (10%) biologically active [65]. As glucocorticoids can readily cross the blood-brain-barrier, the steroids consequently can exert action at both local central nervous system level and systemic peripheral levels, where it induces or represses the transcription of a plethora of target genes [66, 67]. In this way, glucocorticoids orchestrate a vast repertoire of adjustments to adapt to external or internal changes to the environment, ensuring optimal regulation of several interlinked regulatory systems including reproduction, growth, and immunity, and cognitive signalling processes [68]. In addition, fast acting, non-genomic action of glucocorticoids occur, engaging glutamatergic system alterations [69].

Glucocorticoids mediate its own activity and termination via two receptors; the high affinity type 1 mineralocorticoid receptor (MR) and lower affinity type 11 glucocorticoid receptors (GR) [70]. The high affinity of MR to glucocorticoids is thought to be responsible for regulating tonic activity and dictates the basal circadian and ultradian rhythms observed across the day [71]. GR are found widely distributed throughout the brain, with high density found in most neurons and glia. With lower affinity to physiological GC, these receptors only become functionally activated as the stress endocrine hormone cortisol rise following a stressor and play an important role in mediating GC effects on mobilising energy stores, inflammation and neural function [72]. Importantly, it is thought to be primarily responsible for blunting further activity of the HPA stress response. Both receptors act in synergy to mediate HPA dynamics important for several of these systems of homeostasis, eloquently fine tuning the HPA dynamics depending on the necessary action. An example of this is found within cognitive processes, such as learning and memory. GR and MR are both abundantly expressed in neurons of the hippocampus, prefrontal cortex, and amygdala [73] where they are thought to have opposing functions which regulate hippocampal synaptic neuroplasticity during the stress response, important in long term potentiation mechanism of learning and memory. Preclinical rat models have demonstrated that activation of the MRs may be a prerequisite for hippocampal plasticity, while GRs may exert an inhibitory effect of plasticity [74]. Although initial sensitivity to feedback of glucocorticoids due to the high expression of GRs and MRs, after repeated responses seen in acute and chronic stress, the hippocampus down-regulates receptor expression [75]. In line with this, although acute, moderated activation of the HPA axis is a necessary and beneficial response, such chronic activation may lead to tissue damage or receptor desensitisation, resulting in attenuated negative feedback, and ultimately adverse outcomes [76]. Individual differences in vulnerability and resistance to stress, governed by HPA dynamics [77] may thereby contribute to the heterogeneity of the aging, and age-related diseases.

There is evidence that HPA axis dynamics [78] and GR expression levels in areas of the dentate gyrus [79] change over the natural process of aging and are postulated to be a major driver in accelerated aging, with lower diurnal cortisol levels having been associated with longevity [80]. In healthy humans, the HPA axis, and glucocorticoid output typically follows a diurnal pattern. The typical pattern displays a sharp increase upon awakening, known as the cortisol awakening response (CAR), followed by reduced levels throughout the day, until the sleep nadir [81]. Increased basal cortisol output [82], diurnal amplitude flattening [83], and an altered CAR response both in magnitude and in day to day variability [84] have all been associated in aging models [61]. In addition, attenuated negative feedback by glucocorticoid receptors at the level of the paraventricular nucleus, hypothalamus, hippocampus, and prefrontal cortex have been reported [61, 70]. Furthermore, the 11-β hydroxysteroid dehydrogenase enzymatic activity responsible for the interconversion of inactive cortisone to active cortisol has been reported to decrease with age, resulting in the inefficient inactivation of active cortisol to inactive cortisone [85]. The two isoforms of this enzyme have also been suggested as a potential key

mechanism in the acceleration of aging [86]. In addition, the systemic 11-β hydroxysteroid dehydrogenase isoform 1 is predictive of progressive brain atrophy and cognitive decline [87].

Such age-related changes in HPA dynamics are often related with declined cognitive processes, cardiovascular alterations, and psychiatric disease states. For example, a higher 24 h urine output has been associated with Alzheimer's disease [88]. Mechanistically speaking, this may be a result of cortisol induced apoptosis and death of hippocampal neurons [89], as observed by decreased adult hippocampal neurogenesis in an Alzheimer's disease model [90].

7.1 Sex Differences in HPA Axis Regulation

Research suggests there are substantial sex differences in HPA-axis dynamics and cortisol levels during regular diurnal maintenance and in response to stress, which may contribute to differential aging dynamics between the sexes [91]. In vitro models have shown that female rodents exhibit greater basal corticosterone production by the adrenal glands [92] and have a more robust HPA axis response to both physical and psychological stressors, thought to be a result of circulating estradiol levels [93]. Clinical investigations have also provided support for the sexual dimorphism of the HPA axis stress response [94]. The HPA axis stress response in females is characterised by a larger, more sustained secretion of ACTH and cortisol, suggesting enhanced activity and reduced negative feedback [95], while the pulsatile cortisol feedback on ACTH mediated by GC receptors appears to be influenced by sex [96]. In addition, there is evidence indicating that both GRs and MRs are less sensitive to cortisol modulation in females than males, suggesting reduced feedback by autoregulation of these receptors [70].

Although the exact mechanism of action remains to be elucidated, estradiol has been shown to enhance HPA activity, while testosterone appears to have an inhibitory effect by acting upon the hypothalamus [97]. In contrast, there is also evidence to indicate that estradiol, but not testosterone, heightens the cortisol-mediated negative feedback on pulsatile ACTH secretion in both aged men and women [96]. Further research must be conducted to understand this complex interplay between the HPA and HPG axis, across the life-course and considering sexual dimorphisms.

In line with these findings, a clear association between menstrual cycle phase and cortisol basal measurement has been shown. Women in the luteal phase of the menstrual cycle have a similar cortisol response to men, whereas in the follicular phase, attenuation of cortisol production has been shown [98]. Notably, increased CBG is known to occur in cases of estrogen excess (e.g., pregnancy and use of estrogen-containing oral contraceptives, resulting in higher concentrations of total cortisol [99], although free cortisol levels are usually unaltered in states of increased CBG [100].

It is also now appreciated that prenatal exposure to early life stressors and increased levels of glucocorticoids can influence later life behaviour, biological processes, and susceptibility for disease states. Support for sex differences in such early-life programming of the HPA in humans has been demonstrated in rodent models, with evidence for the female HPA axis being more vulnerable to stress and glucocorticoid programming, and female offspring demonstrating an increased HPA reactivity [101]. This may be one mechanism underlying sex differences in later life diseases and sex dimorphisms shown in aging.

7.2 Measurement of the HPA Axis

Glucocorticoids have traditionally been assayed using a range of substrates including blood, saliva and urine that provide a sampling procedure that is simple and relatively non-invasive [102]. More recently, cortisol has been measured using hair sampling, which gives a chronic reading of cortisol levels [103]. Salivary cortisol represents the bioavailable, active form of the molecule that escapes the CBGs in the salivary glands and saliva [65]. In addition, unbound cortisol is excreted in the urine and 24 h measurements correlate well with those in serum in cases of cortisol excess [65]. Hair analysis provides the opportunity to determine past cortisol exposure and represents a retrospective index of cumulative cortisol output which is not influenced by the circadian rhythm of the HPA-axis, or by acute stress. As hair grows at an average rate of 1 cm/month, the 1 cm segment closest to the scalp represents total cortisol levels during the most recent month, with the outer segments representing previous months [103–105]. However, several limitations should be noted, as different hair care routines (e.g., use of hair dye), seasonal variations, and storage length have all been documented to influence readings [106]. The time of sample collection is also a critical consideration as cortisol follows a circadian rhythm of secretion. The cortisol awakening response (CAR) is measured using a minimum of three separate sampling times using blood or saliva (upon awakening, 30 min after waking, and 45 min after waking) [107]. However, the majority of studies employ a ‘minimal protocol’ by analysing one time point only, which has potential implications for the reliability of measurement. For cross-sectional studies it is recommended that up to six consecutive days of samples should be collected to accurately assess single time-points, and that four measures at two consecutive weeks days are required to reliably measure the CAR as a trait measure [108]. HPA axis dynamics and feedback sensitivity can also be measured using a synthetic glucocorticoid, dexamethasone. In clinical testing of suspected hypercortisolemia, the dexamethasone suppression test (DST) can detect abnormal HPA axis activity by a failure to reduce total cortisol production via stimulating the negative feedback mechanism and ACTH production. Interestingly, this has been found to be moderated by genetic variation [109] and use of hormonal contraceptives. The false positive effect found in fertile women using hormonal contraceptives, due to the resultant increased levels of CBG and consequent increase in total cortisol concentrations,

was found to be eliminated after one-week cessation of contraceptive use [110]. Various stressful stimuli that can reliably induce the cortisol response in human research participants can also be used to evaluate HPA dynamics. These include standardised psychosocial protocols, such as the Trier Social Stress Test (TSST), and physical stressors (electrical stimulation).

8 Aging of the Immune System

A functional immune system is critical for protecting the host against infections and malignancies, regulation of wound healing, and ultimately for separation of the 'self' from surrounding organisms that compete for space and resources [111]. The immune system is coordinated into two sub-classifications that govern the immune response. The innate immune system mounts a fast, non-discriminatory response, initiated by macrophages and dendritic cells that produce inflammatory mediators, including histamine, bradykinin, serotonin, leukotrienes, prostaglandins, cytokines and several growth factors and enzymes. This promotes either tissue repair/regeneration with subsequent resolution of inflammation, or tissue remodelling/fibrosis [112]. The adaptive response is initiated if the innate immune response fails to respond sufficiently, creating an immunological memory after the initial response to a specific pathogen. This more sophisticated arm of the immune system requires recruitment of B and T cells, followed by antibody and cell-mediated responses, respectively, and consequently has a targeted, albeit slower action [113].

Like all successful adaptive homeostatic responses, subsequent to the activation of the immune response, an ensuing cessation or resolution of inflammation must occur, returning the tissue environment to the basal state allowing for tissue repair/regeneration. Recruitment of macrophages to an anti-inflammatory phenotype is critical to this process and involves the release of anti-inflammatory cytokines including interleukin (IL)-10 and TGF-β, as well as lipid mediators [114]. An over-activated immune response leads to tissue remodelling (tissue fibrosis), autoimmune and neurodegenerative diseases, rather than tissue regeneration [114, 115]. Changes in inflammatory mediators have been identified in age-related syndromes including type 2 diabetes, Alzheimer's disease and cardiovascular disease [116].

During aging, the immune system loses the ability to protect against pathogens effectively, and fails to support appropriate wound healing [111]. Such a decline in effective immunological functioning results in these subjects not responding efficiently to novel or previously encountered antigens [117]. The adaptive immunological arm undergoes complex changes in the aging process that includes epigenetic and metabolic changes affecting naïve, memory and effector T cells, and B cells [118]. A decrease in the number of naïve T lymphocytes and increase in memory and effector T cells occurs with age, in addition to the reduced diversity in T-cell receptors with consequential diminished functionality of naïve and memory T-cells [116]. Clinically, this results in most vaccines being less immunogenic and efficient in the elderly [119].

Additionally, aging is also associated with progression toward a chronic, subclinical inflammatory state, characterised by elevation of peripheral pro-inflammatory chemical mediators in the absence of overt infection, a term coined 'inflamm-aging' [120]. Several human studies have demonstrated an association between elevated levels of cytokines, especially IL-6, and a decline in innate immune function in older individuals [121]. Such a peripheral inflammatory state has been associated with the susceptibility and progression of many age-related disease states including cancer, diabetes [122], and cognitive and structural brain changes [123]. Moreover, it has been demonstrated as a key risk factor of mortality [124]. In addition, the innate arm of the immune system initiates cross talk with the adaptive arm through antigen presentation, co-stimulatory molecule expression and cytokine production, and consequently can contribute to adaptive immune responses [125]. More recently, it has been discovered that at sites of infection, adaptive immune memory cells can also regulate the innate inflammatory response, by the epigenetic reprogramming of innate immune cells by previous experiences [126]. Interestingly, it has been hypothesised that centenarians exert a counter action to this pro-inflammatory state, by producing increased amounts of anti-inflammatory cytokine mediators including IL-10 and TGF-β [127].

8.1 Sex Differences the Immunological Response

There is a growing body of evidence demonstrating inherent sex differences in the immune response, which includes both the innate and adaptive arms of the immune system. On average, females exhibit a stronger innate and adaptive immune response than males, resulting in improved pathogen clearance, vaccine efficiency, but greater predisposition to autoimmunity and chronic inflammatory disorders [128]. Sex chromosomes contribute to genetic differences due to the fact that many immune-related genes (e.g., FoxP3 and toll like receptors 7 and 8) are present on the X chromosome. As the possibility of incomplete X-chromosome inactivation occurring in females exists, this can lead to over-expression in females of such immune related genes [129, 130]. Sex hormones can also interact with genetic and environmental factors that determine immunity and appear to influence multiple aspects of the immune system, including the contribution to cell differentiation, cytokine profiles, and epigenetic alterations [130, 131]. Estrogen receptors have been shown to regulate cells and pathways in both arms of the immune system in a predominately stimulatory fashion, in addition to immune cell development [132]. On the other hand, androgens have been shown to be primarily immunosuppressive, suppressing immune reactivity and inflammation [133]. Evidence for sex disparity does exists in the immunological response throughout aging, although the majority of studies do not stratify for sex. Females who are in the menopausal period of their reproductive lives show increased peripheral pro-inflammatory markers, higher numbers of natural killer immune cells with reduced cytotoxicity, and reduced numbers of B and T

cells, relative to premenopausal females [134]. Research investigating differential sex influence in the innate immune system is still in its infancy, although data exist suggesting an elevated production of inflammatory cytokines and proteins in females compared to males persists among aged individuals [135, 136]. With regards to adaptive immunity, proliferation of T cells, central to the adaptive immune response, shows a decrease with aging, although this rate of decline was significantly lower in females, than in males [137]. Sex differences have also been noted to occur in response to vaccines protecting against influenza, tetanus, pertussis, shingles and pneumococcal infections, and the efficacy of vaccines in older individuals is consistently higher in females than in males [136].

Collectively, the major changes of the innate and adaptive immune system with aging result in vulnerability to certain infections, and decreased efficacy of many vaccines. Although sex differences in the aging immune system have not been studied extensively, addressing such sex differences may aid in greater effectiveness of vaccines and immunotherapies [130]. As several diseases associated with age are also sensitive to alterations of the immune system, it is important to evaluate potentially- associated sex differences in the underlying pathophysiological processes.

9 Conclusions

The capacity of homeostatic biological systems to continuously adjust for optimal functioning during healthy aging may reflect heterogeneity observed in the aging process. Importantly, such systems including endocrine, immune and cognitive systems appear to be moderated by sex, which may be a defining factor in determining sex-based differences in age-related processes, and diseases. Aging appears to be multi-factorial and characterised by the dysregulation of complex system dynamics, namely the interplay of these homeostatic mechanisms driving the aging process. It can be hypothesised that dysregulation in any of these systems may impose feed-forward alterations on other systems, and dysregulation early in the accelerated aging process may be different for different people. This makes it particularly challenging to research this field, especially early in the aging process. Composite biomarker panels are required at multiple time points in order to target multiple systems and to better understand the chronicity of biomarkers during aging. Moreover, it is critical that future studies specifically address sex-effects when studying the aging process and age-related diseases, to enable sex differences, which are non-modifiable risk factors, to be differentiated from potentially modifiable lifestyle and environmental risk factors. This is crucial to advance therapeutic options that may include sex-based and hormone-based interventions. Policies put forward by the National Institute of Health regarding the requirement of sex and gender inclusion plans in both preclinical and clinical research, underscores this point. Biomarkers of aging cannot escape this necessity.

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Chapter 5
The Therapeutic Potential of Ketogenic Diet Throughout Life: Focus on Metabolic, Neurodevelopmental and Neurodegenerative Disorders

Ann-Katrin Kraeuter, Paul C. Guest, and Zoltan Sarnyai

1 Introduction

Throughout the ages many great minds have prescribed fasting to improve physical and mental health. Nutritional interventions have been used to treat diseases throughout the ages. The Greek philosopher Hippocrates used food as medicine such as lentils for the treatment of ulcers and haemorrhoids. Galen, a Roman philosopher, believed that food could be the cure or the cause of diseases, stating that the excessive consumption of spicy foods (garlic leeks, onions) may cause a fever.

> *Everyone has a physician inside him or her; we just have to help it in its work. The natural healing force within each one of us is the greatest force in getting well. Our food should be our medicine. Our medicine should be our food. But to eat when you are sick is to feed your sickness.*
> Hippocrates

> *Instead of using medicine, rather, fast a day.*
> Plutarch

> *I fast for greater physical and mental efficiency*
> Plato

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Fasting is the greatest remedy, the physician within.
Phillippus Paracelsus

The best of all medicines is resting and fasting.
To lengthen thy life, lessen thy meals.
Benjamin Franklin

Although some of these ideas might be debatable with more recent research, these individuals hypothesised that food might be used as a therapeutic intervention. Fasting is one such nutritional treatment. The energy depots of mammals which enable fasting periods are the liver and adipose tissue [1]. Short periods of fasting or food restriction have been demonstrated to be beneficial in diabetes, cardiovascular disease, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke, cancer and obesity [1]. Periods of fasting result in an elevation of ketones [1]. Over a century ago, a study investigated the effects of starvation as a potential treatment for epilepsy [2]. The increase in ketone bodies, such as acetone and ß-hydroxybutyrate, during starvation periods was first described by Woodyatt in 1921 [3]. Also in 1921, Wilder described that the benefits of fasting could be achieved in a state of ketosis [4].

The ketogenic diet is a form of fasting through altering energy metabolism and producing ketones [5]. The ketogenic diet is a high-fat, moderate protein and low carbohydrate diet, which uses fat as an alternative fuel to glucose. The diet is traditionally composed of three to four grams of long chain-saturated triglycerides for every gram of carbohydrate and proteins [6]. This modern form of fasting reduces circulating insulin and insulin signalling, and results in a metabolic shift to fatty acid utilization [7], which leads to increased production of ketone bodies as an alternative fuel source to glucose [6, 8]. During the ketogenic diet, blood levels of free fatty acids rise [6]. Free fatty acids are transported into the mitochondrial matrix of the liver with the help of carnitine. Acetyl-coenzyme A (acetyl-CoA) is produced through ß-oxidation of the free fatty acids [6]. When glycolysis is predominant, acetyl-CoA is used to fuel the Krebs cycle. However, while in glucose-deprived states, such as ketosis, oxaloacetate is used for gluconeogenesis to maintain serum glucose levels. The decreased availability of oxaloacetate leads to a decreased amount of acetyl-CoA feeding into the Krebs cycle [8]. Consequently, excess acetyl-CoA will form the ketone acetoacetate, which spontaneously degrades into acetone or enzymatically metabolised into ß-hydroxybutyrate. Beta-hydroxybutyrate is formed in a reverse reaction with the help of the nicotinamide adenine dinucleotide hydride (NADH)-dependent mitochondrial enzyme ß-hydroxybutyrate dehydrogenase [8]. These ketone bodies then enter into the bloodstream.

Ketone bodies are not able to cross the blood-brain barrier without the aid of specific transporters. Beta-hydroxybutyrate and acetoacetate can be transported into the brain via the monocarboxylic acid transporter [9]. Interestingly, hippocampal mRNA levels of the monocarboxylic acid transporter are increased during the ketogenic diet [10]. Within the brain, beta-hydroxybutyrate is enzymatically converted by beta-hydroxybutyrate dehydrogenase into acetoacetate. Acetoacetate receives a coenzyme A from succinyl-CoA from the Krebs cycle to form acetoacetate-CoA.

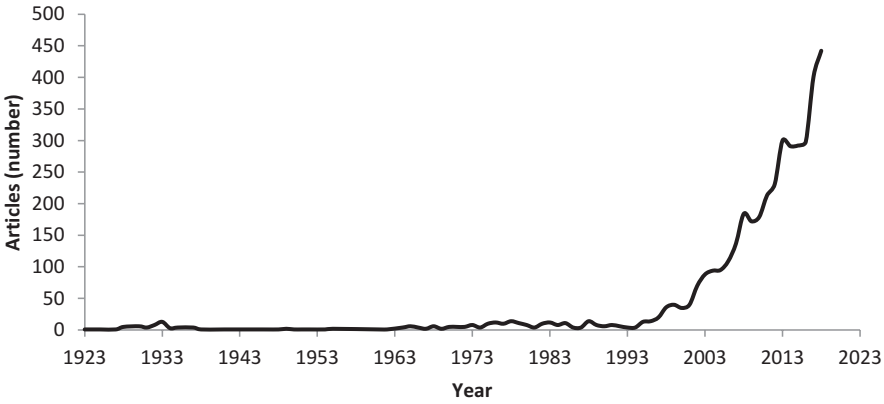

Fig. 5.1 Number of articles published on the ketogenic diet over 1923–2018 (Scopus)

Acetoacetate-CoA will spontaneously degrade to acetyl-CoA, which is able to fuel the Krebs cycle without using glucose or its metabolite pyruvate [8] and excess acetone is rapidly excreted through the breath and urine.

Within the scientific community, the ketogenic diet has received increasing interest in recent years (Fig. 5.1). It has been used in the management of treatment-refractory childhood epilepsy since the 1920s [6]. The potential of this diet as a nutritional therapeutic has been trialled in several disease areas, such as brain cancer [11], brain trauma [12], migraines [13, 14], type II diabetes, as well as neurodegenerative, neurodevelopmental and psychiatric disorders [15, 16]. The neurodegenerative disorders which have been tested in clinical trials using the ketogenic diet include Alzheimer's disease [16], Parkinson's disease [17, 18] and multiple sclerosis [19], and the neurodevelopmental and psychiatric diseases which have been trialled include autism spectrum disorder, anxiety and depression, [20–23], attention deficit hyperactivity disorder [23, 24], schizophrenia [25–28] and bipolar disorder [29, 30].

The pathophysiology of psychiatric conditions is complex and poorly understood [31]. Neurological disorders and substance abuse disorders have increased over recent years and have now closed the gap in terms of prevalence to cardiovascular diseases, which have a well-understood pathophysiology [31]. Current pharmacological treatments for most psychiatric conditions have limited efficacy and can results in considerable side effects. Although these treatments slow down disease progression they do not stop or cure the condition [32]. Therefore, alternative efficacious treatments with more tolerable side effects are needed. In this light, dietary interventions may have fewer side effects and result in better quality of life.

In this chapter, we have reviewed the efficacy of the ketogenic diet in a variety of neurodegenerative, neurodevelopmental and metabolic conditions throughout life. We will start with conditions affecting children, then we will consider metabolic disorders in adults and lastly we will cover conditions affecting the elderly. We have focused on application of the ketogenic diet in clinical studies as well as in preclinical

models and we discuss the known benefits and drawbacks. We also highlight the need for further research in this area aimed at discovery of novel mechanistic targets of the ketogenic diet, which might result in novel treatment options and development of new drugs which maximise the potential benefits/risks ratio.

2 Ketogenic Diet in Childhood

2.1 Epilepsy

Over a century ago, one study investigated the effects of starvation as a potential treatment for epilepsy [2]. In addition, Wilder described for the first time that the benefits of fasting could be achieved in a state of ketosis [4]. It was proposed that a state of ketosis without actual starvation is more sustainable for the treatment of epilepsy in clinical trials [4]. In 1925, Peterman reported the composition of the ketogenic diet as similar to that which is used currently [33]. Acetone and acetoacetate have anticonvulsant effects, which can therefore prevent acutely provoked seizure [6]. This dietary intervention lost its popularity in the 1930s, after the introduction of the first effective anticonvulsant drug, diphenylhydantoin [34]. In 1972 at Johns Hopkins Hospital, Livingston reported the use of the ketogenic diet in 1000 children with epilepsy, resulting in 52% of the patients having complete seizure symptom control and 27% with improved symptoms [35]. In the 1990s, increased drug-resistance to commonly used anticonvulsant drugs resulted in a resurgence of the ketogenic diet as a treatment for epilepsy [36] (Fig. 5.2). Excessive neuronal excitability causes seizures, which may be ameliorated with the ketogenic diet through multiple pathways [37]. A number of recent meta-analyses have

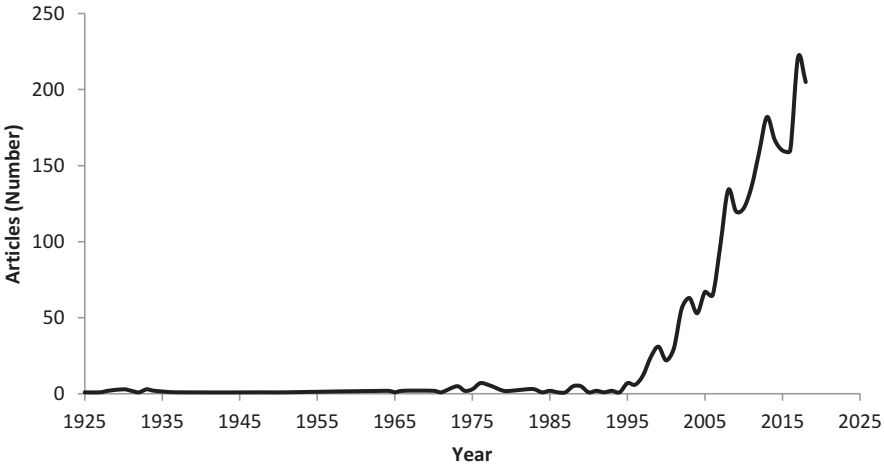

Fig. 5.2 Number of articles published on the ketogenic diet in epilepsy from 1925–2018 (Scopus)

demonstrated the efficacy of the diet in epilepsy [38–40]. In addition, a recent study investigated the efficacy, tolerability and complications of an olive oil-based ketogenic diet treatment in children with epilepsy and found that previous adrenocorticotropic hormone use and the occurrence of constipation at baseline or during treatment were predictors of treatment efficacy [41]. Another study found that the ketogenic diet influenced both the taxonomic and functional composition of gut microbiota in children with severe epilepsy but could not determine whether these changes are involved in efficacy or the side effects of the diet [42]. Finally, a scoping review is underway to determine which outcomes should be measured and reported in studies of childhood epilepsy treated with the ketogenic diet therapy as a means of improving outcomes or helping to monitor side effects [43].

2.2 Attention Deficit Hyperactivity Disorder (ADHD)

ADHD is the most common childhood neurodevelopmental disorder [44–46]. A meta-regression analysis estimated that around 5.29% of children and adolescents might have ADHD [47]. ADHD is characterised by impulsivity, motor hyperactivity, inappropriate behaviour and inattention [44–46]. Diseases associated with ADHD are autism spectrum disorder, communication, specific learning or motor disorders, intellectual disability and tic disorders [45]. In addition, ADHD is accompanied by psychiatric co-morbidities, such as conduct disorder, depression, bipolar disorder and substance abuse disorders [48].

The pathophysiology of ADHD is poorly understood but it has been suggested that abnormal dopaminergic, noradrenergic and serotonergic neurotransmission plays an important role [45]. Current treatment of ADHD includes the use of stimulants of neurotransmitters, dopamine and norepinephrine [45]. Such stimulants increase catecholamine availability and have been shown to be effective in children and adults [45, 49].

Preclinical research investigating the effects of the ketogenic diet in ADHD is limited due to the low availability of appropriate animal models of ADHD. Nevertheless, studies in rodents have found that the diet reduces activity levels compared to control animals [50, 51]. A study in dogs that showed certain behavioural features of ADHD found a decrease in chasing behaviour, but no change in excitability and trainability, after the dogs were fed a diet supplemented with medium chain triglycerides [52].

Thus far, one case report demonstrated that the ketogenic diet improved behaviour, hyperactivity and aggression in a child with ADHD and autism spectrum disorder [23]. Another study which investigated the effects of a ketogenic diet on seizure frequency found improved motor functioning, self-help skills, attention and social skills in children, who had abnormal emotional and behavioural responses at the study onset.

These limited results from human studies suggest that the ketogenic diet may have a potential to attenuate some of the features of ADHD in children.

2.3 Autism Spectrum Disorders

Autism spectrum disorder is a life-long neurodevelopmental condition defined by behavioural abnormalities, such asocial behaviour, communication and repetitive behavioural patterns [53–55]. It is estimated that 1 in 88 children have autism spectrum disorder and is four time more common in males than females [53]. Furthermore, most patients with autism spectrum disorder had intellectual disability (55%) and below average intelligence (17%) in a conventional IQ test [53]. Comorbidities include seizures, gastrointestinal disturbances, motor impairments, auditory disorder, altered sleep, increased anxiety and heightened or reduced sensitivity to stimuli, such as sound or temperature [53, 56].

The aetiology of autism spectrum disorder is unknown but genetic and environmental factors leading to altered neurodevelopment has been suggested [53, 56]. Due to difficulties in identifying individual causative genes, research has focused on environmental factors, such as maternal immune activation, in utero exposure to pesticides, oxidative stress and environmental contaminants [53, 55, 56]. The medical management of autism spectrum disorder includes educational interventions and/or the use of antipsychotic medications [57]. These treatment options are not specific for autism spectrum disorder or its core symptoms and show poor symptom control [58].

Multiple preclinical studies have investigated the effects of the ketogenic diet in animal models of autism spectrum disorder. Improvements have been reported in the core symptoms of autism spectrum disorder with the diet including sociability [59–64], communication [59] and repetitive behaviours [59, 61–64]. Preclinical studies demonstrated that the ketogenic diet alters mitochondrial bioenergetics [65], neurotransmitter signalling [65], hormonal metabolism [65] and the gut microbiome [66], which may contribute to behavioural improvements.

The effects of the ketogenic diet on autism spectrum disorder behaviours have been study extensively in children. Studies have demonstrated that the diet was highly effective in improving mild to moderate symptoms of autism spectrum disorder [20, 22], including sociability, calmness, cognition, language function, stereotypical behaviour, IQ [21], while decreasing hyperactivity and aggressive behaviour [23]. A modified Atkins diet (60% fat, 30% protein, 10% carbohydrates) improved Childhood Autism Rating Scale and the Autism Treatment Evaluation Checklist scores in the areas of speech, sociability and cognition [67]. These promising early results support further research into the possible clinical application of the ketogenic diet in this neurodevelopmental condition.

3 Ketogenic Diet in Adolescence/Early Adulthood

3.1 Schizophrenia

Schizophrenia is a debilitating and life-long psychiatric disorder with an onset typically in early adolescence and is among the leading causes of disability worldwide [68–71]. Schizophrenia affects more than 21 million people (1%) worldwide [72].

Schizophrenia is characterized by three major symptom clusters classified as positive (psychotic), negative and cognitive symptoms [71]. Positive symptoms include visual and auditory hallucinations, disorganized thoughts and language, abnormal and disorganized behaviour and catatonia [73, 74]. Furthermore, individuals with schizophrenia have difficulties with normal emotional responses resulting in social withdrawal, anhedonia and a lack of motivation. These latter characteristics are termed the negative symptoms [71]. Cognitive symptoms are characterized by deficits in working memory [75]. The disease progresses in severity, usually beginning in adolescence with positive symptoms, which are followed later in life by appearance of negative and cognitive symptoms [73]. Schizophrenia has a high mortality rate because of the development of secondary diseases such as cardiovascular disorders and due to an increased proportion of suicides compared to the general population [76–78].

The pathophysiology of schizophrenia is complex. The symptoms are possibly underlined by hyperactivity of the mesolimbic dopamine pathway [79–84], disturbance in glutamatergic functioning [71], decreased activity in mesocortical dopamine projections to the prefrontal cortex [83], genetic factors [75, 85, 86], impairment in cerebral glucose metabolism [87–92] and environmental factors [75]. Current pharmaceutical treatments for schizophrenia include typical and atypical antipsychotic drugs, which act mostly on the D_2 dopamine receptors as antagonists. These are partially effective for positive symptoms but they can have significant side effects.

Several studies have demonstrated through in vivo imaging, post mortem brain analysis and animal studies that schizophrenia might be linked to abnormal cerebral glucose metabolism [91, 93–98]. On the basis of such findings it can be hypothesised that the ketogenic diet might be able to bypass glucose metabolism and use ketone bodies as an alternative fuel source. We demonstrated that the ketogenic diet effectively restored abnormal schizophrenia-like behaviours across the whole range of symptoms in an acute animal model of schizophrenia [99, 100]. Application of the ketogenic diet in an acute animal model of schizophrenia attenuated hyperactivity, stereotypical behaviour and increased sociability, working memory and prepulse inhibition of startle. Another group investigated a genetic mouse model of schizophrenia using the ketogenic diet [101]. The investigators used hippocampal auditory P20/P40 gating to assess the hippocampal inhibitory circuit by implanting depth electrodes into the hippocampus of DBA/2 mice, a translational endophenotype that mimics the inhibitory P50 sensory gating deficits in schizophrenia patients. This enabled determination of the ability to filter early neuronal responses to the second of a pair of identical and repeated auditory stimuli and the results showed that the ketogenic diet significantly improved auditory gating in the mice and reduced the second response.

According to Dohan in 1966 [102], schizophrenia-related hospitalizations decreased, because of limited bread consumption during World War II. Further studies demonstrate that a gluten free diet might be beneficial for patients with schizophrenia [103]. Two early case studies suggested that the ketogenic diet might have clinical efficacy in the management of schizophrenia [25, 26]. More importantly,

two recent case reports showed that the ketogenic diet improved auditory hallucinations, mood, energy, the ability to concentrate and sociability in patients with schizoaffective disorder [27]. This and another recent study showed improvement in the Positive and Negative Syndrome Scales symptom scores following the ketogenic diet [27, 28].

These studies demonstrate the potential positive effects of the ketogenic diet. However, additional larger scale studies are needed to fully evaluate the efficacy in a larger cohort of schizophrenia patients.

4 Ketogenic Diet in Adults

4.1 Obesity and Type II Diabetes

The global incidence of diabetes has increased due to an aging population, urbanization and unhealthy lifestyle choices [104]. It is predicted that by 2030, 439 million people globally will have diabetes, with 90% having type II diabetes. The main modifiable risk factors for type II diabetes are obesity and physical inactivity. Worryingly, type II diabetes has become increasingly prevalent in adolescence and even in children. It is predicted that obesity and overweight prevalence in adults will increase from 33% in 2005 to 57.8% in 2030 [104]. Obesity is the single most important predictor for type II diabetes [104] and a large proportion of patients with type II diabetes are overweight or obese [105].

Obesity is a major health risk associated with cardiovascular complications, such as high blood pressure, dyslipidaemia, abnormal function and size of the left ventricle, disturbed endothelial function and hyperinsulinaemia [106]. Other comorbidities of obesity include metabolic syndrome [107], irregular menstruation due to excess oestrogen levels [108], infertility of both genders [109, 110], non-alcoholic steatohepatitis, arthritis, obstructive sleep apnoea, stroke, arrhythmias and heart failure [108]. Obesity may result in a reduction of the life expectancy of a 40 year-old person by 7 years [111].

For decades, high fat intake has been linked to obesity, diabetes, and heart disease [112]. Interestingly, the debate in the media about which diet is most appropriate for effective weight-loss is still under debate. Furthermore, a lack of knowledge of health practitioners about current therapeutic interventions reduces the likelihood of obese patients receiving the best care of practice to succeed in losing weight [113]. A particular focus has been placed on the increase or decreased consumption of the macronutrients, fats and carbohydrates. However, the question remains to this date which diet is more effective for weight loss and overall health, a low-fat or low-carbohydrate diet.

After a report by the U.S. Senate Select Committee on Nutrition and Human Needs in 1977, which entailed reduced consumption of fats and increased carbohydrate consumption, obesity rose significantly within the U.S. population [112, 114].

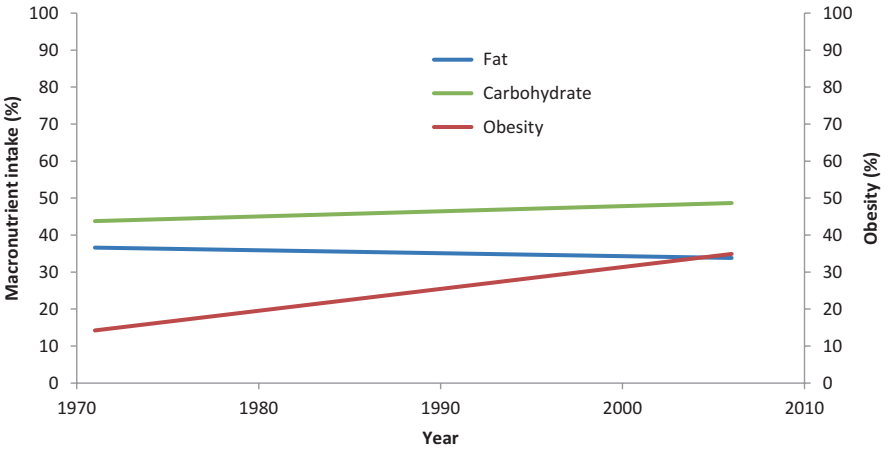

Fig. 5.3 Relationship between obesity and macronutrients, fat and carbohydrates. (Data by Austin et al. (2011))

Furthermore, a study demonstrated the effects of macronutrient change from 1971 to 2006 within the U.S. population [115]. Using the published data from the above study, we found that fat consumption had an inverse relationship with obesity, while carbohydrate intake increased in parallel with obesity (Fig. 5.3). To shed further light on this phenomenon, a prospective urban rural epidemiology study investigated the dietary intake of 13,335 individuals with a median follow-up of 7.4 years [116]. This investigation found that high carbohydrate intake was associated with a greater risk of mortality compared to fat intake. Fat intake was not associated with cardiovascular disease, myocardial infarction, or cardiovascular disease mortality.

Multiple studies have outlined the successful use of the ketogenic diet in obesity through an increase in satiety [117]. The ketogenic diet has gained popularity in obesity since the early 2000s from only two publications in 2002 to 60 publications in 2018 (Fig. 5.4). A recent review outlined the potential effect of the ketogenic diet as a weight loss treatment [118]. All of the studies reviewed showed a reduction in body weight and body fat. Another study compared the effects of a low-carbohydrate diet (70% kcal from fat) with those of a ketogenic (89% kcal from fat) and a control diet in C57Bl/6 mice (all diets maintained at 11.2 kcal/day) [119]. After 100 weeks, the animals fed the low-carbohydrate diet were significantly heavier compared to the ketogenic diet and control groups.

A number of clinical studies have also been conducted which addressed this subject. For example, a recent clinical study investigated 35 sedentary obese patients on the ketogenic diet for 12 weeks, which resulted in significantly reduced appetite and weight loss [120]. Another clinical study of 80 overweight or obese patients demonstrated that those placed on a low-calorie ketogenic diet lost significantly more weight than those on a low-calorie diet [121]. Similarly, another study found a significant weight loss with a ketogenic diet [122].

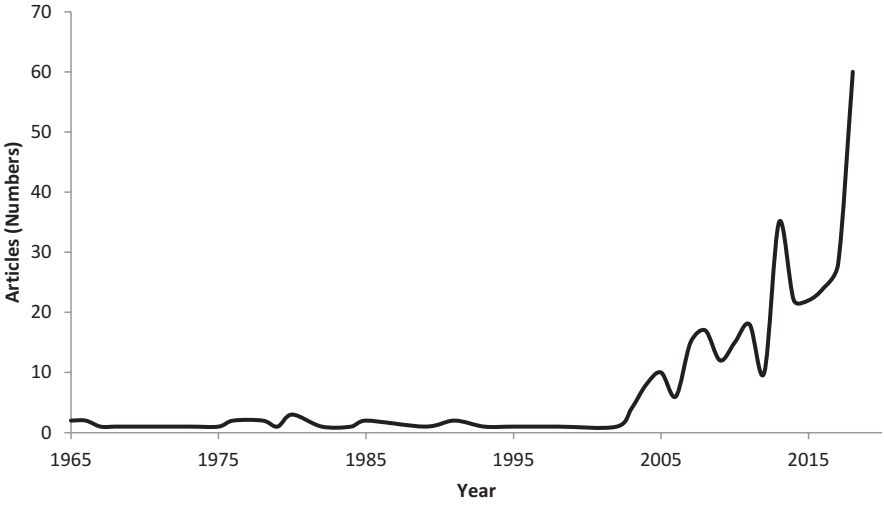

Fig. 5.4 Number of articles published on the ketogenic diet in obesity from 1965–2018 (Scopus)

The ketogenic diet has been shown to have positive effects on metabolic biomarkers. A critical review demonstrated that dietary carbohydrate restriction was beneficial for patients with type II diabetes by decreasing blood glucose levels and having a positive effect on lipid levels [123]. Furthermore, the patients required less insulin and therefore experienced fewer side effects compared to pharmacological treatments. Elevated blood glucose levels have been shown to cause kidney damage, damage of the retina, neurological problems and diabetes mellitus [124]. A low carbohydrate diet can reduce weight and improve glycaemic control [105]. However, long-term reduction of carbohydrates may lead to hypoglycaemia in people taking insulin [105]. A study demonstrated that 8 weeks of the ketogenic diet significantly reduced blood glucose concentrations and insulin levels [120], lowering the risk for metabolic syndrome and type II diabetes [106, 125, 126]. The effect of the ketogenic diet on glucose tolerance and insulin sensitivity has been controversial. One study demonstrated that mice treated for 1 month with the ketogenic diet at 11.2 kcal/day showed impaired glucose tolerance compared to control animals [119]. However, other studies showed that mice fed ad libitum with a ketogenic diet had enhanced glucose tolerance [127]. Another study demonstrated that mice fed previously on a high fat diet for 12 weeks and switched to a ketogenic diet showed a significant improvement in glucose tolerance and insulin sensitivity [128]. Furthermore, Badman et al. showed improved glucose sensitivity in ob/ob mice fed the ketogenic diet [129]. In contrast, a number of studies have shown that the ketogenic diet led to increased hepatic insulin resistance [130] and decreased glucose tolerance [130, 131].

Consistently high levels of glycated haemoglobin (HbA1c) are associated with an increased risk of developing diabetes mellitus [132, 133]. In light of this, admin-

istration of a ketogenic diet for 16 weeks led to significantly decreased levels of HbA1c in humans [132]. In a similar manner, decreased levels of high-density-lipoprotein (HDL) increase the risk for coronary heart disease [134] and 16 weeks on the ketogenic diet led to an increase in circulating HDL levels [132]. On the other hand, lower levels of low-density-lipoprotein (LDL) are associated with decreased risk of cardiovascular disease [134] and 24 weeks on the ketogenic diet was found to result in significantly reduced LDL levels [135]. However, a more recent study found an increase in LDLs in obese patients after 12 weeks of the ketogenic diet [120]. Although cholesterol is important in physiological processes such as forming the scaffolding of cell membranes and production of steroid hormones, vitamin D and bile acid, increased levels of cholesterol may lead to cardiovascular disease [134]. In obese patients, total cholesterol was significantly reduced on the ketogenic diet within 8 weeks [135]. Likewise, triglycerides levels (also associated with cardiovascular disease) were significantly decreased in the obese patients after 24 weeks on the ketogenic diet, despite showing an initial increase [135]. Leptin is important in regulating food intake. Therefore, high levels of leptin may cause obesity [136]. In this light, it is interesting that the ketogenic diet significantly decreased leptin levels [120, 137].

These studies show that the ketogenic diet might be beneficial in the case of obesity. However it is still not clear if the ketogenic diet has benefits for type II diabetes due to the observed conflicting effects on insulin sensitivity and glucose tolerance.

5 Ketogenic Diet in Old Age

5.1 Alzheimer's Disease

Alzheimer's disease is a progressive and ultimately fatal neurodegenerative disorder [138]. In 2015, 46.8 million people worldwide suffered from dementias including Alzheimer's disease [139, 140]. It is predicted that by 2050 the number of people suffering from dementia including Alzheimer's disease will nearly triple to approximately 131.5 million people [139, 140]. Alzheimer's disease is the most common form of dementia characterized by progressive cognitive impairments leading to deficits in speech, thoughts and problem solving, and causing disorientation, confusion, irritability and aggression [140, 141]. Brain imaging studies have shown that hallmark physical features of Alzheimer's disease include neurofibrillary tangles and ß-amyloid plaques, causing cortical atrophy and decreased synaptic plasticity [140, 142]. Excessive neuronal loss will result in loss of bodily functions resulting in death [143].

Current treatment options for mild-moderate Alzheimer's disease include cholinesterase inhibitors, N-methyl d-aspartate (NMDA) receptor antagonists and non-pharmacological interventions [141, 144]. All of the above-mentioned interventions

slow down cognitive decline and temporarily reduce functional deterioration but do not stop the neurodegeneration. Alzheimer's disease is occasionally referred to as Type-3 diabetes due to the association of cognitive impairments and diabetes mellitus [145]. Patients with diabetes have a 65% increased risk of developing Alzheimer's disease compared to patients without diabetes mellitus [145, 146]. Along similar lines, patients with Alzheimer's disease have been shown to be more likely to have insulin resistance [146]. However, the underlying molecular mechanisms for this linkage are not understood. Nevertheless, a number of studies have confirmed that insulin signalling is impaired in Alzheimer's disease and animal models of Alzheimer's disease [146]. Therefore, the ketogenic diet effecting insulin pathways might serve as a novel therapeutic approach for the treatment of Alzheimer's disease [145, 147].

Preclinical evidence has suggested that the ketogenic diet may have some efficacy in Alzheimer's disease. Studies in animal models of Alzheimer's disease have demonstrated behavioural improvements with the ketogenic diet and beta-hydroxybutyrate treatment, such as increased activity [148–151] and improved memory performance [150, 152]. These behavioural improvements were underlined by reduced levels of ß-amyloid plaques [148–150, 152], reduced oxidative stress [148] and improved mitochondrial function [151, 152].

High fat, "Western" diets (HFD- high in carbohydrates and fat) have been implicated as a risk factor for Alzheimer's disease. A longitudinal community-based study from the Chicago Health and Aging Project investigated the effects of various dietary fats on Alzheimer's disease between 1993 and 1997 [153]. The subjects of the study showed a high correlation between consumption of high amounts of saturated and trans-unsaturated fats and development of Alzheimer's disease, whereas participants consuming higher amounts of omega-3 polyunsaturated fatty acids had a significantly reduced risk of developing the disorder. Total fat, animal fat and dietary cholesterol were not associated with an increased risk of developing Alzheimer's disease.

In a clinical study, 23 older adults with mild to moderate memory decline were assigned to either a high or low carbohydrate diet for 6 weeks [154]. Patients on the low carbohydrate diet improved in paired-associated learning after 6 weeks on the diet compared to baseline, whereas no difference was found for the high carbohydrate group. The subjects consuming the low carbohydrate diet showed significantly reduced weight, fasting glucose and fasting insulin levels. No changes in metabolic parameters were found for the high carbohydrate group after 6 weeks on their assigned diet compared to baseline. Finally, a significant positive correlation was identified between ketone levels and memory performance in the low carbohydrate group. This study demonstrated that decreased carbohydrate consumption results in ketosis and decreased insulin levels. However, the authors found no positive correlation between insulin levels and memory performance, which was suggested to be due to the limited sample size. Another clinical study demonstrated that calorie restriction in a larger cohort improved memory performance and they confirmed that this was correlated with decreased fasting insulin levels [155].

A case report demonstrated that supplementing a diet with ketogenic agents, such as ketone monoesters and medium chain fatty acids, resulted in cognitive improvement [156]. The patient rediscovered everyday tasks such as showering and shaving and their memory significantly improved after 6 weeks of treatment with ketone monoesters. The authors showed that the cognitive improvement was closely linked to increased beta-hydroxybutyrate levels. Another case report showed a significant improvement on Montreal Cognitive Assessment scores for a patient with mild Alzheimer's disease placed on the ketogenic diet [157].

Using a small, single-arm pilot clinical trial format, a study showed that a ketogenic diet supplemented with fatty acids resulted in a state of ketosis [16] and the patients showed significantly improvements in their Alzheimer's Disease Assessment Scale-cognitive subscale scores after 3 months. These studies indicate that a ketogenic diet might be beneficial for patients with Alzheimer's disease both from a metabolic and a cognitive perspective.

5.2 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder [158–160], affecting approximately 1% of the population aged 60 years or over [160–162]. Parkinson's disease is characterized by bradykinesia and akinesia (slowness or loss of movement), muscle rigidity, tremor and postural instability [160, 162, 163]. Non-motor symptoms include hyposmia, autonomic dysfunction, sleep dysfunction and a variety of psychiatric symptoms [159]. Genetic predisposition, environmental toxins and oxidative stress play a role in the pathophysiology of Parkinson's disease [163]. The core process of Parkinson's disease is the loss of dopaminergic neurons in the substantia niga region in the midbrain in combination with appearance of Lewy bodies and Lewy neuritis [159]. Patients with Parkinson's disease start showing symptoms when over 50% of the dopaminergic cells are lost [164]. Dopamine replacement therapy, such as L-dihydoxyphenylalanine (L-Dopa) and receptor agonists, is currently the most wildly used treatment [160]. Treatment can mitigate the symptoms but does not delay the neurodegeneration or regenerate the lost neurons [160].

Inflammation is a mayor hallmark of the disease, with an increase in inflammatory cytokines in the brain and the cerebrospinal fluid [158]. The ketogenic diet has been shown to have beneficial effects on inflammation [37] and may therefore might be a novel and safe treatment for patients with Parkinson's disease. Preclinical studies have investigated the effect of the ketogenic diet in a variety of different animal models of Parkinson's disease. Such studies have demonstrated increased motor performance following a ketogenic diet [165, 166] and administration of beta-hydroxybutyrate injections [167]. These studies further demonstrated that the ketogenic diet increased dopamine and its metabolites and normalised neuronal counts in the substantia nigra pars compacta [165, 168]. Likewsie, beta-hydroxybutyrate treatment increased dopamine metabolites, ATP production and mitochondrial oxy-

gen consumption [167]. In addition, the ketogenic diet led to reduced levels of pro-inflammatory cytokines [165].

The ketogenic diet has been trialled in humans with Parkinson's disease and was able to improve resting tremors, freezing, balance, gait, mood and energy levels within 1 month [17]. Two studies demonstrated improvement on the Unified Parkinson's Disease Rating Scale following the ketogenic diet [17, 18]. Importantly, another study demonstrated that the diet did not interfere with the pharmacokinetics of L-Dopa [169].

6 Potential Side Effects of Ketogenic Diet

The ketogenic diet is a therapeutic nutritional intervention and may have some side effects. Some side effects are short or long lasting and can be temporary or lead to permanent changes. These side effects can led to discontinuation of the diet, although appropriate nutritional guidance and adjustments of the diet can reduce or eliminate side effects.

In a randomized controlled trial, 73 patients were recruited and 6 of these poorly tolerated the diet, three withdrew because of parental unhappiness and one child showed increased seizure activity [170]. Kang et al. investigated 129 children with epilepsy and assessed the safety of the ketogenic diet [171]. Minor side effects included extreme drowsiness, dehydration (46.5%), abdominal pain (9%), hunger (22%), lack of energy (24%) and gastrointestinal disturbance such as constipation (33%), vomiting (24%, 27.9%), diarrhoea (13%, 32.6%), constipation (2.3%) and infectious diseases like pneumonia, cystitis and non-specific febrile illnesses (9.3%) and hypercholesterolemia (14.7%), hyperuricemia (26.4%), symptomatic hypoglycaemia (7%), hypoproteinemia (5.4%), hypomagnesaemia (4.7%), repetitive hypernatremia (4.7%) and hepatitis (2.3%). Kang et al. reported that all of the above-mentioned side effects were transient and required only short-term medication. Constipation is caused by the high-fat and low-fibre content of the diet [172]. In line with this, a randomized control trial showed that 33% of children on the ketogenic diet were constipated [170]. On the other hand, the ketogenic diet may cause diarrhoea due to ineffective absorption and the high fat content of the diet [171]. Constipation and diarrhoea can be prevented through administration of vitamins and supplements. Supplement treatment will also guarantee delivery of trace minerals that are reduced within a standard ketogenic diet. The lack of essential minerals may cause reduced energy, which was seen in 24% of patients on the diet [170]. The associated dehydration caused dried mucous membranes [173]. The vomiting, constipation and diarrhoea might be due to the rapid change in diet. Protein malnutrition, especially in children, has to be prevented due to potential stunted growth [174, 175]. With this in mind, a retrospective review of 32 infants showed that 96.4% of patients showed appropriate growth [176].

Kidney stones may occur in 3.1% of patients on the ketogenic diet [171], which can be mediated through increased fluid intake and reduced intake of medication

[177, 178]. A review of 58 children at the Johns Hopkins Hospital between 1980 and 1985 revealed that three patients on the diet developed kidney stones, two had hyperuricemia, one had acidosis and one had hypocalcaemia [179].

At Johns Hopkins Hospital in 1972, 1000 patients with epilepsy on the ketogenic diet showed only a few serious complications [35]. A prospective study of 150 children showed that two children died when on the ketogenic diet, but these children were suffering prior to the study from cardiac dysrhythmia and died of cardiac arrest 1 month after starting the diet. In a further study, two children developed hypoproteinemia, one child developed Fanconi's renal tubular acidosis and two children showed an increased liver function test. Notably, these children were on valproate [180], which has been shown to induce Fanconi's renal tubular acidosis and an increased liver function test [171]. Therefore, it is still not clear whether the ketogenic diet or the anti-seizure medication caused these side effects. Another rare side effect may be acute haemorrhagic pancreatitis, caused by hyperlipidaemia and hypertriglyceridemia [173, 181].

In conclusion, the ketogenic diet is generally safe and unwanted side effects can be eliminated by slightly adjusting the dietary composition. It is rarely required to medicate the side effects or discontinue the diet. However, the level of ketosis must be regulated tightly to ensure efficacy with minimum side effects. A recent study introduced a compact and inexpensive breath sensor to monitor ketosis online [182]. They tested the device on 11 subjects undergoing a 36 h ketogenic diet treatment and the sensor recognized the onset and progression of ketosis, and the readings were consistent with those from capillary blood β-hydroxybutyrate (BOHB) measurements. Interestingly the sensor was capable of detecting those individuals with lower tolerance to the diet. It should be noted that when deficiencies are identified such as depletion of key vitamins and minerals these can be included in the diet as supplements [183].

7 Conclusion

This accumulation of preclinical and clinical evidence indicates that the ketogenic diet could potentially be an effective therapeutic avenue for a variety of disorders with metabolic and inflammatory components. However, current evidence is lacking in randomised, properly controlled, larger scale clinical trials to fully evaluate the efficacy of the ketogenic diet and the potential side effects in the aforementioned diseases and disorders (Table 5.1). The exact mechanism of action of the ketogenic diet has yet to be established in the individual diseases and disorders. The ketogenic diet appears to have fewer side-effects than most current treatment options and might therefore be a potential novel and safer treatment avenue for diseases. Although the ketogenic diet was effective in treating a variety of diseases, it cannot be neglected that the main complication with this diet is non-compliance. Therefore, substances acting like the ketogenic diet and therefore increasing ketosis might have similar beneficial effects as highlighted in this chapter. One recent study showed

Table 5.1 Available evidence of diseases through-out life

Disorder	Preclinical	Case study	Uncontrolled trial	Randomised, controlled trial
Childhood				
Epilepsy	√	√	√	√
ADHD	√	√	√	
ASD	√	√	√	
Adolescence/Early adulthood				
Schizophrenia	√	√		
Adult				
Obesity/Type-2 diabetes	√	√	√	
Elderly				
Alzheimer's	√	√	√	
Parkinson's	√	√	√	

ADHD attention-deficit hyperactivity disorder, *ASD* autism spectrum disorder, √published evidence available

that the ketogenic diet may even have uses as an effective alternative therapeutic strategy for super-refractory status epilepticus patients in intensive care units for reducing seizure frequency and weaning from prolonged mechanical ventilation [184]. However, close monitoring and preventive management of potential adverse effects are critical elements for success. It may even be possible that certain populations respond differently to the ketogenic diet depending on the condition itself as well as certain biomarker readings. Thus, it may be beneficial to carry out further research aimed at identification of such biomarkers. Future research should also be aimed at discovery of novel mechanistic targets of the ketogenic diet, which might result in novel treatment options.

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Chapter 6
Coenzyme Q10 Supplementation in Fibrosis and Aging

Iain P. Hargreaves and David Mantle

1 Introduction

Coenzyme Q10 (CoQ10) is a naturally occurring vitamin-like substance, first characterised in 1957 by Professor Fred Crane at the University of Wisconsin, USA [1]. CoQ10 is a lipophilic molecule which is a member of a group of compounds known as ubiquinones because of their ubiquitous distribution in nature, being found in animal, plants and microorganisms [1]. Ubiquinones are composed of a benzoquinone nucleus and an isoprenoid side chain, which varies in length among the different ubiquinone species with CoQ10 having a side chain composed of ten isoprenoid subunits (Fig. 6.1) [1]. CoQ10 is the predominant ubiquinone species found in human tissues [1]. CoQ10 plays an essential role in cellular energy generation within the mitochondrial respiratory chain (MRC). The role of CoQ10 is of particular importance in tissues with a high energy requirement, such as cardiac muscle. In addition to its role in cellular energy generation, CoQ10 also serves as an important lipid soluble antioxidant and anti-inflammatory agent within the body [1–3]. The objective of this article is to review the potential role of CoQ10 as a biomarker of aging, specifically with regards to the prevention of tissue fibrosis in the heart, which has been implicated in age-related dysfunction of this organ.

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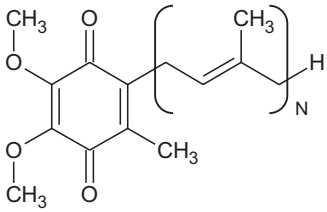

Fig. 6.1 The structure of ubiquinones showing the bezoquinone nucleus and isoprenoid side chain. `N` represents the number of isoprenoid side chain units, which is 10 in Coenzyme Q_{10} (CoQ_{10})

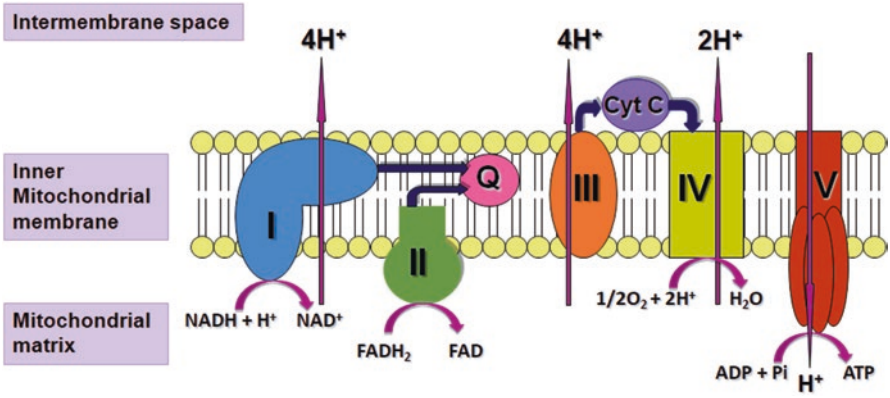

Fig. 6.2 Diagram of the mitochondrial respiratory chain (MRC) and complex V illustrating proton (H+) movement during oxidative phosphorylation. Q: Coenzyme Q_{10}. Cyt C: Cytochrome c

2 Functions of CoQ10

CoQ10 serves as an electron carrier within the MRC (Fig. 6.2), where it is involved in the transfer of electrons derived from complex I (NADH:ubiquinone reductase) and complex II (succinate dehydrogenase) to complex III (ubiquinol cytochrome c reductase), allowing a continuous passage of electrons within the MRC, which is required in the process of oxidative phosphorylation and the concomitant production of ATP, the energy currency of the cell [1]. Tissues with a high energy requirement, especially the heart and skeletal muscles, contain higher numbers of mitochondria within their cells and are particularly reliant on maintaining adequate tissue CoQ10 levels for normal functioning [2]. Thus, the heart and skeletal muscles typically contain about 1000 mg of CoQ10, out of a total body pool of 1500–2000 mg. CoQ10 occurs in cells in two closely related forms, oxidised (ubiquinone) and fully reduced (ubiquinol) [1]. The interconversion between these two forms is essential for the normal functioning of CoQ10, and this interconversion is principally mediated by the selenium-containing enzyme, thioredoxin reductase [4].

CoQ10 is also important within the body serving as a major fat-soluble antioxidant, protecting cell membranes (particularly those of the mitochondria) and circulatory lipoproteins from the damaging effects of free radical species [e.g., reactive oxygen species (ROS)] [1]. CoQ10 is the only lipid soluble antioxidant produced

within the body [5]. The antioxidant function of CoQ10 is attributed to its ubiquinol form, which must be constantly regenerated from CoQ10 to maintain its antioxidant capacity [5]. In addition to directly preventing lipid peroxidation, ubiquinol is able to enhance the cellular antioxidant capacity by maintaining the antioxidants, vitamin C and E, in their active fully reduced forms [6]. Most recently, a gene expression profiling study showed that CoQ10 influences the expression and regulation of hundreds of genes in the human body [7]. In cell culture, CoQ10 has been shown to reduce the activity of inflammatory markers, suggesting CoQ10 may have anti-inflammatory action via gene expression modification, most notably in elderly individuals [8, 9].

3 Synthesis and Deficiency of CoQ10

Although some CoQ10 is obtained from the diet, most of the daily requirement is synthesized within the body, particularly by the liver, although all organs of the body have the capacity to synthesize CoQ10 [1]. Based on a total body pool of 2 g and an average tissue turnover time of 4 days, the human requirement for CoQ10 has been estimated at approximately, 500 mg/day [10]. A relatively small proportion of this daily requirement is obtained from the normal diet, typically up to 5 mg/day [10]. The synthesis of CoQ10 is a complex, multistage process requiring a number of amino acids, vitamins and trace element precursors and cofactors, and a deficiency in any of these can adversely affect the normal production of CoQ10 [1]. It is noteworthy that CoQ10 shares a common biosynthetic pathway with cholesterol [1]. As people age, it has been reported that the capacity of the body to synthesize CoQ10 decreases [5]. Optimal production of CoQ10 occurs around the mid-twenties, with a continual decrease thereafter to approximately 50% at age 70. CoQ10 levels can also be depleted by intense exercise, certain types of prescription medicines, and illnesses [1, 5]. Dietary supplementation with coenzyme CoQ10 therefore provides a mechanism to maintain adequate levels of CoQ10 within the body.

In humans, at least 13 genes are thought to be involved in the biosynthesis of CoQ10, and mutations in 10 of these genes have been associated with primary CoQ10 deficiency, a condition which results from a genetic defect in the CoQ10 biosynthetic pathway [11–13]. The first cases of primary CoQ10 deficiency were reported in 1989 by Ogasahara and colleagues [14]. The two patients were sisters born to unrelated parents and they presented with recurrent rhabdomyolysis, which was associated with developmental delay and seizures [14]. Subsequently, a number of other patients have been described with primary CoQ10 deficiency, which appears to have a heterogeneous clinical presentation. This can be divided into five distinct clinical phenotypes: (1) encephalomyopathic (as in the case of the two siblings described by Ogasahara et al. [14]; (2) cerebral ataxic; (3) infantile multisystem disease; (4) steroid resistant nephrotic syndrome; and (5) isolated myopathy [15].

In most cases of suspected primary CoQ10 deficiency a family history suggests an autosomal mode of inheritance, however, genetic diagnosis is complicated by the fact that the CoQ10 biosynthetic pathway has not been fully elucidated in humans,

and diagnosis generally relies on the determination of the endogenous CoQ10 status of the patient [16]. Interestingly, a CoQ10 deficiency can also result from a disorder not associated with a genetic defect in the CoQ10 biosynthetic pathway [17]. This type of CoQ10 deficiency is known as a secondary CoQ10 deficiency which is thought to be more prevalent than a primary deficiency, and has been associated with diseases such as MRC disorders, cardiovascular disease, Parkinson's disease and sepsis [1, 17]. The cause of secondary CoQ10 deficiency in disease has not yet been fully elucidated and a number of theories have been suggested to account for this loss of CoQ10 including increased oxidative stress-induced catabolism and/or ROS induced inhibition of the CoQ10 biosynthetic pathway enzymes [17].

4 Laboratory Measurement of CoQ10 Status

Quantification of CoQ10 is not usually included in routine biochemical analysis of blood by hospital pathology laboratories. The most common laboratory procedures used to assess CoQ10 status are based on high-pressure liquid chromatography (HPLC) with either ultraviolet (HPLC-UV) or electrochemical detection (HPLC-ED) [16]. CoQ10 levels are usually determined in plasma isolated from blood, with normal plasma levels typically in the range of 0.5–1.7 μM [16]. In view of the fact that CoQ10 levels are dependent upon the circulatory lipoprotein status (lipoproteins are the major carriers of CoQ10 in the circulation), it has been suggested that plasma CoQ10 levels should be expressed as a ratio to total plasma cholesterol status to take into account the lipoprotein status of the blood [1]. Furthermore, dietary intake has also been reported to influence plasma CoQ10 status, contributing up to 25% in some cases of the total amount of this isoprenoid in the circulation [1, 10]. Accordingly, it has been suggested that plasma may not be an appropriate surrogate for the assessment of endogenous CoQ10 status, and skeletal muscle is generally considered as the tissue of choice for this determination [16]. In addition to direct assessment in skeletal muscle, decreased activity of the linked MRC enzymes, NADH:cytochrome c reductase (complex I + III) and/or succinate:cytochrome c reductase (complex II + III) may also indicate evidence of a CoQ10 deficiency since the activity of these linked enzymes is dependent upon the endogenous CoQ10 status of the tissue [16]. However, in view of the invasive nature of a muscle biopsy, blood mononuclear cells or fibroblast skin cells have also been suggested as alternative surrogates to assess endogenous CoQ10 status [16]. With regards to the neurological dysfunction associated with CoQ10 deficiency, the potential to assess cerebral CoQ10 status would be of diagnostic value. However, since it is not possible to directly determine the CoQ10 status of brain tissue, cerebral spinal fluid (CSF) has been used as an alternative surrogate for this evaluation [16]. Tentative references ranges for CSF CoQ10 status have now been established at 1.18–4.91 nM, although highly sensitive mass spectrometry techniques are required for this determination, in view of the low levels of CoQ10 detected in this matrix [18]. Furthermore, considering the increasing number of reported cases of steroid resistant nephrotic syndrome associated with CoQ10 deficiency, Yubero et al. [19] have

developed a reliable method for the determination of the CoQ10 status of kidney epithelial cells isolated from urine and it is hoped that such an approach will prevent the need for an invasive needle biopsy to assess the CoQ10 status of this organ.

5 Aging and Fibrosis

Fibrosis is the formation of fibrous connective tissue, particularly collagen, in response to an injury. Fibrosis is an adaptive response to tissue injury, and is an essential part of the normal processes of wound healing and tissue repair [20]. In younger individuals, such fibrous material is replaced over time by new functional tissue. However, in older people, tissue scarring tends to persist and may continue to form and accumulate. Uncontrolled continuation of fibrosis can result in scarring and the permanent remodelling of the organ which can result in an eventual loss of function [21]. Progressive fibrosis is a hallmark of the aging process and has been implicated in the pathogenesis of diseases of the heart, lungs, liver, kidneys and bone marrow [22]. It has been estimated that organ fibrosis may be the root cause of over 800,000 deaths per year, approximately 50% of the total number of human deaths [20]. However, at present there is no effective treatment for fibrosis or information available for designing appropriate therapeutic strategies [21, 22].

5.1 Fibrosis Mechanism

Fibrous connective tissue such as collagen is produced by specific types of cells called fibroblasts, which are present in most organs. Under normal circumstances, fibroblasts produce collagen in a controlled manner, to provide a "scaffold" for the structural support of the various types of functional cells in the different organs. Following tissue injury, fibroblasts are activated via inflammatory cytokines to produce collagen as part of the scar formation/healing process [23]. It is well established that fibrosis is linked to inflammation, and there is evidence that even low-grade but persistent inflammation is sufficient to promote, for example, cardiovascular fibrosis where an increased amount of collagen can contribute to the increased stiffness of both arteries and the heart wall [21].

5.2 Preclinical Studies on CoQ10 Supplementation and Fibrosis

A number of studies have demonstrated the beneficial effects of CoQ10 supplementation on fibrosis related parameters (including oxidative stress/inflammation) in various animal models of fibrosis. These include dimethylnitrosamine-induced liver fibrosis in mice [24]. In this model, CoQ10 treatment was found to block the

activation of the stella cells (which is a central event in liver fibrosis) via activation of the nuclear factor, erythroid 2–like (Nrf2) which also resulted in an increased expression of the enzymes involved in the synthesis of the antioxidant, glutathione (GSH) in the hepatic cells. In sub-optimal nutrition-induced liver fibrosis in rats, CoQ10 supplementation was found to reverse fibrosis via suppression of the expression of cytokine transforming growth factor beta 1 (*Tgfβ1)* via a mechanism involving the upregulation of Nrf2-antioxidant response element (ARE)-associated genes [25]. In a study by Chen et al. which investigated the effect of CoQ10 supplementation on doxorubicin induced cardiac fibrosis in rats, it was found that CoQ10 was able to ameliorate the fibrosis by decreasing the expression of both *Tgfβ1 and* connective tissue growth factor *(CTGF*) [26].

In a study which assessed isoprenaline induced cardiac fibrosis in rats, CoQ10 was found to inhibit fibrosis in the heart as well as the kidney via its ability to reduce oxidative stress and prevent inflammatory cell infiltration in the tissues. However, the details of the mechanism of action of CoQ10 were not elucidated in the study [27]. In a recent study, Xue et al. [28] reported that CoQ10 supplementation was able to suppress the activation of mouse pancreatic stellate cells (PCSs), which has been associated in the development of pancreatic fibrosis, by its ability to decrease intracellular ROS levels and induce the mammalian target of rapamycin (mTOR) cell signalling pathway. CoQ10 supplementation was also found to ameliorate methotrexate induced lung and liver fibrosis in rats by the attenuation of hepatic oxidative stress which, contrary to the previous study [28], was found to result in a down-regulation of mTOR expression and increased evidence of autophagy [29]. The reasons for the disparity between the two studies are uncertain, but may represent a species or organ specific mechanism of fibrosis induction. Interestingly, in the aforementioned studies, CoQ10 appears to attenuate fibrosis by its ability either directly or via activation of cell signalling pathways to ameliorate cellular oxidative stress.

5.3 Cardiovascular Fibrosis

In the normal heart, contractile cells (myocytes) occupy approximately 75% of the tissue volume, with the remainder comprising other cell types, which are predominatly fibroblasts [21]. Collagen secreted by fibroblasts forms a supportive scaffold for the myocytes, as well as providing a means of transmission for myocyte generated force within the heart [21]. With regard to the normal ageing process, postmortem analysis of cardiac tissue from human subjects without cardiovascular disease found that the collagen content increased by approximately 50% between the third and seventh decades of life [30].

Unlike other organs, the heart has a limited regenerative capacity following injury, with the repair process involving the removal of necrotic cells, followed by fibrotic scar tissue replacement [21]. Two common aspects of cardiovascular disease in which fibrosis plays a major role are myocardial infarction and heart

failure [20]. Myocardial infarction is caused by the blockage of the coronary arteries, which results in the death of the cardiomyocyte cells responsible for contraction. This in turn causes fibroblasts to produce excess collagen, which helps to preserve the structural integrity of the tissue, but which stiffens cardiac muscle, impairing heart contractility and relaxation, ultimately leading to impaired cardiac function and eventual heart failure [31]. Excessive production of collagen by cardiac fibroblasts can also cause fibrotic thickening of the heart valves, again ultimately resulting in heart failure [30, 31].

6 CoQ10 and the KISEL-10 Study

In the KISEL-10 randomised controlled clinical trial, normal elderly individuals were supplemented with coenzyme Q10 (200 mg/day) and selenium (200 μg/day) for 4 years. The supplemented individuals showed a 53% reduction in cardiovascular related mortality risk compared to placebo [32]. Supplemental selenium was included in this study as this trace metal functions as a co-factor for the enzyme thioredoxin reductase, which is responsible for the interconversion of the ubiquinone and ubiquinol forms of CoQ10. In addition, there is evidence suggesting that a selenium deficiency may be prevalent within the general populations of a number of European countries, including Sweden [37]. Furthermore, selenium is also required for the biological activity of the antioxidant enzyme, glutathione peroxidase which together with ubiquinol contributes to the cellular antioxidant defense [38]. A more recent study by Alehagen et al. [33] using data derived from the KISEL-10 study has now identified significant reductions in the blood levels for a range of biochemical markers of fibrosis, including cathepsin S, endostatin, galectin-3, growth differentiation factor 15 (GDF-15), matrix metalloproteinase-1 (MMP1), MMP9 and tissue inhibitor of metalloproteinase 1 (TIMP1). It is therefore concluded that the improvement in heart function and reduced risk of cardiovascular mortality following supplementation with coenzyme Q10 and selenium results from a reduction in cardiovascular fibrosis. It is of note that the reduction in systemic fibrosis markers may also help to prevent fibrotic degeneration of other tissues such as the lungs and liver [33].

7 Safety of Coenzyme Q10 Supplementation

The safety of CoQ10 has been investigated by Hidaka et al. [34] and Hosoe et al. (2007) [35]. These authors reported that CoQ10 is generally well tolerated, with no serious adverse effects being detected in long term use. Rarely, some individuals may experience mild gastrointestinal disturbances, although this is not dose related. There are no known toxic side effects, and CoQ10 cannot be overdosed. CoQ10 is well tolerated in healthy adults at an intake of 900 mg/day, and in rats at a dose of

up to 1200 mg/kg/day. In addition, Yamaguchi et al. [36] reported that CoQ10 had no genotoxic activity. The safety of CoQ10 has been confirmed in more than 200 randomised controlled trials, over a wide range of disorders including cardiovascular disease, Parkinson's diseases and mitochondrial disease [1].

8 Requirements for Coenzyme Q10 Supplementation

CoQ10 is a lipid soluble substance absorbed from the digestive tract in the same manner as other dietary fats. Because of its hydrophobicity and large molecular weight, absorption of CoQ10 is in general slow and somewhat limited. Oil based formulations show the highest bioavailability. Absorption of CoQ10 is non-linear, with increasing doses absorbed to a decreasing degree. CoQ10 is therefore best administered in split doses (typically 100 mg two or three times daily).

When first manufactured, CoQ10 is produced in a crystalline form which cannot be absorbed from the digestive tract. In CoQ10 supplements, this crystalline form must be further treated to break it down into individual molecules to enable absorption and, most importantly, crystals should not re-form within the capsule. Supplement manufacturers vary in their ability to fulfil these requirements, and previous clinical trial studies reporting lack of benefit in a variety of disorders may have failed because of insufficient dosage and/or lack of bioavailability of the particular supplement used.

9 Conclusions

Oral supplementation with CoQ10 and selenium provides a means of correcting dietary deficiencies to which older individuals may be subject. In addition, supplementation significantly reduces the levels of fibrotic markers in elderly individuals, thereby reducing the extent of cardiovascular fibrosis to which older individuals are subjected, and improving cardiovascular function and reducing risk of cardiovascular associated mortality. Although the factors responsible for the anti-fibrotic action of CoQ10 have yet to be fully elucidated, its antioxidant and anti-inflammatory functions are thought to be major contributors to its clinical efficacy. Further studies may lead to its use as an intervention to promote healthy aging. This is particularly pertinent to the treatment of idiopathic pulmonary fibrosis (IPF), a progressive and ultimately fatal lung disorder disproportionately affecting the elderly, with two-thirds of patients who present with IPF being older than 60 [39]. The management of IPF via prescription-type drugs has proved to be particularly refractory, and alternative therapeutic strategies for the treatment of this disorder are clearly warranted and CoQ10 may be an appropriate consideration.

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Chapter 7
Dietary Restriction, Cardiovascular Aging and Age-Related Cardiovascular Diseases: A Review of the Evidence

Behnaz Abiri and Mohammadreza Vafa

1 Introduction

Calorie restriction (CR) and alternate-day fasting (ADF) (or intermittent fasting) are two of the various forms of dietary restriction (DR) [1]. CR, explained as a decrease in calorie intake without malnutrition, has been demonstrated to increase life span, ameliorate many functional indexes, and decrease metabolic risk factors for chronic metabolic disorders in several mammalian species [2, 3]. Calorie restricted diets have included decreasing food intake to 60–85% of daily energy requirements [1]. In addition to noticeable life span prolongation, CR has significant impacts on age-related physiological and pathophysiological changes [4–9]. CR is also expected to decrease cardiovascular morbidity and mortality by creating pleiotropic cardiovascular preservation [6–9]. Several investigations have demonstrated that CR significantly reduces oxidative damage in the aging heart [7]. The concentrations of 8-oxo-2′-deoxyguanosine were found to be lower in cardiac mitochondria acquired from rats receiving CR than in those from *ad libitum* (AL)-fed groups [7]. Recent studies demonstrated that CR makes an active defense response that enhances survival during stressful states [8, 10]. At the core of this response are the so-called longevity regulatory pathways, which consist of insulin/insulin-like growth factor 1, the mammalian target of rapamycin (mTOR), AMP-activated kinase (AMPK), and

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NAD-dependent deacetylases, which are also called sirtuins. These signaling pathways make a network with positive and negative feedback control [11, 12]. They also play a major role in the evolution of CR-induced cardiovascular health.

ADF is another dietary regimen that has also been investigated. ADF regimens commonly imply a "feast day" on which food is eaten ad libitum and this is changed with a "fast day" on which food is not consumed or is decreased. The feast and fast phases are typically 24 h each, but they may differ. An important point about the ADF approach is that total calorie intake is not to be restricted. Instead, the frequency of food intake is changed [1]. Findings from both animal and human studies demonstrate that ADF may reduce the risk of cardiovascular diseases (CVDs), while results from animal experiments propose a protective impact on cancer risk. In regards to diabetes prevention, animal models show a beneficial impact. Indirect evidence proposes that the two diets may share similar mechanisms of action. These findings propose that ADF may function by promotion of resistance to oxidative attack, which is a key characteristic of the stress resistance theory.

Because of the elevation cardiovascular risk factors with age and also due to the anti-aging influences of restricted diets, this paper reviews the evidence that associates DR, cardiovascular aging and age-related cardiovascular diseases, along with implications for strategies to prevent or retard age-related cardiovascular diseases in the elderly population.

2 Methods

The data were obtained according to the results of original articles associated with CR, ADF, cardiovascular aging, age-related CVD, cardiovascular protection, age-related cardiomyopathy and atherosclerosis. For this purpose, we mostly used the online PubMed database and Google scholar search engine, with the use of following keywords: "calorie restriction", "alternate-day fasting", "cardiovascular aging", "age-related cardiovascular diseases", "cardiac aging" and "aging cardiomyopathy". Then, we chose the pertinent free- access full texts and reviewed the appropriate articles. We contemplated suitable published articles in the English language with no restrictions regarding the dates of articles. Our review also incorporated animal and human studies. In addition, we searched some of the references of the selected articles to make the associated topics more clear as appropriate. We summarized the information about the some of the most important papers in Tables 7.1 and 7.2.

3 Probable Indicators of DR in Cardiovascular Diseases

The prevalence of left ventricular hypertrophy (LVH), atrial fibrillation (AF), and congestive heart failure (CHF) increases significantly with aging [13]. The poor consequences of CVD in old age can be described, at least in part, by cardiac

Table 7.1 Results from studies of the impact of calorie restriction (CR) on left ventricular (LV) function and cardiac aging

First Author (reference no)	Taffet [29]	Shinmura [30]	Ahmet [33]	Yan [34]
Species	B6D2-F1 hybrid mouse	Ficsher-344 rat	Ficsher-344 rat	129/Sv mouse
Age at the time of investigation	30–35 month old	30 month old	24 month old	24 month old
Length of time of CR	28–33 months	22 months	22 months	2 months
LV diastolic function	Improved	Improved	Improved	Not mentioned
LV systolic function	Unchanged	Unchanged	Improved	Improved
Cardiac fibrosis	Not investigated	Unchanged	Reduced	Reduced
Cardiomyocyte apoptosis	Not investigated	Reduced	Not investigated	Reduced
Cardiomyocyte size	Not investigated	Reduced	Reduced	Reduced
Total cardiomyocyte number	Not investigated	Not investigated	Unchanged	Unchanged

senescence both at the cellular and organ levels. To investigate the probable indicators for DR in age-associated CVDs, a discussion of the impacts of DR on atherosclerosis and vascular aging is required. Atherosclerosis and vascular aging are dependent on each other and are specific constituents to what is currently mentioned as clinical vascular disease [14]. Thus, aged blood vessels produce the milieu in which atherosclerosis can burgeon and DR can hinder both atherosclerosis and vascular aging [6, 9].

Moreover, LVH and CHF are diseases related to cardiac aging [13]. So, the prevention of CHF related to LVH, which is described as heart failure with preserved ejection fraction (HFpEF), is considered as a good indicator for DR. Next to population aging, obesity and overeating are common health problems in developed countries. The aggregation of visceral adipose tissue related to overeating and obesity results in metabolic syndrome and type 2 diabetes mellitus and, consequently, an elevation in morbidity and mortality from atherosclerotic diseases [15, 16]. Evidence demonstrates that overeating and obesity speed up the aging process, while DR can delay this. Thus, CVDs related to metabolic disorders might be the best indicator for DR.

There is also evidence that CR can decrease the incidence of neurodegenerative disorders in animal models of Alzheimer's disease, Huntigton's disease, amyotrophic lateral sclerosis, Parkinson's disease and spinal muscular atrophy [11]. These results lead to the proposal that DR can prevent the progression of genetic and/or degenerative disorders regardless of aging or obesity status. Hence, it has been speculated that DR may delay the progress of genetic cardiomyopathies such as mitochondrial myopathy. While, no experimental investigations have been done regarding this issue, the subject remains unresolved.

Table 7.2 Results from studies of the impact of calorie restriction (CR) on myocardial ischemia-reperfusion (I/R) injury and ischemic precondition

First Author (reference no)	Species	Age	Length of time of CR	Impacts of CR
Shimura [6]	C57BL6 mouse	6 month old	3 months	Decreased myocardial I/R injury
Abete [40]	Wister rat	6 month old 24 month old	5 months 12 months	Restored the impact of ischemic preconditioning in aged hearts
Broderick [41]	Wister rat	10 month old	8 months	Decreased myocardial I/R injury
Chandrasekar [42]	Ficsher-344 rat	12 month old	11.5 months	Decreased myocardial inflammation after I/R
Long [44]	Sprague Dawley rat	20 month old	8 months	Restored the impact of ischemic preconditioning, but decreased cardiac performance after I/R injury
Shimura [46]	Ficsher-344 rat	4 month old 24 month old	5 weeks 5 weeks	Decreased myocardial I/R injury in both investigated ages of rats
Shimura [48]	C57BL6 mouse	4 month old	5 weeks	Decreased myocardial I/R injury
Shimura [47]	Ficsher-344 rat	12 month old	6 months	Decreased myocardial I/R injury and restored the impact of ischemic preconditioning
Edwards [43]	B6D2F1 mouse	30 month old	6 months	Decreased myocardial I/R injury
Sung [49]	C57BL6 mouse	4 month old	5 weeks	Decreased myocardial I/R injury
Peart [45]	C57BL6 mouse	12 month old	3 months	Decreased myocardial I/R injury

4 Impacts of DR on Vascular Aging and Atherosclerosis: Evidence from Experimental Animal Studies

Evidence reveals that increased production of reactive oxygen species (ROS) results in endothelial dysfunction with aging in animal models [6, 9, 12]. One of the results of elevated oxidative stress with aging is functional inactivation of nitric oxide (NO) due to high levels of superoxide generation. Damaged NO bioavailability due to age-associated oxidative stress in the coronary circulation and other vascular beds leads to severe dysfunction of vasodilation [6, 9, 12]. In addition to keeping normal organ blood flow, NO generated by endothelial cells exerts vasoprotective and cardioprotective impacts, consisting of inhibition of platelet accumulation, inhibited adhesion of inflammatory factors to endothelial cells, disturbance of pro-inflammatory cytokine-induced signaling pathways, apoptosis inhibition,

conservation of endothelial progenitor cell function and control of tissue energy metabolism [6, 9, 12]. The disruption of NO bioavailability becomes worse due to an age-associated decrease in endothelial NO synthase (eNOS) expression, decreased availability of tetrahydrobiopterin, reduced availability of intracellular L-arginine and disturbed balance between eNOS and inducible NO synthase (iNOS). Age-associated impairment of NO bioavailability is likely to lead to elevated vascular inflammation and atherogenesis and result in a cellular energetic imbalance [6, 9]. In addition to inactivation of NO and the resulting macromolecular harm, ROS play major roles in the pathophysiological signaling pathways of endothelial and smooth muscle cells [6, 9].

CR regulates and activates eNOS, promotes NO bioavailability and ameliorates endothelial dysfunction in aged animals and humans [6, 9, 12, 17, 18]. Moreover, decreases in the age-related enhancement in oxidative stress are also believed to contribute to the anti-aging action of CR [6, 7, 9, 12]. Indeed, recent investigations have shown that CR effectively reduces the vascular production of ROS in aged animals [6, 7, 9, 12].

Considerable experimental and clinical evidence demonstrates that aging is related to chronic low grade inflammation that makes the vasculature susceptible to atherosclerosis. Recent studies have revealed important crosstalk between inflammation, ROS generation, and endothelial dysfunction in the pathogenesis of vascular senescence [6, 9]. Among the agents involved in this crosstalk, activation of nuclear factor- kB (NF-kB) has an important role in endothelial activation and pro-inflammatory gene expression with advancing age [6, 9, 19]. In this regard, CR decreases vascular NF-kB production and endothelial activation in aged rats [19]. CR can also disturb other pro-inflammatory signaling pathways, consisting of c-Jun N-terminal kinase (JNK), p38 kinase, and activator protein-1 DNA-binding activity [6, 9].

The sirtuin family has been demonstrated to play a notable role in different aspects of CR [6–10]. Sirt1 is expressed in high abundance in the cardiovascular system and its production is promoted by CR [6, 7]. Pharmacological over-expression or activation of Sirt1 by resveratrol exerts significant anti-oxidative and anti-inflammatory influences, which appear to result in decreased cellular senescence [6, 7, 9, 10, 20]. Furthermore, endothelial-specific over-expression of Sirt1 greatly decreases the atherosclerosis progression in mice with apolipoprotein (Apo)-E deficiency [21]. CR may control both eNOS expression and activity by Sirt1 activation. Recent investigations have shown that Sirt1 and eNOS localize in endothelial cells and Sirt1 deacetylates eNOS, promoting eNOS activity and enhancing NO in endothelial cells [20].

There have been few studies that assess the influence of ADF on vascular aging and atherosclerosis in rodents. Rodents and monkeys kept on ADF show increased insulin sensitivity [22]. Rodents maintained on ADF demonstrate reductions in resting blood pressure (BP) and heart rate (HR) [22, 23]. These results suggest that ADF can also prevent vascular aging and atherosclerosis. However, more evaluations are required to compare the influence of ADF with that of CR on vascular aging and atherosclerosis.

5 Impacts of CR on Vascular Aging and Atherosclerosis: Evidence from Human Studies

Elevated thickening and stiffness of large arteries and endothelial dysfunction in apparent healthy old individuals, along with enhance in systolic blood pressure and pulse pressure, forecast a higher risk for progression of clinical atherosclerosis, acute coronary syndrome (ACS) and stroke [14]. Such age-associated vascular alterations can be attributed to vascular aging. In this context, vascular aging is a risk factor for possible clinical disease manifestation.

Atherosclerosis and the following cardiovascular difficulties are considerable causes of death worldwide. The risk factors for atherosclerosis consist of hypertension, type 2 diabetes, higher concentrations of serum total and LDL cholesterol and smoking. Aging is also a major risk factor for atherosclerosis. Early or accelerated vascular aging can be increased by cardiovascular risk factors and cellular senescence is also seen in patients with atherosclerosis [14]. Thus, atherosclerosis is a disorder resulting from both organismal aging and cellular senescence.

CR has been demonstrated to delay vascular aging in both rodents and humans [6, 9, 12, 17, 24–27]. The underlying mechanisms of the beneficial cardiovascular impacts of CR are multifaceted, consisting of ameliorations in systemic risk factors for atherosclerosis such as dyslipidemia and insulin resistance and BP lowering, along with direct anti-aging impacts on the vasculature [6, 9].

6 Impacts of ADF on Vascular Aging and Atherosclerosis: Evidence from Human Studies

ADF includes an AL feed day alternated with a 25% calorie intake fast day. While the intervention duration has been 12 weeks at most, ADF was found to be beneficial for weight loss (−6.5%) and for ameliorating the atherosclerosis risk factors in normal weight and overweight adults [28]. The ADF protocol appears to be hopeful for treating overweight persons and preventing the progress of type 2 diabetes in patients with CVDs.

7 Impacts of DR on Cardiac Aging and Age-Related Left Ventricular Diastolic Dysfunction: Animal Calorie Restriction Experiments

For the first time, Taffet et al. [29] indicated that long term CR ameliorates age-related alterations in late diastolic function in mice. In another study, Shinmura et al. [30] used Fischer-344 rats in an evaluation of the influence of long term CR on

cardiac senescence, with the diet initiated at the age of 8 months and continued until the age of 30 months. The results of this study showed that long term CR ameliorated diastolic function in the aged myocardium by improving the age-related failure in myocyte relaxation. Moreover, the results demonstrated that a reduced size of cardiomyocytes may help by improving left ventricular (LV) diastolic dysfunction in rats on CR diets. In addition, the results suggested that CR impacts on age-related changes in cytoskeletal proteins, resulting in the amelioration of LV diastolic function.

Dhahbi et al. [31] indicated that long term CR decreases the myocardial collagen and extracellular matrix content and reduces cardiac fibrosis related to aging. Therefore, CR-induced alterations in cardiac connective tissue may help in the improvement of diastolic function, particularly late diastolic function, as reported by Taffet et al. [29]. Oppositely, a study carried out by Shinmura et al. [30] did not find a significant reduction in cardiac fibrosis in the hearts of rats receiving CR. These results proposed that long term CR may improve the age-related failure of early diastolic function by keeping the function of the sarcoplasmic reticulum (SR). These findings are different from those of Taffet et al. [29] as this latter group of researchers found no amelioration in early diastolic cardiac function in mice. However, the results reported by Shinmura et al. [30] are consistent with those of Seymour et al. [32], who found that CR ameliorated cardiac remodeling and diastolic dysfunction in Dahl-ss rats.

Recently, another research group reported the impact of long term CR on cardiac aging and assessed LV function in Fischer-344 rats at an earlier age. In comparison to AL-fed rats, 24-month old rats receiving CR had decreased levels of cardiac and aortic fibrosis, elevated density of cardiomyocytes that had smaller size, decreased diastolic dysfunction, and normal systolic performance and arterioventricular coupling [33]. Taken together, these results demonstrated that CR prevents age-related LV dysfunction by retarding cardiac aging.

On the other hand, Yan et al. [34] found that short term CR may reverse age-related LV dysfunction. When short term (2 months) CR was initiated after age-related LV dysfunction was generated in 20-month-old mice, leading to reduced cardiac function, and increased LV weight, the myocardial fibrosis and apoptosis were reversed and LV performance was found to be similar to that of young mice or mice that had been on CR diets from younger ages. The researchers also reported that short term CR prevented changes in cytoskeletal proteins which led to aging cardiomyopathy, but not the reduction in cardiomyocyte number related to advancing age [34].

8 Mechanisms by Which CR Delays Cardiac Aging

The mechanisms by which long term CR delays cellular aging and diminishes the physiological function of organs have not been completely clarified. Aging happens, in part, due to the aggregation of oxidative harm caused by oxidative free

radicals that are produced continually during the metabolic processes [7]. Oppositely, CR reduces the age-related aggregation of oxidative harm to lipids, proteins and DNA. Also, expression of protein carbonyls is lower in hearts of rats receiving CR diets compared with hearts of AL-fed rats [7, 30]. It is probable that long term CR delays cellular aging and decreases age-associated functional losses by diminishing oxidative harm in the aged heart. However, there is still no concrete evidence indicating that the diminishing of oxidative harm is the first means by which CR prevents cardiac aging.

Another probable mechanism by which long term CR may delay cardiac aging is through the elevation of autophagy [7, 30]. Autophagy under basal states has a housekeeping role in the turnover of cytoplasmic components. Increased autophagy during CR has been suggested to be protective because this process leads to degradation and removal of harmed organelles. When autophagy is not functioning perfectly in the aged heart, the aggregation of damaged SR and mitochondria may be lethal by leading to diastolic dysfunction. Damaged autophagy in the aged heart may lead to the aggregation of lipofuscin, as well as in the inhibition of autophagy. It was reported that long term CR decreases the aggregation of lipofuscin [30] and the researchers proposed that long term CR disturbs this cycle in the aged heart. Furthermore, it was demonstrated that increased autophagy is related to suppression of the mTOR pathway in the heart [30]. The activation of mTOR leads to a negative regulatory impact on the induction of autophagy [10]. Recently, a proteome analysis showed that either 10 weeks of CR or rapamycin treatment ameliorated protein turnover in aged hearts [35]. It is possible that the inhibition of mTOR signaling during CR conditions has a key role in the making of CR-induced cardioprotection.

9 Impacts of DR on Cardiac Aging and Age-Related Left Ventricular Diastolic Dysfunction: Animal ADF Experiments

While the protective impact of CR against age-related cardiac dysfunction has been accepted as proven in rodents, the impact of ADF on cardiac senescence and cardiac function remains unclear. Castello et al. [36] demonstrated the impacts of long term ADF, from 2 to 24 months of age, on cardiac fibrosis and oxidative stress related to aging in rats. The results showed that ADF significantly decreased cardiac oxidative harm, the expression of pro-inflammatory cytokines, NF-kB DNA binding capacitance, and cardiac fibrosis, compared to the same parameters in AL-fed 24 months old rats. The researchers also reported that ADF decreased cardiomyocyte hypertrophy in aged rats, possibly by repressing extracellular signal-regulated kinase 1 and 2 and restoring repressor of cytokine signaling 3 and signal transducer and activator of transcription 3 activities, alterations which have been associated with advancing age [37].

In contrast, a study by Ahmet et al. [38] concluded that ADF has an adverse impact on cardiac function. In this study, ADF was initiated at 4 months and kept until 10 months of age. ADF led to 9% lowering in cardiomyocyte diameter and a 3-fold elevation in myocardial fibrosis. Also, the results showed a significant lowering in LV diastolic compliance and a defective elevation in systolic pump function. The mechanism by which ADF may lead to the diastolic dysfunction with a decreased cardiac reserve remains unclear, although the researchers proposed that the defect in important nutrients or the activation of the renin-angiotensin-aldosterone system may be involved. Due to these discrepancies across studies, further studies are warranted.

10 Impacts of Dietary Restriction on Cardiac Aging and Age-Related LV Diastolic Dysfunction: Human Calorie Restriction Trials

In one study, Meyer et al. [39] showed that CR is useful for LV diastolic function in humans. The E/A ratio (the ratio of peak velocity of blood flow due to gravity in early diastole to the peak velocity flow caused by atrial contraction in late diastole) was greater in the CR group than in the group receiving a Western diet, although the differences were not significant. The researchers assumed that the useful impact of CR on LV diastolic function resulted from reductions in systolic BP and systemic inflammation caused by CR.

Although ADF effectively decreases body weight even in humans, more studies investigating the impact of this diet on cardiovascular functions are required before it can be applied in clinical studies in humans.

11 Impacts of Calorie Restriction on Myocardial Ischemia-Reperfusion Injury

Lifelong CR significantly reduces myocardial oxidative stress and the inflammatory response related to advanced age [4, 7–9]. In this regard, some researchers have investigated the impact of long term CR on the grade of myocardial ischemia-reperfusion injury and the progress of ischemic preconditioning (PC) [6, 40–49]. Broderick et al. showed that CR for 8 months ameliorated recovery of cardiac function after 25 min of ischemia in rat hearts [41]. The researchers also demonstrated that the cardioprotective impact of CR was related to amelioration in mitochondrial respiration. Abete et al. [40] showed that CR can restore the cardioprotective impact of ischemic PC in the hearts of aged rats. CR did not ameliorate the recovery of LV function after ischemia-reperfusion but considerably restored the PC impact in aged hearts. Long et al. [44] showed that CR for 6 months restored the PC impact in

middle-aged rat hearts. However, in their study, recovery of cardiac output and aortic flow was decreased in CR fed rats compared to AL-fed rats.

In another study [47], 6 month old rats were exposed to CR for 6 months to determine whether or not this diet can ameliorate myocardial ischemic tolerance and restore the progress of ischemic PC. The results showed that 6 months of CR ameliorated the recovery of LV function after ischemia-reperfusion and decreased infarct volume in the hearts of the 12 month old rats. Moreover, CR restored the ischemic PC impact in middle-aged rats. Altogether, the results of recent studies supported the potential of CR intervention aimed at lowering myocardial harm to decrease ischemic stress in humans.

Although, several studies have shown that fasting decreases myocardial ischemia-reperfusion injury in rodents, it remains unclear whether or not ADF decreases myocardial ischemia-reperfusion injury.

12 Impacts of DR on Myocardial Infarction and Post-Infarct LV Remodeling

The influences of DR on infarct volume after permanent coronary ligation and post infract LV remodeling were investigated in vivo. After 3 months of receiving an AL or ADF diet, myocardial infarction (MI) was made by permanent coronary ligation in 5 month old rats. Infarct volume was investigated by use of triphenyltetrazolium chloride spotting 24 h after MI and found to be significantly smaller in rats receiving ADF [50]. In addition, post-infarct LV remodeling, myocardial infarction extension and LV function assessed by echocardiography 10 weeks after MI were significantly ameliorated in ADF fed rats. Thus, ADF may protect the myocardium against ischemic damage and decrease post infarct LV remodeling, potentially via anti-apoptotic and anti-inflammatory mechanisms. Oppositely, this research group indicated that 3 months of CR prior to coronary ligation could not protect the hearts against ischemic damage or post infarct LV remodeling in 5 month old rats [33].

When ADF was initiated 2 weeks after permanent coronary ligation, the survival of rats after MI was considerably enhanced [51]. Moreover, ADF decreased post infarct remodeling and ameliorated LV function after myocardial infarction. Molecular investigations showed an up-regulation of angiogenic agents consisting of hypoxia-inducible factor 1α, brain-derived neurotrophic factor, and vascular endothelial growth factor in the hearts during fasting states. These results suggested that the protective influence of ADF against post infarct LV remodeling was greater than that of CR.

13 Dietary Restriction to Protect Against Metabolic Cardiomyopathy Related to Obesity and Type 2 Diabetes Mellitus

Overeating and obesity are serious health problems in societies throughout the world. Obesity-related metabolic disorders, including metabolic syndrome and type 2 diabetes, quicken atherosclerosis and vascular aging, leading to the progression of ischemic heart disease (IHD), stroke, peripheral arterial disease, and other CVDs [15, 16]. In addition, metabolic disorders lead to cardiac hypertrophy, HFpEF, and pulmonary artery hypertension. Hence, the impact of DR on cardiac dysfunction related to metabolic disorders is of considerable clinical interest.

Ob/ob mice demonstrate noticeable obesity, insulin resistance, cardiac hypertrophy, mitochondrial impairment and ectopic lipid aggregation, resulting from leptin deficiency. In a study, Sloan et al. [52] compared the impact of CR on myocardial metabolism and function in 4 week old ob/ob mice to that of leptin supplementation. While CR led to normal body weight and glucose tolerance, fat mass content and circulating lipid concentrations remained elevated in CR fed ob/ob mice. Palmitate oxidation in the heart remained increased in ob/ob mice receiving CR and became normal following intraperitoneal or intracerebroventricular leptin application. These findings suggest that damaged hypothalamic leptin signaling can elevate myocardial fatty acid oxidation in spite of CR.

Moreover, the useful impacts of CR were impaired in ob/ob mice when CR was initiated from middle age. AlGhatrif et al. [53] showed that CR can restore LV diastolic function, reverse myocardial steatosis, and ameliorate insulin sensitivity when this diet was initiated at 2 months of age in ob/ob mice, but none of these alterations were reported when CR was initiated at 6–7 months of age. However, CR resulted in reduced cardiac oxidative stress and normal NOS activity.

Recently, some clinical studies have indicated that CR combined with significant body weight loss has cardiac-specific impacts and improves LV diastolic function in healthy individuals [39], and also in patients with type 2 diabetes [15]. Therefore, the clinical use of CR and the progress of CR mimetics have potential as novel therapeutic approaches in the treatment of subjects with diastolic dysfunction. However, the mechanisms by which CR ameliorates cardiac diastolic dysfunction in humans remain unclear. Recent evaluations have led to the proposal that myocardial triglyceride content is an independent predictor of diastolic function in old age [54] and in subjects with type 2 diabetes [15]. The reduction in myocardial triglyceride content due to CR is related to amelioration in LV diastolic function [15, 54]. It is probable that elevated autophagy aids the degradation of fatty acid intermediates that are potentially toxic.

14 Impacts of Dietary Restriction on Autonomic Nerve Function

An age-related reduction in autonomic nervous system function is partly responsible for the elevation in arrhythmias and syncope with aging [55]. Some researchers have investigated the impact of DR on autonomic nerve function in rodents or humans [23, 55]. To investigate the impact of DR on beat-to-beat heart rate variability (HRV) and diastolic BP variability (DBPV), Sprague Dawley rats inserted with telemetric transmitters were exposed to AL, ADF or CR feeding at 3 months of age [23]. Both ADF and CR led to elevations in the low frequency (LF) and the high frequency (HF) constituents of the HRV spectrum within 1 month. ADF, but not CR, reduced the LF and LF/HF ratio of the DPV spectrum. These results suggested that DR leads to reduced sympathetic nervous system activity and increased parasympathetic tone. Furthermore, the impact on parasympathetic tone appears to be more powerful from ADF than that from CR.

In one study, Stein et al. [55] assessed HRV in 22 subjects aged 35–82 years following CR compared to 20 age-matched controls eating a Western diet (WD). All HRV values were significantly greater in the CR group compared to the same values in the WD group. These results propose that CR reduces sympathetic tone, elevates parasympathetic tone and restores circadian rhythm variance. In conclusion, DR may exert direct systemic impacts that oppose the expected age-related alterations in autonomic nervous system function in humans.

15 Conclusions

In summary, dietary restriction consists of calorie restriction and and alternate day fasting as approved nutritional interventions that manifest anti-aging impacts and delay the aging process. Dietary restriction also shows protection in all cell tissues and organs including the heart and vasculature bed. It also impacts on energy and oxygen radical metabolism, inflammatory agents and cellular stress response systems. Through these mechanisms, dietary restriction can protect the cells and organs against the damaging impact of aging. Because aging is associated with serious chronic disorders such as cardiovascular diseases and type 2 diabetes, and the proportionate population of elderly people has had an elevating trend over the last century, achieving the most effective nutritional intervention that achieves healthier aging with fewer disabling problems can ameliorate the public health state and enhance quality of life.

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Chapter 8
Gut Microbiota and Microbiota-Related Metabolites as Possible Biomarkers of Cognitive Aging

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1 Gut Microbiota and Cognitive Aging

1.1 The "Gut-Brain Axis" in Dementia

In the last decade, several studies have highlighted that the intestinal microbiome, i.e. the ensemble of bacteria symbiotically living with the host in the gut lumen, may influence the physiopathology ofa large number of human diseases, not involving only the gastrointestinal system [1–3]. For example, alterations of the composition of gut microbiota may promote the progression of chronic liver disease [4], chronic renal failure [5] and even the formation of kidney stones [6]. By modulating insulin sensitivity, anabolism and systemic inflammation, the gut microbiota could also have relevance in the onset of age-related bone diseases and sarcopenia [7, 8].

In this context, the existence of a possible "gut-brain axis" influencing cognition in aging has been hypothesized [9, 10]. A large number of animal studies, recently reviewed elsewhere [10], have demonstrated that alterations of the gut microbiota composition, the so-called dysbiosis, can be associated with reduction of performances at cognitive tasks and, conversely, the administration of probiotics or

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functional foods can prevent cognitive decline in transgenic models of Alzheimer's disease. The hypothesized mechanisms involved in these phenomena include bacterial production of amyloid proteins able to promote β-amyloid deposition in the brain tissue, modulation of vagal nerve activity, production of bacterial metabolites that may act as endocrine modulators or neurotransmitters, modulation of neuroinflammation and microbiota-mediated transformation of nutrients into substances exerting toxic or protective activity on brain cells [10, 11].

Therefore, the current scientific literature suggests that the intestinal microbiome can influence the onset of dementia and its progression in multiple ways, making the "gut-brain axis" a promising area of research for the future.

1.2 Clinical Relevance of the "Gut-Brain Axis" in Humans with Dementia

Despite the results of pre-clinical studies, the translation of the "gut-brain axis" concept into human health is still uncertain. In fact, only a few investigators have comprehensively compared the intestinal microbiota composition of older patients with cognitive complaints (ranging from mild cognitive impairment to overt dementia) with that of healthy active older subjects (Table 8.1). The results of these studies suggest the presence of reduced gut microbiota biodiversity (i.e., lower numbers of species) and specific compositional signatures, such as over-representation of *Bacteroides* and Enterobacteriaceae and under-representation of *Dialister*, in subjects with cognitive symptoms [12–15]. In the largest of these studies, comparing the fecal microbiota composition of 34 older patients with dementia and 94 age-matched controls, dementia was associated with increased biodiversity, reduced representation of *Bacteroides*, and an increased Firmicutes/Bacteroidetes ratio [16]. These associations were independent of other known biomarkers of dementia, including ApoE ε4 polymorphism, presence of lacunar infarcts at brain computed tomography (CT) or amyloid deposition detected by positron-emission tomography (PET) [16].

These findings, although promising, should be interpreted with caution. Most of the existing studies were observational, with a cross-sectional design preventing to draw solid conclusions on causal inference. Low sample sizes of these studies also represent an issue (Table 8.1) and generalizability to the general population with cognitive impairment or dementia is not automatic.

These limitations could be overcome by rigorous and well-designed intervention studies, implying the manipulation of the intestinal microbiome by administration of probiotics or functional foods. However, despite the large number of human trials with pre- or probiotics, studies specifically focused on dementia are still lacking [17]. The few studies published to date included only subjects without cognitive complaints [17] or with advanced dementia [18–20], and thus had a low chance of detecting significant variations in cognitive performance. Moreover, the investigators

Table 8.1 Overview of the main human studies comparing the fecal microbiota composition between subjects suffering from dementia or cognitive symptoms with healthy older subjects

First author, journal, year, [Ref]	Setting and country	Number and characteristics of participants	Mean age	Gut microbiota-related parameters differing between cases and controls	Implications
Cattaneo A, Neurobiol Aging, 2017 [12]	18 memory clinics in Northern Italy	73 patients with dementia (40 amyloid-positive and 33 amyloid-negative) 10 controls	71 ± 7 (cases) 68 ± 8 (Controls)	Abundance of *Eubacterium rectale*(anti-inflammatory) Abundance of*Escherichia/Shigella* (pro-inflammatory)	The fecal microbiota of patients with dementia has a higher pro-inflammatory activity than controls
Vogt NM, Sci Rep, 2017 [13]	Memory Clinic and Registry for Prevention of Alzheimer's disease in the United States	25 patients with Alzheimer's disease 25 controls	71 ± 7 (cases) 69 ± 8 (controls)	Overall biodiversity (α-diversity) Inter-individual variability (β-diversity) Abundance of the Firmicutes and Bacteroidetes phyla Abundance of 13 taxa, including *Bifidobacterium, Alistipes, Bilophila* and *Clostridium*	Subjects with Alzheimer's disease have a distinct composition of fecal microbiome, with reduced biodiversity and abundance of putative anti-inflammatory taxa
Manderino L, J Int Neuropsychol Soc, 2017 [14]	Community recreation and wellness center in the United States	43 healthy community dwellers (25 with intact cognitive functions and 18 with previously unknown mild cognitive impairment)	64 ± 7	Abundance of 4 bacterial phyla (Bacteroidetes, Firmicutes, Proteobacteria, Verrucomorbia)	The gross composition of fecal microbiota in terms of phylum abundance is correlated with cognitive performance; the strongest correlation is observed for Verrucomorbia abundance
Zhuang ZQ, J Alzheimers Dis, 2018 [15]	4 hospital outpatient clinics in China	43 patients with dementia 43 controls	70 ± 9 (cases and controls)	Inter-individual variability (β-diversity) Abundance of several species including *Bacteroides, Actinobacteria, Ruminococcus, Lachnospiraceae, Selenomonadales*	Subjects with dementia have a distinct fecal microbiota composition than controls at both community and species level
Saji N, Sci Rep, 2019 [16]	Memory clinic in Japan	34 patients with dementia 94 controls	76 (interquartile range 69–81)	Overall biodiversity (α-diversity) Firmicutes/Bacteroidetes ratio Abundance of *Bacteroides, Bifidobacterium* and Lactobacillales	The gut microbiota composition was associated with the status of suffering from dementia independently of other known and well-established biomarkers of dementia

in these studies concentrated on microbiological and inflammation-related, rather than on clinical outcomes [18–20]. In the only study which assessed cognition as the primary outcome, the intervention consisted in a 12-week resistance training program associated with the administration of a *Bifidobacterium* spp blend, so that the improvement of executive functions detected in the intervention arm could not be attributed with complete certainty to the benefits of gut microbiome manipulation [21].

In this scenario, the possibility of obtaining a clinically relevant modulation of cognitive symptoms with microbiome-targeted interventions in older patients still remains speculative.

1.3 Gut Microbiota at the Cross-Road Between Environment and Human Pathophysiology

An increasing body of evidence suggests that the human microbiome composition is strongly influenced by environmental factors [1]. These factors include dietary patterns and habits [2], exercise [22], drugs [23, 24], the presence of diseases [23, 24], climate and pollution [25]. Moreover, the gut microbiota composition is shaped in each human being according to his or her genetic background, and is greatly influenced by immune system functionality [1, 20]. Aging itself represents a process able to influence the gut microbiota composition in an independent way, implying a progressive reduction of biodiversity and resilience to stressors [26]. Finally, growing evidence indicates that the output of metagenomics analyses of fecal microbiota is dependent on the bacterial load, which is ultimately associated with stool moisture [27, 28]. Intestinal motility, number of bowel movements and hydration status are thus important variables influencing the microbiome composition.

In the largest population-based study to date, investigating the human gut microbiota composition and its determinants, the authors were able to identify a long list of environmental factors associated with a distinct microbiological signature [29]. These results put into question the role of the intestinal microbiome as an independent mediator of human health and disease. They also suggest the existence of complex relationships between the environment and the host physiopathology, with gut microbiota possibly positioned at the cross-road [8, 30]. In fact, it is very difficult to consider all of the possible environmental factors influencing the microbiome composition, acting as potential confounders in pathophysiological associations [1, 30].

From a clinical perspective, this means that the disease-associated microbiome alterations most likely represent only one component of a large network of pathophysiological factors [30]. It also helps to understand why microbiome-targeted interventions, including administration of probiotics and functional foods, rarely provide clinically significant results outside the field of gastroenterology. If the microbiome is a cross-road mediator between the environment and host pathophysiology, then environmental interventions have a greater potential of influencing dis-

ease onset and course than microbiome manipulations targeted to a single or a limited number of bacterial species.

Despite these possible limitations, the intestinal microbiome remains a promising target for anti-aging interventions [31], particularly in the field of cognitive disorders, in which studies conducted in mouse models have provided encouraging results [10]. However, much research must still be done before we can recommend therapeutic strategies centered on the microbiome for patients with cognitive impairment, mainly because of objective difficulties in identification and preparation of probiotic blends or functional foods able to induce significant modification in the overall structure of gut communities [32].

Possibly, the nearest goal in this field of medicine is the use of gut microbiota composition and gut microbiota-related metabolites as biomarkers of disease. The existing studies exploring the relationship between microbiome and dementia highlight that a certain number of abnormalities in microbiome composition and functionality may be of potential interest as biomarkers. To be useful as biomarker of cognitive function, a physiological parameter should be measurable, correlated with cognitive performance or other indicators of cognitive impairment, able to predict the onset and course of cognitive impairment as well as the therapeutic response to treatments [33, 34]. Correlation with other measures of disease and prediction of outcomes are thus necessary before a biomarker can enter into clinical practice [33, 34]. Unfortunately, most of the research on the gut-brain axis has been focused on correlations rather than predictions [1]. However, there are many microbiome-related parameters deserving careful evaluation as possible biomarkers of cognitive aging in future studies.

2 Gut Microbiota Composition as Biomarker of Cognitive Function

2.1 Microbiome Biodiversity

The latest next-generation sequencing techniques (16S rRNA microbial profiling, shotgun metagenomics) allow detection of virtually all different taxa harbored in biological samples [35, 36]. After bioinformatics processing, the output of these analyses retrieves several indexes of biodiversity, such as the Shannon Index, the Simpson Index or the Chao1 Index [37]. In microbial ecology, biodiversity (also called alpha diversity) is defined as species richness and relative species abundance in space and time [38]. Thus, the more elevated are biodiversity indices, the higher is the number of species harbored in the fecal sample [37].

A high fecal microbiome biodiversity is generally considered to be a marker of good health status, implying a virtuous symbiotic equilibrium between microbial communities and host [3]. Conversely, reduced fecal microbiome biodiversity is generally associated with poor health status, and represents the main feature of gut

microbiota dysbiosis [3]. For example, studies performed in patients with critical illnesses admitted to intensive care units globally show a high level of fecal microbiota dysbiosis, with reduced species richness [39]. Moreover, older patients with prolonged hospital stay who develop *Clostridium difficile* infection, one of the most frequent healthcare-associated infections, exhibit a reduced level of fecal microbiota biodiversity, compared with those who have milder health problems and shorter hospital stays [40].

However, an elevated fecal microbiota biodiversity is not necessarily associated with a healthy microbiota. When patients are treated with systemic antibiotic therapy, a transient increase in gut microbiota biodiversity may be observed due to overexpansion of microbial populations, mainly pathobionts that are not sensitive to the administered treatment [41, 42]. Stressful events such as exercise to exhaustion, may also promote increased gut microbiota biodiversity due to increased colonization and expansion of opportunistic pathogens at the expense of symbionts [22]. Thus, a finding of increased biodiversity in fecal microbiota should be carefully interpreted, especially in the light of which taxa are present and their relative abundance.

In mouse models of senescence or dementia, the association between gut microbiota biodiversity and cognitive performance has provided inconsistent results. For example, in a group of senescence-accelerated mouse prone 8 (SAMP8) mice exhibiting symptoms of severe cognitive dysfunction, the gut microbiota biodiversity measured by Chao1 and Shannon indices was significantly reduced in comparison with controls [43]. However, another study showed that older mice with mild symptoms of cognitive dysfunction exhibited an increased, rather than decreased, gut microbiota biodiversity in comparison with younger mice [44]. Antibiotic-induced reduction of gut microbiota biodiversity resulted in worsened cognitive performance of mice in one study [45] but was associated with reduced Aβ amyloid deposition in transgenic mouse models of Alzheimer's disease [46, 47]. In germ-free mice, harboring no intestinal microbiota, a massive reduction of Aβ amyloid brain deposition was also documented in comparison with controls [48].

In humans, the association between gut microbiota biodiversity and cognitive functions has been assessed in different settings. In patients with cirrhosis undergoing liver transplantation, a high gut microbiota diversity was associated with reduced risk of encephalopathy and thus cognitive symptoms [49]. However, species richness in gut microbiota was unable to predict long-term cognitive outcomes in a group of unselected patients with cirrhosis [50]. In obese subjects, the Shannon index of fecal microbiota was correlated with anatomical and functional parameters of brain magnetic resonance imaging (R2∗ and fractional anisotropy of the hypothalamus, caudate nucleus and hippocampus) and cognitive performance related to speed, attention and flexibility [51].

In a group of 85 HIV-infected subjects naïve to antiretroviral therapy, fecal microbiota biodiversity was significantly lower in the presence of HIV-associated neurological disease with cognitive symptoms [52]. However, this difference was not independent of educational level and HIV-infection-related clinical data, such as CD4 T-cell count, suggesting that gut microbiota dysbiosis is not pathophysiologically involved in the cognitive dysfunction [52].

In the largest human study to date, Verdi et al. [53] analyzed the fecal microbiota of 1551 individuals over the age of 40 and correlated microbial variables with the cognitive performance assessed by several tests (verbal fluency, Mini-Mental State Examination, Deary-Liewald Reaction Time test, Paired Associated Learning from the Cambridge Neuropsychological Test Automated Battery). Among the results of these tests, only the Deary-Liewald Reaction Time test score was inversely associated with alpha diversity independently of covariates, suggesting that the possible link between gut microbiota biodiversity and cognition may be mediated by several other environmental and host-related factors [53].

Fecal microbiota biodiversity was analyzed in patients with dementia in only three studies [13, 16, 54]. Araos et al. analyzed the fecal microbiota of 85 long-term-care facility residents with advanced dementia, demonstrating a very high level of dysbiosis with reduced species richness in all subjects [54]. Their results suggest that advanced dementia may be associated with low alpha diversity indices but the absence of a control group in the study design prevents solid conclusions. In another study performed in 25 patients with dementia and 25 controls, the Shannon index was significantly reduced in cases (Table 8.1) [13], supporting the possible association between reduced alpha diversity and cognitive symptoms. However, in the most recent and largest study comparing alpha diversity between 34 demented patients and 94 controls, Saji and colleagues documented that the Shannon index was significantly lower in controls, rather than in demented patients (Table 8.1) [16].

Overall, the current literature state-of-art does not support the use of fecal microbiota biodiversity indices as biomarkers of cognitive aging. The studies performed on this topic are too scarce, with reduced sample sizes, and have therefore provided inconclusive results with interpretations that may be challenging. However, they do suggest that an association between alpha diversity and cognitive symptoms may exist, although this is not independent of covariates. Future studies should better investigate this association, considering larger sample sizes, thorough selection of covariates and the use of longitudinal designs to assess whether variations in gut microbiome composition can predict variations in cognitive function.

2.2 Firmicutes to Bacteroidetes Ratio

Bioinformatics processing of next-generation sequencing metagenomics output allows identification of the number of bacterial taxa harbored in fecal samples as well as assignment of each of these to the corresponding taxonomic level and calculation of their relative abundance [35, 36]. These data are generally used to determine the relative abundance of the two most represented phyla in fecal microbiota, i.e., Bacteroidetes and Firmicutes, and their ratio. The Firmicutes/Bacteroidetes ratio is an index representing the overall qualitative composition of the fecal microbiota. The intestinal microbiota can in fact physiologically assume different states, called enterotypes, with a predominance of either Bacteroidetes or Firmicutes,

according to individual factors and dietary habits [55]. The Firmicutes/Bacteroidetes ratio increases from infancy to adult life and then declines again with senescence [56]. The predominance of Firmicutes in fecal microbiota composition has also been associated with the presence of metabolic imbalances, especially obesity, insulin resistance and metabolic syndrome [57, 58]. In fact, it generally increases with increasing body mass indices [59]. The Firmicutes/Bacteroidetes ratio has thus become an important parameter in the evaluation of the relationship between gut microbiota, obesity and obesity-related disorders [57].

An altered Firmicutes/Bacteroidetes ratio can however also represent an index of dysbiosis in other diseases, including dementia. In mice genetically prone to dementia (3xtg breed), gut microbiota analyses revealed a marked elevation of the Firmicutes/Bacteroidetes ratio compared to controls [60]. In mice with diet-induced obesity, over-expression of Firmicutes and reduced expression of Bacteroidetes was associated with impaired cognitive performance, especially in recognition memory and spatial memory tasks [61]. Finally, in older mice, an increase in the Firmicutes/Bacteroidetes ratio was associated with compromised cognition and increased anxiety behaviors, and this was not observed in younger mice [44].

The Firmicutes/Bacteroidetes ratio was calculated in human beings with dementia in only two studies, where it appeared significantly higher than in healthy controls [15, 16]. However, in a population of cognitively healthy older adults, Manderino et al. showed that greater proportions of Firmicutes and smaller proportions of Bacteroidetes in fecal microbiota were associated with better performances at cognitive tests [14].

Thus, the current literature state-of-art seems to support the concept that the presence of cognitive impairment may be associated with variations in the Firmicutes/Bacteroidetes ratio in fecal microbiota, making this parameter a promising potential biomarker of cognitive aging, deserving investigation in future studies. The capacity of this ratio to predict the onset and worsening of cognitive symptoms in human beings, and its association with obesity and metabolic imbalance should be particularly assessed.

2.3 Abundance of Specific Taxa in Fecal Microbiota

Apart from the overall composition of the fecal microbiota, variations in the representation of specific taxa may show a significant association with the presence of cognitive symptoms. The outputs of metagenomics analyses and their bioinformatics elaborations generally retrieve, for each detected bacterial taxon, a ratio of relative abundance, representing the proportion of bacteria belonging to that taxon on the total bacterial load of the sample [28]. Thus, these relative abundances do not represent the absolute quantities of bacteria harbored in each sample, and this concept should be considered for interpreting any result [28]. However, relative abundances could represent promising biomarkers of health status, especially if their association with diseases is reproducible across different studies.

The main bacterial taxa found over-represented or under-represented in fecal samples of either patients or animal models with cognitive symptoms, cognitive impairment or dementia, according to the current literature state-of-art, are summarized in Table 8.2. The findings of studies on mouse models [43, 60, 62–64] showed some degree of inconsistency. For example, in mice with cognitive impairment, Christensenellaceae and Ruminococcaceae were found to be depleted in one study [43] and over-represented in another [60]. However, depletion of Bifidobacteria and over-representation of Anaeroplasmatales were both associated with dementia in two studies [60, 63]. The largest study which compared senescence-accelerated mouse prone 8 with senescence-accelerated mouse resistant 1 breeds, showed that the presence of cognitive symptoms was associated with depletion of 26 taxa and over-representation of only one taxon [43].

Human studies which investigated the relative abundances of specific microbial taxa in fecal microbiota composition in relation to cognitive performance were performed in healthy subjects [19, 53, 65, 66], patients with cirrhosis [67–69], Parkinsonism [70, 71], or dementia/cognitive impairment [12–16]. An overview of taxa that resulted in significant differences between subjects with and without signs of cognitive dysfunction is presented in Table 8.2. In a large population of twins aged 40 or older, Verdi and colleagues showed that under-representation of taxa belonging to the Burkholderiales order was associated with worse performance at cognitive tests independently of covariates such as frailty and treatment with proton pump inhibitors [53]. These findings suggest that microbiota depletion of Burkholderiales may represent a promising biomarker of cognitive aging, although the cross-sectional design of the study prevents drawing any conclusions on prediction of cognitive outcomes. In their studies on patients with cirrhosis, Bajaj and colleagues found other putative microbial biomarkers of cognitive impairment [67–69], namely Alcaligenaceae, Porphyromonadaceae and Enterobacteriaceae with positive associations, and Lactobacillales and Lachnospiraceae having negative associations with the condition (Table 8.2). These studies provide interesting data on the possible usefulness of gut microbiota-related parameters for predicting cognitive outcomes. However, they were conducted using a population of patients exhibiting a high burden of gut microbiota dysbiosis, due to cirrhosis. Therefore, their results may not be immediately transferred to the elderly population with cognitive impairment or dementia.

Decreased relative abundance of Lachnospiraceae, *Butyricicoccus* and *Clostridium* XIVb, and increased relative abundance of Lactobacillaceae and Christensenellaceae were associated with the presence of cognitive impairment in two distinct studies performed on patients with Parkinsonism [70, 71]. Interestingly, a decreased relative abundance of Lachnospiraceae associated with cognitive symptoms was also observed in patients with cirrhosis and, most importantly, in one of the few studies specifically conducted in the field of human dementia [15, 69]. Similarly, a decreased relative abundance of *Clostridium* XIVb was also detected in the study by Vogt and colleagues [13], in which the fecal microbiota of 25 patients with Alzheimer's disease was compared with that of 25 healthy controls (Table 8.1).

Table 8.2 Overview of the main bacterial taxa found as over- or under-represented in the fecal microbiota of animal models or human beings with cognitive symptoms, cognitive impairment or dementia

Mouse studies		Human studies	
Over-representation in cognitive impairment/dementia	Under-representation in cognitive impairment/dementia	Over-representation in cognitive impairment/dementia	Under-representation in cognitive impairment/dementia
Anaeroplasmatales [60, 64]	*Bifidobacterium* [60, 64]	Alcaligenaceae [67]	*Adlerkreutzia* [13]
Anaerostipes [64]	Christensenellaceae [43]	*Alistipes* [13]	Bacteroidaceae [15, 16]
Christensenellaceae [60]	*Desulfovibrio* [43]	*Bacteroides* [13]	*Bifidobacterium* [13]
Cyanobacteria [64]	Lachnospiraceae [43]	*Bilophila* [13]	Burkolderiales [53]
Erysipelotrichiaceae [63]	*Prevotella* [43]	*Blautia* [13]	*Butyricicoccus*[70]
Helicobacter [65]	Ruminococcaceae [43]	*Caldiserica* [66]	*Clostridium* XIVb [13, 69, 70]
Odoribacter [65]	*Subdoligranulum* [43]	Christensenellaceae [71]	*Dialister* [13]
Proteobacteria [63]		Enterobacteriaceae [68]	*Eubacterium* [12]
Ruminococcaceae [60]		Enterococcaceae [15]	Lachnospiraceae [15, 69, 71]
Sutterella [63]		*Escherichia/Shigella* [12]	Lactobacillales [68]
Tenericutes [64]		*Gemella* [13]	*Parabacteroides* [19]
Turicibacter [60]		*Helicobacter* [72]	*Phascolarctobacterium* [19]
		Lactobacillaceae [71]	Ruminococcaceae [19, 69]
		Phascolarctobacterium [13]	*Turicibacter* [13]
		Porphyromonadaceae [67, 68]	Veillonellaceae [15]
		Prevotellaceae [65]	Verrucomorbia [14]
		Proteobacteria [14]	
		Ruminococcaceae [15, 65]	
		Tenericutes [66]	
		Thermodesulfobacterium [66]	

Some taxa are listed both as over- or under-represented because of some inconsistency across the results of different studies

Thus, the abundance of Lachnospiraceae and Clostridium XIV b may deserve greater attention in future studies as possible biomarkers of cognitive aging.

The study by Vogt and colleagues [13] was also the most accurate one to date in the identification of microbial taxa with altered relative abundance in Alzheimer's disease, and with significant correlation of these with other biomarkers, including p-tau and β-amyloid deposition. Specifically, the relative abundance of *Blautia*, *Bacteroides*, *Phascolarctobacterium*, *Alistipes*, *Bilophila* and *Gemella* was increased in Alzheimer's disease, while the relative abundance of *Clostridum*, *Bifidobacterium*, *Dialister*, *Adlerkreutzia* and *Turicibacter* was decreased [13]. Future, larger studies should better clarify whether these bacteria may assume the role of microbial biomarkers of dementia or cognitive impairment.

It is also noteworthy that a significant association between the representation of some taxa in the fecal microbiome and imaging biomarkers of cognitive dysfunction [66] or genetic risk factors for Alzheimer's disease (APOE polymorphisms) [65] has been detected in two distinct studies. Other investigations recently reviewed by Franceschi et al. [72] also suggest that the presence of Helicobacter pylori in the intestinal microbiome could be involved in the development of Alzheimer's disease, by promoting systemic chronic inflammation or molecular mimicry mechanisms. These aspects will need further confirmation in future studies but may represent the bases for the discovery of other microbial biomarkers of dementia and cognitive impairment.

In summary, the current literature state-of-art suggests a list of possible gut microbiota-related biomarkers of cognitive aging, shown in Table 8.2. However, the evidence is too scarce to recommend the use of fecal microbiota analyses as a method to identify the risk of cognitive impairment or dementia. In addition, these studies were conducted in heterogeneous settings, included small numbers of participants and had a cross-sectional design, preventing study of the association of microbiome composition with clinical outcomes. Finally, some inconsistencies are present among the results of different studies, reinforcing the need to develop larger and sounder investigations in this field.

3 Microbiota-Related Metabolites as Biomarkers of Cognitive Function

3.1 The Role of Microbial Metabolites as Biomarkers in the Gut-Brain Axis

Many substances synthesized by components of the gut microbiota, either as metabolic byproducts or constituents of bacterial structures, may have a role in influencing the gut-brain axis functionality in cognitive function [10]. These substances, their characteristics and possible pathophysiological relevance have been recently reviewed by many authors [73–77]. These include neurotransmitters [73, 74],

metabolites derived by amino acid catabolism [75], short-chain fatty acids (SCFAs) and other lipids [76], components of the outer membrane of bacteria [76] and even bacterial amyloid proteins [77]. Despite their possible involvement in the development and course of dementia, only a limited number of them could have potential usefulness as biomarkers of cognitive aging. In fact, neurotransmitters, such as acetylcholine, norepinephrine, histamine and γ-aminobutyric acid, are also synthetized by the host, and their microbial origin cannot be easily detected with standard laboratory methods [73]. Bacterial amyloid proteins may be involved in the pathophysiology of Alzheimer's disease by promoting the formation of β-amyloid protein aggregates in the brain by molecular mimicry but they cannot easily be detected in biological samples [77]. Metabolites derived by amino acid catabolism and SCFAs have recently received considerable attention by researchers and show, in some cases, correlations with cognitive function [75, 76]. However, some technical issues associated with laboratory methods of detection are currently limiting their study and implementation in clinical practice.

Along with substances synthesized by the intestinal microbiota, there are also a number of substances derived from diet and transformed by certain metabotypes of gut microbiota that may have a relevant pathophysiological involvement in the gut-brain axis [8, 10]. However, since the intake of the precursors of these substances is variable and not easy to standardize in the diet, their use as biomarkers is in doubt. The most known of these substances is trimethylamine N-oxide (TMAO), which is produced by the metaorganismal metabolism of dietary choline and has been implicated in the pathogenesis of several human diseases, including cardiovascular, cerebrovascular and metabolic diseases [78]. Other microbial products of nutrients introduced with diet include polyphenol metabolites and urolithins [8]. Finally, recent studies link cognitive performance to vitamin K status, which is partly produced by the intestinal microbiota [79], so that vitamin K metabolism may represent another promising area of research in the field of cognitive aging.

The putative biomarkers of cognitive aging derived by microbiota metabolism can be detected in several biological samples, ranging from blood to cerebrospinal fluid to feces. In fact, most of the substances produced by gut microbiota are absorbed into the systemic circulation and can be detected in blood. This phenomenon is particularly enhanced in the presence of a "leaky gut". This means that increased intestinal mucosa permeability iscaused by inflammatory conditions of the gastrointestinal tract or by the presence of an extreme gut microbiota dysbiosis [80].

From the systemic circulation, some microbiota-derived substances may reach the central nervous system and may be best detected in cerebrospinal fluid samples. Some investigators have also studied metabolic byproducts of intestinal bacteria in stool samples, since their concentration may be directly related to the microbiota composition and functionality. SCFAs are among the most studied microbiota-derived biomarkers of disease in stool samples [81]. However, these molecules show low stability in fecal samples leading to huge intra-individual variability, depending on the timing of analyses after sample collection [81].

3.2 Structural and Functional Components of Bacteria as Biomarkers of Cognitive Aging

The structural and functional components of gut microbiota that have been detected in biological samples of the host and related with the onset of cognitive dysfunction or dementia, include lipopolysaccharide (LPS) and rhamnolipids (Table 8.3). LPS can be detected in human blood samples at low concentrations even in the absence of bacteremia or sepsis. This phenomenon is an expression of gut microbiota

Table 8.3 Overview of the main bacterial products or metabolites that may be potentially useful as biomarkers of cognitive aging and dementia, according to the current scientific literature

Possible biomarker	Category/Origin	Biological sample of detection	Comment
Lipopolysaccharide	Constituent of bacterial wall, exotoxin	Blood Cerebrospinal fluid Brain biopsies	Increased in case of altered gut mucosa permeability or gut microbiota dysbiosis; Enhances neuroinflammation
Rhamnolipids	Constituent of bacterial wall, exotoxin	Blood Cerebrospinal fluid	Found in cerebrospinal fluid and blood in only one study, promote inflammation
Kynurenine Kynurenic acid 3-hydroxy-kynurenine	Products of amino acid metabolism (tryptophan)	Blood	Only indirectly influenced by gut microbiota metabolism of tryptophan
Indole-3 acetic acid Indoxyl sulfate	Products of amino acid metabolism (tryptophan)	Blood	Products of microbiota-driven degradation of tryptophan; Uremic toxins found in advanced chronic kidney failure
NADH:Ubiquinone reductase	Bacterial enzyme	Feces	Enzyme related to bacterial synthesis of tryptophan through the shikimate pathway
Acetate, butyrate, propionate	Short-chain fatty acids	Blood Feces	Products of bacterial metabolism of carbohydrates; Show neuroprotective, anti-inflammatory and pro-anabolic effects
Deoxycholic acid	Bile acid	Blood Feces	Concentrations depend on the gut microbiota metabolism of bile acids
Trimethylamine-N oxide	Product of microbial metabolism of dietary choline	Blood Cerebrospinal fluid	Marker of increased cardiovascular and cerebrovascular risk
Polyphenol metabolites	Product of microbial metabolism of nutrients	Blood	Effect depends on the individual metabotype (composition and functionality of gut microbiota)
Vitamin K	Bacterial cofactor	Blood	Possibly influencing cognitive function; role not yet assessed in relation to microbiota

dysbiosis or altered intestinal permeability, and causes chronic subclinical activation of inflammation that is involved in the pathophysiology of several non-communicable diseases, including atherosclerosis, diabetes and obesity [82]. Since inflammation is also an important mechanism triggering age-related cognitive impairment and the onset of dementia, some researchers have investigated the possible association between the presence of LPS in biological samples of the host and dementia [10].

In mice with diet-induced obesity, an association between reduced performance at recognition and spatial memory tasks and increased serum levels of LPS was detected [61]. These findings were confirmed by an intervention study where genistein was administered in association with a high-fat diet to mice [83], suggesting that LPS levels may be inversely correlated with cognitive performance. However, the intracerebral injection of LPS in mouse models of Alzheimer's disease did not induce significant alterations in cognitive function [84].

Human serum levels of LPS have not been associated with cognitive performance by any study to date. However, investigations on brain biopsies have demonstrated that lysates from the hippocampus and superior temporal lobe neocortex of patients with Alzheimer's disease had significantly higher concentrations of LPS than brain lysates from non-demented controls [85–87]. Therefore, the relationship between LPS and cognitive performance in humans deserves more investigations and LPS could represent a promising biomarker of cognitive health.

Andreadou et al. have also demonstrated that rhamnolipids, other bacterial virulence factors possibly derived from the gut microbiota, exhibit higher serum and cerebrospinal fluid levels in patients suffering from Alzheimer's disease than in non-demented controls [88]. These findings have not been replicated in other studies but the role of rhamnolipids as a possible biomarker of cognitive aging and pathophysiology of dementia should be investigated.

3.3 Microbiota, Amino Acid Metabolism and Cognitive Aging

Studies performed in mice have demonstrated that the decline of cognitive performance is associated with a distinct metabolic profile in serum and in the brain [44, 60]. In mouse genetically prone to dementia, an overabundance of ketone bodies, lactate and amino acids and depletion of unsaturated fatty acids and choline, was demonstrated in serum and feces, suggesting a role of the intestinal microbiota metabolism in driving these changes [60, 65]. Brain metabolomics of aged mice showed significantly lower concentrations of amino acids (including methionine, phenylalanine, cysteine and creatine) and lipidic cofactors (including hydroxycholesterol, prostagrandins, phosphocholine and docosapentaenoate) than control young mice [44]. Part of these brain metabolic signatures may be directly influenced by the gut microbiota. In fact, older mice with signs of cognitive impairment had significantly higher brain levels of two metabolites related to microbial metabolism of the amino acid tryptophan, i.e., 3-indoxyl-sulfate and phenol sulfate,

compared to young mice [44]. Interestingly, these metabolites have been associated with neurological toxicity and inflammation [89]. In patients who underwent bone marrow transplantation, the levels these molecules were predictive of clinical outcomes, reflecting the presence of gut microbiota dysbiosis [90].

The metabolism of tryptophan has also been associated with brain function in humans. Aging is associated with reduced serum levels of tryptophan and increased activation of the main human metabolic pathway of tryptophan degradation, i.e. the kynurenine pathway [91]. The serum levels of some kynurenine pathway intermediates, such as kynurenic acid and 3-hydroxykynurenine, have also shown a positive association with the level of cognitive impairment [91]. In a recent large population-based study, the kynurenine-tryptophan ratio was inversely associated with the performance at the Kendrick Object Learning Test and Controlled Oral Word Association Test [92]. These findings can be explained by a possible neurotoxic effect of kynurenine pathway intermediates.

Gut microbiota can contribute to tryptophan degradation with its own metabolic pathways. The most studied intermediate is indole-3 acetic acid (IAA), which is known as one of the main uremic toxins in patients suffering from chronic kidney failure [93]. Serum IAA levels were found to be negatively associated with Mini-Mental State Examination test score in patients undergoing chronic hemodialysis [94] and with depressive symptoms in subjects with severe chronic kidney disease not undergoing hemodialysis [95]. Another intermediate of microbial tryptophan degradation is indoxyl sulfate, which showed serum concentrations that were associated with poor executive functions in a group of 199 patients with early-stage chronic kidney disease [96]. Interestingly, indoxyl sulfate is able to induce apoptosis in human astrocytes, suggesting a possible involvement of this microbiota-derived metabolite in the pathophysiology of dementia [97].

Moreover, tryptophan is autonomously produced by bacteria through the shikimate pathway, a seven-step metabolic route allowing the synthesis of aromatic amino acids from two intermediates in carbohydrate metabolism, i.e., phosphoenolpyruvic acid and erythrose 4-phosphate. In a study performed on 20 patients with Alzheimer's disease and 5 healthy controls, Paley et al. were able to identify gut bacterial gene sequences unique to patients with Alzheimer's disease, belonging to the enzyme NADH:Ubiquinone reductase that is related to the optimal functioning of the shikimate pathway [98]. These findings suggest that the gut microbiota of patients with Alzheimer's disease may have an enhanced production of tryptophan through the shikimate pathway and that this phenomenon may have relevance in the physiopathology of dementia.

In summary, the current scientific literature does not provide sufficient data to recommend the adoption of bacterial products of amino acid metabolism as biomarkers of cognitive aging. However, the complex interplay between the gut microbiota and the host in tryptophan metabolism may have relevance in the field of cognitive impairment and should be further investigated in the future. Kynurenine, kynurenic acid, 3-hydroxykynurenine, IAA and indoxyl sulfate could represent good candidates as biomarkers of cognitive aging.

3.4 Microbiota-Derived SCFAs and Cognitive Aging

SCFAs, namely acetate, butyrate and propionate, are among the most studied metabolic products of the gut microbiota. They are derived from dietary carbohydrates and absorbed into the systemic circulation, where they can exert a wide range of physiologic functions, including control of body weight, regulation of insulin sensitivity, regulation of inflammation and immune system activation [99, 100]. These mechanisms may have particular relevance for central nervous system diseases, in which inflammation is also involved in the pathogenesis of Alzheimer's disease [101]. Butyrate has been implicated as a central mediator of the possible gut-brain axis, and the effects of the administration of butyrate or butyrate-producing bacteria on brain functions have been investigated through several animal studies, recently reviewed by Stilling and colleagues [102]. However, few of these studies were focused on dementia or cognitive impairment and, therefore, their clinical relevance remains uncertain.

In a study performed in mice, Fröhlich and colleagues demonstrated that the administration of antibiotics to induce a deep gut microbiota dysbiosis, was associated with cognitive dysfunction and lower colonic concentrations of SCFAs compared to administration of placebo [45]. Conversely, the administration of a symbiotic (*Enterococcus faecium* plus inulin) in a mouse model of cognitive aging was associated with an increase in the fecal concentration of butyrate and better cognitive performances than the placebo [103]. The administration of a butyrate-producing species, Clostridium butyricum, as a probiotic to mouse models of vascular dementia and cerebral ischemia/reperfusion injury resulted in improved cognitive function or reduced neurologic deficits [104, 105]. In another study, the administration of *Bifidobacterium breve* strain A1, a metabolically active strain able to produce acetate, to an Alzheimer's disease mouse model resulted in improvements in cognitive function or in a less pronounced cognitive decline [106].

These data globally indicate that SCFAs, and particularly butyrate, could play an important physiopathological role in cognitive impairment, and thus may represent good biomarkers of cognitive aging. Fecal samples of patients with Alzheimer's disease are generally abundant in butyrate-producing bacteria [107] but no comparison with healthy controls is currently available on this specific point. The role of SCFAs as biomarkers of cognition in older subjects should thus be more investigated in future studies. Unfortunately, the volatile nature of these compounds makes them difficult to detect in human samples, and analyses generally show a low degree of reproducibility [81].

3.5 Other Microbiota-Derived Compounds and Cognitive Aging

3.5.1 Bile Acids

Bile acids are not a primary product of intestinal microbiota but their metabolism is influenced by the microbiome composition and functionality. In fact, dysbiosis can be associated with important alterations of bile acid metabolism, contributing to the

pathogenesis of several diseases [108]. Tauroursodeoxycholic acid, a bile acid found in bears and subject to gut microbiota metabolism, showed neuroprotective properties especially in animal models of Huntington's disease [109]. These properties have also been recently demonstrated in mouse models of dementia, in which the administration of tauroursodeoxycolic acid was able to slow down amyloid precursor protein processing and amyloid-β deposition [110, 111].

These data have raised speculations that other bile acids produced by humans and subject to gut microbiota metabolism, may have a role in the pathophysiology of dementia and could serve as biomarkers of cognitive aging. Olzarán and colleagues were able to identify a metabolic fingerprint of Alzheimer's disease by analyzing plasma samples of a large group of patients with mild cognitive impairment or dementia [112]. Interestingly, they found that the concentrations of one bile acid (deoxycolic acid) were independently associated with the presence of cognitive symptoms [112]. Another recent study showed that specific bile acid profiles in serum were associated with other well-established biomarkers of Alzheimer's disease normally found in the cerebrospinal fluid (amyloid $\beta_{1\text{-}42}$ and p-tau$_{181}$) and with imaging markers of brain atrophy [113], reinforcing a possible role of bile acids as microbiota-derived biomarkers of cognitive aging.

3.5.2 TMAO

TMAO is a metabolic product derived from microbial metabolism of dietary choline and serum levels of this molecule have been associated with an increased risk of atherosclerosis in animal models and human beings [78]. Thus, it has been considered as a marker of risk of vascular dementia [78]. However, recent studies have highlighted that it may also have relevance in the pathogenesis of Alzheimer's disease. First, the administration of exogenous TMAO to mice genetically prone to dementia resulted in an increased number of senescent cells in hippocampus, reduced synaptic plasticity-related protein expression and reduced cognitive performance [114]. Moreover, in a large sample of older patients, either with Alzheimer-type dementia, mild cognitive impairment or no cognitive symptoms, the levels of TMAO detected in cerebrospinal fluid were significantly different according to cognitive status and related with other biomarkers of Alzheimer's disease (i.e., phosphorylated tau, total tau and neurofilament light chain protein) [115]. Thus, the elevation of TMAO in cerebrospinal fluid may represent a promising biomarker of cognitive dysfunction.

3.5.3 Polyphenols

Dietary polyphenols have been associated with reduced risk of a large number of cardiovascular disorders, based mainly on studies in vitro [116]. These compounds are generally metabolized by the gut microbiota into a large number of substances that may exert a protective function against neurodegeneration, and there is

interest in these molecules as potential biomarkers of cognitive aging. For example, 3-hydroxybezoic acid and 3-(3′-hydroxyphenyl)propionic acid, two metabolites derived from microbiota degradation of anthocyanidins contained in grape seeds, accumulate in brain tissues of rats and exhibit the capacity of interfering with the assembly of β-amyloid into neurotoxic aggregates [117]. Polyphenol metabolites can also modulate cognitive resilience in mice, but this capacity is dependent on the gut microbiota composition and functionality, the so-called metabotype [118]. Therefore, the administration of polyphenols as food supplements or nutraceuticals may have different effects in different individuals, depending on the gut microbiota.

An example of this inter-individual variability of polyphenol metabolism, depending on gut microbiota composition, is the ellagitannins, a group of polyphenol compounds found mainly in pomegranates and walnuts [119, 120]. In human beings, two different gut microbiota metabotypes (A and B) have been identified, and each of these is able to produce different metabolites from ellagitannins, called urolithin-A and urolithin-B, respectively [119, 120]. Urolithin-A has been associated with reduced cardiometabolic risk factors and with beneficial health effects, unlike urolithin-B [119]. Thus, the gut microbiota urolithin-related metabotype may contribute to the observed variability of health benefits of pomegranate extracts found in humans [120].

A recent study performed in APP/PS1 mouse models of Alzheimer's disease has demonstrated that the administration of urolithin A ameliorated cognitive performance and positively influenced several pathophysiological mechanisms of dementia, including amyloid-β deposition, neuronal apoptosis and neuroinflammation [121]. Urolithin A also seems to be able to enhance mitophagy, which is a mechanism protecting against cognitive impairment [122]. In a small human randomized trial, the administration of 8 ounces of pomegranate juice for 4 weeks to older patients reporting cognitive complaints resulted in a significant improvement in verbal memory tasks, which was paralleled by a significant increase in plasma concentrations of urolithin A-glucuronide [123]. These data underline the point that urolithin A and urolithin A-metabotype of gut microbiota may represent interesting biomarkers of cognitive aging viaidentification of a subset of patients that are susceptible to nutritional intervention.

3.5.4 Vitamin K

Vitamin K metabolism and the administration of vitamin K antagonists as oral anticoagulants have been recently studied as possible risk factors for cognitive decline in older individuals [124, 125]. Some studies support a positive association between vitamin K levels and cognitive performance [79, 124], while the administration of vitamin K antagonists to rats resulted in altered cognition [126]. However, the effect of vitamin K antagonists on cognitive function may depend on the pathophysiological mechanism of dementia, as it may be protective in cerebrovascular forms of dementia [125] and probably detrimental in neurodegenerative forms [79, 124]. Patients with cognitive impairment who need the administration of vitamin K antagonists are more likely to have multimorbidity and more severe forms of cognitive

dysfunction at baseline, so that the putative association between vitamin K antagonists and progression to dementia may be spurious [127]. In this scenario, the intestinal microbiome could play an important role, since vitamin K is physiologically produced by intestinal bacteria [128]. However, no study has investigated the correlation between production of vitamin K by the gut microbiota and cognitive outcomes to date. Vitamin K could however represent another promising biomarker of cognitive aging meriting future investigations.

4 Conclusions

Gut microbiota composition and microbiota-derived metabolites or substances represent a promising area of research for the identification of novel biomarkers of cognitive aging. However, the current literature state-of-the-art does not support the implementation of microbiome-related biomarkers of cognitive aging for use in clinical practice. The study of the relationship between gut microbiota composition and functionality in mild cognitive impairment and different types of dementia should be translated from animal models to patients. In addition, these studies should be focused on alpha diversity, Firmicutes/Bacteroidetes ratio, abundance of specific key taxa and microbial metabolism of amino acids, SCFAs, bile acids, vitamin K and nutrients such as polyphenols and choline.

The use of new microbiome-related biomarkers of cognitive aging could bring to several advantages. If these biomarkers show alterations in the early phases of dementia, they could assist physicians in the diagnostic process, or even lead to early diagnosis. They could also help identifying those patients with a quicker evolution towards dementia, deserving more aggressive treatments and strict follow-up. From the patient's perspective, it could bring improved management of cognitive diseases and give important information on neurological diseases, facilitating a comprehensive classification of health status. Finally, it could also have economic relevance, since the microbiome analyses are generally less expensive than brain imaging examinations. For all these reasons, more research is urgently needed in this field.

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Chapter 9
The Impact of Herbal Products in the Prevention, Regeneration and Delay of Skin Aging

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1 Introduction

The skin is the outermost and the largest organ of the human body, representing one sixth of the total body weight [1]. Skin has a role as a physical barrier against harmful microorganisms, toxic substances and ultraviolet radiation, and it regulates water loss and body temperature [2, 3]. Changes in the skin will affect an individual not only physiologically but also psychologically, which are both significant for quality of life. Along with aging, the skin like all human organs changes progressively. This natural change is called chronological aging. However, as the outermost organ and as a body barrier, the skin will also be subject to exposure to stressors and dangers from the environment outside the body. Changes in the skin due to external factors are referred to as premature aging, and because they are often caused by Ultra Violet radiation (UVR), this is also called photoaging [4–6].

Skin reflects the intrinsic and extrinsic aging process in the human body, which further causes changes in its function and structure [7]. The dermatological concern correlated with skin aging has grown as the aging population in the world has increased. Skin aging not only affects appearance but it can also influence an individual's social behavior and reproductive status [2]. Thus, some people pay a considerable amount of expense for cosmetics and pharmaceuticals that could prevent, regenerate and delay skin aging [8].

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Aging phenomena are known but the aging process is not clearly understood. Aging is associated with a decrease in function and character of physiological, physical and appearance of the body [9–11]. In 1956, Harman developed the theory of free radicals (reactive oxygen species; ROS) as a cause of aging. An imbalance in the amount of exposure and elimination of ROS by antioxidants will affect many physiological processes in the body. Environmental insults to the skin such as those caused by UVR and pollutants can cause the formation of ROS [5, 6]. ROS are unstable molecules that can react easily with electron acceptors, such as oxygen, and turn into free radicals. The accumulation of ROS that are not eliminated by the antioxidant system in cells produces what is called oxidative stress. Clinical manifestations of oxidative stress on the skin due to accumulation of ROS include increased skin weakness, xerosis, wrinkles, pigmentation irregularities, benign growths (such as seborrheic keratosis) and/or malignant neoplasms such as basal or squamous cell carcinoma. At the cellular level, there can be a decrease in keratinocyte proliferation, reduced generation of the stratum corneum, lower regeneration of the protective layer caused by reduced lipid synthesis, and thermoregulation damage due to changes in responsive blood vessels and the autonomic nervous system.

The rate of skin aging is affected by intrinsic and extrinsic factors and almost every aspect of biological function [3]. Intrinsic skin aging factors can be affected by ethnicity, anatomical variation and hormonal changes in cutaneous tissues. Some of the extrinsic factors in skin aging include poor nutrition, smoking, nicotine, pollution, and UVR exposure.

Herbal extracts are complex mixtures of natural compounds with various structures and origins. These have been used in skin care and cosmetic products since ancient times. The strategies of herbal product for the treatment and prevention of aging skin are variously based on their potential activities as antioxidants [1], anti-photo-aging agents [12, 13], anti-inflammation activity [14, 15], as skin cell proliferation promotors [16], modulators of collagen and elastin synthesis [17], or as inhibitors of melanin production [18].

The increasing concerns regarding quality of life and appearance have led to an increase in the market of natural compounds with anti-aging and photoprotective properties [19]. Plant extracts and natural compounds are able to protect or ameliorate the deleterious effects on aged skin, and many of them are used as either oral dietary supplements or topical cosmetic formulations. The importance of cosmetical preparation is not only related to improving the overall appearance of the skin during aging but it also offers a better quality of life through prevention and treatment of skin disorders related to the aging process [20].

This review will summarize the skin aging characteristics in each layer, the usage of herbal products in the prevention, regeneration and delay of the skin aging process, and herbal formulations to increase the effectivity of cosmeceutical products.

2 Characteristic of Aging Skin

Aged skin has the characteristics of being thinner and pigmented, with increased laxity, coarseness, wrinkling, sallow coloration, telangiectasia (apparent widened venules), dryness, fragility, ease of bruising, and cutaneous malignancies. Intrinsic skin aging is a normal physiological process that occurs due to genetic or metabolic factors [3]. The most noticeable histological changes occur in the basal cell layer as the epidermis becomes thinner, the dermal-epidermal junction (DEJ) area decreases, and basal cell proliferative capacity weakens [21].

The skin consists of different cell types and compartments with different functions [22–25]. The salient features of aged skin occur throughout the epidermis, dermis and subcutaneous tissue, which in turn manifest in the alteration of skin topography. The basal layer contains pigment-producing melanocytes, which determine skin color and possess photo-protecting properties. The DEJ connects the epidermis to the underlying dermis, which contains dermal fibroblasts and appendages such as hair follicles, sebaceous glands and sweat glands. As shown schematically in Fig. 9.1, the aged skin dermis is relatively avascular and acellular compared with younger skin. It also has lower production and disorganization of collagen, fragmented elastic fibers, as well as lower glycosaminoglycans and melanocytes.

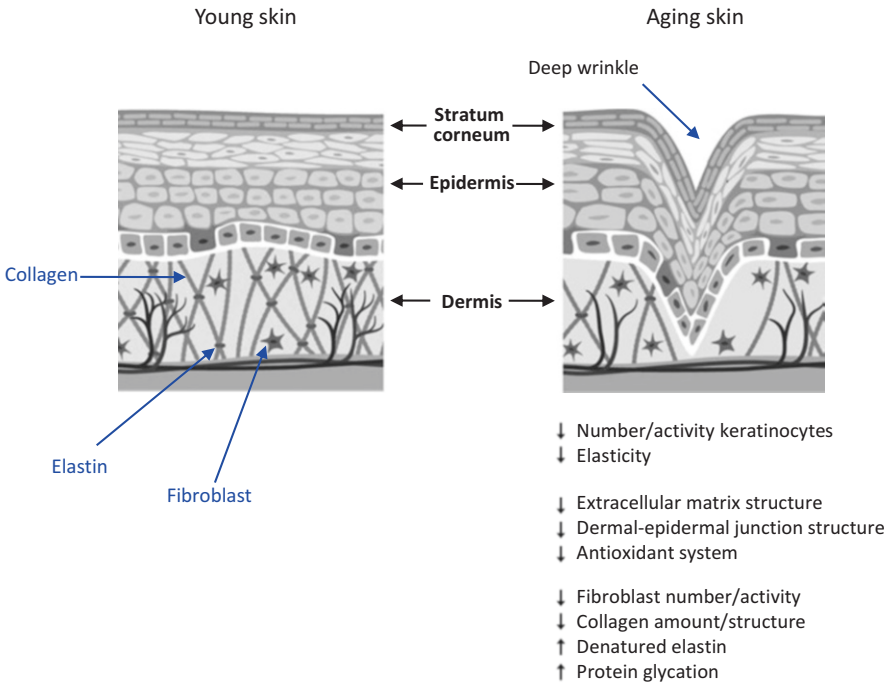

Fig. 9.1 Clinical manifestation of aged skin

Signs of skin aging include thin and dry skin, coarse wrinkles, decreased elasticity, laxity, aberrant pigmentation and a rough-textured appearance. Decreased proliferation and renewal capacity of basal keratinocytes and reduced epidermal stem cell number are also several factors that can cause epidermal, DEJ and dermal thinning [3]. The extracellular membrane (ECM) generates fibroblasts, which are responsible for maintaining skin integrity and elasticity in the dermis area. Therefore, alterations and degradation of the ECM during dermal thinning can cause increased numbers of wrinkles and decreased skin elasticity [26].

2.1 Epidermis

The epidermis is the outermost part of the skin, composed of four strata known as the basal, spinous, granular and cornified layers [4, 5, 27]. The dominant cell types in the epidermis are keratinocytes and melanocytes. It has a function to prevent water loss, protect the body from toxic chemicals and pathogenic microorganism, abrasions and UVR.

Regeneration of the epidermal cells occurs continuously in young skin. This regeneration begins with multiplication of proliferative cells in the deepest layer, and these are pushed out by differentiation and cell division [4, 5, 21, 28]. Differentiation causes molecular, structural and functional changes of keratinocytes so that these will occupy different layers. The peak of differentiation will produce corneocytes which are dead cells. Corneocytes, which are rich in proteins, are embedded in the matrix, which is composed of ceramides, cholesterol and fatty acids [23, 29].

In the cornification process, calcium has an important role. The lowest calcium level is found in the basal stratum and increases up to the stratum corneum. In young skin, the stratum granulosum has high calcium levels. In old skin, the distribution of calcium in each layer is so irregular that the composition of the protective proteins of corneocytes changes. This is also thought to be a mechanism of protection against aging [4, 5]. The speed and regeneration ability of epidermal cells is influenced by age. Aging leads to decreased numbers of cells that proliferate in each layer and dead cells accumulate so that the regenerative capacity of the tissue is lower. The structure and shape of cells change to become more porous and the functions of structural organizations become less effective [28].

The normal aging process does not affect dermis thickness, but there are noticeable changes in skin structure. For example, the skin becomes more susceptible to irritation and has lower permeability, which results in reduced trans-epidermal water flux [23, 29]. In some cases, the whole epidermal layer will gradually decrease in thickness with age at a rate of approximately 6.4% per decade. In photoaged conditions, this reduction in thickness will occur faster. The form of keratinocytes also becomes shorter and fatter, while the corneocytes become larger as a result of decreased epidermal turnover. In aged skin, there is a decreased water binding capacity due to changes in amino acid composition and lipid levels so that the

capacity of natural skin moisturizing factors are reduced. Disorders due to the aging process can also affect the barrier function of the skin, due to the global reduction of lipids in the stratum corneum, which will affect corneocyte binding in the matrix. In addition, the DEJ can be flattened, causing reduced interdigitation between the epidermis and dermis which results in reduced supply of nutrients and oxygen.

Melanin has a role as sun protector in the skin [23]. In photoaging, the production of melanin decreases due to a decrease in the functional amount of melanocytes in the basal layer of the epidermis (stratum basale). Therefore, older people are more susceptible to UVR exposure which can cause sun-induced cancer [23]. Aging of the skin causes uneven pigmentation with a tendency towards hyperpigmentation, even though there is a decrease in the number of melanocytes. In addition, there is also loss of melanocytes in certain areas and changes in interactions between melanocytes and keratinocytes [27].

2.2 Dermis

The dermis contains nerves, blood vessels, lymphatics and secretory organs [27]. Fibroblasts are the main cell type found in the dermis and these function to synthesize and degrade the ECM. The ECM structure consists of highly organized, elastic and reticular collagen fibers. The three primary structural components of the dermis are collagen, elastin and glycosaminoglican (GAG). Collagen functions to give skin its strength and maintain tissue integrity, whereas elastin provides elasticity and resilience. The extrafibrillar matrix consists of a complex mixture of proteoglycans, glycoproteins, glycosaminoglycans, water and hyaluronic acid.

Many of the components and characteristics of the dermis decrease with age, such as thickness, vascularization and cellularity, number of mast cells and fibroblasts, as well as the levels of glycosaminoglycans, hyaluronic acid produced by fibroblasts and the number of interfibrillary ground substances [22, 25, 27, 29]. Decreased elastin and collagen turnover caused by a decrease in fibroblasts and collagen synthesis also occurs due to aging. Photoaged skin shows disorganized collagen fibrils, fragmented elastic fiber and accumulation of abnormal elastin-containing material. The clinical manifestations of dermal aging include increased skin stiffness because the molecular integrity of the dermis is lost, decreased extensibility of torsion, reduced elasticity, and increased susceptibility to tearing-type injuries [29].

2.2.1 Collagen

Collagen is the most abundant protein in skin, constituting about 70% of dry skin mass, and it is responsible for conferring strength and gives support to human skin. Normal aging is characterized by atrophy of epidermis and dermis and also flattened rete ridges [23]. Aged skin has thickened fibrils in rope-like bundles and

it has a decreased collagen synthesis level [23, 30, 31]. The ratio of collagen types in human skin is also an indicator of the skin age, because this ratio is altered in aged skin. In young skin, 80% of collagen is type I and 15% is type III. In contrast, old skin contains more type III collagen due to decreased levels of type I collagen. In photoaged skin, studies have shown that there is 59% reduction of type I collagen. Collagen IV also plays an important role as the framework for other molecules and it is also responsible for maintaining the mechanical stability of DEJ, which is assumed to be correlated with wrinkle formation. However, there are no significant differences in collagen type IV levels in sun-exposed and uv-exposed skin. Collagen VII functions by anchoring the fibrils that attach the basement membrane to the underlying pappilary dermis. Sun-exposed skin also shows significantly lower numbers of anchoring fibrils compared to normal skin. Therefore, it is assumed that wrinkles may form as a result of a weakened bond between the dermis and epidermis, due to degradation of anchoring fibrils. Loss of collagen type IV and VII marks the base region of the wrinkle. Overall, the collagen abundance in the skin decreases at a rate of approximately 1% per year.

Skin aging, either caused by extrinsic or intrinsic factors, is marked by an elevation of activator protein-1(AP-1) activity, matrix metalloproteinase (MMP) gene expression, inhibition of transforming growth factor (TGF)-β signalling, reduction of collagen synthesis and increased collagen degradation [32]. UVR exposure has been found to up-regulate the synthesis of MMPs through increased amounts of transcription factors such as c-jun and c-fos. MMPs are increased even without UVR exposure and this leads to the formation of AP-1. AP-1 then activates the MMP genes and stimulates the production of collagenase, gelatinase and stromelysin. Thus, AP-1 activation and (TGF)-β signaling inhibition mediates collagen degradation via increased proteolysis. MMPs, especially collagenase and gelatinase, are known to be produced within hours of UVB exposure. Long term elevation of MMPs likely leads to disorganized and clumped collagen, and it is assumed that this causes the lower collagen I levels in photoaged skin [23, 30, 31].

2.2.2 Elastin

Photo-aged skin causes the accumulation of amorphous elastin material, called elastosis [23, 30, 31]. UV exposure induces elastin fibre thickening and coiling in the pappilary dermis and reticular dermis, reduction of microfibril numbers, increases in the interfibrillar area, complexed shaped and arrangement of the fibres and increased numbers of electron-dense inclusions. Aged skin also has small amounts of sugar and lipids and abnormally high levels of polar amino acids. MMP-2 is thought to be the protease responsible for degradation of elastin.

Sun exposure level affects the magnitude of the hyperplastic response or an increased amount of elastic tissue. Photo-aged skin has been found to have lower

elasticity and resiliency [31]. This skin is characterized by changes in the normal pattern of oxytalan fibers in the papillary dermis by the formation of the fibrous network in young skin that ascends perpendicullary from the uppermost section of the papillary dermis to just beneath the basement membrane. The loss of elasticity manifests as sagging skin in the elderly person [23, 30, 31].

2.2.3 Glucosaminoglycans

Glucosaminoglycans (GAGs) are polysaccharide chains with repeating disaccharide units attached to a core protein, which are responsible for conferring the outward appearance of the skin by its capability to bind water uo to 1000 times their volume [31, 33]. Hyaluronic acid (HA), dermatan sulphate, and chondroitin sulphate are types of GAGs, which give skin its plump, soft, and hydrated appearance. They also help to maintain the salt and water balance. There are conflicting reports regarding the effect of UVR exposure on GAG concentrations in the skin, especially regarding HA. Some studies have reported that the GAG concentration in the skin decreased following UVR exposure, but others have suggested that there are no UVR-induced changes in GAG levels. It is assumed that this discrepancy may have occurred because GAGs are produced in both the epidermal and dermal areas. In intrinsically aged skin, the HA level in the dermis remains stable but the epidermal HA diminishes almost completely. Reduced levels of HA can be observed in the photoaged skin and scars. HA can be found in young skin at the periphery of collagen and elastin fibers and also at the intercept of these fibers in the both dermal and epidermal area, but not in the stratum corneum or stratum granulosum [34]. Wrinkle formation, sagging skin, reduced turgidity and capacity to support microvasculature in the skin correlate with decreased HA levels.

2.3 Vasculature

Blood flow to the skin is decreased in aged skin, with the reduction reported to be around 40% between the ages of 20 to 70 years [31]. This results in lower nutrient exchange, inhibition of thermoregulation, decreased temperature in the skin surface and skin pallor. The anatomical and physiological changes in the microcirculation that have been reported include impaired microvascular reactivity, increased vascular stiffness, decreased vascular density and impaired vascular organization [35]. Acute and chronic UV radiation stimulates skin angiogenesis through upregulation of vascular endothelial growth factors and inhibition of thrombospondin-1, a potent angiogenesis inhibitor. In particular, a decreased vascular network appears in the papillary dermis, as shown by the disappearance of the vertical capillary loops which is the cause of the effects described above [36].

2.4 Subcutaneous Tissue

The subcutaneous layer maintains the structural integrity and function of blood vessels needed by the dermal and epidermal layers [30, 31, 36]. Subcutaneous fat is higher in females compared to males. In females, it represents 85–90% of the total body fat [36]. Subcutaneous fat is highly partitioned in distinct, independent compartments separated by septal barriers [37]. Fat redistribution in the aged skin results in a reduced subcutaneous:visceral fat ratio [38], which has been found to be correlated with physiological alteration of adipocyte metabolism and adipokine synthesis [39–41]. Skin aging also caused diminished subcutaneous fat in the face, as well as in the dorsal area of the hands and shins.

3 Herbal Products for Skin Anti-aging Strategies

The use of oral and topical exogenous antioxidants is one means of preventing and repairing oxidatively-damaged skin. Various plant species have been widely used as sources of oral and topical antiaging agents. In addition, a number of plant secondary metabolites have activities that may lessen the effects of aging on the skin, such as antioxidants, photoprotective agents, anti-inflammatory molecules, modulators of collagen/elastin synthesis and inhibitors of melanin synthesis.

3.1 Antioxidants

The secondary metabolites in plants include antioxidant compounds (especially phenolic compounds; catechins, isoflavones, proanthocyanidins, and anthocyanins), phenolic acids (benzoic, gallic, and cinnamic acids) and stilbenes [42–44]. These are derived from plants such as tea, grape, bergamot, fernblock, rooibos, grapefruit, and red orange, and are currently widely used in cosmetic formulations. Based on their structure, polyphenol compounds can act as antioxidants through radical scavenging or as oxidized prooxidants via phenoxyl reactive radicals or intermediate quinone or quinone metides. The antioxidant activities of polyphenols appear to be more effective than vitamin E and vitamin C. Their use in topical formulas has been shown to be effective as antiaging agents through antioxidant activities [42]. Flavonoids and phenolic acids have the activity of capturing free radicals and acting as chelating metal ions such as iron and copper, which can initiate the formation of reaction free radicals. In addition, flavonoids can inhibit the activity of some redox enzymes so they can inhibit cell damage caused by free radicals [45, 46].

Genistein and daidzein are effective antioxidant compounds. Both of these compounds are isoflavonoids, estrogen-like molecules, which are found in soybeans [45]. The glycoside group is not estrogenically active and can be used for topical

applications. Also, the epigallocatechin-3-galate (EGCC) contained in tea leaves is a polyphenol with strong antioxidant activity and topical preparations of tea leaf extract containing this compound can inhibit the effects of UVR exposure, including protecting the body from UVR-induced immunosuppressant activity. Various plant species from the coffee family also contain polyphenols such as proanthrocyanidins, quinic acid, caffeic acid, caffeine and chlorogenic acid, which are also powerful antioxidants [31, 45]. Ferulic acid is another antioxidant found in whole grains, spinach, parsley, grapes, rhubarb, and cereal grains (especially wheat) [47]. Topical use of grape seeds has been proven to prevent or modrate UVR-related damages, such as the thickening of the epidermal layer, erythema, pigmentation, inflammatory neutrophil infiltration and collagen degradation, as well as the increased expression of genes associated with skin aging such as those for cyclooxygenase 2 (*COX-2*), nuclear factor erythroid 2–related factor 2 (*NRF-2*) and heme oxygenase 1 (*HO-1*). The main content of grape seed is resveratrol which is a natural stilbene compound. There are also other antioxidant compounds which may have use as skin anti-aging products, such as quercetin, catechin, epi-catechin, gallic acid and oligomeric proanthocyanidins [43].

3.2 Anti-photo-aging Agents

It is well established that UVR can cause skin disorders such as sunburn and non-melanoma skin cancer. A photoprotective agent can be used to minimize the harmfull effects of UVR exposure to the bare skin. Sun protection factor (SPF) describes the effectiveness level of bioactive compounds that act as UV protectants. It is defined as the minimal erythema dose ratio between sunscreen-protected and unprotected skin [48]. Sunscreens slow the progression of photo-aging and prevent aging effects on the skin such as wrinkles, actinis keratosis reduction, solar elastosis and squamous skin carcinoma. Sunscreen preparations contain organic UV filters that block either UV-A and/or UV-B radiation by absorbing the radiation [49, 50].

Organic sunscreens that have been commonly used are aminobenzoate (e.g., p-aminobenzoic acid esters), cinnamate (e.g., octinoxate), salicylate, actocrylene and benzophenone (e.g., oxybenzone), avobenzone and mexoryl SX [48]. Many plant polyphenols have been evaluated as potential sun protection agents, since they are able to absorb UV-B radiation [12–14]. For example, the phenolic compounds of Schinus terebinthifolius crude extract, such as ethyl gallate, gallic acid and several flavonoid compounds, may be useful as natural sunscreen products [12]. In addition to their antioxidant activities, EGCG and resveratrol also have activities that can reduce the size of erythema caused by UVR exposure. High dosages of flavanol compounds from cocoapowder have a similar capability. Finally, carotenoids such as β-carotene, lycopene, canthaxan-thin, and lutein derived from tomatoes, carrots and algae, are antioxidants that exhibit photoprotective activity and are capable of reducing the size of erythema cause by UVR exposure [43].

3.3 Anti-inflammatory Agents

As noted above, UVR induces oxidative stress in epidermal cells which causes lipid peroxidation and cell damage. In this scenario, the complement system recognizes the oxidation-specific epitopes and causes inflammation, which leads to macrophage infiltration and activation that removes the damaged cells and oxidized lipids [51, 52]. Macrophage activation leads to MMP release that degrades the ECM. Thus, long term exposure to UVR can lead to overactivation of the complement system, which causes DEJ damage and overburdens macrophages with oxidized lipids, which further causes chronic inflammation and long-term damage to the dermis due to proinfammatory cytokines and ROS release [51, 53]. In cases of inflammation caused by photoinduced oxidative stress, phenolic compounds can help by attenuating the activity of inflammatory mediators such as interleukin 6 (IL-6) and prostaglandin-E2 (PGE2). In addition, the natural compounds such as veratric acid [15], dihydrochalcone phloretin, afzelin [14] and luteolin [54] can decrease expression of the inflammation-related molecules.

3.4 Promotion of Skin Cell Proliferation

Epidermal skin cells possess the ability for self-renewal and dead cell replacement with turnover times of 40–56 days, but aging makes the capacity decline [55]. Bioactive agents from herbal products can be used to restore this regenerative capacity to some extent. Several plants that had been proven to have skin cell renewal capacity include Populus nigra buds, oak woodm mate leaf, and benjoin resin [16]. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) analysis of Populus nigra buds extracts revealed the presence of 6 phenolic acids (caffeic, -coumaric, isoferrulic, di-O-methylcaffeic, cinnamic), three flavonoids (pinocembrin, pinobanksin, and its derivate) and salicin [16], which all have potent antioxidant, antiinflammatory and skin regeneration activity. This extract modulates transcription of genes, such as those for Kruppel-like factor 10 (*KLF10*), E2F-4 transcription factor (*E2F4*), and epidermal growth factor (EGF) response factor 1(*ZFP36L1*), which are involved in skin regeneration through effects on proliferation, differentiation, survival, and DNA synthesis. Expression of the catalase (*CAT*) gene was also found to be up-regulated, which is responsible for antioxidative effects, and the chemokine ligand 5 (*CCL5*) gene down-regulated, which is linked to inflammatory processes. The ellagitannin compounds of oak wood, caffeoyl derivatives from mate leaf and phenolic acids in benjoin resin have also been identified as compounds capable of modulating skin-aging related genes. All of these compounds have a demonstrated ability to down-regulate the proinflammatory *CCL5* gene and up-regulate proliferation marker genes (*KLF10*, *E2F4* and *ZFP36L1*) [16].

3.5 Modulation of Collagen and Elastin Synthesis

The ECM proteins such as collagen and elastin are increasingly proteolyzed with aging by the MMPs. MMPs in normal cells have been found to be regulated at the transcriptional level by specific protein inhibitors. The homeostatic imbalance of synthesis and degradation of the ECM causes loss of skin integrity, and may result in wrinkle formation. However, inhibition of MMP activity facilitates maintenance of skin structure and the phenolic compounds are known to preserve skin structure through this mechanism [56].

Crude grape pomace from white wine contains phenolic compounds such as cathecin, epicatechin, procyanidins B1 and B2, gallic acid, caftaric acid, quercetin glycosides and stilbene trans-resveratrol which all act as inhibitors of MMPs. The strongest activity was found in a fraction containing gallic acid, and this was followed by procyanidins and catechin which had proteolytic inhibitory activity, especially to collagenase [56]. Unfortunately, the use of these compounds is limited due to their high molecular weights and low permeabilities.

Gallic acid and elaeocarousin in an Emblica officinalis Gaertn ethanolic extract has been reported to enhance proliferation and collagen production in fibroblasts [17]. The extract was capable of minimalizing wrinkle formation through collagen synthesis, MMP inhibition and revival of damaged collagen fibers. Tannins such as emblicanin, pedunculagin and punigluconin are assumed to have similar activity as green tea tannin (EGCG), which modulates the expression and production of MMPs via AP-1 and nuclear factor kappa B (NF-κB) activation [57].

Depletion of collagen production in the aged skin can also be treated with collagen synthesis enhancers, such as vitamin C and glycolic acid, which are claimed to have anti-wrinkle capacities, although this has not been proven.

3.6 Melanin Production Inhibitors

Melanin is capable of absorbing 50–75% of the UVR exposure to the skin [58, 59]. Therefore, it can play a significant role as a skin protector. However, the hyperpigmentation caused by melanin overexpression can also lead to dermal disorders. Melanin is natural pigment produced by melanosomes in melanocytes and it is transferred to keratinocytes through dendrites. Melanin is a tyrosine derivative that forms during oxidative stress reactions through the activity of the tyrosinase enzyme. Melanin synthesis is also regulated by tyrosine-related protein 1 and 2 (TRP-1, TRP-2) and cellular signaling.

Melanin inhibitors work through inhibition of tyrosinase [60, 61]. Phenolic and flavonoids which have a structure similar to tyrosine, with regards to the presence of aromatic purine rings, and these can act as substrate analog inhibitors in melanogenesis [62, 63]. The compounds Brazilin and isoflavonoiud-4-O-methylsappanol were identified in a Sappanwood methanolic extract as having the ability to inhibit melanogenesis [64] in faskulin-treated HMV-II human melanoma cells. Resveratrol

and oxyresveratrol are also tyrosinase inhibitors [65, 66]. Other herbal products that have melanogenesis inhibitor activity include Morus alba leaf extract [67], Arthrophytum scoparium [18], and Trifolium nigrescens Subsp. The petrisavi [68] and Morus alba leaf extract inhibitory effects are due to the presence of 20 phenolic compounds (8 benzofurans, 10 flavonoids, one stilbenoid and one chalcone) [67]. Catechol and tetrahydro-isoquinoline in Arthrophytum scoparium are responsible for the inhibition of tyrosinase and TRP-1 as well as down-regualtion of the tyrosinase microphthalmia-associated transcription factor (*MITF*) and melanocortin 1 receptor (*MC1R*) genes [18]. In addition, trifolium nigrescens phenolic compounds showed inhibitory effects on mushroom tyrosinase, and one of these containing a glucoside residue was found to have the highest activity [68].

4 Herbal Skin Care Product with Anti-aging Properties

The increasing proportion of the aging population globally correlates with the increasing demand of anti-aging products. The need for a beautiful, healthy and youthful appearance has become a trend and the cosmeceutical industry has become an ever-growing market in order to meet these demands. In line with this, new technologies and innovations have been brought into play to increase quality and effectivity of these products, and to attract more customers.

Presently, there are many topical and oral skin care products that function by improving or maintaining skin health [69–72]. Topical products are known as "cosmeceuticals", while oral products are known as "nutricosmeceuticals". Both of these are backed by scientific evidence of effectiveness and safety although these do not follow such strict rules as those demanded in the traditional drug discovery industry (Fig. 9.2).

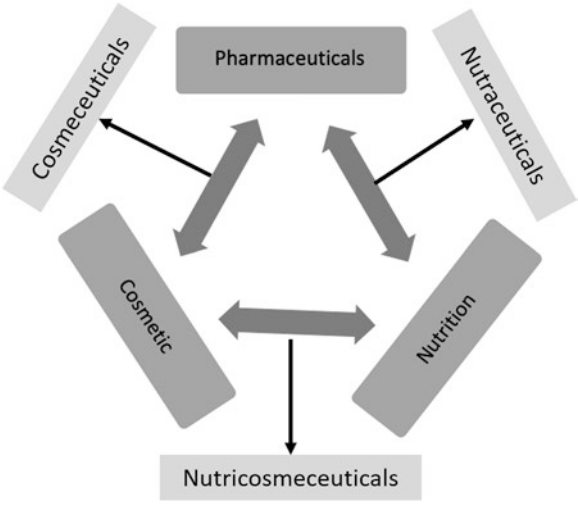

Fig. 9.2 Intersept between pharmaceuticals, cosmetics and nutrition

4.1 Cosmeceutical Products

Cosmeceutical is a portmanteau of the words cosmetic and pharmaceutical, which means that it is a cosmetic preparation that also has a pharmaceutical function (Fig. 9.2) [69–74]. Cosmeceuticals are intended to improve and reduce skin imperfections, compared to cosmetics that only hide or mask such imperfections. Similar to cosmetic products, cosmeceutical products are applied topically and intended for daily use. Such products are used to resolve facial aging problems, in particular wrinkles, mottled pigmentation, roughness of skin, rhytides, erythema, skin tone loss, dryness, sallowness, furrows, solar elastosis and black spots. The bioactive ingredients can comprise sunscreens and antioxidants to enhance the protection level of the skin against ROS and thus prevent photodamage and improve skin malignancies [74–77].

There are various forms of cosmeceutical products for antiaging treatments including lotions, creams, gels and liquids. These products usually function as moisturizers, antioxidants, anti-wrinkle agents, whitening agents, skin supplements (vitamins) or growth factors (Fig. 9.3). These products are often intended for daily use and thus the efficacy and safety aspects must be guaranteed. Apart from this requirement, there are several things about the quality that must also be fulfilled, such as it should not be irritative, it should be suitable for various or selected skin types [74, 76, 77].

Aged skin is more vulnarable to dryness. This is due to decreased production and increased degradation of GAG which reduces the ability of the skin to bind water. To improve this condition, regular use of a moisturizer could protect and strengthen the skin. It has been shown that this hydration ability of cosmeceutical products makes the skin softer and smoother [1, 76, 78, 79].

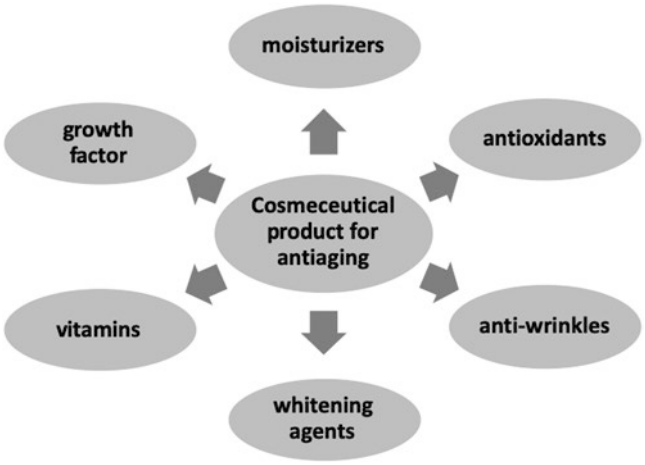

Fig. 9.3 Cosmeceutical product activity

In order to meet these consumer demands, the cosmeceutical is aiming for product specifications with higher efficacy, immediate effects and guaranteed levels of safety. Firstly, the material should have complete function in the form of natural product extracts, active compound isolates or synthesized or semi-synthesized compounds [25, 77]. Secondly, there is the need for development of new technologies for transdermal delivery systems so that the active ingredients can easily and quickly traverse the skin. Finally, the development of technologies that can objectively measure the penetration and effectiveness of the material should be developed in parallel as a means of quality and efficiency monitoring [74, 77, 79].

In the development of product cosmeceuticals, the mechanism of penetration into the skin layer is the basis for developing a drug delivery system. In general, the drug enters the target site through damaged skin However, the active ingredients must be able to penetrate into normal skin in the same manner as cosmetic products. Of the three main skin layers described earlier, the stratum corneum acts as a barrier layer against penetration. The choice of the drug delivery system technology applied to the material must be enable the active ingredient to traverse this barrier [11, 77].

Besides the skin penetration properties, the purpose of a drug delivery system is to increase the duration of action and stability, prevent incompatibility with other ingredients in the formulation and prevent the occurrence of undesirable effects either locally or systemic [73, 79, 80]. Various forms of drug delivery systems that are widely used include vesicles (liposomes, niozomes, nanosomes, phytosomes, herbosomes, marinosom, oleosom, aquasom, ultrasom, photozom, ethosome, transferosome, sphingosome, colloidosomes), emulsions (microemulsions, nanoemulsions, liquid crystals) and particulates (microparticles, nanoparticles, solid lipid nanoparticles, structured nano lipid carriers, microcapsules, nanocapsules, microspheres, nanocrystalline and cyclodextrin). The development of a drug delivery system for cosmeceutical products has been shown to increase its usefulness, but because the product is used daily for a long time, attention should be paid regarding the possibility of unwanted side effects. Therefore, product development must also take in to account the safety aspects [79, 80].

4.2 Nutricosmeceutical Products

The desire to have a youthful appearance may be met with good nutrition in line with the pharase, “beauty from inside”. This has led to new innovations regarded as “nutricosmeceuticals”, which are nutritional products that have both cosmetic and antiaging effects (Fig. 9.2) [70, 81–83]. As physical beauty is reflection of skin health status, the nutricosmeceutical industry aims to develop food products that have been proven to maintain healthy bodies and also have good effects on the skin. Recently, a number of skin food products have been developed, containing carotenoids, polyphenols, vitamins, essential fatty acids and pre-and probiotic substances, which have been used previously via topical applications to improve skin conditions (Fig. 9.4) [1, 82].

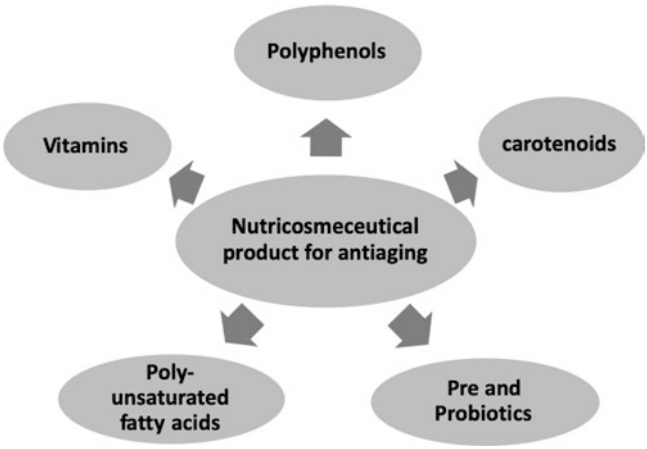

Fig. 9.4 Ingredients in nutricosmeceutical products

Since free radical formation appears to be linked to aging, antioxidant intake may help to restore homeostasis. Foods that are rich in antioxidants have also been shown to have a role in maintaining health and in delaying the aging process [75, 78, 81, 82]. These were shown to have a protective effect against free radicals found in the skin, through activation of endogenous antioxidants including enzymatic (reduced glutathione, superoxide dismutase and catalase) and non-enzymatic (vitamin E isoform, vitamin C and ubiquinol) ones. Thus both exogenous and endogenous antioxidants may have an important role in counteracting oxidative stress. In line with this, several endogenous antioxidants are known to be stimulated by a number of nutritional factors derived from fruits and vegetables.

Some carotenoid derivatives of vitamin A, such as β-carotene, astaxanthin, lycopene and retinol, are known to be effective anti-oxidants [1, 75, 78, 81, 82]. Tomatoes, watermelons, guavas, rosehips, and pink grapefruits can reduce oxidative damage because these all contain lycopene which acts as a singlet oxygen quencher. In addition, carrots, pumpkins, yams, mangoes and papayas, which are sources of β-carotene, can protect the skin from erythema caused by UV induction. The structure of polyphenols also shows strong anti-oxidant properties. Other molecules that have the basic structure of polyphenols include phenolic acids, flavonoids, stilbenes and lignans. Polyphenol compounds are found in fruits, vegetables, cereals, chocolate, dried beans and vegetables, as well as in drinks such as fruit juice, tea, coffee and red wine. Food sources containing linolenic acid and linoleic acid include fish and shellfish, flaxseed, flax oil, soybean oil, canola oil, chia seeds, pumpkin seeds, sunflower seeds, leafy vegetables, walnuts, sesame seeds, avocado seeds, avocados, salmon and albacore tuna. These foods can act as anti-inflammatory agents and thereby provide significant protection against UV radiation-induced erythema, for example. Probiotics and prebiotics can be consumed in various forms of food and beverage products, and studies have shown that these products can accelerate the recovery of human skin immune homeostasis after UV-induced immunosuppression.

The association between nutrition and skin conditions has led to the use of nutricosmeceutical products to maintain and regain body homeostasis through nutrients that enter the body, so that these can improve skin health and beauty. A complete and balanced nutrition can also positively affect the condition and appearance of the skin. Therefore, nutritional supplements in the form of nutricosmeceutical products can be used to help optimize diets and improve quality of life.

5 Conclusions

Chronological aging naturally affects and causes changes in the body, especially in the appearance and characteristics of the skin. Exposure to external factors will worsen this change. Considerable efforts have been made to improve, maintain and delay the effects of aging due to both intrinsic and external factors through invasive and non-invasive methods. In line with this, antiaging products have developed rapidly. The consumers demand products that can show the effects of rapid change, but they also realize that the results will not be as fast as surgery. Nevertheless, they prefer antiaging products that can "improve", "maintain" and "delay" the effects of aging on their appearance. Products that can meet these demands are nutricosmeceuticals and cosmeceuticals, which offer a complementary means of acquiring skin health from inside and outside.

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Chapter 10
Epigenetic Clock: Just a Convenient Marker or an Active Driver of Aging?

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1 Introduction

Why we age has been a question of heated debate for a very long time. More or less common view today is that aging has multiple causes [1]. In a recent seminal paper, nine hallmarks of aging were proposed to contribute to aging and collectively to determine the aging phenotype [2]. Epigenetic systems control gene activity and thus, directly or indirectly, affect all other hallmarks. Once differentiated, all cells in metazoan organisms must "remember" their appropriate pattern of gene expression for the organism to survive and function normally. Studies of developmental biology have established a role for epigenetic systems in establishing and maintaining these differentiated states of cells. For many years the epigenome was thought of as a static entity; once a cell becomes differentiated, and its genome is appropriately methylated and chromatin configured, no further changes in the epigenome were supposed to occur. Unexpectedly, multiple studies in the last years showed that epigenome is a dynamically regulated system involved in aging, or at least affected by it, and responsive to various external and internal factors [3–5].

An ability of epigenetic systems to affect all other hallmarks of aging puts them into a key position to affect the basic mechanisms (driving forces) of aging. On the other hand, active components of the epigenetic systems are encoded in the genome and, thus, are themselves under epigenetic control. Many links, both feedback, and feed-forward exist between different parts of the epigenome. Transcripts of genes encoding proteins of DNA methylation and histone modification systems could be targeted and controlled by miRNAs, whereas expression of genes encoding miRNAs are controlled by cytosine methylation and histone modification. DNA

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methylation and histone modification systems are known to be coupled at both gene expression and target recognition levels. Which alterations in DNA methylation and other epigenetic marks play a causal role in aging and which are just manifestations of aging have yet to be elucidated. The links between epigenome and aging are complicated and probably mutual. The epigenome is changed by various environmental and internal factors that affect aging, but it also could directly or indirectly affect aging. Similar to other cell systems, the epigenome is prone to gradual degradation due to genome damage, stressful agents and other aging factors. However, unlike mutations and other hallmarks of aging, age-related epigenetic changes could be fully or partially reversed to a "young" state.

2 Changes in DNA Methylation Levels During Aging

The first experimental proof of age-dependent changes in DNA methylation was obtained in our lab about half a century ago. In humpback salmon and rat, levels of cytosine methylation (5mC/C) in DNA of different organs have been found to gradually decrease with age [6, 7]. In mammals, the age-dependent loss of methylation mainly affected the heavily methylated repeat fraction of the genome [8] possibly leading to a global genome destabilization, since this fraction predominantly consists of transposable elements. Repression of such elements is widely believed to be one of the main defensive functions of DNA methylation. Indeed, activation of retrotransposons has been shown to occur at late cellular senescence leading to an interferon type I (IFN1-type) inflammation response, probably contributing to aging-associated chronic inflammation (inflammaging) – a known hallmark of aging [9, 10].

DNA methylation levels in different mice organs have shown a correlation with the chronological age [11, 12]. The rates of methylation loss upon aging in DNA of two mouse species was found to be inversely correlated with their maximal lifespans. A progressive decrease in DNA methylation levels was also observed in mouse, hamster and human fibroblast cultures [13]. In these cases, the rates of DNA hypomethylation were also inversely correlated to the cell lifespans (maximal number of the cell population doublings before senescence). Errors in the maintenance DNA methylation were considered to be the main cause of the progressive 5mC loss in dividing cells since the fidelity of the maintenance DNA methylation is ~95% per cell generation [14, 15]. Such loss of 5mC, essentially stochastic as it seemed to be, could affect aging at both cellular and organismal levels by increasing the transcriptional noise and eventually leading to aberrant transcription of various genes. However, no DNA hypomethylation was observed in dividing immortal cell lines [13]. Since the fidelity of the maintenance DNA methylation could hardly be higher in these immortal cell lines compared with their normal counterparts, the age-related loss of 5mC could not be explained by the maintenance methylation errors alone. Indeed, similar losses of 5mC upon aging occurred in mouse tissues with widely different proliferative activities (liver, brain, and small intestine mucosa) [11].

Moreover, it was found that besides global DNA hypomethylation, selective hypermethylation of some genes occurs upon aging [16–18]. The tumor suppressor genes, known to be hypermethylated in tumor cells, are also hypermethylated in normal cells upon aging [17, 18]. The promoter CpG island (CGI) sequences of *LOX*, *CDKN2A* (also known as *p16*, *INK4a* or $p16^{INK4a}$), *RUNX3*, and *TIG1* genes were found to be mainly unmethylated in human normal stomach epithelial cells before the 50-year age but progressively methylated between 50 and 80 years [19]. Such progressive methylation could explain the increase in tumor occurrences known in aged people. Since the age-dependent increase in DNA methylation levels was non-linear in this case, it evidently should be caused by some specific process, not just an accumulation of stochastic errors.

Discordance in DNA methylation profiles between monozygotic (MZ) twins can be used to discriminate between stochastic and "programmed" age-associated changes in DNA methylation. The methylation levels of total lymphocyte DNA were found to be practically identical between MZ twins in 65% of the pairs and significantly different in 35% of the pairs [20]. Identical DNA methylation levels were typical in younger pairs, whereas aged pairs usually had the most divergent DNA levels. DNA methylation differences between MZ twins gradually increased with age. Most discordant DNA methylation patterns were characteristic of the older twin pairs that lived separately and had different lifestyles. Thus, phenotypic and epigenetic differences could be due both to the effects of various factors (smoking, physical activity, dietary preferences) and stochastic methylation errors (epigenetic drift).

3 Changes of Genome Methylation Patterns Upon Aging

DNA methylation patterns in solid human tissues were found to be dependent both on tissue and age [21]. A distinct correlation between age and methylation was observed, loci in CGIs gained methylation with age, whereas loci not in CGIs lost it. This pattern was consistent across tissues and in blood-derived DNA. Gene-loci that earlier have been reported to be associated with aging, *ESR1*, *GSTP1*, *IGF2*, *MGMT*, *MYOD1*, *RARB*, and *RASSF1*, displayed significant methylation alterations (mainly increases) with age. Genes involved in epigenetic regulation (*LAMB1*, *DNMT1*, *DNMT3B*, *HDAC1*, *HDAC7*), telomere maintenance (*TERT*, *ERCC1*, *RAD50*), and premature aging (*WRN*), all showed age-related methylation alterations.

Out of approximately 3600 mouse genes studied, 21% displayed hypermethylation, and 13% displayed hypomethylation with age in small intestine cells [22]. Among genes devoid of the promoter CGIs, 7% displayed hypermethylation and 11% hypomethylation. The genes known to be hypermethylated in colon cancer (*Cdh13*, *Dok5*, *Esr1*, *Igf2*, *Myod1*, *Nkx2-5*, *Cdkn2a*, *Pgr*, and *Tmeff2*) showed age-related hypermethylation in normal small intestine. Globally, the hypermethylated gene group was enriched for genes involved in development and differentiation,

whereas the hypomethylated gene group was not enriched for any specific functional category. These age-dependent gene methylation patterns were partially conserved between humans and mice, and the conserved genes were mainly among the hypermethylated ones.

About 350 CpG loci were found to be differentially methylated with age (aCpGs) in human peripheral blood leukocytes (PBLs), with approximately 200 hypermethylated (hyper-aCpGs) and 150 hypomethylated (hypo-aCpGs) [23]. More than 95% of aCpGs were located within 500 bp of transcriptional start sites (TSS). More than 60% of hyper-aCpGs found in PBLs were also present in purified fractions of CD14+ monocytes (short-lived cells of the myeloid lineage) and CD4+ T-cells (long-lived cells of the lymphoid lineage). Unlike hyper-aCpGs, the hypo-aCpGs set was significantly reproduced in T-cells but not in monocytes. The conservative hyper-aCpGs group was enriched for genes with tissue-specific functions, including neural cell-related processes. Most of these genes are inactive or moderately active in PBL. These hyper-aCpGs significantly overlap with genes that are hypermethylated in various human cancers. Similarly to cancer cells, their methylation could decrease differentiation potential and increase the self-renewal of stem cells. Possibly, this is one of the mechanisms that enhance cancer frequency at advanced ages.

Of 476,366 CpGs covered by the Illumina Human Methylation450 BeadChip, about 29% were found to be differentially methylated across ages 14–94 years in PBLs [24]. Of these aCpGs, 60.5% were hypomethylated and 39.5% hypermethylated with age. For most sites, DNA methylation changed approximately linearly with age. The strongest positive correlation of the methylation level with age was seen for a CpG site located in the *ELOVL2* gene promoter CGI (methylation levels were from 0.35 in the youngest to 0.89 in the oldest individuals). Among the sites that become hypermethylated with age, 81.4% were located in CGIs, compared to only 2.8% for the hypomethylated sites. A larger fraction of CpGs located in gene bodies and 3′-un-translated regions (UTRs) became hypomethylated with aging. The genes with hypermethylated CGIs were over-represented in molecular functions of DNA binding and transcriptional regulation, developmental processes, and neurological functions. The genes that became hypomethylated with age were not enriched for any specific functional categories. Interestingly, both genes that became hypermethylated and hypomethylated with age were more likely to decrease than increase expression with age. Thus, both hypermethylation and hypomethylation with age can decrease expression, probably via different regulatory mechanisms.

In contrast to total PBLs, 12,275 and 48,876 aCpGs were identified in purified CD4+ and CD8+ T cell samples [25]. The majority of hyper-aCpGs were located in CGIs at silent gene promoter regions that were enriched for repressive histone methylation marks. Hypo-aCpGs were located more at the borders of CGIs and in gene bodies. In a subset of genes expressed in CD8+ T cells, a negative correlation between DNA methylation and transcription levels was observed. Many genes that are essential for the differentiation and function of T cells were among these, explaining possible causes of the age-related decline in the immune response.

In brain samples from variously aged human donors, 589 CpGs showed age-dependent methylation in one division, 167 in two, 86 in three, and only 10 in all four divisions studied [26]. Of all aCpGs found, approximately 82% were located within CGIs, 11% were not within CGIs, and 7% were in regions that could not be unequivocally defined as CGIs or non-CGIs. All 10 aCpGs that showed significant correlations with age in four brain divisions were located within CGIs, and their methylation levels were increased with age. Four of these sites were identified previously as aCpGs in other tissues [21, 23]. A positive correlation between methylation level and age was observed for the majority (>95%) of aCpGs. Of the aCpGs located within CGIs, 98% were hypermethylated at advanced ages. Compared with other tissues, the brain contains mainly hyper-aCpGs and most of these are located in the promoter-associated CGIs of genes that encode transcription factors. Apparently, the age-dependent hypermethylation of specific genes could be a part of the epigenetic program affecting genome expression in aging cells, whereas the age-dependent loss of DNA methylation seems to be a manifestation of stochastic epigenetic drift. Indeed, intergenic regions and CpG poor promoters were found to be more likely to undergo changes in methylation during early life in MZ twin pairs, whereas the methylation of CGIs and CpG-rich promoters was most stable [27]. Since environmental and lifestyle differences between MZ twins are mostly absent at these ages, the divergence of their DNA methylation patterns should be a result of stochastic methylation errors.

The sites located within CGIs were found to be predominant among hyper-aCpGs in various human organs (brain, blood, kidney, and skeletal muscle), whereas hypo-aCpGs were most characteristic beyond CGIs, and their methylation levels were more variable between tissues [28]. Hence, age-related variations in methylation common between different tissues are mainly observed in CGIs and represented by increases of methylation levels. On the other hand, tissue-specific variations in methylation are more characteristic of CpGs located beyond CGIs and represented by decreased methylation levels. Among the genes hypermethylated with age in various tissues, most are functionally connected with the regulation of transcription and control of morphogenesis. Skeletal muscle showed the lowest overlap in total aCpGs with other tissues and had the strongest association of aCpGs with tissue-specific gene expression. In a study on human skeletal muscle approximately 6000 CpGs were found to be differentially methylated between the younger (18–27 years) and older (68–89 years age) male adults [29]. Of these aCpGs, 92% (5518) were hyper-aCpGs, while the remaining 8% were hypo-aCpGs. Interestingly, in aging human skeletal muscle, a strong preference was found for the aCpGs to be located within gene bodies, while no preference was observed for promoter regions.

In epidermal samples of variously aged humans, only 58,995 CpGs of those covered by the Illumina Human Methylation450 BeadChip showed a statistically significant change in methylation [30]. The large majority of these aCpGs showed only minor (<0.1) quantitative methylation differences. Of different genomic substructures (CGIs, shores, shelves, and open sea regions), only CGIs showed robust hypermethylation in older samples. Of the 2000 most variably methylated aCpGs, about half showed the consistent age-related difference. Interestingly, a substantial

fraction of age-related methylation changes accumulated during narrowly defined time windows. Step-wise hypomethylation of 256 CpGs occurred between 30 and 50 years, whereas step-wise hypermethylation of 768 CpGs occurred in two pronounced peaks around 40 and 55 years. Within-sample methylation variance for all aCpGs was higher in younger samples, indicating that the dynamic range of skin DNA methylation becomes reduced with age. The methylation patterns appeared highly homogeneous among the younger epidermis samples, but correlation coefficients were markedly reduced between older samples. Similar correlation analysis for specific gene networks showed a distinct age-related loss of correlated gene expression. Skin aging appeared to be accompanied by a reduced fidelity of epigenetic regulation. Age-related erosion of DNA methylation patterns manifested itself in two distinct features. The first one is that sharply demarcated regions of almost complete and almost absent methylation of young methylomes appeared to be less clearly defined in old methylomes, which is reflected by the significantly reduced variance and spatial correlation within methylomes. The second one is that inter-individual similarity between young methylomes was essentially lost in older methylomes. Thus, DNA methylation patterns become more homogeneous with age within individuals but more heterogeneous between them. These findings define important features of the epigenetic drift associated with aging. The age-related erosion of methylation patterns is accompanied by a reduced fine-tuning of the transcriptional circuitry.

A comparative study of DNA methylation changes in blood cells of mice, rhesus monkeys, and humans showed that similar epigenetic changes occur in all three species [31]. Age-related gains of methylation were most pronounced in CGI sites that were unmethylated in young individuals, whereas age-related hypomethylation occurred at highly methylated non-CGI sites. Ingenuity® Pathway Analysis (IPA®) of hypermethylated genes showed enrichment for developmental processes, cancer and cardiovascular disease, cell development, signaling, growth, and maintenance, in all three species. For 328 human genes that changed expression by more than two-fold upon aging, for which promoter methylation data were available, a significant negative correlation between changes in methylation and gene expression was observed. Genes showing gains of expression with age were mostly hypomethylated, whereas those with reduced expression were often hypermethylated. When the rates of epigenetic changes were compared between three species, a negative correlation with respective lifespans was observed, at approximately 5% per year in mice, 0.5% per year in monkeys and 0.1% per year in humans. The effects of calorie restriction (CR) on methylation were analyzed in 2.7–3.2-year-old mice exposed to 40% CR starting at 0.3-years, and in rhesus monkeys exposed to 30% CR starting in middle age (7–14 years) and analyzed at 22–30 years (CR treatment period – 15–21 years). CR-exposed rhesus monkeys showed attenuation of the age-related methylation changes compared to *ad libitum* (AL)-fed controls by approximately 7 years. Even stronger effects were seen in CR-mice that had a 0.8-year DNA methylation age at a 2.8-year chronological age. The effects of CR in mice were detectable in different tissues, although significant variability in the extent of the age-related changes and CR impact was observed.

No changes of global DNA methylation level were observed with age in mouse hippocampus in either CpG or CpH contexts [32]. Age-related changes in methylation at individual CpGs (aCpGs) were equally distributed between hypermethylation and hypomethylation events, the majority (>90%) of these being different between males and females. Age-related differentially methylated CpHs (aCpHs) were observed in males and females, with a greater number of hypermethylated sites. Most of these were also sex-specific. Unlike in other organs, enrichment of aCpGs outside of CGI units in intergenic regions and introns, was evident in hippocampus, whereas CGIs and promoter regions were under-represented among hypermethylation events. Similar enrichment patterns were found for aCpHs.

A small increase of global methylation with age was found in a recent whole-genome bisulfite sequencing (WGBS) study of DNA methylation in mouse liver [33]. Out of 3176 aCpGs found, 1945 gained and 1231 lost methylation. Mapping of these aCpGs relative to gene bodies showed 241 hypomethylated and 275 hypermethylated genes. Several hyper-aCpGs were found in the body of the *Elovl2* gene, known to be hypermethylated with aging in humans and mice. Age-related hypermethylated genes showed functional enrichment for the regulation of transcription, circadian rhythm, regulation of ERK and Ras signaling, and response to growth factor signaling, whereas hypomethylated genes were enriched for regulation of the MAPK cascade, xenobiotic, amino acid, and lipid metabolism. In CR mice, only 2250 aCpGs were found, with 1512 hypermethylated and 738 hypomethylated. Globally, an amelioration of aCpGs by CR was observed. Of 571 aCpGs significantly ameliorated by CR, 439 were hypo-aCpGs and 132 hyper-aCpGs. CR-ameliorated aCpGs were enriched in genes involved in the regulation of transcription and ketone, acetyl-CoA, and long-chain fatty acid metabolism. Notably, aCpGs, especially hypo-aCpGs, were strongly enriched in open chromatin domains (containing histone marks H3K9ac, H3K4me1 and H3K27ac). Hypo-aCpGs were strongly enriched in distal and intragenic active enhancers, active promoters, and a wide range of transcription factor binding sites. Thus, open chromatin domains appear to be most prone to methylation changes, especially methylation loss. Hyper-aCpGs were found to be significantly enriched in bivalent chromatin domain CGIs containing both activating H3K4me3 and repressive H3K27me3 marks. Bivalent CGIs were also enriched for CR-ameliorated aCpGs. Hyper-aCpGs were also enriched in repressed H3K27me3- domains, whereas no enrichment was evident for CR-ameliorated aCpGs. Globally, the observed changes in DNA methylation with aging were not associated with concomitant changes in the expression of differently methylated genes. In old CR mice, 56 genes showed an inverse relationship between gene expression and DNA methylation. These genes code for several key enzymes of hepatic lipid metabolism and are direct targets of the transcription factors Srebf1 and ChREBP, two well-known regulators of hepatic lipogenesis. The *Srebf1* gene itself was hypermethylated and downregulated upon CR treatment. Hence, long-term CR-induced changes of DNA methylation and gene expression are specifically associated with hepatic lipid homeostasis.

Genome-wide age-related changes in DNA methylation in blood cells of variously aged mice (between 3-month-old “young adults” and 35-month-old

10%-survival adults) were studied by reduced representation bisulfite sequencing (RRBS) [34]. A slight, but significant decrease of global methylation with age was found. Out of approximately 800,000 CpGs studied, 21.2% were found to be correlated with age, including 10.2% that gained methylation and 11% that lost it. Of these aCpGs, 86% gained (41.6%) or lost (44.3%) methylation predominantly in late life, and 14% changed methylation during early adulthood. Sites with increasing methylation were characterized by the low methylation status, and those with decreasing methylation by high methylation, compared with the average methylation levels. A strong tendency to gain methylation with age was observed in CGIs, 5′-UTRs, first exons, and promoters. Introns and 3′-UTRs tended to lose methylation. Regulatory elements, such as miRNA and lncRNA targets, DNase-hypersensitive sites, and transcription factor binding sites tended to gain methylation. Increasing methylation in evolutionarily conserved regions and decreasing methylation in intergenic and repetitive regions (retrotransposons) were also observed. The gene pathway analysis showed 102 pathways to be associated with loss of promoter methylation and 1162 pathways associated with its gain. Among the former ones were pathways related to DNA repair, immunity, and inflammation, whereas the latter showed pathways related to development regulation. In addition, pathways were significantly enriched related to aging and prolongevity interventions, such as the response to IGF and TGFβ, MAPK, WNT and Notch signaling, stem cell regulation, estradiol response, fatty acid metabolism, and transcriptional regulation. The DNA methylation pathway was also in that group, including the *DNMT1* gene. CR starting at the age of 4 months led to a rapid shift in methylation of 14,516 sites, and most of these were in the same direction as during aging. Long-term effects of CR were essentially opposite, slowing down the influence of aging on the methylome.

When the rates of DNA methylation changes with aging were studied in mammalian species with highly variable maximal lifespans, an inverse correlation was observed [35]. This relationship was observed not only between extremely different in longevity species, such as mouse and human, but also for species that have more similar lifespans, such as dog, naked mole rat, and rhesus macaque. Moreover, this relationship held when two different breeds of dogs were compared, namely the miniature long-haired dachshund (average lifespan 12–15 years) and flat-coated retriever (average lifespan 8–10 years). In a transchromosomic mouse strain that harbors a freely segregating human chromosome 21, the rate of change of methylation with age for the same 15 aCpGs in mouse was about 21-fold faster than in humans. Thus, aCpGs on a chromosome from a human showed greatly accelerated methylation dynamics in mouse, indicating that such aCpGs could serve as sensors of the aging rate.

The human lifespan is widely believed to be genetically determined by 20–30% [36]. In the general human population, the searches for gene loci affecting longevity have been essentially unsuccessful. However, analysis of sibling pairs surviving to beyond 100 years has shown that the genetic influence upon survival increases with older and older ages, particularly beyond the one percentile of survival [37]. Moreover, in a genome-wide association study of extreme human longevity comprising 801 centenarians with a median age of 104 years and 914 genetically

matched controls, 281 independent single nucleotide polymorphisms (SNPs) associated with extreme human longevity were discovered [37]. The possible significance of DNA methylation as a factor controlling lifespan in humans was studied in leukocytes from female centenarians, as well as their daughters of approximately 70-years old selected from pairs, where the father was also long-lived (>77 years), females of approximately 70 years old, whose parents were both non-long-lived, and a control group of young (17–34 years) women [38]. The clinical histories showed that in general, the centenarians' daughters have a much better health status than daughters of non-long-lived parents. Evidently, the probability of becoming long-lived is inheritable to a considerable degree. Global DNA methylation levels were significantly decreased in all three aged groups compared with the control group but to different extents. Maximum hypomethylation was observed in daughters of non-long-lived parents, minimal in centenarians' daughters, and intermediate in centenarians themselves. About 700 CpGs located in approximately 600 genes were hypermethylated in all three aged groups – to similar extents in both daughter groups, and to a greater extent in centenarians. This set of hypermethylated loci was enriched for genes functionally related to organ development, cell differentiation, and transcriptional regulation. A total of 330 CpGs located in 326 genes were hypomethylated in aged groups – to similar extents in both daughter groups and to a greater extent in centenarians. This set of hypomethylated loci was enriched for genes involved in defense responses, acute inflammation, and signal transduction. Approximately 150 CpGs located in 124 genes were significantly hypermethylated in daughters of centenarians compared with daughters of non-long-lived parents. This set was enriched for genes functionally related to nucleotide metabolism, nucleic acids synthesis, and cellular signaling. On the other hand, 67 CpGs located in 65 genes were significantly hypomethylated in the centenarians' daughters compared with daughters of non-long-lived parents. This set was enriched for genes related to downstream processes of the signal transmission. The most strongly pronounced differences in methylation levels between the two daughter groups were found for twelve CpGs (ten hypermethylated and two hypomethylated in centenarians' daughters) located in nine genes. Six of the hypermethylated genes (*SLC38A4*, *SLC22A18*, *MGC3207*, *ECRG4*, *ATP13A4*, and *AGPAT2*) are involved in metabolic processes, one hypermethylated (*DUSP22*) is a tumor-suppressor gene, and another hypermethylated gene (*ZNF169*) encodes a zinc finger DNA binding protein with unknown function. The function of the only hypomethylated gene (*FLJ32569*) is also unknown. Obviously, genome methylation in the daughters of centenarians is more stable compared with daughters of non-long-lived parents. Thus, epigenome stability and a more robust epigenetic control for nucleotide metabolism, nucleic acids synthesis, and signal transduction may contribute to increasing lifespan and healthy aging in centenarians.

Interestingly, the longevity-promoting mutation Ames dwarf $Prop1^{df/df}$ in mice has been found to increase the stability of the liver DNA methylome through chronological aging [39]. Significantly more age-related differentially methylated regions (aDMRs) were found in the wild-type mice compared with $Prop1^{df/df}$ mice. At shared hyper-aDMRs, the magnitude of the methylation increase with age was about the

same in wild type (WT) and dwarf mice. At hypo-aDMRs restricted to WT mice, young and old dwarf mice showed methylation levels comparable to young WT mice, while at the hypo-aDMRs restricted to Ames mice, young and old WT mice showed methylation levels comparable to old mutant mice. At shared hypo-aDMRs, dwarf mice showed consistently higher methylation than WT in both age groups, while the magnitude of the change with age was about the same. In dwarf mice, hypo-aDMRs were biased towards a higher methylation level. At hypo-aDMRs shared between WT and dwarf mice, the latter ones exhibited a higher initial level of methylation in young animals, thus potentially buffering them against the effects of age-associated hypomethylation. When hypo-aDMRs were compared between ad libitum (AL)-fed, CR and rapamycin-treated young and old mice, the methylation loss with chronological aging was suppressed by CR and, to a lesser extent, by rapamycin. At hyper-aDMRs, the methylation gain was also suppressed by CR. The protective effect of rapamycin was not significant in the whole population of hyper-aDMRs but was detectable in some individual aDMRs.

4 DNA Methylation as an Age Predictor

The aging rates are non-equal in different persons. Women are known to have a longer average lifespan compared with men. Aging can be accelerated by unhealthy life habits, such as smoking, or slowed down by the good ones, such as physical training. Molecular markers of biological, rather than chronological age, are needed to evaluate more precisely the degree of age-dependent deterioration in physical welfare. The age-related variance of DNA methylation seems to be a good contender for this role. Unfortunately, the age-related DNA methylation changes are masked by methylation variations caused by other factors or stochastic errors. In saliva samples of MZ twin pairs, 88 CpGs were found to have methylation levels significantly correlated with age, with 69 of these being positively correlated and 19 negatively correlated [40]. Most (83%) of these aCpGs were located within promoter CGIs. Three genes that showed a most clear correlation with age and had the widest range of methylation values, *Edaradd*, *NPTX2* and *Tom1L1*, showed a clear correlation with age in men, but only two (*Edaradd* and *Tom1L1*) were correlated in women. The methylation levels of *Edaradd* and *Tom1L1* were linearly decreased with age, whereas the methylation level of *NPTX2* was increased. Based on the methylation levels of just two CpGs (*Edaradd* and *NPTX2*), the age of the test subjects could be predicted with a 5–6 year accuracy, whereas the addition of one more site (located in *ELN* gene) reduced the mean absolute error (MAE) to ~3.5 years. No epigenetic drift was detected in the promoter regions for the CpGs studied.

Comparative studies of age-related methylation patterns in various tissues showed these patterns to be highly tissue-specific. Nevertheless, some loci have methylation levels significantly correlated with age in various tissues. These common methylated loci could be of the highest relevance to the internal mechanisms of aging, and their methylation status could be used as an epigenetic signature to

estimate the biological age. The first non-cell-type-dependent epigenetic aging signature was deciphered based on the DNA methylation datasets from several independent studies that used the Illumina HumanMethylation27 BeadChip platform [41]. Of more than 450 aCpG sites found, most were hypermethylated with age and only 25 hypomethylated. This is in accord with the view noted above that hypermethylation is a dominant trend of specific methylation changes upon aging, whereas hypomethylation is less stringently regulated. The most accurate age predictions were obtained when a set of four hyper-aCpGs (*TRIM58*, *KCNQ1DN*, *NPTX2* and *GRIA2*) were used. To further enhance the prediction accuracy, a hypo-aCpG (*BIRC4BP*) was added to the set. When all five loci were used, the average prediction accuracy across all datasets was ±12.7 years, whereas the use of only three of the most reliable ones (*NPTX2*, *GRIA2*, *KCNQ1DN*) enhanced the accuracy to ±11.4 years. It should be noted that in this case the age prediction applied to various tissues and was gender-independent, whereas in the study described above [40] the prediction was based only on saliva samples. When the blood samples were investigated, the set of aCpGs with high predictive capability could be narrowed down to just three (*ITGA2B*, *ASPA* and *PDE4C*), and the accuracy of age prediction was ±4.5 years [42]. Similar accuracy was obtained with blood samples using another set of five aCpGs (*ELOVL2*, *C1orf132*, *TRIM59*, *KLF14* and *FHL2*) [43]. These same CpGs appeared to be informative to predict age in blood, saliva and buccal swab samples simultaneously with high accuracy (~4 years) [44]. Even the use of two of the most informative aCpGs located in *ELOVL2* enabled age prediction in blood cells with reasonable accuracy (MAE = 7 years) [45]. For reasons that are still unknown, the methylation of a few CpG sites in *ELOVL2* appeared to be the most robust single-gene predictor of age [46]. In a study of methylation in whole blood, DNA samples obtained from variously aged (9–99 years) subjects and cord blood samples, the highest correlation (0.92) with age was observed for CpG sites in the promoter CGI of the *ELOVL2* gene, followed by promoter CGIs of *FHL2* (0.80) and *PENK* (0.63). *ELOVL2* showed a progressive increase in methylation beginning from the first stages of life and displayed the widest methylation range – from 7% to 91%. Respective ranges for *FHL2* and *PENK* were 12–53% and 1–27%. *ELOVL2* encodes an enzyme involved in the synthesis of long ω3 and ω6 polyunsaturated fatty acids (PUFAs). Taking into account the important role that PUFAs play in the regulation of metabolism, inflammation and membrane integrity, it would be interesting to elucidate the possible role of *ELOVL2* expression in aging.

In a simple model that used a quadratic regression for *ELOVL2* methylation and linear regressions for methylation of *ASPA*, *PDE4C* and *EDARADD*, highly accurate age predictions for blood (MAE = 3.75 years) and tooth (MAE = 4.86 years) samples were obtained [47]. An epigenetic clock with reasonable accuracy (MAE~5 years) was built for human semen samples using linear regression of methylation levels of just three CpGs [48].

In a large-scale investigation using the Illumina HumanMethylation450 BeadChip platform, methylation levels of 485,577 CpG sites were analyzed in blood DNA from more than 650 volunteers of 19- to 101-years old [49]. An age prediction model (“Hannum clock”) was built using a set of 71 aCpGs. The mean

error of age prediction by this model was ±3.9 years (96% correlation between the chronological age and the predicted age). Nearly all markers in the model were located within or near genes with known functions in age-related conditions, such as Alzheimer's disease, cancer, tissue degradation, DNA damage and oxidative stress. The model was capable not only of predicting the age but also of revealing the factors that affect the personal rate of aging. For example, gender was found to affect age significantly, with epigenetic "aging" in men being approximately 4% faster than in women. Body mass index (BMI) was found not to affect the aging rate of blood, adipose and muscle cells, whereas age acceleration by approximately 2.2 years per each additional 10 BMI units was observed in the liver [49, 50]. When the model was used to estimate the age of tumors, these appeared to be around 40% more aged than respective normal cells of the same person. The age prediction model worked with DNA samples from other organs (breast, lung, kidney, and skin) with the same accuracy as with blood samples, when a linear offset specific for each organ was used. When the epigenetic predictive models were constructed using the same algorithm but based on the age-related methylation data from other organs (breast, lung and kidney), the main difference was in the sets of most informative CpG sites chosen. Only two CpGs near *ELOVL2* appeared to be common. Not only the methylation levels of aCpGs were changing with age, but also the variation limits of these methylation levels between different persons became larger for most sites. For any specific person, the extent of deviation of these values from the population averages appeared to be a fairly accurate measure of the individual aging rate.

An "ultimate" biological age predictor ("Horvath clock") was built based on all publicly available data concerning age-related variations of DNA methylation in various tissues and cell lines (nearly 8000 samples, 51 tissues and cell types) [51]. A total of 353 methylation sites were chosen by elastic net regression analyses that allowed the most reliable age prediction for various tissues and cells (96% correlation to chronological age; MAE = 3.6 years). Blood cells that have different life spans, CD14+ monocytes (myeloid lineage) living several weeks at the most, and CD4+ T cells (lymphoid lineage) living for months to years, were shown to have identical epigenetic ages in blood samples from healthy male subjects. Hence, epigenetic age reflects some internal methylome features, related to the chronological age of the person, not just the age-dependent peculiarities of the respective blood cells. The mean epigenetic age was highly correlated with the chronological age in most tissues. The variations in epigenetic age between different tissues of the same person were small. The notable exceptions were breast tissue in women (epigenetically older compared with other tissues) and sperm in men (epigenetically younger compared with other tissues). Surprisingly, though all brain regions of the same person have similar epigenetic ages in subjects younger than 80 years, the cerebellum and to a lesser extent the occipital cortex exhibit progressively negative epigenetic age acceleration in the older persons, i.e., these brain regions are younger than others [52]. This relative resistance of cerebellum to aging correlates with overexpression of 1239 genes compared with other brain regions, two RNA helicase superfamilies (SF1 and SF2) being the most enriched among these genes.

As could be expected the epigenetic age of embryonic stem cells (ESCs) is close to zero [51]. Interestingly, induced pluripotent stem cells (iPSCs) do not differ from ESCs by their epigenetic age. Therefore, epigenetic age is reset when iPSCs are produced from differentiated somatic cells. When cells are maintained in culture, ESCs and iPSCs included, their epigenetic age increases with each passage. However, the epigenetic age is clearly not a reflection of the mitotic age, since it tracks chronological age in tissues that are widely different in proliferative potential, including post-mitotic neurons. It is also not related to cell senescence since its correlation with chronological age is observed in immortal cell lines, such as ESCs.

For reasons that are still unclear, the pan-tissue epigenetic clock appeared not to be a good predictor for the age of skin fibroblasts. Moreover, no aging acceleration was found by this clock in fibroblast lines derived from the skin of patients with Hutchinson Gilford Progeria Syndrome (HGPS) – a disease with obvious manifestations of accelerated aging. By analyzing DNA methylation data sets obtained from human fibroblasts, keratinocytes, buccal cells, endothelial cells, blood and saliva, a new version of the epigenetic clock, referred to as the "skin & blood clock," was built [53]. A total of 391 CpGs were selected, of which 45 were shared with the blood-based Hannum clock and 60 CpGs – with the pan-tissue Horvath clock. This new clock outperformed the pan-tissue clock in fibroblasts, microvascular endothelial cells, buccal epithelial cells and keratinocytes. It also showed that fibroblasts from HGPS cases exhibit accelerated epigenetic aging. In blood samples, it appeared to be more accurate than both "classic" Horvath and Hannum clocks. It also correctly estimated gestational age when applied to cord blood samples. Regarding other cell types and tissues, including sorted neurons and glial cells, brain samples and liver samples, the new clock performed similarly to classic Horvath clock. The new clock correctly estimated the age of neonatal fibroblasts to be close to zero, whereas the pan-tissue clock estimated their age to be >10 years. The proliferation of human fibroblasts or keratinocytes in culture led to a continuous increase in epigenetic age measured by both new and classic clocks but the accuracy of the new clock was clearly better.

While the popular epigenetic clocks by Hannum and Horvath produce fairly good estimates of chronological age, their predictive power of aging acceleration associated with many age-related diseases is limited. This feature is probably a natural consequence of their having been built based on chronological age as the reference for selection of the most relevant CpGs. By definition, CpGs with methylations that do not display strict correlations with chronological age were excluded. On the other hand, the most relevant methylation sites to predict biological age could be those that signal the departure of biological age from chronological age and thus account for differences of health and physiological status between individuals of similar chronological ages. Such differences could be quantitatively described by "phenotypic age" derived from various clinical biomarkers that strongly predict relative risks of mortality, physical and cognitive disability, and visual manifestations of aging between same-aged individuals. Along these lines, a mathematical model of the phenotypic age calculation was elaborated based on nine clinical variables and chronological age [54]. When validated on a 12 years

follow-up cohort of more than 6200 individuals, phenotypic age was highly correlated with chronological age (r = 0.94). A 1 year increase in phenotypic age compared with chronological age was associated with a 9% increase in the risk of all-cause mortality and mortality from aging-related diseases, a 10% increase in the risk of mortality from cardiovascular disease, a 7% increase in the risk of mortality from cancer, and a 20% increase in the risk of mortality from diabetes. Furthermore, phenotypic age was highly correlated with comorbidity count and physical function measures. Thus, a more powerful epigenetic biomarker of aging could be developed by replacing the prediction of chronological age with the prediction of phenotypic age. A "PhenoAge" epigenetic clock has been constructed by selecting 513 CpGs whose methylation in blood samples appeared to be most predictive of phenotypic age [54]. Of these 513 CpGs, 41 were common with the classic Horvath clock, 6 were common with the Hannum clock and 5 with both clocks. The strongest correlation to age was observed for149 CpGs located in CGIs. Among genes that tended to be over-expressed in epigenetically older individuals, functional enrichment was observed for pro-inflammatory, response to nutrients, and growth hormone signaling pathways, multicellular organism growth, and regulation of DNA methylation. Among genes that were down-regulated in epigenetically older persons, enrichment was observed for processes involving the transcriptional and translational machinery, and damage recognition and repair. Epigenetic PhenoAge was highly correlated with phenotypic age and significantly associated with mortality risks when tested in five independent large human cohorts. A 1 year increase in epigenetic PhenoAge was associated with a 4.5% increase in the risk of all-cause mortality. In persons representing the top 5% (fastest agers) the risk of mortality was around 1.6 times higher than in the average person of similar chronologic age and approximately 2.6 times higher than in the bottom 5% (slowest agers). Irrespective of chronological age, higher epigenetic PhenoAge was associated with an increased number of coexisting morbidities, decreased likelihood of being disease-free, increased physical disabilities and increased coronary heart disease. It was significantly different between those who had never smoked, current smokers, and former smokers. Epigenetic PhenoAge appeared to differ significantly between racial groups, non-Hispanic blacks having the highest PhenoAge, and non-Hispanic whites the lowest. Individuals with higher education and income appeared younger. Increased exercise and fruit and vegetable consumption (rich in carotenoids) were associated with lower PhenoAge. PhenoAge acceleration was positively correlated with the circulating levels of C-reactive protein (CRP), insulin, glucose and triglycerides, and with the waist-to-hip ratio. Thus, the epigenetic PhenoAge clock appears to capture organismal functional state, beyond the that explained by chronological age, and accurately predicts morbidity and mortality risks.

Another epigenetic clock, DNAm "GrimAge," was built using 1030 unique CpGs to predict with maximal accuracy the negative outcomes of smoking and a selection of plasma proteins that have previously been associated with mortality or morbidity (adrenomedullin, CRP, PAI1, and GDF15) [55]. Among the CpGs selected, significant enrichment was found for 28 functional pathways, including MHC class II receptor activity, cytokine-mediated signaling pathway, response to

IFNγ, regulation of protein sumoylation, endoderm formation, epigenetic regulation of gene expression and fatty acid transmembrane transport. A novel measure of epigenetic age acceleration, AgeAccelGrim, was defined as the difference between the DNAm GrimAge and chronological age. A positive value of AgeAccelGrim indicates that the DNAm GrimAge is higher than chronological age. In a 13.7 years follow-up study assessing time-to-death due to all-cause mortality, DNAm GrimAge was superior relative to all other epigenetic clocks in lifespan prediction. Previous epigenetic clocks showed a negative correlation between epigenetic aging acceleration and plasma biomarkers of vegetable consumption, but AgeAccelGrim was more accurate in this aspect. The self-reported proportion of carbohydrate consumption was associated with lower AgeAccelGrim, whereas an increased proportion of fat intake was associated with increased AgeAccelGrim. Triglyceride, insulin, glucose and plasma CRP levels were positively correlated with AgeAccelGrim, whereas HDL cholesterol levels were negatively correlated. BMI and waist-to-hip ratio were associated with increased AgeAccelGrim, whereas higher education and income were associated with lower AgeAccelGrim. AgeAccelGrim appeared to be superior in detecting the beneficial effects of physical exercise. Polyunsaturated omega-3 fatty acid intake was negatively correlated with AgeAccelGrim, the effect being more pronounced in males than in females.

A special version of the epigenetic clock was devised to predict the gestational age (GA) at birth based on DNA methylation in umbilical cord blood samples [56]. Of 148 aCpGs chosen by elastic net regression, only six were in common with those in classic Horvath clock. The accuracy of GA prediction by the clock was within 1.5 weeks (MAE = 1.24 weeks), which is about the same as errors of clinical estimates of GA based on ultrasound investigation.

Since "classic" versions of the epigenetic clock showed age-related DNA methylation to change more rapidly during childhood and adolescence, a special version of the clock "fine-tuned" to predict the age of children and adolescents with maximal precision was built [57]. Of 6350 aCpGs from the epigenome-wide association analysis, 116 of the previously known age-related sites were confirmed in children, but 83 novel aCpGs were selected as the best predictors. These sites collectively predict the chronological age in children and adolescents with high accuracy (correlation = 0.93, MAE = 0.62 years). This higher accuracy compared with "adult" clocks is most likely a consequence of rapid changes of aCpG methylation levels during childhood and adolescence noted above. Besides, children and adolescents have less confounding factors with regards to lifestyle.

As noted above, the age of sperm cells cannot be predicted accurately with the classic multi-tissue epigenetic clock [51]. It has been shown that sperm cells have a distinct pattern of age-associated changes in DNA methylation [58]. A longitudinal study of sperm DNA methylation in fertile donors at time points 9–19 years apart showed 139 aCpGs that lose methylation and only 8 aCpGs that gain methylation with age. The average methylation levels of these aCpGs were used to construct a linear regression model of age prediction [59]. Testing the model on several independent sperm samples showed a high accuracy for age prediction, with an average

standard deviation of only 0.877 years. When sperm ages were compared between individuals who had never smoked, smokers and long-term (>10 years) smokers of similar chronological ages, an increase of around 1.5% was observed in all smokers and an increase of 2.5% in long-term smokers compared with those who had never smoked. Thus, the model is capable not only of correctly predicting age based on sperm DNA methylation but also in predicting the aging-acceleration effects of smoking.

The epigenetic age could serve as a convenient marker in the assessment of the rejuvenation treatments efficiency. A comparative analysis of DNA methylation levels of peripheral blood mononuclear cells between semi-supercentenarians (mean age: 105.6 years), their offspring (mean age: 71.8 years), and age-matched controls (meanage: 69.8 years) showed that the offspring of semi-supercentenarians have a lower epigenetic age than age-matched controls (epigenetic age difference = 5.1 years), and that the epigenetic age of centenarians is approximately 8.6 years lower than their chronological ages [60]. The aging rates of different tissues of the same person can be used to identify those with diseases, such as cancer. Even more, the increased epigenetic aging in blood cells appeared to be highly predictive of future cancer [61–64]. An interesting example of the application of the epigenetic age concept was an estimation of the mortality risk [65]. It was found that a 5-year acceleration of epigenetic aging versus chronological aging results in a 16% increase of mortality risk, irrespective of general health, lifestyle and genetic factors. Similar results were obtained in another study which found that a 5-year higher epigenetic age compared with chronological age was associated with a 15–20% increase of all-cause, cancer and cardiovascular disease mortalities [66]. Generally, the epigenetic age appears to be a more robust predictor of biological aging and all-cause mortality compared with chronological age [67]. HIV infection, both chronic and recent, was shown to increase the epigenetic age by about 5 years in blood cells [68, 69] and by 7.4 years in brain tissue [68]. Down syndrome is known to be associated with segmental manifestations of accelerated aging, such as declining immune function and precocious Alzheimer's disease. It was found that Down syndrome subjects exhibit a significant epigenetic age acceleration (by 6.6 years on average) in blood and brain tissue but not in the buccal epithelium [70]. In addition, a strong correlation between epigenetic age acceleration in brain tissue and features of Alzheimer's disease was observed [71]. A significant epigenetic aging acceleration was also observed in blood samples of Parkinson's disease patients [72] and senior women with insomnia [73]. Memory decline, especially episodic memory, is strongly associated with aging. A lower epigenetic age was observed both at baseline and after a 15-year follow-up in subjects with maintained memory functions compared to those with memory decline [74]. Moreover, epigenetic age at follow-up was significantly predictive for the dementia occurrence, whereas chronological age was not. Excessive and chronic stress is widely believed to be associated with accelerated aging and increased occurrence of aging-related pathologies, such as cardiovascular disease, immune dysfunction and neuropsychiatric disorders. In a highly traumatized cohort of African Americans aged 18–77 years, cumulative life-time stress, but not childhood trauma or current stress

alone, was found to be associated with accelerated epigenetic aging [75]. Since glucocorticoids are primary molecular mediators of the stress responses, the authors further studied their possible role in epigenetic aging acceleration. Of the 353 classic Horvath clock CpGs, 110 showed significant methylation changes 3 h after oral exposure to dexamethasone, with 98 of these decreased and 12 increased. Werner syndrome is one of the most extensively studied progeroid syndromes with multiple features of accelerated aging in early adulthood. As expected, a study showed that was also associated with significant acceleration of epigenetic aging by both Hannum and Horvath clocks [76].

Diets rich in whole grain and dietary fibers, fish and omega-3 fatty acids, fruits and vegetables, as well as increased physical activity, moderate alcohol consumption, and higher educational attainment have all been linked to good health and longevity. The effects of these lifestyle factors on the rate of epigenetic aging in blood cells were studied using both the Horvath (internal epigenetic aging) and Hannum (external epigenetic aging) clocks [77]. Small but statistically significant acceleration of epigenetic aging by the Hannum clock was correlated with plasma levels of triglycerides, CRP, insulin, glucose and γ-tocopherol, as well as with systolic blood pressure, waist-to-hip ratio and BMI. An inverse correlation was observed between Hannum age acceleration and fish intake, alcohol consumption, plasma levels of carotenoids, such as α- and β-carotenes, lutein and zeaxanthin, and β-cryptoxanthin, and with education, income and exercise. The Horvath epigenetic aging rate showed weaker correlations with dietary and lifestyle factors.

A meta-analysis of blood DNA methylation data from 16,245 individuals from 18 cohort studies showed an association of education, smoking, obesity, alcohol intake and physical activity with the aging rate using the number of stochastic epimutations (SEMs) and three versions of the epigenetic clock [78]. The total number of SEMs takes into account whole-genome epigenetic deregulation during aging (epigenetic drift), whereas epigenetic clocks serve as markers of biological age. The level of education was significantly associated with the four DNA methylation markers investigated. Individuals with a lower education level had a higher number of SEMs, and higher epigenetic aging acceleration as assessed by the Horvath, Hannum, and PhenoAge clocks. The observed associations were still significant after the adjustment for smoking, BMI, alcohol and physical activity, but the estimated effects were moderately reduced. Current smokers had higher numbers of SEMs and higher Hannum and PhenoAge epigenetic aging acceleration compared with those who had never smoked. Former smokers had intermediate outcomes. The estimated effect size of the association between smoking and epigenetic aging biomarkers was comparable to those observed for education, except for the magnitude of the association with PhenoAge, which was significantly higher. A similar pattern of associations was observed for the effects of obesity. Obese individuals (BMI $\geq$ 30) had higher Horvath, Hannum, and PhenoAge epigenetic aging acceleration. The estimated effects of obesity were comparable to those of education, except in the case of PhenoAge, which was significantly higher. No significant difference was found between alcohol abstainers and occasional drinkers, but habitual drinkers had higher Horvath, Hannum and PhenoAge epigenetic aging acceleration. As observed

for the other risk factors, the higher estimated effects were observed by the PhenoAge clock. A negative effect of low physical activity was detectable with Horvath clock only. No significant differences in associations of each risk factor with epigenetic aging were detected between men and women. A significant interaction with age was found for the association of SEMs with education, smoking and obesity, with stronger effects in older individuals.

The predictive accuracy of epigenetic clocks can be enhanced when less universal models are used. For example, for blood cells, this reached a value of 2.6 years when 17 aCpGs were used [79]. In a follow-up study of the same persons 8 years later, the predicted increases in methylation levels of hypermethylated sites and decreases in methylation levels of hypomethylated sites were observed. Recently, longitudinal dynamics of the epigenetic age estimates by Horvath clock has been studied using two Finnish follow-up groups – Vitality 90+ (age 90 years at baseline, 4-year follow-up) and Young Finns (age 15–24 years at baseline, 25-year follow-up) [80]. The most notable finding was that the epigenetic age acceleration was stable across the lifespan, its rate being set before adulthood. At the age of 15 years, the slope of epigenetic aging was already set at an individual level that remained essentially the same over the 25-year follow-up period. At older ages, the rate of epigenetic aging was also steady.

A genome wide analysis of DNA methylation in liver biopsies from 45 donors aged between 13 and 90 years using the Illumina HumanMethylation450 BeadChip platform, showed that 8823 CpGs were differentially methylated with age, and most of these (5772) were positively correlated with age [81]. These hyper-aCpGs were enriched in bivalent chromatin domains. Multidimensional scaling of 8823 aCpGs showed a linear correlation with chronological age up to 60 years, while a trend to a plateau was observed at older ages. The same trend was confirmed when epigenetic age of liver samples was estimated by the Horvath epigenetic clock. Thus, slower epigenetic aging of the liver occurs after 60 years of age. In search of compact regions containing multiple aCpGs, 75 differentially methylated regions (aDMRs) were identified that contained 687 aCpGs in 89 genes, all positively correlated with aging. Not unexpectedly, the top-ranking aDMR mapped to the CGI of the *ELOVL2* gene. The age-dependent DNA methylation signature identified appeared to be liver-specific, although for some aDMRs the age-association was similar between liver and several other tissues (for example, the CGI of *ELOVL2*). Of 687 aCpGs in the 75 aDMR signature, 80 aCpGs in 25 aDMRs were significantly associated with BMI. Lean subjects (BMI < 25) had lower DNA methylation levels compared with obese subjects of the same chronological age. Since all 75 aDMRs were hypermethylated with age, this result showed accelerated epigenetic aging in livers of obese subjects. Transcriptome-wide analysis of gene expression in the same liver samples showed 56 genes to be differentially expressed with age (aDEs). Of these, 47 were positively correlated with age. Unlike the methylation data, multidimensional scaling of the expression values of the 56 aDEs presented an almost linear association with chronological age between 13 and 90 years. Several functional gene sets were identified to be significantly enriched among positively correlated aDEs, namely those involved in allograft rejection, IFNγ response,

inflammatory response, epithelial-mesenchymal transition (EMT) and myogenesis, whereas genes with a negative correlation with age were enriched in metabolic processes. Of 56 aDEs, 11 genes that displayed age-associated changes in both methylation and expression were identified. Of these 11 genes, only three (*ZIC1*, *TSPYL5*, and *FZD2*) showed a significant correlation between methylation and expression levels.

To build an epigenetic clock model in the humpback whale, methylation of CpG sites in regulatory regions of eight genes, known to be differentially methylated with age in other mammals, was studied in skin DNA in a small population of whales with known ages [82]. Three sites were chosen that showed the strongest correlation with age, namely *TET2*_CpG + 31 (methylation decreases with age), *CDKN2A*_CpG + 297 and *GRIA2*_CpG + 202 (methylation increases with age) (positions relative to the start codon). The predictive accuracy of the clock appeared to be fair (MAE = 3.75 years). The precision of the PyroMark system used to measure methylation levels was estimated to be about 2.2 years. Thus, a substantial share of the clock error could be attributable to error in measurement of methylation levels. Hence, it would be higher if more accurate methods of the methylation quantitation are used.

An epigenetic clock model for canid species was built by RRBS analysis of genome wide DNA methylation patterns in blood DNA samples of variously aged domestic dogs and gray wolves [83]. Statistically significant conservation of aCpGs between dogs, wolves and humans was observed for around 9000 CpGs. After an initial pre-selection of sites with the absolute correlation between methylation and age above 0.3, an elastic net regression was used to build an age-predictive model. The correlation between predicted and actual ages was 0.8, and the MAE was 0.8 years. The average number of CpGs in the individual regression models was approximately 120.

Several epigenetic clocks have been constructed based on DNA methylation data of mice. Using RRBS DNA methylomes of mouse blood samples, a robust epigenetic clock (mDNAm clock) was defined by a weighted average of DNA methylation of 90 CpG sites [84]. The mean square difference between the epigenetic age and the chronological age was ~16 days. The CpG sites contributing to the mDNAm clock were distributed across the chromosomes and did not match the sites observed in human clocks. Interventions known to increase lifespan and delay age-dependent phenotypic changes in mice (CR, growth hormone receptor knockout, Snell dwarf mutation) showed consistent reductions of epigenetic age compared with chronological age. Several iPSC lines obtained from kidney and lung fibroblasts of 10-week-old mice were calculated to be less than 1 month old by the mDNAm clock, whereas the original fibroblasts were about 300 days old by the same mDNAm clock. Thus, the mDNAm clock measured the biological age rather than the chronological age.

Using 107 liver methylomes of variously aged mice, a subset of 148 aCpGs was selected as an epigenetic clock in mouse liver [85]. Analysis of methylomes from mice subjected to lifespan-extending conditions (*Prop1df/df* dwarf mutation, CR, rapamycin treatment) showed lower epigenetic ages compared with age-matched

controls. Since previous studies have shown that these interventions not only extend lifespan but also improve tissue and physical functioning with age, the slowing of the epigenetic clock in livers appears to reflect the slowing of biological aging.

A multi-tissue epigenetic clock was constructed based on RRBS DNA methylomes of liver, lung, muscle, spleen, heart and brain samples from newborn to 31–41-week-old mice [86]. Only a small share of the age-correlated methylation changes was common to all tissues, suggesting that changes in DNA methylation are primarily driven by tissue-specific processes. The final multi-tissue epigenetic clock was based on methylation levels of 329 aCpGs. The clock performed well across all tissues and ages, with a MAE of around 3 weeks that corresponded to less than 8.5% error relative to the oldest age analyzed. Similar to human clocks, the performance of mouse age-predictor was more accurate in younger animals.

Four versions of the new epigenetic clock were built using combined data on DNA methylation in mouse tissues obtained from several RRBS and WGBS studies [87]. The first two clocks were built based on all aCpGs using two different regression methods. Two other clocks were constructed using the same two regression methods but based on 952 aCpGs located in highly conserved stretches of DNA. The accuracy of the chronological age prediction appeared to be maximal in clocks built by the elastic net regression on all aCpGs (MAE = 2.5 months). Two clocks based on conserved aCpGs were clearly inferior to those based on all CpGs. Nevertheless, their accuracy was impressive (MAE = 3.8 months). All epigenetic clocks appeared to be of use for multiple tissues, estimating with high accuracy the age in all tissues. One of the most notable conclusions of this study was that many clocks of similar performance could be constructed based on different sets of aCpGs. The data from 3 CR experiments showed significant slowing of aging by both versions of the elastic net regression clocks, but the largest anti-aging effect was observed with the ridge regression clock based on all aCpGs. This latter clock also detected a delay of epigenetic aging in three types of long-lived dwarf mutant mice (growth hormone receptor knock-out, Snell dwarf, and Ames dwarf).

When nine genomic regions of published predictors [84, 86] that contain compact groups of multiple aCpGs were analyzed by pyrosequencing in blood samples from variously aged (11–117 weeks) mice, the best correlation with age was observed for 15 aCpGs from five regions (R^2 = 0.99; mean absolute deviation (MAD) = 2.76 weeks) [88]. Moreover, using just three aCpGs in the *Hsf4* gene, it was possible to predict mouse age with high accuracy (R^2 = 0.95; MAD = 5.24 weeks). Similar results were obtained for three of the most informative aCpGs in the *Prima1*, *Hsf4* and *Kcns1* genes. Thus, age predictions based on a few CpG sites have similar precision, as previously described using multi-CpG predictors [84–86].

Since the mouse epigenetic multi-tissue clock [86] was tuned for tissues of younger mice, a new version was built based on RRBS DNA methylomes of different tissues collected from mice aged from 1 week to 35 months [89]. This new epigenetic clock included methylation of 435 CpGs and showed high accuracy in mice of both sexes across the entire lifespan (R^2 = 0.89, MAE = 72.7 days). Most new clock CpGs (58%) were located in open sea, 5% in shelves, and 11% in shore regions of the genome, and 26% were localized to CGIs. Of different genes in the

mouse genome, *Kcns1* contained clock CpGs present both in the new clock and blood epigenetic clock [80]. This gene was represented by 6 CpGs in the blood clock and 11 CpGs in the new multi-tissue clock. As noted above, the CpGs of this gene have a high correlation with age [88]. By using DNA methylation data from variable sets of samples, 100 varieties of the new multi-tissue clock were built along the same lines. Of the first version multi-tissue clock of 435 CpGs, five CpGs were found in the blood clock [84] and multiple new varieties of the multi-tissue clock. A CpG site with the highest absolute weight, chr8:110168311, was found in all new clocks as well as in the blood clock. This site is located in the promoter region of the *CALB2* gene. Three other sites from the same region were found in the new multi-tissue clock and had high weights. The CpG site chr2:164168131, which appeared in the new multi-tissue clock and the three-CpGs pyrosequencing-based epigenetic clock [88] was found in 95 clock varieties out of 100. At the same time, the majority of the new multi-tissue clock sites were detected in less than half of the 100 clock varieties. Thus, globally, methylation changes during aging allow for selection different subsets of aCpGs informative as epigenetic clocks.

5 Epigenetic Clock Resetting: A Root to Rejuvenation?

Unlike many other hallmarks of aging, age-related epigenetic changes could be fully or partially reversed to a "young" state. A natural example of the full reversal of aging is fertilization, a process when the fusion of haploid oocyte and sperm cells results in a diploid cell (zygote) that has a zero age. A similar age reset occurs upon somatic cell nuclear transfer (SCNT) when the nucleus of a somatic cell is transferred to cytoplasm of an enucleated oocyte, resulting in the development of a new individual. The oocyte cytoplasm seems to possess a capability to erase all of the aging features that have accumulated in the nucleus of the somatic cell. Thus, age-related features of the cell nuclei, whatever their nature, are principally reversible.

Regarding epigenetic aging markers, the main difficulty in their resetting is probably the "needle in a haystack" problem. The epigenome of any cell is a complex mosaic of markers, in which the age-related ones are intermixed with a plethora of others. An easy way to reset the age-related epigenetic changes is to fully erase all existing epigenetic information and then rebuild it from scratch in a form corresponding to the zero age. Such erasure occurs during the first hours following fertilization [90, 91].

Another example of epigenetic age resetting is the production of induced pluripotent stem cells [92]. Recent observations have shown that iPSCs generated from senescent cells or centenarian donor cells rejuvenate telomeres, gene expression profiles, oxidative stress, and mitochondrial metabolism to the levels characteristic of ESCs, and can re-differentiate into fully rejuvenated cells [93–95]. Also, such iPSCs have an epigenetic age close to zero, as noted earlier [51]. Interestingly, fibroblasts from the extremely long-leaved rodent naked mole rat were found to be highly resistant to reprogramming into iPSCs [96]. Not only was the frequency of

iPSCs colonies extremely low, but their ability to induce teratomas when injected into immunodeficient mice was much lower compared with mouse iPSCs. It is likely that this stable epigenome contributes to the extreme longevity and cancer resistance of the naked mole rat.

In all cases described above, resetting of the aging clock was coupled to cell dedifferentiation. This raises the question of is it possible to epigenetically reprogram cells to a more youthful state without disturbing its differentiation status? Two distinct transcriptional waves or phases have been distinguished during cellular reprogramming. The first stochastic phase is driven by c-Myc and Klf4, and the second deterministic phase is driven by Oct4, Sox2 and Klf4 [97, 98]. Importantly, changes in active and repressive histone markers, such as H3K4me3 and H3K27me3, occur during the first phase, changes in miRNA expression are observed during both phases, and alterations in DNA methylation take place mostly during the second phase [98]. Early in the reprogramming process, the four factors induce epigenetic changes by a stochastic mechanism, leading to an intermediate, partially reprogrammed state. Activation of endogenous *Sox2* that occurs eventually in a small fraction of intermediate state cells, triggers a gene activation cascade that drives these cells to the pluripotent state. Since epigenetic reprogramming of somatic cells to iPSCs involves an intermediate state, it is possible that a partial rejuvenation could be achieved at this stage without complete reprogramming to iPSCs. Indeed, short-term expression of the Yamanaka factors Oct4, Sox2, Klf4 and c-Myc in fibroblasts derived from a premature aging mouse model has been found to ameliorate multiple age-associated hallmarks of aging, including the accumulation of DNA damage, cellular senescence markers ($p16^{INK4a}$, $p21^{CIP1}$), epigenetic dysregulation (changed levels of H3K9me3 andH4K20me3) and nuclear envelope defects, without loss of their cellular identity [99]. Although Yamanaka factor expression *in vivo* has been shown to lead to cancer development, their short-term cyclic induction ameliorated hallmarks of aging and extended the lifespan in a mouse model of premature aging. Additionally, this improved the regenerative capacity of pancreas and muscle tissue following injury in physiologically aged mice. To understand whether or not the partial reprogramming with Yamanaka factors is indeed a *bona fide* rejuvenation approach, the dynamics of epigenetic age was evaluated [100]. To this end, subpopulations of human dermal fibroblasts successfully transformed after Yamanaka factor transfection and expressing the TRA-1-60 antigen (a specific marker of hPSCs) were isolated and analyzed for gene expression and DNA methylation at regular time points over 49 days. A steady decrease of epigenetic age by 3.8 years per day was observed starting on day 3, which reached a zero level at day 20. While at first glance these data contradict those cited above [98], the populations of potential target sites of DNA methylation were different between these two studies. Partially reprogrammed cells at days 7 and 11 are known to express pluripotency markers at high levels but also have a high propensity to revert to the somatic state [101]. Since the epigenetic age is already significantly decreased at these time points, partial reprogramming of aging without affecting cellular identity is possible in principal.

In an experimental procedure known as heterochronic parabiosis, a shared circulatory system between young and old mice is established, thus exposing an old mouse to factors present in young serum. Heterochronic parabiosis was found to restore the activity of the Notch signaling pathway, as well as the proliferative and regenerative capacity of the aged skeletal muscle satellite cells [102]. Furthermore, heterochronic parabiosis increased proliferation of aged hepatocytes and restored their cEBP-α levels to values seen in young animals. Thus, age-related decline of progenitor cell activity could be modulated by systemic factors that change with age. Another study showed that the number of newly born neurons, proliferating cells and neural progenitors in the dentate gyrus of hippocampus decreased in the young heterochronic parabiont mice and increased in the old ones [103]. These findings suggest that the systemic milieu appears to affect the biological age of cells. In the adult brain, neural stem cells (NSCs) reside in a heterogeneous niche where they are in direct contact with blood vessels and the cerebrospinal fluid. The vasculature influences neural stem cell proliferation and differentiation by providing signaling molecules secreted from endothelial cells and by delivering systemic regulatory factors. In the aged niche, the vasculature deteriorates with a consequent reduction in blood flow and the neurogenic potential of NSCs declines, leading to reduced neuroplasticity and impaired cognition. In a mouse heterochronic parabiosis model, remodeling of the aged cerebral vasculature in response to young systemic factors was observed, producing noticeably higher blood flow [104]. Thus, circulating factors have diverse positive effects in aging mice, including enhancing neurogenesis and improving the vasculature in the cortex and other parts of the brain. It has been shown that the age-dependent decrease in adult neurogenesis in mouse hippocampus and concomitant cognitive impairment could be caused by loss of Tet2 activity and reduced hm5C levels [105]. An increase of Tet2 has been found in older parabionts after exposure to young blood compared with age-matched isochronic parabionts exposed to old blood. As noted above, hypermethylation of promoter-associated CGIs in genes encoding transcription factors is a prevailing feature of the aged brain methylome [26]. Thus, reactivation of Tet2 DNA demethylase appears to be relevant to brain rejuvenation.

About 70 circulatory proteins from young parabiont mice were found in muscle tissue of old parabiont mice [106]. Many of these proteins possess rejuvenating promyogenic properties and are expected to synergize when reaching the old muscle tissue simultaneously. LIF-1 has been shown to enhance the repair of injured muscle. Cripto and cerberus1 act as antagonists of TGFβ1 that increases with age and this inhibits regeneration of old muscle. GDF5 is a TGFβ family member that promotes muscle innervation known to decline with age. Follistatin might counteract the effect of myostatin known to inhibit muscle stem cell proliferation. Cerberus1 and DKK-1 antagonize the age-elevated Wnt pathway activity. These "young" proteins also have known beneficial effects on other tissues. Of special note, leptin was identified as one of these "young" proteins and it broadly regulates hormonal networks, including those controlling reproduction and metabolism, and it has numerous anti-aging effects. Leptin interacts with oxytocin in its positive effects on the health of muscle, bone, brain and in reduction of obesity. Moreover, since both

leptin and oxytocin activity decline with aging, the increase of leptin/oxytocin axis might represent a key event in mammalian aging that is rescued by heterochronic parabiosis.

Genome-wide microarray analysis of hippocampi showed a distinct gene expression profile between aged isochronic (aged-aged) and aged heterochronic (aged-young) parabiont mice [107]. Furthermore, genes related to synaptic plasticity signaling pathways, including *Creb*, were among the top gene ontology enrichment categories associated with heterochronic parabiosis. These changes in gene expression were correlated with increased numbers of cells expressing the immediate early genes *Egr1* and *c-Fos* and with increased dendritic spine density on granule cell neurons in the dentate gyrus of heterochronic compared with isochronic parabionts. Collectively, the data described above show that exposure to young blood counteracts aging at the molecular, structural, functional and cognitive levels in the aged brain.

Effects of the temporal inhibition of NF-κB activity on cell aging were tested in chronologically aged transgenic mice with the *NF-κB* gene conditionally repressed in epidermal skin cells [108]. Comparison of the global expression profiles between young and old skin samples revealed 414 genes that were significantly altered, with most of these upregulated, in old skin. About 50% of these age-dependent genes were putative direct targets of NF-κB, consistent with the proposed role of NF-κB in aging. Upon 2-week *NF-κB* blockade in old skin, the expression of 225 of the 414 age-related genes returned to levels indistinguishable from those in the young skin samples. Globally, the expression profile of the NF-κB-quenched aged skin was more similar to that of young skin than to the control aged skin. Thus, NF-κB activity is required to maintain a substantial portion of the global gene expression program induced with age in murine skin. In addition to alterations in gene expression, aged skin is characterized by epidermal atrophy, decreased proliferative capacity and increased frequency of cellular senescence. Aged murine tissues also exhibit increased expression of SA-β-gal and $p16^{INK4A}$, two markers of cellular senescence. The *NF-κB* blockade increased epidermal thickness in old skin to a degree intermediate between young and old skin, increased cell proliferation and significantly decreased expression of SA-β-gal and $p16^{INK4A}$. In addition, skin constitution and general condition improved. Importantly, a reversal of skin cell senescence and increased proliferative capacity induced by *NF-κB* blockade occurred with preservation of normal tissue homeostasis and differentiation. Cell proliferation occurred predominantly in the basal layer of the epidermis, the normal proliferative compartment, and the spatial organization of the mature epidermal stratification was intact. Thus, NF-κB activity is required to maintain cellular senescence associated with chronological aging in murine skin. These data suggest that many features of mammalian aging may not be due to the passive accumulation of stochastic cellular damages and errors but rather are actively enforced by special gene expression programs and therefore can be substantially reversed by selective gene expression interventions. The NF-κB action in skin aging appears to be cell-autonomous since the reversion of the aging features was possible in limited patches of the epidermis in otherwise old animals. Since NF-κB is a transcription factor responsive to oxidative

stress, DNA damage, growth signals and immune activation, and it acts on a number of target genes, it seems to be in an ideal position to transduce diverse extracellular signals to adaptive changes in gene expression and tissue homeostasis. The contribution of specific NF-κB target genes to aging remains unknown but it seems likely that its biological effects may be mediated by combined effects of a large number of the NF-κB responsive genes.

HSC activity decreases with age, manifesting in reduced self-renewal, hematopoiesis and lymphopoiesis [109]. In mammals, the target of rapamycin (mTOR) pathway integrates multiple signals from nutrients, growth factors, and oxygen to regulate cell growth, proliferation and survival [110]. Conditional deletion of the *Tsc1* gene in HSCs of young adult mice drove them from quiescence into a state of rapid cycling, increased mitochondrial biogenesis and elevated levels of reactive oxygen species [111]. Importantly, this deletion dramatically reduced both hematopoiesis and self-renewal of HSCs and led to constitutive activation of mTOR. In murine HSCs, mTOR activity increases with age, whereas treatment of old mice with the mTOR inhibitor rapamycin significantly increased their life span [112]. Moreover, the treatment caused a significant increase in the proliferative activity of HSCs and a decrease in the expression of $p16^{Ink4a}$ and $p19^{Arf}$, known markers of cell aging. In addition, old mice have impaired B cell generation due to decreased numbers of pre-B cells and rapamycin treatment enhanced the generation of B cells due to a four-fold increase in the number of pre-B cells.

Thus, the rejuvenation of differentiated or committed cells can be achieved without disturbing their differentiation programs. These results suggest the possibility of targeted therapies to reverse individual features of aging and to alleviate age-related pathologies in the elderly.

6 Conclusions

Both epigenetic drift and the epigenetic clock contribute to age-related changes in DNA methylation but in fundamentally different ways [113]. Epigenetic drift is a result of essentially stochastic events and, therefore, leads to increased discordance between individual epigenomes across the lifespan. Conversely, the epigenetic clock refers to programmed changes in methylation of specific CpG sites that are consistently related to age between individuals of the same species. Despite this fundamental difference, both kinds of DNA methylation changes could be affected by environmental and internal stimuli or occur without any obvious cause. Environmental exposures are generally believed to be highly variable between individuals, leading to diverged epigenetic patterns. On the other hand, shared experiences may lead to similar epigenetic changes that influence the rate of the epigenetic clock. However, the inter-individual consistency of epigenetic clocks suggests that they are a manifestation of some special “epigenetic program of aging.” Both kinds of DNA methylation changes with age probably affect age-related phenotypes. Epigenetic drift leads in a global decrease in precision of epigenetic regulation with

age, probably resulting in increased transcriptional noise and tissue dysfunction. Epigenetic clocks could be useful in multiple ways as an accurate molecular biomarker of aging. The crucial question is whether epigenetic clocks are just manifestations or driving forces of aging. An appealing hypothesis is that epigenetic drift is just a by-product of the aging process, whereas epigenetic clocks are at the heart of the aging mechanisms, reflecting an evolutionary selected programmed mechanism. Methylation changes underlying epigenetic clocks start well before the time when the phenotypic manifestations of aging are evident. The genes most closely linked to epigenetic clocks are enriched for those involved in development and differentiation, such as polycomb targets and bivalent chromatin domains. The apparent continuity of epigenetic clocks across development and aging may reflect a common program of development that involves an aging step at older ages. Since, both phenotypic manifestations of aging and epigenetic age can be alleviated by prolongevity treatments, such as CR, it is tempting to suggest that slowing of the clock contributes to lifespan extension. Studies in which rejuvenation has been achieved via targeting the epigenome [99, 100] support this view and make epigenetic intervention appear to be one of the most promising ways to long life and healthy aging.

Aging is generally believed to be an inevitable and essentially irreversible process in all living organisms. However, multiple studies described above have shown that it could be completely reversed at the cellular level and reversed to a considerable extent at the organismal level. Cellular reprogramming has been shown to reset the age of somatic cells to zero. On the whole organism level, manipulations of specific signaling pathways, such as the insulin/IGF-1, NFκB, mTOR, AMPK and sirtuin networks, and external interventions, including CR, physical activity and functional foods, were shown to extend the life span of model animals and slow down epigenetic aging. Heterochronic parabiosis experiments have shown that at the organismal level aging not only could be slowed down but also reversed to a significant extent, at least in some organs. It would be interesting to determine whether these rejuvenating effects of parabiosis are caused by epigenetic changes.

Studies of the aging details using predictive epigenetic models could have many practical implications, from health assessment to forensic analysis. As the predictive accuracy of the models improves, it seems likely that the biological age, measured by epigenetic clocks, might become more useful in clinical practice than chronological age. Finally, epigenetic clocks could become indispensable as markers of biological age in evaluating the efficiency of the new rejuvenation procedures.

Conflict of Interest The authors confirm that this article content has no conflict of interest.

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Chapter 11
From White to Brown – Adipose Tissue Is Critical to the Extended Lifespan and Healthspan of Growth Hormone Mutant Mice

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1 Introduction

The growth hormone (GH) and insulin-like growth factor 1 (IGF-1) axis (collectively known as the somatotropic axis) was demonstrated to be a major determinant of mammalian longevity more than 20 years ago [1, 2]. Since then, numerous laboratories have attempted to elucidate mechanisms underlying the role of this axis in longevity, leading to an ever-growing list of possible mechanisms to disentangle [3, 4]. During the same timeframe, the growing obesity epidemic in the developed world has resulted in a dramatic increase in adipose tissue (AT) research. Since GH plays integral roles in the physiology of AT, there has been a surge in research attempting to understand how AT impacts longevity in GH-mutant mice. This review is aimed to give an overview of AT, GH, and how the interplay between the two influences longevity.

2 Adipose Tissue

Traditionally, AT was believed to be metabolically inactive, solely acting as a tissue to store excess calories. However, paradigm-shifting studies demonstrated that AT secretes adiponectin [5], leptin [5, 6] and resistin [7], which paved the way for

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Table 11.1 Different types of adipose tissue. The major similarities and differences between white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue

	WAT	Beige AT	BAT
Color	White	White	Brown
Lipid storage	Unilocular	Unilocular	Multilocular
Mitochondria	Few	Some	Many
Innervation	Normal	Increased	High
Thermogenic capacity	Low	Medium	High

future work on AT as an endocrine organ. Since then, our understanding of AT has been greatly expanded. We now know that there are at least three distinct types of adipose tissue: white adipose tissue (WAT); brown adipose tissue (BAT); and beige AT. As predicted, each type of AT depot has specific functions. Further differentiating these types of AT is the significant cellular heterogeneity within an AT depot itself [8]. Despite this heterogeneity, AT is largely made-up of postmitotic adipocytes and their replicative precursors, termed preadipocytes. The differentiation of preadipocytes into mature adipocytes is transcriptionally controlled through the coordination of CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor gamma (PPARγ) [9]. This section is dedicated to defining the similarities and differences between WAT, BAT and beige AT, which are summarized in Table 11.1.

2.1 WAT

The defining characteristic of WAT in both humans and mice is the storage of excess energy. Morphologically, WAT is characterized by a large, unilocular lipid droplet, with few mitochondria. WAT has both similarities and differences in mice and humans. In mice, WAT is present in superficial subcutaneous depots, mainly in the scapular and inguinal regions. WAT is also present in the intra-abdominal region of mice in the form of perigonadal (epididymal and paraovarian in males and females, respectfully), mesenteric, and retroperitoneal AT. In humans, WAT is more widely distributed and is present subcutaneously in the gluteal, femoral, clavicular, and abdominal regions. WAT in humans is also present intra-abdominally in intraperitoneal, retroperitoneal, mesenteric, and omental AT depots. The major difference in WAT distribution between mice and humans is the large perigonadal depot in mice, and the large omental depot in humans. Most mouse studies in the context of metabolism and aging use inguinal WAT (iWAT) to represent subcutaneous AT, and perigonadal AT to represent the so-called visceral AT. Although this practice is widely used and accepted, it is worth mentioning that some investigators prefer a more stringent use of the term "visceral" to include only AT that directly drains into the portal vein, rather than any intra-abdominal AT depot. By this definition, only mesenteric AT in mice would be considered "visceral" [10].

In obese subjects, there are critical changes in WAT physiology. AT itself is surrounded by a thick extracellular matrix (ECM). During weight gain, the ECM in WAT must expand to accommodate hypertrophic adipocytes. However, this results in poor vascularization [11] and subsequent hypoxia. Hypoxia in the adipocyte is just one of several instances that cause an increased secretion of proinflammatory cytokines to be released from WAT during obesity [12]. Another unfavorable impact of obesity on WAT is ectopic lipid distribution. For example, intra-myocellular lipids [13–17] and intra-hepatic lipids [18–21] are associated with insulin resistance, while epicardial fat is associated with an increased risk of coronary artery disease [22–24].

2.2 Bat

The differences between BAT and WAT begin during development, as BAT comes from a mesoderm lineage that is myogenic factor 5 (MYF5) positive [25]. Unlike WAT, BAT is characterized by multilocular lipid droplets, many mitochondria, and is rich in both innervation and microvasculature. These anatomical traits are important for the main function of BAT, thermogenesis. Sympathetic nerves provide a source of norepinephrine (NE) to stimulate thermogenesis, blood vessels provide nutrients to the tissue as well as aid in heat dissipation, and multilocular lipid droplets have an increased surface area to facilitate an increased rate of lipolysis. The thermogenic circuit relies on several transcriptional inputs including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [26] and PR domain containing 16 (PRDM16) [27]. Moreover, cues from other transcriptional machinery such as thyroid hormone receptor [28] and retinoic acid receptor [29] play important roles in the thermogenic circuit. Readers interested in learning more about the transcriptional control of the thermogenic program are directed to the following reviews [30–32]. Central to the function of BAT is uncoupling protein 1 (UCP1), which dissociates the electron transport chain to release chemical energy in the form of heat.

Thermogenesis itself begins with the release of NE from the sympathetic nervous system, which acts on β3-adrenergic receptors, which are associated with G-protein coupled receptors (GPCRs) of the Gs subtype [33, 34]. Subsequently, a rise in cytosolic cAMP results in the activation of protein kinase A (PKA) [35], which has several functions including activating mitogen-activated protein kinase (MAPK) p38 [36] and increasing cytosolic free fatty acid (FFA) levels in the cell by phosphorylating perilipin [37]. This, in turn, causes the release of comparative gene identification-58 (CGI-58) to activate adipose triglyceride lipase (ATGL), the major triglyceride lipase in BAT [38–40]. The breakdown of triglycerides into FFAs in BAT is critical for two reasons. First, the resulting FFAs can be shuttled into the mitochondria where they can undergo β-oxidation to produce ATP and reduced electron carriers to maintain thermogenesis [30]. Second, the FFAs act as activators of UCP1 [41, 42]. Conversely, purine nucleotides act as inhibitors of UCP1 [41].

It was long believed that BAT in humans was non-existent past adolescence. However, a decade ago, seminal studies that "rediscovered" BAT in adult humans proved otherwise [43–45]. Because a few hundred milligrams of BAT can oxidize up to 60% of consumed glucose and lipids in a cold-acclimated mouse [46, 47], investigators became interested in using BAT therapeutically to combat the growing obesity epidemic. Although humans do possess BAT, it differs in several ways from BAT in mice. Mice have several distinct BAT depots including the large interscapular depot, as well as the axillary, cervical, paraaortic, cardiac, and perirenal depots. In humans, brown adipocytes appear interspersed in WAT, mainly in the supraclavicular region, but are also present in the para-aortic, cervical, axillary, perirenal, and paravertebral regions. It is worth noting that recently a supraclavicular BAT depot was discovered in mice [48]. There is an interscapular BAT depot in humans, although it disappears during adolescence. Currently, the gold standard for assessing the location and activity of BAT in humans is positron emission tomography coupled with computed tomography (PET/CT) during the infusion of radiolabeled 18-fluoro-deoxyglucose (18FDG) in a patient either wearing a "cold vest" or receiving a β3-agonist, such as mirabegron [49]. However, this method does not always accurately reflect BAT activity, and has led to vastly different estimates of the total volume of BAT present in humans, ranging over two orders of magnitude from only a few, to a few hundred milliliters [50]. The measurement of BAT in humans, along with a detailed description of its pitfalls has been reviewed elsewhere [49]. Regardless, we know that BAT is present in adult humans and has a significant impact on metabolism. A clear example of this is a study in which type 2 diabetics spent several hours a day over a 10-day period at 15 °C, which resulted in a significant increase in their glucose infusion rate (GIR) during a euglycemic clamp [51].

One of the biggest advances in our understanding of thermogenic AT in the past few years is the presence of UCP1-independent thermogenic mechanisms. For example, both brown and beige AT thermogenesis can occur through a creatine-based substrate cycle [52–54]. Moreover, beige AT thermogenesis can be controlled through ATP-dependent calcium cycling [55]. These findings can begin to explain why UCP1 null mice only become obese under thermoneutral temperatures [56, 57], while BAT-deficient mice are obese and insulin resistant at standard room temperature [58, 59]. To-date, however, these UCP1-independent forms of thermogenesis have not been examined in GH mutant mice.

2.3 Beige AT

Beige AT is distinct from both WAT and BAT. Beige adipocytes reside within WAT depots, contain mitochondria expressing UCP1, and are therefore thermogenic. Under basal conditions, thermogenic output of beige adipocytes is relatively low, however, stimulants such as cold exposure, exercise, or treatment with PPARγ agonists significantly increase the expansion and energy expenditure in these cells in a

process termed beiging. Although beiging was described more than 30 years ago [60, 61], only recently have the specific lineages and molecular regulators that give rise to beige AT been worked out [62, 63]. It is worth noting that this is an area of ongoing investigation, with no clear consensus. For example, some studies have shown that beige AT derives from a lineage distinct from BAT that is positive for myosin heavy chain 11 (MYH11) and platelet derived growth factor receptor alpha (PDGFRα) in mice [64–67], while other beige adipocytes have been found to be positive for paired box 3 (PAX3) and MYF5 [68]. Adding further complexity are conflicting studies in which some investigators demonstrate that beige adipocytes are formed de novo in response to external cues [66, 69], while others argue they arise from the transdifferentiation of white adipocytes [70, 71]. Regardless of the developmental origin of beige adipose tissue, during cold-exposure, thermogenic beige adipocytes replace non-thermogenic white adipocytes, which is reversible when cold-acclimated mice are placed at thermoneutrality [72].

3 Properties of Adipose Tissue

3.1 Adipose Tissue Heterogeneity

Beyond the previously discussed inter-depot differences, AT is highly heterogenous within each depot. By volume, the majority of AT is composed of mature adipocytes. These adipocytes have a turnover rate of approximately 10% annually in humans, with a much faster turnover rate of 5% daily in mice [73, 74]. Because of this, preadipocytes, or committed adipocyte progenitors, are critical to the AT niche. Work has already been done to identify markers of white, brown, and beige adipocytes, however, studies identifying novel cell surface markers of preadipocytes that give rise to these adipocytes is mostly lacking, although some markers have been identified [75]. For example, sorting preadipocytes with high CD29 expression enriches for a population of preadipocytes that differentiate into cells with a high expression of UCP1 [75]. Preadipocytes are also a major source of tumor necrosis factor α (TNF-α), suggesting their role in AT extends beyond acting as a precursor cell [76].

Immune cells are another cell type with a large role in AT. It has been appreciated for years that macrophages are present in AT, and that their presence increases with obesity. However, considerable improvements have been made to our understanding of different subpopulations of macrophages in AT [77–79]. For example, there is good evidence that both M1 (referred to as classically activated) and M2 (referred to as alternatively activated) macrophages exist within AT [80, 81]. Along with macrophages, natural killer cells are recruited to AT during obesity, causing insulin resistance [82]. Changes in resident immune cells may also play a beneficial role in the physiology of AT. For example, cold-exposure causes an influx of M2 macrophages and eosinophils that aid in thermogenesis [83–85].

AT stores energy as triglycerides during caloric excess, and must liberate FFAs during periods of caloric demand through lipolysis. Since lipolysis is stimulated by the sympathetic release of NE, it makes sense that nerves are a core component of AT [86]. To transport nutrients to AT, or dissipate heat and FFAs (in BAT and WAT, respectfully), there must also be the presence of microvasculature, including endothelial cells and smooth muscle cells [8]. Certainly, AT heterogeneity has garnered attention in recent years. With advances in technologies such as single cell RNA-sequencing, the makeup and functional importance of the AT niche is sure to be further developed in the near future.

3.2 Adipose Tissue as a Secretory Organ

Studying WAT as an endocrine organ began 20 years ago with the discovery of leptin, resistin, and adiponectin being secreted from WAT [5–7]. WAT has also been known to secrete proinflammatory cytokines such as TNF-α and IL-6, which facilitate the development of insulin resistance during obesity. What has been much less studied, however, is the role of BAT as a secretory organ. Recently, this has changed as secreted factors from BAT (referred to as batokines) have been demonstrated to have both autocrine/paracrine and endocrine effects. For example, the lipokine 12,13-diHOME has been demonstrated to have an autocrine effect on BAT that results in increased lipid uptake [87]. Other factors that have paracrine/autocrine action in BAT are vascular endothelial growth factor A (VEGFa) and nitric oxide (NO), which increase angiogenesis [88, 89]. Moreover, fibroblast growth factor 2 (FGF2) and nerve growth factor (NGF) increase innervation and the recruitment of preadipocytes [90–92]. Endocrine factors that are secreted from BAT include insulin-like growth factor-binding protein 2 (IGFBP2) [93], WNT10b [93], and FGF21 [94]. BAT also secretes microRNAs. For example, both mice and humans show an inverse relationship between BAT activity and circulating levels of miR-92a [95]. Readers interested in learning more about the secretory function of BAT are encouraged to read a relevant review [96].

3.3 Alterations in AT during Aging

The main changes in WAT during aging is the gradual decline in tissue mass, the redistribution from subcutaneous to intra-abdominal depots, and the ectopic distribution of lipids in organs such as the liver and muscle [97–100]. Metabolically, aged WAT has a decline in its sensitivity to insulin and fatty acids [97, 101–103]. Moreover, aged WAT has an increased secretion of harmful proinflammatory cytokines such as TNF-α and IL-6 [104, 105]. There does appear to be an increase in macrophage infiltration in subcutaneous WAT, although this does not seem to apply to intra-abdominal WAT [106]. A final means through which WAT changes during

aging is through the preadipocyte pool. Preadipocytes from aged tissue have lower levels of the transcription factors PPARγ C/EBPα, and their target genes [107, 108], which may explain the reduction in their capacity to differentiate into mature adipocytes. Moreover, senescent preadipocytes accumulate in aged WAT, which could contribute to metabolic impairment and increased systemic inflammation [97]. Readers interested in learning more about WAT remodeling during aging are directed to a relevant review [97].

Some of the changes mentioned above apply to BAT. For example, BAT mass decreases with age. Although senescence in BAT has been understudied, it is plausible to assume that brown preadipocytes also senesce, and lose the ability to differentiate into mature brown adipocytes with age. Many of the age-related changes in BAT relate to impaired thermogenic capacity. One mechanism for this is through the increased visceral AT expression of forkhead box protein A3 (FOXA3), which impairs BAT mass and function [109]. Interestingly, deletion of FOXA3 increases BAT late into life and extends longevity [109]. Another deleterious change in aged BAT is the presence of sympathetic neuron-associated macrophages, which chelate NE, resulting in decreased thermogenic output [110]. Finally, the well-documented age-dependent mitochondrial dysfunction impairs thermogenesis. The decline in the thermogenic function of BAT likely plays a critical role in the metabolic impairment and obesity observed during middle-age.

4 Growth Hormone

GH is a 22 kDa peptide hormone that is secreted from somatotrophs in the anterior pituitary. Its secretion is induced by the release of growth hormone releasing hormone (GHRH), and inhibited by the release of somatostatin (SST), both of which are released from the hypothalamus. GH has negative feedback on GH release from the pituitary, as well as on GHRH from the hypothalamus. Another level of feedback is through IGF-1 which acts on both the pituitary and hypothalamus [111]. Ghrelin, a “hunger” hormone is another factor that stimulates the release of GH [112]. AT can regulate GH production through FFAs and leptin which inhibit and stimulate GH production, respectively [113, 114].

In circulation, GH acts by binding to growth hormone receptor (GHR) on target tissues. Mainly, GH acts on the liver to stimulate the production of IGF-1, but GH can also act on other tissues such as muscle and AT [115]. Therefore, GH can elicit direct effects, or indirect effects through the action of IGF-1. Once bound to a homodimerized GHR, there is a conformational change in the receptor structure which brings together the associated janus kinase 2 (JAK2) domains together, allowing for transactivation [116] and subsequent phosphorylation of signal transducer and activator of transcription 5 (STAT5). Activated STAT5 can then enter the nucleus and act as a transcription factor [117]. GH has been demonstrated to signal through other non-canonical pathways including mammalian target of rapamycin (mTOR) and extracellular signaling-regulated kinase (ERK) [118].

5 Examples of Altered Growth Hormone Action

5.1 Humans

The two main ways that GH is altered in humans is through its overproduction in acromegaly and GH resistance, or through its under production in GH deficiency. Patients with acromegaly suffer from increased GH secretion, and subsequent increases in IGF-1 production. The increased secretion of GH is oftentimes the result of a pituitary adenoma. Acromegaly patients are more prone to cancer [119–121], diabetes [122], and are often short-lived compared to people with normal GH secretion [123, 124]. GH deficiency has multiple etiologies that influence the age of the onset of disease. In children, congenital GH deficiency is usually the result of mutations in genes encoding GH, GHRH, or other pituitary factors involved in the secretion of GH [125]. In adults, acquired GH deficiency is typically the result of hypopituitarism or irradiation of a pituitary adenoma [126]. Beyond deficiency, patients can be resistant to GH through mutations in the gene encoding GHR [127]. This, disease, termed Laron syndrome, causes patients to have low IGF-1, with elevated levels of GH [128]. Patients with both GH deficiency and resistance demonstrate decreased height, increased obesity, decreased bone mineral density, and altered lipid metabolism [3]. Interestingly, patients with Laron syndrome appear to be protected from cancer [129, 130] and diabetes [131], although Laron syndrome patients from cohorts in Israel and Turkey appear to still develop diabetes [132, 133], making the "protected" status from diabetes less clear.

5.2 Mice

To further understand the impact of GH signaling, several transgenic lines overexpressing GH have been created, the most commonly used being the bovine GH (bGH) transgenic line [134, 135]. These mice have a transgene that ectopically expresses GH under a strong promoter such as phosphoenolpyruvate carboxykinase (PEPCK). bGH mice are noticeably larger than their control littermates, and exhibit increased muscle mass. bGH mice demonstrate increased insulin resistance, and severe hyperplasia and hypertrophy of their hepatocytes [136]. Many of these mice die of hepatic cancer. Aging in these mice appears to be accelerated, and lifespan is reduced to around 1 year-of-age [135, 137].

Ames dwarf mice were first described in 1961, and suffer from a spontaneous mutation in the gene encoding prophet of pituitary factor 1 (Prop1) [138]. Snell dwarf mice were first described in the 1929, and suffer from a spontaneous mutation in the gene encoding pituitary factor 1 (Pit1) [139]. Since Prop1 is a transcription factor for the Pit1 gene, the phenotypes of Ames and Snell dwarfs are essentially identical, with both strains of dwarf mice lacking the production of GH, thyroid-stimulating hormone (TSH) and prolactin [140]. The downstream consequences of

these mutations are a decreased production of IGF-1 and the thyroid hormones, T3 and T4. Both Ames and Snell dwarf mice are extremely long-lived, with an ~50% increase in longevity in Ames dwarf mice (1) and ~42% increase in Snell dwarf mice [141]. Along with increased longevity, these animals have a decreased incidence of cancer [142], increased insulin sensitivity [143] and increased adiposity, particularly in the subcutaneous depot [144].

Growth hormone receptor knockout (GHRKO) mice were developed to replicate Laron syndrome in mice by disrupting the gene encoding GHR/GH binding protein [145]. As with Laron syndrome patients, GHRKO mice are small, have increased adiposity, and have increased circulating GH with low circulating IGF-1 [145]. GHRKO mice also live ~38% longer than control mice [146]. Studies of cognitive function [147, 148] and tissue histopathology [149] revealed that GHRKO mice have a delay in aging, similar to that of Ames and Snell dwarf mice. To study the effects of GH action in specific tissues, many tissue-specific lines of GHRKO mice have been created [150–153]. The first line attempting to knockout the GHR gene in AT was done using Cre driven by the adipocyte protein 2 (Ap2) promoter (termed FaGHRKO) [154]. Unlike the global GHRKO mice, these animals had an increase in IGF-1 and had no improvement in insulin sensitivity [154]. It has since been reported that the Ap2 promoter is "leaky" and is expressed in macrophages, endothelial cells, and in the brain [155, 156]. Therefore, an AT-specific GHRKO mouse (termed AdGHRKO) was generated using the more specific adiponectin promoter-driven Cre [157]. These animals show no difference in GH or IGF-1, have increased fat mass, decreased liver triglyceride content, and are insulin sensitive [157].

Other mouse lines such as the GHRH knockout (GHRHKO) mice live ~45% longer than their control littermates, and have decreased IGF-1 production and increased adiposity [158]. "Little" mice have a mutation of the GHRH receptor gene, live ~25% longer than their normal littermates, and develop increased adiposity [141]. Several current reviews discuss mechanisms of altered longevity in all these mouse lines in more detail [4, 159, 160].

6 Adipose Tissue in Mice and Humans with Altered Growth Hormone Action

What is clear is that a defining phenotype of GH-related mutations is alterations in adiposity. For example, patients with acromegaly and bGH mice have decreased adiposity [161–164], while patients and mice that are GH-deficient or GH-resistant have increased adiposity [144, 165–167]. Particularly noteworthy is that the iWAT depot in GHRKO mice is equal in mass to that of their control littermates, despite GHRKO mice weighing approximately 66% less [166, 168]. It has also been demonstrated that dwarf and GHRKO mice maintain a higher extra- to intra-peritoneal distribution of lipids [169], which is the opposite of acromegaly patients which have a higher ectopic distribution of lipids [170]. Beyond alterations in mass and

distribution, there appears to be an alteration in the endocrine function of AT in GH-mutant animals. For example, leptin and adiponectin are decreased in bGH mice, and increased in dwarf and GHRKO mice [171, 172]. In fact, altered endocrine function may partially explain an unexpected phenotype, where surgical removal of the epididymal WAT (eWAT) in dwarf and GHRKO mice results in decreased insulin sensitivity [173, 174]. This finding may be due to the fact that eWAT in long-lived GH-mutant mice secretes less pro-inflammatory cytokines such as TNF-α and IL-6, while secreting more adiponectin [173, 174]. The secretory function of BAT in these animals has not yet been assessed. Lowering senescent cell burden can extend longevity [175]. Therefore, it is important that 18-month-old dwarf and GHRKO mice have a reduced senescent cell burden, and that their preadipocytes demonstrate an increased differentiation capacity, suggesting that their AT has a "younger" phenotype [169]. Senescence in BAT of long-lived GH-mutant mice has not yet been investigated.

Although many studies have been conducted on WAT in GH-mutant mice, far less has been done relating to the BAT of these animals. To date, we know that BAT in GHRKO and Ames dwarf mice has an increased expression of Ucp1, and that BAT in bGH mice has a decreased expression of Ucp1 [176, 177]. This is accompanied by an increase or decrease in BAT mass in Ames dwarf/GHRKO and bGH mice, respectively [176, 177]. The highly active BAT observed in Ames dwarf and GHRKO mice may at least partially explain the increased rate of energy expenditure and reduced respiratory quotient observed in these animals [178]. Particularly curious in Ames dwarf mice is the increased BAT activity despite having a depleted thyroid hormone axis. This suggests that other circulating factors may be responsible for the increased BAT activity, although this hypothesis remains to be tested. It does appear that the increased BAT activity of these animals may play a role in several biomarkers of healthy aging. For example, placing Ames dwarf mice at thermoneutrality (30 °C) eliminates differences in oxygen consumption rate and respiratory quotient, and reduces their enhanced insulin sensitivity [179]. Further testing will need to be done to determine if thermoneutral housing also impacts longevity in these animals.

7 Final Thoughts

GH has a critical role in metabolism due to its profound effects highly metabolic tissues such as the liver, muscle and AT. Increased adiposity is associated with comorbidities ranging from diabetes to Alzheimer's disease. Therefore, it is of major consequence that the AT in long-lived GH-mutant mice functions in a more metabolically beneficial way. These differences include AT distribution (extra- instead of intraperitoneal), endocrine function (shift from pro- to anti-inflammatory cytokines), and replication and senescence status (differentiates well and has a low senescent cell burden), as well as an increase in thermogenesis and BAT activity. In terms of whole-body physiology, these alterations cannot be understated. For example, both WAT and BAT in these animals most likely significantly contribute to

improved glycemic control, which is believed to be a major factor in their improved healthspan and lifespan. There are still areas that should be examined in the context of AT in GH animals. For example, we now know that AT secretes many proteins, metabolites, lipids, miRNAs, and is a rich source of exosomes. Examining how these are alteredin GH-mutant mice is a currently unexplored area. Moreover, extrapolating these findings to human subjects would be of considerable interest.

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Chapter 12
The Cytoskeleton as a Modulator of Aging and Neurodegeneration

Konstantinos Kounakis and Nektarios Tavernarakis

1 Introduction

The cytoskeleton is a cellular entity, encompassing a multitude of filamentous proteins, forming structures that impart mechanical strength, allow intracellular transport and spatial organization, connect the cell to its environment, and generate forces that permit movement [1]. The ubiquitous nature of the cytoskeleton and the breadth of its functionality make it one of the most fascinating aspects of cellular biology, as well as one that is always worth considering when researching or discussing phenomena that affect the cells. In this review, we discuss the relevance of the cytoskeleton to the processes of aging and neurodegeneration, and provide examples that demonstrate its importance.

1.1 Components of the Cytoskeleton

Three types of cytoskeleton polymers have been defined: actin microfilaments, microtubules and intermediate filaments [1, 2]. We briefly describe their structure and function below.

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1.1.1 Actin Microfilaments

Actin filaments (also commonly referred to as F-actin) are about 7 nm in diameter and consist of monomers of globular (G-actin) that interact head-to-tail with each other. G-actin can bind ATP, and this promotes its polymerization to F-actin. ATP is subsequently hydrolyzed to ADP. Actin filaments are polarized with a positive (+) and negative (−) end. Polymerization can occur at both ends but is significantly faster at the + end. The filaments can organize into higher order structures with the help of crosslinkers (Fig. 12.1). Highly aligned actin bundles are responsible for the formation of narrow cell protrusions, such as filopodia, while highly branched bundles take part in larger cellular movements, such as those that occur in phagocytosis. The polarity of the filaments also allows them to support a family of ATP driven motor proteins, the myosins, that contribute to actin network organization and force generation [1, 2].

In neurons, actin forms patches in the initial segment of the axon and at points along its length [3, 4]. It also forms, in association with the actin capping protein

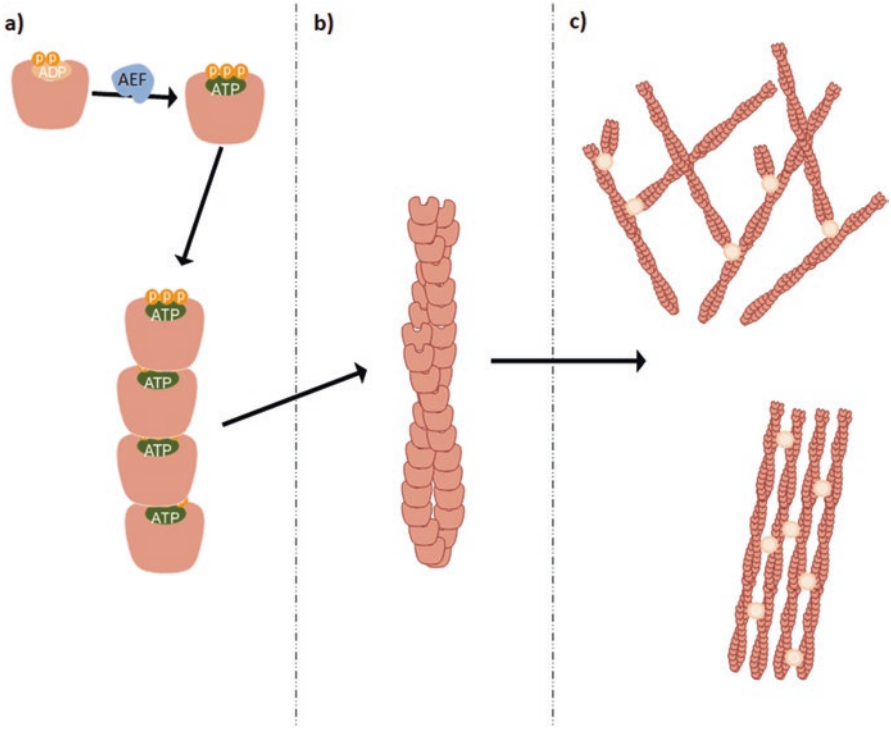

Fig. 12.1 Organization of actin microfilaments. (**a**) G-actin polymerization. Actin monomers are loaded with ATP with the help of a protein with ATP exchange factor (AEF) activity. This induces their polymerization. (**b**) F-actin. (**c**) Larger scale F-actin organization facilitated by crosslinker proteins

adducin, a series of periodic rings spaced by spectrin that are wrapped around the axonal shaft [5]. Actin is also a major contributor the motility and guidance of the neuronal growth cone. The growth cone has three domains: the central (C), the peripheral (P) and the transition (T) domain [6]. Actin is rich in the P and T domain and its polymerization and recycling allows for the formation of exploratory filopodia. In addition, myosin 2 generates forces that assist in propelling the growth cone forward and steer it towards its targets. Inhibition of these functions does not prevent axonal growth but it significantly reduces its speed and abolishes its ability to respond to guidance cues [7, 8]. It can also act as the driving force for axonal branching, as actin filament patches can initiate the formation of protrusions that subsequently are invaded by microtubules to create new collateral branches that allow the same axon to interact with multiple targets [9]. Furthermore, actin has been connected to synaptic signaling, as it has been implicated in the regulation of synaptic vesicle pools, vesicle docking to the active zone, and even endocytic retrieval of vesicle membranes [10]. Finally, actin contributes to dendritic spine organization [11–13] and, in collaboration with microtubules and the receptor-associated protein gephyrin, contributes to postsynaptic receptor clustering [14].

In oligodendrocytes, the glial cells that are responsible for myelinating the axons of the CNS to facilitate fast action potential conduction, the actin cytoskeleton plays a critical role by allowing these cells to alter their morphology during development [15]. These cells possess protrusions with actin rich filopodia and lamellipodia. Actin in these structures is organized in a fashion mostly similar to growth cones [16]. It acts as a necessary driving force that allows these protrusions to extend towards their target axons and wrap around them [17–20]. Subsequently, actin depolymerization allows these protrusions to convert into sheets by reducing surface tension, enabling proper myelin spreading [19].

1.1.2 Microtubules

Microtubules (MTs) are cylindrical bundles of parallel protofilaments comprised of α- and β- tubulin. These bundles can have 10–16 individual filaments, with 13 being the most common. They have a typical diameter of about 25 nm. Both tubulins can bind GTP, which promotes polymerization, but eventually hydrolyze it to GDP, weakening their affinity. This leads to what is described as “dynamic instability”, as microtubules can switch between stable growth and rapid depolymerization. Microtubules are polarized, with a + and a − end. This polarity becomes particularly apparent during a phenomenon known as treadmilling, during which tubulin is simultaneously removed from the − end of the filament and polymerized to the + end (Fig. 12.2). This polarity also allows microtubules to support ATP driven motor proteins, the kinesins and dyneins, which are responsible for the guided transport of cellular cargo. Microtubules interact with a group of proteins known as MAPs (microtubule associated proteins) that influence their stability and interactions with other cellular components. A subset of MAPs, the +TIPs (plus-end-tracking proteins) interact specifically with growing microtubule ends. There are also − end

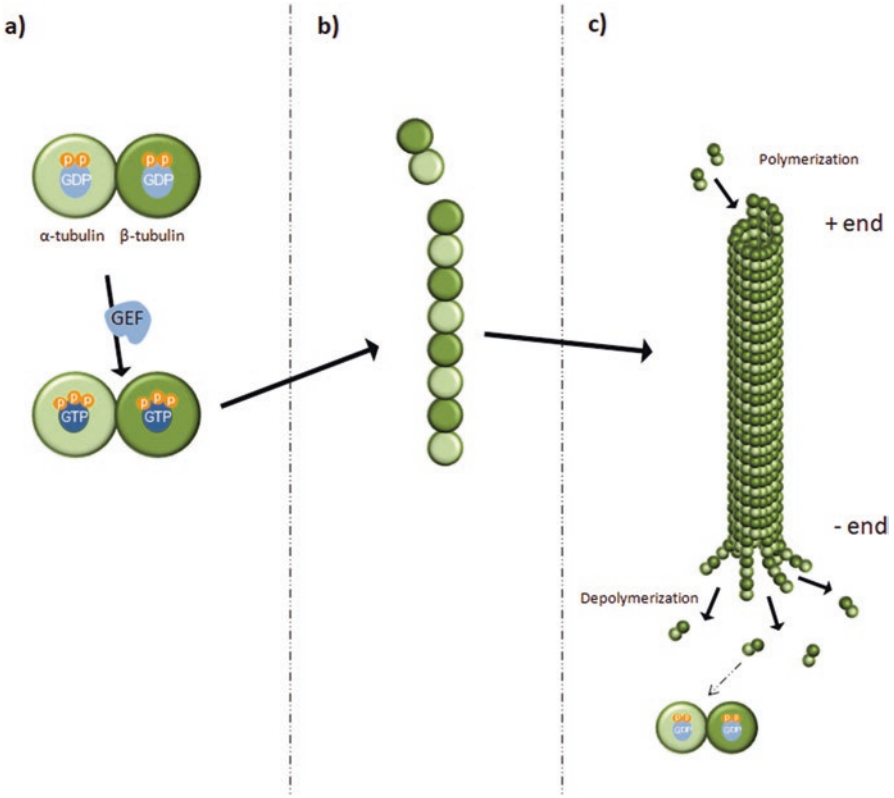

Fig. 12.2 Organization of microtubules. (**a**) GTP is loaded to α- and β- tubulin with the help of a protein with GTP Exchange Factor (GEF) activity. (**b**) A tubulin filament. (**c**) Microtubule structure and dynamics

capping proteins that can prevent depolymerization. Microtubule nucleation often needs to start at a Microtubule organization center (MTOC) where γ-tubulin interacts with α- and β- tubulin, providing a base for the start of filament extension [1, 2, 21]. The MTOC of mammalian cells is known as the centrosome. It consists of two perpendicular tubulin structures known as centrioles that are surrounded by a centrosomal matrix of proteins involved in microtubule nucleation, anchoring and release. The duplicated centrosome is responsible for the formation of the mitotic spindle, the microtubule structure that segregates chromatids during cell division [22].

In mature neurons, microtubules are arranged with the − end towards the cell body and the + end extending outwards, along the axon. They are discontinuous, with multiple start and stop sites [21]. In this context, there is evidence that microtubules cease to rely on the centrosomal MTOC for their organization [23, 24]. Axonal microtubules extend into growth cones, where they localize primarily in the C domain. However, they can extend even further and they are known to interact

with actin, particularly actin bundles that form filopodia in the P zone [6]. These microtubules have dynamic ends and are crucial to growth cone steering [25]. Outside of the growth cone, in cases of interstitial axonal branching formation, some axonal microtubules are reorganized and interact with the newly forming protrusion [26]. Microtubules also extend between the cell body and dendrites. In this case they adopt a mixed orientation, with + and − ends facing towards both directions [27].

1.1.3 Intermediate Filaments

Intermediate filaments (IFs) constitute a diverse family of cytoskeletal proteins that are expressed differentially across cell types. All of these proteins share structural similarity and organize in similar ways to provide mechanical strength and stability to most cell types, especially against tensile forces. IF subunits consist of a globular N-terminal head, an α-helical core and a variable C-terminal domain. Intermediate filament monomers tend to coalesce in pairs, forming parallel coiled coil dimers. Two antiparallel dimers can also associate to form a tetramer (Fig. 12.3). The higher scale organization depends on the tissue and the actual filament components but typically leads to a filamentous polymer of ~10 nm in diameter. Examples of IFs include keratin, vimentin, the lamins of the nuclear skeleton, α-internexin, peripherin, synemin, nestin or the light, medium and heavy neurofilaments (NF-L, NF-M and NF-H, respectively). Intermediate filaments are not polarized and therefore do not support molecular motors [1, 2, 28, 29].

Neuronal intermediate filaments (which will be referred as neurofilaments or NFs from now on) represent the main cytoskeletal element of mature neurons. They consist of NF-L, NF-M, NF-H and occasionally α-internexin and peripherin, with

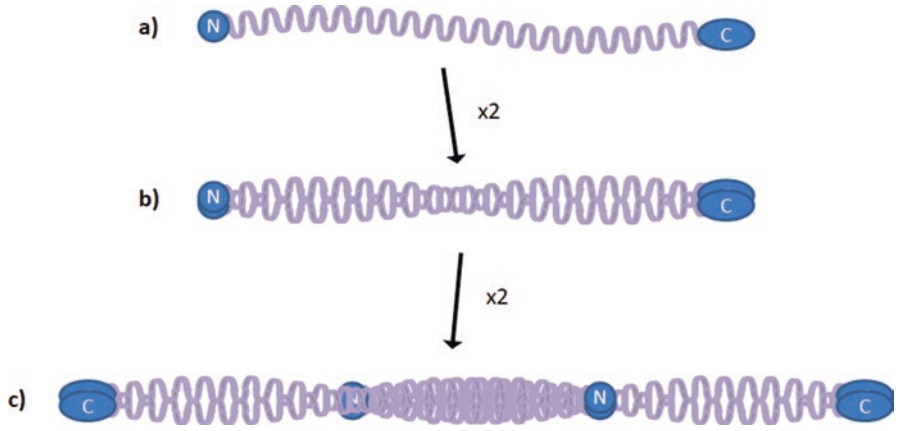

Fig. 12.3 Intermediate filament organization. (**a**) Monomer. (**b**) Dimer of parallel monomers. (**c**) Tetramer of antiparallel dimers

the actual composition varying by organism or even stage of development. The NF-M and NF-H C-terminal domains are notable for the presence of a large number of lysine-serine-proline (KSP) repeats that represent targets for regulatory phosphorylation. Neurofilaments reside in axons and act as regulators of axonal caliber, which has implications in myelin thickness and the rate of axonal conduction. They are also associated with axonal growth and regeneration [2, 29, 30].

2 The Importance of the Cytoskeleton to Aging and Neurodegeneration

2.1 Cytoskeleton and Organismal Aging

There is ample experimental evidence connecting the cytoskeleton with the processes of cellular and organismal aging. In yeast, actin has emerged as a regulator of lifespan by regulating the inheritance of mitochondria. During budding, actin cables create a retrograde flow from the bud towards the mother cell, driven by polymerization and myosin activity. This flow pushes mitochondria away from the bud, forcing them to "swim upstream" and ensuring that only healthy mitochondria can reach the new cell, granting it a longer lifespan and healthspan [31, 32].

In C. elegans, the actin cytoskeleton has been observed to deteriorate with aging. HSF-1, the master regulator of the heat shock response that provides thermotolerance and also contributes to organismal longevity, has been shown to act against this deterioration. This effect is mostly mediated through the upregulation of the expression of the calcium binding protein PAT-10. Most notably, loss of pat-10 is sufficient to decrease organismal lifespan and thermotolerance, while overexpression enhances thermotolerance and promotes longevity [33, 34].

In mammals and particularly in humans, oocyte fertility is reduced in aging. This is in part due to deterioration of meiotic spindle integrity. Spindle microtubules lose their ability to accurately interact with meiotic chromosomes and separate them, thus causing aneuploidies. The deterioration of the spindle can be attributed to the reduced activity of enzymes that are responsible for centrosome and microtubule maintenance [22, 35]. Centrosome defects have also been proposed as a possible explanation for the age-related decline of stem cell division [36].

The myelin sheath is crucial to adult neuron performance. Unfortunately, even healthy aging is accompanied by the emergence of defects in myelin composition and structure [37–39]. Thus maintenance mechanisms need to be activated to protect the axons. In the case of CNS oligodendrocytes, there is evidence indicating that the cytoskeleton is a fundamental constituent of these processes. Septins, a family of cytoskeleton associated scaffold proteins, have been shown to form filaments along with anillin in mice that support the myelin sheath and loss of these proteins leads to defects in myelin structure [40]. Additionally, it has been shown that de novo myelination pathways in the CNS remain active in adulthood through

new oligodendrocytes [41–43], the maturation of which is guided by cytoskeleton dynamics. This de novo myelination has been mostly associated with plasticity related adaptations but could also participate in maintenance.

In humans, aging is associated with increased aortic stiffness. This is often considered to preclude myocardial infarction, renal disease or even cognitive decline. A significant part of this aortic stiffness is attributed to vascular smooth muscle cells and particularly to their non-muscle actin cytoskeleton that is responsible for their connection to the extracellular matrix. Decoy peptides that inhibit actin polymerization or the interaction of the actin associated proteins talin and vinculin have been shown to be a potential method for counteracting aortic stiffness [44].

Another issue that emerges with human aging is the deterioration of heart health. Heart failure in particular is one of the most prominent causes of death and disability in the elderly [45]. Actin is critical to heart health, as actin fibers constitute a major component of sarcomers, the mechanical units that drive cardiomyocyte contraction [46]. Experiments in mice and rats have shown a conserved activation of actin remodeling by vinculin during aging. Further experiments in Drosophila have suggested that this is an anti-aging mechanism that improves heart function and overall organismal lifespan [47]. Actin is also relevant to heart health due to its association with the proliferative capacity of cardiac fibroblasts. Aging fibroblasts exhibit reduced levels of the LOX-1 receptor, lose their proliferative capacity and exhibit a disorganized actin network. Restoration of LOX-1 levels re-establishes fibroblast proliferative potential and reinstates actin organization [48].

2.2 Cytoskeleton and Neurodegeneration

Considering the prominent presence and important functionality of cytoskeletal proteins in neurons, it comes as no surprise that they have also been heavily implicated over the years in processes underlying their dysfunction. Below, we discuss experimental data that connects the cytoskeleton to neurodegenerative diseases, as well as injury induced neurodegeneration.

2.2.1 Tau Associated Pathologies

Alzheimer's disease (AD) is characterized by extracellular deposits of Aβ peptides and intracellular filamentous aggregates of Tau, a major microtubule associated protein [49–52]. Beyond AD, Tau aggregation has emerged as a common form of phenomenon in more than 20 different types of neurological disease, including Pick's disease, progressive supranuclear palsy, chronic traumatic encelopathy, argyrophilic grain disease, frontotemporal dementia with parkinsonism-17, corticobasal degeneration and Parkinson's disease (PD) [49, 53]. In the human brain, Tau has six isoforms with either 3 or 4 microtubule binding repeats at its C-terminal domain (3R and 4R Tau respectively) [49, 54, 55]. The protein is typically a dipole but

post-translational modification, especially phosphorylation, can affect its charges and disrupt its ability to bind microtubules [56–58]. In addition to its microtubule binding abilities, it has been shown to interact with the plasma membrane [59]. It is also capable of interacting with actin, induce its polymerization and promote microtubule and actin co-alignment [60].

Tau assembles into filaments through its repeats forming a cross-beta structure. Thus, the microtubule binding regions are trapped in the core of the aggregate, rendering physiological interaction with microtubules impossible [61–64]. Tau aggregates are commonly referred to as Neurofibrillary Tangles (NFTs), but their actual morphology can vary across different diseases, leading to their sub-characterization into paired helical filaments (PHFs), straight filaments (SFs) and twisted ribbon-like lilaments (TRFs) [49, 51]. Tau is abnormally hyperphosphorylated in all of its aggregates. This has led to the belief that phosphorylation is toxic and induces Tau aggregation. However, this might not be the case as human tauopathies have not been linked to defects in kinases or phosphatases, and kinase inhibition has not been shown to be an effective treatment option [49, 51]. Furthermore, there is evidence of Tau phosphorylation acting in a benign fashion in the process of hibernation [65, 66], without fibril formation and with reversibility.

There are several possible explanations on the causes of Tau associated neuropathology; Tau aggregation could lead to an effective LoF phenotype by preventing the protein form exercising its normal roles [67]. For instance loss of Tau in mouse models of AD (over-expressing mutant APP, the precursor of the Aβ peptide) aggravated neurodegeneration and exhibited axonal swellings full of cellular debris and mislocalized organelles, vesicles and even presynaptic terminal components [68]. In addition, Tau KO mice exhibit intracellular iron accumulation, substantia nigra neurodegeneration, brain atrophy and parkinsonism. Supplementation with an iron chelator rescued this phenotype. These observations were attributed to reduced transport of APP onto the neuronal membrane (APP in conjunction with ferroportin acts as the sole iron export system in neurons) due to the altered microtubule dynamics that arise from lack of Tau [69]. Another indication supporting this idea is the observation that microtubule stabilizing drug treatment has had some effectiveness in ameliorating tauopathy [70–72]. An alternate explanation could be that Tau (normal, mutant and/or phosphorylated) represents a toxic threat to cells in a gain of function (GoF) fashion. The protein has, for instance, been implicated in the disruption of mitochondria through the induction of mitochondrial fusion, inhibition of mitophagy and a reduction of ATP production [73, 74]. There are indications suggesting that a GoF threat might arise from non-filamentous forms of Tau [51], as experiments have demonstrated that truncated/cleaved Tau can be toxic [75, 76]. In addition, neurodegeneration can occur before or without Tau filament formation [77, 78] and tangle formation can persist in rescued animal models [79]. In the latter case, NFT formation might act as an attempt from the cell to quarantine dangerous Tau forms. Arguably, it is possible that both explanations are true on a disease by disease basis, or even simultaneously, with aggregation acting as the “lesser evil” that initially protects neuronal cells from toxicity but eventually ends up being deleterious through dysregulation of the cytoskeleton or other effects.

The aforementioned Tau-actin interaction [60] might have a functional implication in neurodegenerative disease, as experiments in Drosophila melanogaster and have shown that mutant forms of Tau associated with human tauopathies are capable of inducing the formation of actin rich structures resembling Hirano bodies (actin aggregates that occur in human patients). Actin was necessary for Tau toxicity in these instances. Tau phosphorylation, as well as transgenic Aβ42 expression, exacerbated actin aggregation and neuronal death [80]. Recently it was reported that tau can accumulate and form tangles in the medial temporal lobe and particularly in the entorhinal cortex as a pure consequence of normal "healthy" aging indicating a possible mechanism for the aging-associated loss of episodic memory [81].

2.2.2 Other Microtubule Associated Pathologies

The implication of microtubules in neurodegenerative disease extends beyond the role of Tau. Part of the neurotoxicity in Huntington's disease (HD) can be attributed to defects in microtubule based axonal transport, and MT stabilizing acetylation is potentially beneficial [82]. Very similar observations have been made in a model of Charcot-Marie-Tooth disease (CMT) [83]. Experiments in a PD model have shown that intracellular transport could be disrupted due to the reduction of microtubule dynamics, and that this might preclude mitochondrial damage and caspase 3 activation [84]. Disrupted mitochondrial dynamics, along with reduced levels of MAP expression, can also be observed in amyotrophic lateral sclerosis (ALS) patients and models, and pharmacological MT stabilization can delay the progression of the disease in mice [85–87].

2.2.3 Actin Associated Pathologies

ALS is a neurodegenerative disorder associated with the loss of motor neurons in the cerebral cortex, the brainstem, and the ventral horn of the spinal cord [88]. The disease is mainly linked with alterations in genes such as superoxide dismutase 1 (SOD1), fused in sarcoma (FUS) and TAR DNA binding protein (TARDBP / TDP-43) [89]. Spinal muscular atrophy (SMA) is a disorder with phenotypical similarity to ALS that exhibits motor neuron loss exclusively in the ventral horn of the spinal cord [88]. SMA is attributed to loss of function (LoF) of the survival of motor neuron 1 gene (SMN1) [90]. Both ALS and SMA have been linked with altered cytoskeletal dynamics or mutations in known regulators of the cytoskeleton [91–97]. Notably, the actin regulators profilins have been implicated in both diseases [93, 96–98]. Profilins are a family of proteins that can bind monomeric G actin and facilitate the exchange of ADP for ATP. Depending on the cellular conditions, profilins have been suggested to act as either a promoter of actin polymerization and F-actin stabilizer, or as a sequester of G-actin and F-actin destabilizer [88, 99]. Profilin binding activity can be inhibited through phosphorylation by the RhoA kinase (ROCK), an important regulator of actin dynamics [100, 101]. It has been

shown that SMN1 binding to profilin 2 reduces its inhibitory effects and promotes actin polymerization [93]. It has also been suggested that this binding protects profilin from ROCK phosphorylation and that the source of cytoskeletal defects in SMA is the loss of this protection [88]. In ALS, profilin 1 has been suggested to contribute to disease pathology through the formation of TDP-43 associated aggregates [97, 102, 103], through loss of its ability to interact with stress granules [104], or through dysregulation of actin dynamics [105, 106]. It is worth mentioning that profilin has also been shown to interact with the polyglutamate protein Huntingtin and inhibit its aggregation. The prevention of profilin inhibition by ROCK has also been demonstrated as a potential therapeutic approach for HD [101]. Beyond its aforementioned potential association with Tau, another connection of actin with AD pathology was revealed recently. The actin cytoskeleton was shown to be compromised in transgenic mouse models early in disease progression in conjunction with dendritic spine effects and a decline of AMPA signaling [107].

Microglia can act as a line of defense against AD by migrating towards extracellular Aβ42 aggregates, binding them and phagocytosing them. However during aging, Nogo/Ngr signaling reduces the ability of microglia to migrate and adhere to Aβ42 through Rho-GTPases that regulate actin dynamics and end up preventing protrusion extension and cell polarization [108]. On the other hand, the cytoskeleton might also have an inhibitory role in this interaction, as it has been reported that cytosolic phospholipase A2 (cPLA2), a factor that mediates the Aβ-induced response in glial cells, acts to reduce the cytoskeletal-membrane connectivity that represents a physical barrier against Aβ endocytosis [109].

Alterations in actin dynamics may also play a role in PD, as a-syn has been shown to inhibit cofilin, an actin destabilizer, in experimental models and patients. This leads to actin overstabilization, with potential negative implications for synaptic signaling [110]. Cdc42 is a Rho-GTPase that is involved in the regulation of actin dynamics. Some variants of variants of CMT have been associated with a mutation in Frabin, the GTP exchange factor of Cdc42 [111, 112].

2.2.4 Neurofilament Associated Pathologies

Neurofilaments have also been associated with neurodegenerative disease. Abnormal neurofilament aggregation has been observed in various disorders, such as AD, PD, CMT and ALS. It seems to be connected with deviations from the exact correct NF component stoichiometry, as it can occur in response to both down-regulation or up-regulation of individual NF genes [113–115].

In AD, neurofilaments are another major component of Tau NFTs [116]. In these tangles, they adopt a paired helical filament conformation [117], and exhibit extensive levels of phosphorylation [118].

Neurofilaments are also a primary component of the Lewy bodies, the characteristic protein inclusions of PD [119, 120]. They are extensively phosphorylated in this instance as well [121]. Patient tissues exhibit down-regulation of NF-L and NF-H expression [122]. Mutations in the gene that codes NF-L have emerged as a

cause for CMT. These mutations lead to defects of axonal transport, neurofilament disorganization, and usually aggregation [123–131]. Some of the NF-L mutations that cause CMT lead to neurofilament aggregation due to the abolition of protective phosphorylation [129–132].

ALS is characterized by intraneuronalaxonal NF aggregation [133–135]. This is also the case in mice expressing mutant human SOD1, the gene mostly associated with familial cases of ALS [136]. This aggregation might be dispensable for the eventual progression of the disease [137] but its reduction might still be somewhat beneficial. Perhaps unexpectedly, overexpression of NF-H [138–140], or NF-L [138] or downregulation of NF-L [141] were all successful in imparting a partial protective effect that is attributed to a redirection of NF accumulation from the axon to the cell body/perikaryon. The exact mechanism of this protection is, however, uncertain.

2.2.5 Neurodegeneration Due to Injury

Injured axons of CNS neurons degenerate, in a process known as Wallerian degeneration. Fragmentation of microtubules is possibly the earliest step in this process [142]. Axons that are retracting due to injury exhibit a disorganized microtubule network [143]. In cases where axonal regeneration is possible (such as the peripheral nervous system), it is driven by microtubules and requires tubulin deacetylation, a modification that decreases their stability [144, 145]. The levels of expressed and axonally transported neurofilaments are also reduced, and are only restored in axons that can regenerate [146–154]. Microtubule destabilization accompanied by energy depletion precludes neurofilament defects, mitochondrial swelling and axonal degeneration. Artificial energy repletion is effective at stopping this process [155]. Dendrites also degenerate after injury. Experiments in D. Melanogaster showed that this requires microtubule severance by the ATPase fidgetin [156].

3 Conclusions

Despite decades of research, our knowledge on the cytoskeleton remains incomplete. There are still numerous questions that need to be addressed regarding cytoskeletal contributions to pathology. In this regard, the cytoskeleton represents a clear challenge for future research, and for the development of potential therapeutic strategies relevant to aging and neurodegeneration.

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Chapter 13
Metabolic Biomarkers in Aging and Anti-Aging Research

Paul C. Guest

1 Introduction

Human life expectancy has increased by approximately two-fold over the last 200 years, which has resulted in a significant increase in the proportion of elderly individuals in the population [1]. This increase in lifespan has been predicted to continue rising to reach an anticipated life expectancy of more than 85 years by the year 2030 for people in the developed world [2]. However, it comes as no surprise that advanced age is associated with a decline in physiological status, leading to an increase in age-related diseases [3]. Thus, the increase in life span is significantly associated with increased prevalence of diseases such as diabetes, metabolic disorders, cardiovascular disorders, cancer and neurodegenerative disorders [4]. Reducing the negative impacts of advanced age and increasing the human healthspan has therefore been an important goal of aging and anti-aging research throughout the world [5–7]. With this in mind, we are now beginning to understand the physiological mechanisms underlying aging and the next steps will be to identify and validate biomarkers and to target the underlying cellular and molecular determinants.

Aging can be defined as the time-dependent malfunctioning of molecular and physiological mechanisms in organisms, causing defects such as shortening of telomeres and increased production of damaging reactive oxygen species (ROS), which lead to increased senescence of cells and deterioration of the tissues as well as of the entire organism. Cellular senescence is one process by which cells cease to divide and this is thought to contribute to both tissue and organismal aging, and to be a

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protective factor against cancer and tumor cell proliferation [8, 9]. Senescent cells have been found to accumulate in various tissues in mice in an age dependent manner [10]. The presence of these cells in tissues can cause dysfunction of the surrounding cells due to release of pro-inflammatory factors. Thus, aging is characterized by systemic degeneration over time and interventions that counteract this degeneration are expected to augment both the healthspan and the lifespan.

Several differing types of aging interventions have been tested in experimental models as a means of extending healthspan and for prevention or slowing of age-related diseases. For example, caloric restriction has been shown to decrease age-related diseases in nonhuman primates [11]. In addition, clinical trials of calorie restriction over a 2 year period found evidence of reduced oxidative damage, suggesting that this approach could also reduce the risk of age-related diseases in humans [12]. As we are now unravelling the mechanisms of how aging occurs, a number of compounds are currently undergoing testing for effectiveness in slowing the aging process in preclinical studies. This includes sirtuin activators [13], mammalian target of rapamycin (mTOR) inhibitors [14] and mitochondrial inhibitors [15].

The mitochondria are now known to be important in regulation of the aging process [16]. The primary function of these organelles is to generate energy for the organism in the form of adenosine triphosphate (ATP) but they are also involved in other physiological processes which have links to aging, such as apoptosis, autophagy and production of reactive oxygen species (ROS). Reduced mitochondrial function and generation of declining amounts of ATP have been observed in various organs and tissues, including skeletal muscle [17], heart [18] and brain [19]. This age-related mitochondrial impairment can be seen at multiple levels including mitochondrial number and morphology, electron transport chain (ETC) activity and ROS formation [20].

This chapter reviews how the complex process of aging may be regulated at the physiological, cellular and molecular levels and how this information is being unravelled by research using model organisms. At present, the most promising results have come from studies of the molecular pathways involved with caloric restriction, insulin/insulin-like growth factor signalling and mitochondrial ROS production, in nematode, fly and rodent models.

2 Biomarkers of Aging in Caenorhabditis elegans

C. elegans is a popular model organism for aging and anti-aging studies due to its short lifespan, fully annotated genome and age-dependent physiological changes [21]. Early research found that one gene associated with aging was *DAF-2*, which encodes a homologue of the mammalian insulin/insulin-like growth factor (IGF) family [22, 23]. Decreased DAF-2 signaling leads to translocation of the fork-head transcription factor DAF-16 into the nucleus, and this leads to activation of numerous genes associated with stress response, lipid metabolism, immunity and longevity

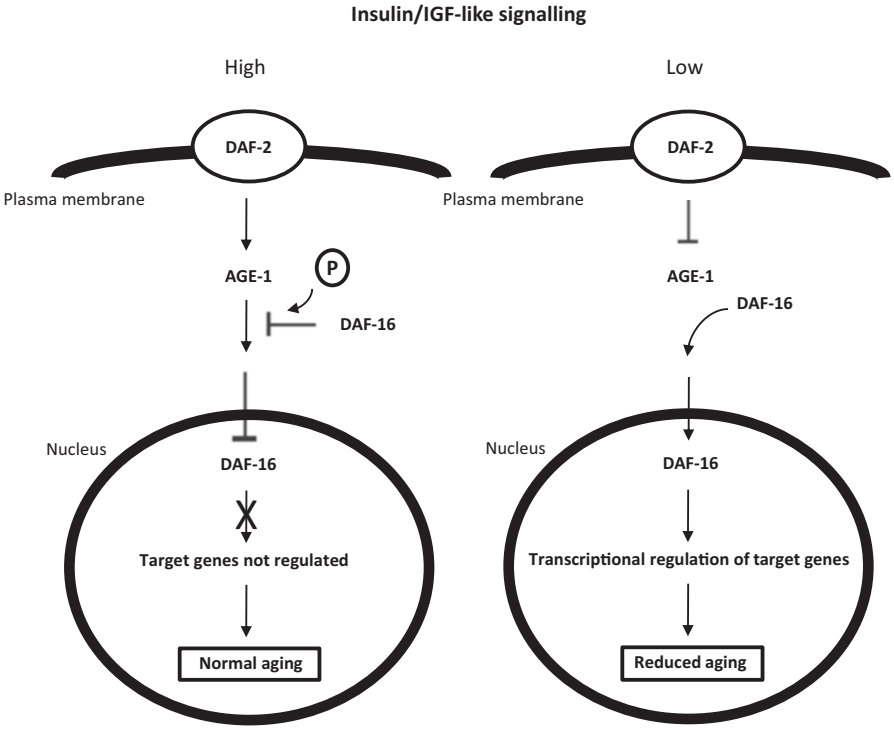

Fig. 13.1 Effects on aging via through the insulin/IGF-lik) in C. elegans. Under conditions of high levels of DAF-2 signalling, the transcription factor DAF-16 is phosphorylated and cannot enter the nucleus to activate target genes. Under conditions of low DAF-2 signalling, un-phosphorylated DAF-16 can enter the nucleus and turn on the target genes

(Fig. 13.1). The end result is a worm that can live twice as long as its natural counterparts [22]. There are also several models involving caloric restriction which lead to increased lifespan in C. elegans. EAT-2 mutant animals have dysfunctional pharynges, which results in decreased food intake along with a lifespan increase that is approximately 50% greater than wild type animals [24]. The effects of caloric restriction or impaired insulin/IGF-1-like signalling partially overlap in their downstream signalling processes, which include activation of pathways such as mitochondrial autophagy and inhibition of the mammalian target of rapamycin (mTOR) [23].

Other studies have linked caloric restriction to an improved oxidative stress response, which is mediated by the oxidoreductase enzyme thioredoxin 1 [25]. Another research group identified four genes that extend lifespan specifically in DAF-16 mutants but not in the EAT-2 mutants. These were the genes encoding S-adenosyl methionine synthetase (*SAMS*), Rab-like GTPase (*RAB-10*), dietary restriction response of unknown function (*DRR-1*) and a putative RNA-binding protein (*DRR-2*) [26].

As indicated above, the insulin/insulin-like growth factor-1 (ins/IGF-1) signalling pathway is involved in regulation of longevity and resistance to oxidative stress in C. elegans [27–29]. This is achieved via regulation of the downstream DAF-16 transcription factor [30, 31], which targets genes associated with these pathways during aging of long-lived C. elegans mutants (Fig. 13.1) [32, 33]. Under high insulin/IGF-like signalling conditions, such as following a meal high in fats and sugars, DAF-16 is phosphorylated and cannot enter the nucleus to activate target genes. Under conditions of low insulin/IGF-like signalling, un-phosphorylated DAF-16 can enter the nucleus and turn on the target genes. Intersetingly, a number of these studies have found increased ATP concentrations with reduced insulin/IGF-1 signalling and lower respiratory rates [34–38]. In addition, intracellular ROS are removed more efficiently under these conditions due to the higher activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase [34, 39].

The C. elegans life cycle consists of larval, dauer larval and adult stages. In the case of the dauer state, no feeding occurs [40, 41]. During the normal larval stages (L2-L4), cell growth and proliferation are driven by the tricarboxylic acid (TCA) cycle and young adult worms have a high tolerance to anoxia and protection against ROS [42, 43]. Significant decreases in oxygen consumption and metabolic rate have been seen in normal worms after these worms reach adulthood, consistent with decay in muscle function [44–46]. A recent study showed that expression of the cyclooxygenase (COX) assembly protein [mammalian gene homolog sco-1 (*SCO2*)] gene was increased with aging in wild type worms [47], suggesting that mitochondrial components are damaged by ROS during the aging process, inducing a shift from the TCA cycle to aerobic glycolysis. Conversely, the finding of an age-related increase in the levels of phosphoenolpyruvate carboxykinase (PEPCK) induced by calorie restriction in wild type C. elegans, indicates a shift in the balance to gluconeogenesis [47]. PEPCK plays an important role in energy production throughout various life stages of the worm and other invertebrates. The C. elegans PEPCK enzyme is involved in regulation of metabolism associated with cataplerosis, which is the removal of intermediate metabolites from gluconeogenesis and other pathways in anaerobic environments [48, 49]. Yanase et al. found that the upregulation of gluconeogenesis during aging in C. elegans was associated with reduced mitochondrial respiration and increased expression of PEPCK and the NAD-dependent protein deacetylase sir-2.1 [47, 50].

In C. elegans, exposure to the hyperoxia accelerates senescence so that the levels of intracellular ROS increase [51–53]. Mutations of the C. elegans *MEV-1* gene, encoding the large subunit of the cytochrome b succinate dehydrogenase, result in increased production of the mitochondrial superoxide anion (O2-), resulting in a shortened lifespan [54]. Likewise, the levels of cellular metabolites such as lactate and pyruvate are correlated with the switch from mitochondrial respiration to glycolysis during aging [47, 55]. These findings indicate that the cytochrome b succinate dehydrogenase plays an important role in energy metabolism as well as in superoxide anion production that is involved in sensitivity to atmospheric oxygen. Thus, further studies of the physiological and molecular changes in the *MEV-1* mutants might help to elucidate the pathological mechanisms of aging.

3 The Role of the Mitochondria in Aging

Mitochondria are the main site of energy production in most eukaryotic cells. This is where the processes of glycolysis and beta-oxidation of lipids occur in the process of generating ATP. This occurs via reduction–oxidation (redox) reactions along the oxidative phosphorylation (OXPHOS) complexes within the inner mitochondrial membrane (Fig. 13.2a) [56]. This is also one of the principal sites of aging as the progressive accumulation of cell damage has been proposed to be due to over-production of reactive oxygen species (ROS) (Fig. 13.2b) [57, 58]. Catalase is a peroxisomal enzyme which works together with mitochondrial glutathione peroxidise in antioxidant reactions preventing against ROS formation [59]. Studies in mice have shown that targeting of catalase to the mitochondria can result in reduced ROS damage and increased lifespan [60], along with an improvement in exercise performance [61]. With these results in mind, mitochondria-targeted catalase gene therapy has been proposed as a potential co-treatment approach in cases of Duchenne muscle dystrophy, in which the muscle tissues have high levels of ROS production [62]. This approach may also prove useful to slow the effects of sarcopenia, characterized by loss of muscle mass, which can occur during aging. Superoxide SOD2 is

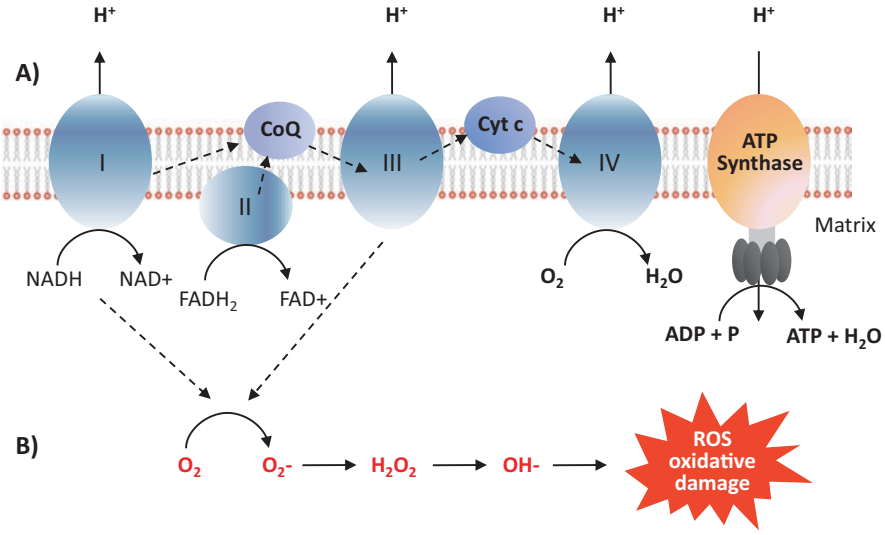

Fig. 13.2 (**a**) Generation of ATP via reduction–oxidation (redox) reactions along the oxidative phosphorylation (OXPHOS) complexes within the inner mitochondrial membrane. (**b**) Reactive oxygen species (ROS) production via the electron transport chain. Anions are produced at complexes I and III producing superoxide (O_2-). Hydrogen peroxide (H_2O_2) is converted into the hydroxyl radical (OH−). Superoxide and the hydroxyl radical are ROS that cause oxidative stress to macromolecules and organelles. Arrows with dashed lines indicate the direction of electron transfer. CoQ coenzyme Q, Cyt c cytochrome c, NAD+ oxidized nicotinamide adenine dinucleotide, NADH reduced nicotinamide adenine dinucleotide, FAD oxidized flavin adenine dinucleotide, $FADH_2$ reduced flavin adenine dinucleotide

a mitochondrial enzyme that converts superoxide species to H_2O_2 and O_2 [63]. In a heterozygous SOD2 ($SOD2^{+/-}$) knockout model, aged mice had significantly increased oxidative stress in their smooth muscle cells, resulting in a pathological stiffness, similar to that which occurs in atherosclerotic plaque formation during aging [64]. In a human study, suboptimal brain aging was found in subjects with a specific SOD2 variant [65]. These findings indicate an essential role of SOD2 in prevention against oxidative damage.

Coenzyme Q is a component of the mitochondrial electron transport that functions as an electron transporter between oxidative phosphorylation complexes, leading to ATP synthesis and it also serves as an antioxidant factor [66]. Given this, coenzyme Q supplementation has been tested with some success as a potential treatment of a number of disorders, such as cardiovascular disease, metabolic syndrome, neurodegenerative diseases and inflammation [67]. It has also been shown to prevent oxidative stress in a senescence-accelerated mouse model and in aged mice and rats [68, 69]. Along the same lines, the mitochondria-targeted form of coenzyme Q (MitoQ) was found to reduce cognitive decline, oxidative stress and loss of synapses in a mouse model of Alzheimer's disease [70] and to extend the lifespan of a C. elegans Alzheimer's disease model [71].

Caloric restriction appears to work by lowering mitochondrial O_2 consumption, leading to reduced generation of damaging ROS. This has been linked to changes in the mammalian target of rapamycin (mTOR) and sirtuin pathways [72]. Inhibition of mTOR has been found to extend lifespan in multiple species [73–76]. Sirt1 (the mammalian orthologue of Sir2) has been associated with neuroprotection [77], reduction of fat storage [78, 79] and insulin secretion from pancreatic-β cells [80]. Sirt1 increases expression of genes involved in fatty acid oxidation in response to low glucose, thereby providing a switch from glucose to a fatty acid oxidation metabolism under low caloric conditions [81, 82]. Some dietary activators of Sirt1 have been identified such as resveratrol and melatonin [83]. Another sirtuin family member (SIRT3) is thought to be involved in increasing the mitochondrial glutathione antioxidant defense system under caloric restriction conditions [84, 85]. One study of a mouse model lacking the p66 adapter protein found that the resulting increase in lifespan might be linked to improved metabolic homeostasis via regulation of Sirt3 activity [86]. Conversely, a Sirt3 knockout mouse ($Sirt3^{-/-}$) model showed increased oxidative stress and mitochondrial protein dysfunction [87].

Caloric restriction has also been shown to reduce the incidence of metabolic disease, cancer and brain atrophy, as well as all-cause mortality in non-human primate species [88, 89]. For example, a two- year caloric restriction study of healthy individuals found that this diet led to enhanced resting energy efficiency and lower systemic oxidative damage, compared to the effects seen for a control group on a normal caloric diet [12]. Another study of healthy elderly subjects found that a caloric restriction diet improved memory performance [90].

In summary, caloric restriction in a number of species has been shown to counteract age-related decline and increase lifespan by inducing a shift from carbohydrate to fatty acid metabolism, enhancing mitochondrial energy production and activating antioxidant defence mechanisms.

4 Redox Stress and Aging

The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is an essential component in the synthesis of fatty acids, cholesterol and deoxynucleotides as well as being a protective factor against redox stress. The levels of NADPH decrease with age [91, 92] due to oxidative stress resulting from the effects of accumulated mitochondrial electron transport chain dysfunction and inflammation [93, 94]. The mitochondrial theory of aging suggests that aging is associated with the accumulation of damage from increased mitochondrial ROS [58]. The redox theories of aging have proposed the idea that aging results from changes in the redox balance of molecules such as the oxidized and reduced forms of NADP (NADP+/NADPH) and glutathione (GSSG/GSH), as well as by changes in cell signalling [95, 96].

A number of studies have found that the rate of mitochondrial superoxide generation and phospholipid fatty acid saturation levels is negatively-correlated with lifespan in various species [97, 98]. The mitochondrial inner membrane is enriched in certain phospholipids essential for electron transport chain and ADP/ATP transport functions and these phospholipids are vulnerable to damage by ROS [99]. The loss of NAD+ and NADPH appears to be a factor in the aging of Drosophila melanogaster and C. Elegans [100, 101] and addition of NAD+ [102] or nicotinamide riboside [103] to the culture media has been found to extend lifespan. The decreased levels of NAD+ in aged mouse muscle leads to stabilization of the hypoxia inducible factor-1α (HIF-1α) and decreased c-Myc-induced expression of mitochondrial genes involved in electron transport chain function [104]. This leads to increased mitochondrial NADH and decreased NAD+ levels, as well as a reduction of the proton-motive force across the inner mitochondrial membrane. In turn, this would lead to decreased levels of glutathione reductase and, therefore, increased GSSG/GSH ratios. The increased oxidation within these pathways also results in oxidation of other redox-related molecules [105], thereby leading to oxidation of lipids, proteins and nucleic acids, culminating in the tissue dysfunction associated with the aging process.

Redox stress also appears to be associated with most age-related disorders such as diabetes and cardiovascular conditions. The NADP+/NADPH ratio is the strongest known redox determinant in age-induced oxidation with redox potentials ranging from −400 to −20 mV [106] and this ratio shifts to a more oxidized state with aging [107]. Quantification of oxidative stress in model systems can be performed by measurement of the GSSG/GSH ratio and the oxidation state can be determined in intact cells using the genetically encoded fluorescent probes, such as roGFP or roGFP2 [108]. However, these studies have demonstrated that both oxidizing and reducing changes can affect aging and longevity [109]. These findings suggest that redox measurements in the cytoplasm and mitochondria are important. In C. elegans, Reduced function of the NADPH-generating enzymes in C. elegans has been found to both increase and decrease longevity, most likely due to activation or inhibition of different compensation pathways. Reduction of the cytoplasmic NADP+/NADPH ratio has mostly resulted in increased longevity but this can also result in

reductive stress leading to mitochondrial oxidation and increased ROS generation. Thus, considerable further work is required in this area to fully elucidate the role of the redox potential in aging and longevity.

5 I'm Not Dead Yet (INDY)

INDY is a non-electrogenic solute transporter that transports di- and tri-carboxylates across the plasma membrane, as described in studies of D. melanogaster [110]. Reduced expression of INDY has been found to enhance longevity in a way that is similar to the effects of calorie restriction [111, 112]. Knockout of the mammalian homologue of INDY (the sodium-coupled citrate transporter; NaCT) led to protection from obesity and insulin resistance and this effect was found to be mediated by altered mitochondrial metabolism and reduced hepatic lipid generation [113]. Citrate is a vital metabolite that links multiple metabolic pathways such as glycolysis, gluconeogenesis and lipid synthesis [114–118]. Citrate is also an intermediate involved in the TCA pathway, which leads to generation of energy in the form of ATP. Transcription of INDY is regulated by the nutritional status. Calorie restriction was found to reduce expression of INDY in D. melanogaster [119, 120] and of the INDY homologue in mice [113]. On the other hand, administration of large amounts of olive oil increased INDY expression in rats [121]. Other studies have shown that regulation of INDY may occur via epigenetic mechanisms [122–124].

Mutations in the INDY gene are associated with lower levels of body fat, reduced circulating insulin-related proteins and decreased ROS and lifespan extension [111, 119, 125]. Genetic deletion or pharmacological inhibition of INDY in model organisms has been found to reduce the effects of a number of metabolic conditions such as non-alcoholic fatty liver disease (NAFLD), obesity and insulin resistance [113, 126–128]. For this reason, INDY is considered a potential drug target for metabolic diseases [129]. For example, it has been shown that knockdown of INDY expression using a liver-selective siRNA approach resulted in improved insulin sensitivity and reduced triglyceride accumulation [130]. Future studies are required to determine the effectiveness of INDY-directed compounds in the treatment of metabolic diseases and other diseases affected by metabolic disturbances including diabetes, obesity and cardiovascular disorders, as well as neurodegenerative and psychiatric disorders [131]. Ultimately, such compounds should be investigated to determine whether or not they promote healthier aging and increased longevity.

6 Obesity and Aging

Obesity has now become a global epidemic with a prevalence that has tripled over the last 30 years [132]. Obesity can increase the risk of numerous disorders such as diabetes, cardiovascular diseases and cancer, thereby increasing the mortality rate

[133–135]. To further our understanding of the effects of obesity, and to identify novel therapeutic approaches, a number of epidemiological approaches have been undertaken including population-based studies, case-control and clinical trials, which have to identification of risk factors, metabolic impacts and potential new treatments [136, 137]. However, such human-based studies are limited by factors such as underreporting and difficulty of discerning the impact of specific components of diets [138, 139]. As an alternative, animal-based studies can be more carefully controlled and these also allow the analysis of different tissues for determination of metabolic and molecular effects [140]. In addition, the pathways that regulate energy balance linked with weight control are highly conserved across the animal kingdom.

There are different types of high fat diets, such as those including 30–85% of the calories coming from fats [141], and these produce physiological effects such as obesity and insulin resistance [139, 142]. Weight gain is commonly used as a simple biomarker for monitoring the outcome of the diet or for determining the effects of an intervention, although body fat composition can give more precise information [139]. For example, one study found that administration of a 40% fat diet for 10 weeks caused rats to gain 10% in body weight but 35–40% in body fat [143]. In addition, the types of dietary fat can be important as several studies have now shown that lard-based diets are more obesogenic compared to oil-based diets [142, 144, 145].

Other studies have used a cafeteria-based diet approach as this closely mimics the "Western diet" [146]. Cafeteria-based foods include biscuits, cheese, processed meats, cakes, chocolate and peanut butter, as examples [147], which tend to induce hyperphagia [148, 149]. Therefore, compared with the high fat diet, the cafeteria diet can induce higher weight and abdominal fat gains, thereby inducing more damage to tissues such as the heart and liver, as well as leading to inflammation, hyperinsulinemia, hyperglycemia and glucose intolerance [150, 151]. Furthermore, a diet that combines both sugar and fats may be more efficient in eliciting metabolic changes and obesity in comparison to high fat diets [152]. The obesogenic effect of the high sugar/high fat diet may be due to the increased levels of saturated fatty acids that are less available as an energy source but instead are acetylated into triacylglycerol and stored in adipose tissue at increased levels [145, 153]. This is compounded by the insulinogenic effect of the rapidly absorbed simple sugars, resulting in a rapid decrease in blood sugar levels [154]. This triggers a neurochemical craving response similar to that seen in cases of addiction [155]. A model of the high sugar high fat diet which incorporates fructose and sweetened condensed milk as a source of sugar and beef tallow as a source of fat resulted in a greater body weight and abdominal fat gain, compared to controls along with induction of metabolic syndrome, and changes in the function of organs, such as heart, liver, and kidneys [156].

Taken together, these findings indicate that increasing our understanding of how obesity can alter cellular physiology and metabolic function could open new therapeutic avenues to extend the period of healthy aging.

7 Conclusions and Future Perspectives

Results from epidemiological studies have shown that most of the healthcare costs in developing countries are accounted for by age-related disorders and these costs are expected to increase along with the increasing proportion of the elderly population in developed countries. This is mainly due to the fact that the increase in average life expectancy has not been paralleled by a corresponding increase in healthspan [157]. Thus, considerable research has been underway to understand the process of healthy aging at the physiological and molecular levels. Results from preclinical models and data from human studies suggest that insulin/IGF signalling and efficiency of mitochondrial energy production are key regulators of this process. In addition, studies of these pathways have provided both rationales and potential drug targets for therapeutic interventions. A number of investigations along these lines have already been completed in animal models with the aim of finding a way of slowing the aging process and extending the human healthspan. Interventions such as caloric restriction and exercise, and administration of nutritional compounds and drugs such as antioxidants, omega-3 fatty acids, metformin and aspirin, target the mitochondria to delay or counteract the effects of aging [158]. Many of these approaches have shown early promise and have led to the identification of key biomarkers that can be used for monitoring the effects of aging as well as the efficacy of emerging anti-aging interventions. It is likely that such aging interventions will also delay the development of chronic diseases and thereby extend both the healthspan and the lifespan.

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