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Part III Cellular Aging and Lower Animal Models

“5. Cellular aging and metabolites in aging”

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Abstract

Diversity is observed in the wave of global aging, as aging is a complex biological process exhibiting individual variability. To assess aging physiologically, markers for biological aging is required in addition to the calendar age. The discovery of replicative senescence by Hayflick developed into telomere research, while stress-induced senescence (SIS) is known as a telomere-independent event. SIS serves as physiological barrier to oncogenesis *in vivo*, while it activates senescence-associated secretory phenotype (SASP), inducing chronic inflammation. Thus, beside telomere length, p16, p53 and inflammatory cytokines have been utilized as biomarkers for cellular senescence. From a metabolic perspective, the aging hypothesis includes the mitochondrial and the calorie restriction (CR) hypothesis. However, little is known whether CR is applicable to human longevity, as human aging is greatly affected by a variety of factors. Comprehensive analysis of the human blood metabolome captures complex changes with individual difference, detecting metabolites for aging or aging-relevant conditions. These information are valuable for future clinical applications in diseases relevant to aging.

Keywords

Telomere, stress-induced senescence, SASP, metabolites, frailty, fasting, antioxidant

Introduction

Currently, Japan is the only country in the world with a societal aging rate (% of population over 65 yr old) exceeding 25% (global average = 12.38%) (ONU, 2015). However, a wave of global aging is steadily approaching, and the global average is expected to reach 23.4% by around 2050. Indeed, many European and Asian countries will reach aging rates of more than 25%, while Japan is expected to exceed 42.5% by that time (ONU, 2015).

In such super-aging society, we observe greater “diversity of aging”. As the number of healthy elderly people increases, the number of bedridden patients and people who need long-term nursing care also increases. Because “diversity of aging” reflects individual variability, clinical symptoms among the elderly are also diverse. Thus, “aging” is multi-faceted and complex, and variability is characteristic of aging.

Calendar age has commonly been used as an indicator of human aging. Calendar age defines the elderly as over 60 or 65 years old, proposed by the United Nations in 1956. In addition to calendar age, the importance of “biological age” has also been proposed, mainly based on findings in basic aging research, as we are now facing with diversification of the elderly in globally aging society.

As redefinition of “elderly” itself is becoming necessary (proposal of the Japanese Geriatrics Society in 2017), here we overview the progress both in the cellular aging and the metabolomic aspects of ageing.

Historical theory and replicative senescence

In 1882, August Weismann proposed the “wear and tear” theory as the earliest ageing

model (Kirkwood and Cremer, 1982). In this theory, the major cause of organismal ageing is speculated to be accumulated damage due to stresses, followed by functional exhaustion of organs and cells; during juvenile stages, the ability to recover from damage is preserved, which gradually declines and vanishes in adulthood.

Weismann also presented the first experimental evidence of germ cell immortality (Weismann, 1892). First, he cut off the tails of young mice before they achieved sexual maturity. When later bred, the tails of their offspring were perfectly normal. He repeated this process for twenty-two generations, and observed that the acquired tail abnormality was never inherited. He drew the conclusion that the modulated properties in somatic cells *in vivo* were never inherited due to their definite lifespans, while the genetic properties in reproductive germ cells are transmitted from generation to generation due to their potential immortality. Terminally-differentiated somatic cells *in vivo* could be vulnerable to various stresses and eventually enter senescence state, during ageing. In 1891, Weismann proposed that once somatic cells are isolated from the body, they do not senesce.

When cell culturing *in vitro* became feasible, Carrel and Ebeling tested Weismann's idea. They reported that fibroblasts from fetal chicken hearts could grow on laboratory glassware for at least 34 years under certain conditions (Carrel and Ebeling, 1921),

supporting Weismann's hypothesis. However, their experiments proved to be irreproducible because their primary cell cultures became contaminated by unnoticed cells in the culture medium, which was changed once a week. This created the appearance of continuous cell growth for several decades (Witkowski, 1980).

Later, Hayflick also addressed the question of whether cells senesce *in vitro*. He observed that during serial culturing *in vitro*, human primary fibroblasts divided a certain number of times, but eventually stopped dividing. This phenomenon became known as the Hayflick limit or "replicative senescence" (Hayflick, 1965).

Telomere-dependent and -independent senescence

Around the same time, James D. Watson, who discovered the DNA double helix, predicted that genomic DNA is shortened during chromosome replication (Wellinger, 2014). He proposed that the ends of genomic DNA are not replicated during cell division, since the machinery for DNA replication progresses only in the 5' to 3' direction on the "antiparallel" DNA strand. This notion regarding the behavior of chromosome end structure led to the concept of "telomeres".

In 1986, Cooke et al. reported that the ends of sex chromosomes are shortened in senescent human somatic cells, consistent with Watson's idea (Cooke and Smith, 1986).

Strikingly, repetitive "TTAGGG" nucleotide sequences were found in the ends of

chromosomes (Blackburn and Gall, 1978). Telomeric sequences become shorter as cells undergo senescence, implying that the Hayflick limit would be linked to shortening of telomeres. Later, telomerase was identified, which functions for maintenance of telomere length by adding a specific DNA sequence to telomeres (Greider and Blackburn, 1985). Finally, Shay and Wright reported that ectopic expression of telomerase immortalizes human primary cells (Bodnar et al., 1998), providing definite evidence that telomere length is the causal factor for cellular senescence. Thus, telomeres have come to be called "biological clocks" or "tickets for ageing."

Identification of telomere raises another question. Telomere length varies greatly, depending on the species; 25-50 kb for mice and 10-15 kb for humans (Whittemore et al., 2019). However, the *in vitro* lifespan of rodent primary cells is much less than that of human cells, indicating that telomere shortening is not the only factor for limiting lifespan. Indeed, even in primary cells with sufficient telomere length, cellular senescence is induced by stress due to harmful substances and by environmental factors, such as DNA damage, acid, oxidative stress, histone deacetylase inhibitors, culture stress, etc. These telomere-independent factors collectively cause stress-induced senescence (SIS) (Sherr and DePinho, 2000).

Ras-val12 is a point mutation often observed in cancer cells. Manuel Serrano et al. noticed

that in sharp contrast to normal cells, the arrested primary cells by the introduction of Ras-val12 displayed a “fried egg” morphology with a huge nucleus and enlarged cytoplasm, resembling senescent cells. Consistently, Ras-val12 provoked premature senescence, called oncogene-induced senescence (OIS), a subtype of SIS (Serrano et al., 1997).

Double-edged sword of SIS

Cancer cells exhibit multiple oncogenic mutations. In the experimental settings, the combination of activated Ras-val12 and ablated tumor suppressor p53 renders the primary cells escape from senescence, like cancerous cells. The reasonable explanation for this senescence-bypassing event is that OIS, a subtype of SIS, is a biological protective mechanism that suppresses carcinogenesis by preventing cells with oncogenic mutations from progressing into cancer cells. However, its physiological impact *in vivo* was doubted when Ras-val12 knock-in mice were reported as cancer prone (Johnson et al., 2001). Indeed, both types of tumor, benign and malignant, coexist in Ras-val12 knock-in mice. Benign tumors consist of senescent cells, while senescent cells disappeared in malignant lesions (Collado et al., 2005). These findings *in vivo* are consistent with the *in vitro* inhibitory effect against cancerous immortalization by SIS or OIS.

Recently, a close association between SIS and chronic inflammation has been discovered. Senescent cells produce more inflammatory cytokines (e.g. IL-1 and IL-6) than non-senescent cells, a state defined as “senescence associated secretory phenotype” (SASP) (Coppe et al., 2008). SASP promotes senescence of neighboring cells. In such contexts, “chronic inflammation” provoked by cellular senescence has a significant influence on organismal ageing (Freund et al., 2010). Thus, SIS serves as “double-edged sword”; suppression of cancer *in vivo* vs promotion of deleterious chronic inflammation. Interestingly, Nuclear Factor- κ B (NF- κ B) was identified as a highly activated transcription factor during cellular senescence (Fig. 2B). NF- κ B activation induces anti-apoptosis genes, Bcl-2 and Xiap, followed by resistance of senescent cells against cell death. However, NF- κ B also activates inflammatory responses in senescent cells (Adler et al., 2007). Thus, “chronic inflammation due to cellular senescence (SASP)” exerts causal effect on the progression of age-related diseases.

Senescence markers

The irreversible cell cycle arrest with a metabolically active state is one of the prominent features of cellular senescence (Campisi, 2013). In senescent cells, the enzymatic activity

of cyclin-dependent kinase (CDK), a driver for cell cycle, is greatly diminished (Campisi and d'Adda di Fagagna, 2007). Comparative analysis of protein profiles between senescent and non-senescent cells identified CDK inhibitors, p21 and p16Ink4a protein, as senescence markers (Fig. 1). Thus, in senescent cells, accumulated p21 and p16 Ink4a inhibits cell cycle progression and causes cell cycle arrest.

The other several markers for cellular aging were also identified, in addition to telomere length and "fried egg" like morphology. Senescence-associated beta-galactosidase (SA- β -gal) staining efficiently detects senescent cells (Dimri et al., 1995). Senescent nuclei are frequently accompanied with phosphorylated histone 2AX variant (H2AX γ) foci as results of DNA damage and senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003). Accumulated lipofuscins are sometimes observed in senescent cytoplasm, representing aggregates of ubiquitinated proteins (Moreno-Garcia et al., 2018). These cellular senescence markers are useful device, to evaluate proliferative limits of senescence as an index. In addition, inflammatory cytokines are also recognized as markers of both senescence and chronic inflammation.

The aging hypothesis relevant to metabolic profiles

Oxidative stress has been proposed as a cause of aging and more recently as a factor involved in chronic inflammation. "Free radical theory" is one of the most popular aging hypotheses (Harman, 1956). Damage to macromolecules, including telomeric DNAs, caused by free radicals is known as "oxidative stress" (von Zglinicki, 2002). In addition, as more than 90% of free radicals has been attributed to mitochondria (Balaban et al., 2005), the "Mitochondrial Aging Hypothesis" was proposed (Harman, 1972) (Fig 2). Consistently, the genetically-manipulated mice with the extended life span, super Arf / p53 mice, displayed the increased levels of antioxidants, including glutathione (Matheu A et al 2007).

Calorie restriction (CR) by 20-30% extended life span by 20% or more in many model organisms, such as mice, flies, fish, spiders, etc (McCay CM, 1934). CR activates or modulates several signal modules, such as Tor kinase, AMPK kinase, Sirtuin, and FOXO transcription factor (Haigis and Guarente, 2006) (Onken and Driscoll, 2010) (Stanfel et al., 2009) (Fig 2). FOXO upregulates several radical scavengers. Consistently, oxidative stress is reduced during CR, indicating its antioxidative roles (Sohal et al., 1994).

Strikingly, these CR-relevant signaling pathways could be modulated by several metabolites, which are effective both for extension of lifespan in experimental models and for treatment of human aging-related diseases (Fig 2). Rapamycin inhibits the activity of Tor kinase, utilized as an immunosuppressor or anti-cancer drug, while metformin stimulates AMPK kinase for diabetic therapy (Mouchiroud et al., 2010). In addition to resveratrol, NAD⁺ also activates Sirtuins. The lifespan is extended by overexpression of adipose-tissue-specific Nampt, which catalyzes the biosynthesis of NAD⁺ (Yoshida M et al 2019). However, it is still controversial whether modulation of FOXO would be

efficacious in clinical applications, as FOXO overexpression compromises metabolic regulation in mice (Nakae et al., 2002).

Moreover, the mitochondrial aging hypothesis has been challenged, since partial impairment of mitochondrial functions provoked longevity in nematodes and fly (Houtkooper et al., 2013). Consistently, certain limited, but defined overload of mitochondrial oxidative stress prolonged lifespan in nematodes, known as “mitohormesis” (Yang and Hekimi, 2010). These opposing outcomes on oxidative stress and longevity cannot be ignored. In human clinical trials, supplementation with the antioxidants, beta-carotene, vitamin A, or vitamin E, exacerbated mortality (Bjelakovic et al., 2014). In sharp contrast to the success of "calorie restriction hypothesis" in experimental models, some epidemiological studies suggest that slightly overweight people live longer (Corrada et al., 2006). Compared to that in model organisms, aging research in human beings may require alternative approach, in part due to its variability and complexity.

Besides CR, supplementations of other metabolites are under examination as intervention approach against human aging. Clinical trial of NMN (nicotinamide mononucleotide), a precursor of NAD⁺, have been started (Irie J et al. 2020). The combination of exercise and supplementation for branched-chain amino acids improved muscle strength in aged frailty (Ikeda T et al 2016). Thus, metabolites themselves could be potentially one of promising strategies.

Metabolomic approach for human whole blood

Metabolites are defined as small organic compounds, produced by the metabolic activity in living organisms, from bacteria to humans. The metabolome detects and quantifies numerous metabolites with e.g., liquid chromatography-mass spectrometer

(LC-MS). Analysis of the metabolome (metabolomics) is a growing branch of chemistry, not only to search for biomarkers, but also to provide valuable metabolic information on physiology and pathophysiology.

Among other things, blood reflects physiological states *in vivo* affected by epigenetics, genetics, life-style, and health (Blackburn et al., 2015). Thus, metabolomics of human blood presents extensive evidence on metabolic aspects not only of physiological responses, health, aging, and diseases, and but also of biological effects of chemicals, nutrients, stressors, and environmental factors (van der Greef et al., 2013).

Blood contains cellular and non-cellular components: red blood cells (RBCs), white blood cells (WBCs), platelets, and plasma. Various investigations have been mainly conducted on plasma or serum, the non-cellular component (Srivastava, 2019). This is probably due to the difficulty in handling cell-derived metabolites, part of which are rather labile (Gil et al., 2015).

We have established novel method for analyzing whole blood, plasma, and RBCs. The whole blood metabolome includes about 130 metabolites, comprising over 14 subgroups (nucleotides, nucleosides, nucleobases, vitamins and coenzymes, nucleotide-sugar derivatives, sugar phosphates, sugar derivatives, choline and ethanolamine derivatives, organic acids, anti-oxidants, amino acids, methylated compounds, acetylated compounds, carnitines, etc.) (Chaleckis et al., 2014). These compounds reflect diverse cellular metabolisms; energy production, DNA and RNA synthesis, lipid metabolism, mitochondrial respiration, redox homeostasis, protein synthesis, and so on. Consequently, amounts of these compounds are influenced or regulated by various activities of tissues,

activation or inactivation, secretion or absorption, regeneration or degradation, contraction or relaxation, digestion or condensation, accumulation, or excretion, etc.

Among the ~130 compounds assessed by whole blood metabolomics, 50-60 are enriched in RBCs (Chaleckis et al., 2016). Based on the coefficient of variation (CV: SD divided by the mean), individual differences of metabolites are classified into two subgroups: those that are rather invariable (CV < 0.4) and others with large variability (CV >0.4 or higher). The former covers vitally essential metabolites (ATP, glutathione, phospho-sugars, etc), while the latter include dietary compounds such as caffeine, carnosine, ergothioneine, 4-aminobenzoate, and others (Chaleckis et al., 2016). Thus, whole blood metabolomics comprehensively captures complex changes with individual differences, and is useful for human aging research. Indeed, whole blood metabolomics have revealed metabolites that serve as biomarkers relevant to aging, fasting, and frailty (Chaleckis et al., 2016) (Teruya et al., 2019) (Kameda et al., 2020).

Blood metabolites for aging markers

Although targeted metabolomic analysis has identified aging-relevant compounds (Srivastava, 2019), a comparative, non-targeted analysis of the whole blood metabolome was carried out to compare healthy young and elderly people (Chaleckis et al., 2016). Among the 126 metabolites, there were statistically significant differences in 14 metabolites (11%) between young (29 ± 4 years old) and elderly people (81 ± 7 years old), which can be regarded as aging markers. Six of them are RBC-enriched, implying that whole blood metabolomics is an efficient device for human aging research.

Nine of the 14 metabolites decreased in the elderly, while five of them increased (Table 1). The former include 1,5-anhydroglucitol (1,5-AG), acetyl-carnosine, carnosine, ophthalmic acid (OA), leucine, isoleucine, nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate (NADP^+), and UDP-acetyl-glucosamine, while the latter comprise citrulline, pantothenate, dimethyl-guanosine, N-acetyl-arginine, and N6-acetyl-lysine.

1,5-AG, the 1-deoxy form of glucose, is derived from many foods. Circulating 1,5-AG is excreted from renal glomeruli, but reabsorbed through renal proximal tubules. As glucose is a competitive inhibitor against reabsorption of 1,5-AG, diabetic patients with high blood glucose levels show lower 1,5-AG levels. Thus, 1,5-AG is known as diabetes marker. However, levels of HbA1c and serum glucose, other diabetic parameters, are normal in both healthy young and elderly in this study; hence, it is possible that reabsorption of 1,5-AG in the kidneys is reduced with aging even in healthy people (Chaleckis et al., 2016). Carnosine and acetyl-carnosine are dipeptides with antioxidant properties, which are abundant in muscles and neurons (Park et al., 2005).

OA is a tripeptide analog of glutathione, and L- γ -glutamyl-L- α -aminobutyrylglycine is synthesized by the same enzyme (glutathione synthase) utilized for glutathione production. Because glutathione is one of the most abundant antioxidative compounds in cells, OA is known as an oxidative stress marker (Soga et al., 2006). NAD^+ and NADP^+ are coenzymes involved in various redox reactions. Leucine and isoleucine are essential amino acids involved in maintenance of skeletal muscle (Buse and Reid, 1975). Recent blood metabolomics study consistently identified reduction of essential amino acids in elderlies (Chen et al., 2020). UDP-acetyl-glucosamine is a substrate for glycosyl-based

modifications and is involved in synthesis of proteoglycans and glycolipids (Hirschberg and Snider, 1987).

Among metabolites that increase in the elderly, citrulline is synthesized in the urea cycle, and is involved in excretion of nitrogen. Dimethyl-guanosine and N-acetyl-arginine are also related to nitrogen metabolism (Niwa et al., 1998) (Mizutani et al., 1987). These results suggest that urinary discharge of nitrogen-related metabolites is possibly reduced in the elderly. Pantothenate is a precursor of coenzyme CoA, involved in the TCA cycle and β -oxidation.

Correlation analysis among these 14 aging compounds suggests strong correlations among metabolites with declining values in elderly, and also among those that increase in the elderly. Interestingly, there was no negative (reverse) correlation between these two subgroups, indicating at least two distinct subgroups for aging metabolites.

Blood metabolites for fasting markers

The evidence on CR and longevity has been well documented in experimental models. Recent reports suggest that intermittent fasting, a cycle of fasting and feeding, enables *C. elegans* to live about 50% longer than conspecifics on a normal diet (Honjoh et al., 2009). Thus, CR and intermittent fasting have overlapping roles in prolongation of lifespan (Fig 2).

Although little is known about the link between fasting and aging in humans, it is well known that humans can withstand 30-40 days of fasting if dehydration is avoided, in sharp contrast to the vulnerability of mice to hunger (average survival of only several

days of starvation). For example, Gandhi experienced hunger strikes of up to 21 days, 14 times or more in his lifetime as a form of political protest (Peel, 1997).

Historically, research on fasting physiology has focused particularly on energy substitution (Owen et al., 1969). Glucose normally constitutes the major fuel source under non-fasting conditions, but during fasting, glycogen stores are rapidly exhausted in an effort to maintain minimum glucose levels in the blood. After depletion of glycogen storage, in addition to gluconeogenesis, fasting forces the human body to utilize various non-carbohydrate metabolites, such as lipids and branched-chain amino acids (BCAAs) as energy sources (Cahill, 2006). Hormonal changes stimulate lipolysis in white adipose tissue (WAT) and liver. First, 3-hydroxybutyrate (3-HB) increases 30 to 60-fold and is converted into acetyl-CoA in the brain or other tissues as an alternative energy source (Owen et al., 1969). Second, elevated acylcarnitines during fasting are essential for lipid transport into mitochondria (Hoppel and Genuth, 1980). Third, increased concentrations of branched-chain amino acids (BCAAs), mainly released from muscle, are also utilized in the mitochondrial TCA cycle or in liver lipogenesis (Pozefsky et al., 1976). Thus, elevation of butyrates, BCAAs, and acylcarnitines in circulating blood (quantified by targeted metabolomics or other techniques) serve as indicators of fasting.

we conducted an exhaustive analysis of nearly 130 metabolites from blood of four healthy young people for 58 hours of starvation. In this study, over one-third of metabolites increased, indicating much greater metabolic activation during fasting than expected. Among 44 increased metabolites, well-known fasting markers figure prominently (ketone bodies, carnitines, and BCAAs), consistent with previous findings (Hoppel and Genuth, 1980) (Fig 3).

In addition, several novel aspects of fasting were discovered involving (1) TCA cycle metabolites, (2) antioxidants, and (3) signaling molecules (Figure 3). First, an increase in TCA cycle metabolites reflects activation of mitochondrial function throughout the body during starvation, since red blood cells are not equipped with mitochondria. Increases of well-established fasting metabolites, ketone bodies, carnitines, and BCAAs, also support mitochondrial activity during fasting. Second, an increase in antioxidant compounds was also observed; in addition to urate, xanthine, carnosine, OA, and ergothioneine (ET). Urate is one of the most abundant antioxidants in blood (El Ridi and Tallima, 2017), the precursor of which is xanthine. ET is abundant in mushrooms and fungi. In yeast, ET also increases in a low-glucose environment (Pluskal et al., 2014). Additionally, four metabolites (6-phosphogluconate, glucose-6-phosphate, pentose phosphate, and sedoheptulose-7-phosphate) generated via the pentose phosphate pathway (PPP) increased in plasma, but not in RBCs during fasting. Activation of the PPP produces NADPH, which is essential for redox control (Patra and Hay, 2014). As sugar phosphate compounds in blood are enriched in RBCs, PPP metabolite increases only in plasma suggest that responses in tissues are largely responsible for these altered profiles during fasting. Collectively, one of the most significant metabolic changes during starvation is antioxidant enhancement.

Third, whole blood metabolomics identified purines and pyrimidines (GTP, CTP, ADP, IMP, cytidine, and adenine) and some signal-modulating metabolites (3-hydroxybutyrate and 2-oxoglutarate) as fasting markers (Teruya et al., 2019). It is conceivable that fasting provokes global remodeling of transcriptional networks to adapt to metabolic changes. Increased purines and pyrimidines may support anabolic metabolism for RNA and protein synthesis. 3-hydroxybutyrate (3-HB), a major energy

substitute during fasting, is also known as a histone deacetylase inhibitor (Shimazu et al., 2013), while 2-oxoglutarate activates 2-oxoglutarate oxygenase, functioning in demethylation of histones and nucleic acids, and destabilization of transcriptional factors (Loenarz and Schofield, 2008). Fasting may genetically or epigenetically modify transcriptional networks via such metabolites (Loenarz and Schofield, 2008).

Interestingly, four metabolites that increase during fasting (carnosine, OA, leucine, and isoleucine) overlap with the decreased metabolites in elderly (Table 1 and Figure 4). It is possible that fasting may exert anti-aging or rejuvenile effect through upregulation of these aging metabolites.

Frailty markers for anti-oxidation, cognition, and mobility

Many aging-related diseases are increasing in our globally aging society, including lifestyle diseases (hypertension, diabetes, obesity, osteoporosis, atherosclerosis, etc), dementia, cancer, and others. Among other factors, the severity of frailty is most closely correlated with health risks of advanced age (Li et al., 2020). Frailty is a vulnerability to stressors, due to the declining physiological capacity of organs as a result of aging (Fried et al., 2001). Because it results from age-related deterioration of multiple organ systems, frailty displays complex features, including cognitive dysfunction, hypomobility, and impaired daily activity.

Frailty is currently defined by three major diagnostic tools (Dent et al., 2017b). The Fried Cardiovascular Health Study Index (CHS) is useful to detect physical frailty (Fried et al., 2001), while the Rockwood Frailty Index covers its multimorbidity (Rockwood et al., 1999). In contrast, the Edmonton Frailty Scale (EFS) or Tilburg Frailty Indicator,

efficiently evaluates both physical and psychosocial aspects of frailty (Rolfson et al., 2006).

Four recent studies reported the metabolomic analysis of blood from frail elderly subjects (Pujos-Guillot et al., 2018) (Marron et al., 2019) (Rattray et al., 2019) (Livshits et al., 2018). However, these reports drew divergent, non-overlapping conclusions, stemming from different experimental designs. For example, the former two reports applied the Fried CHS index as a diagnostic tool, which lacks cognitive assessment, while the latter two utilized the Rockwood Frailty index. Since these studies were based on serum samples, we performed whole blood metabolomics for frailty. Our study was designed to cover multiple domains of frailty, by applying the EFS, the Japanese version of the Montreal Cognitive Assessment (MoCA-J) (Fujiwara et al., 2010) and the Timed Up & Go Test (TUG) (Podsiadlo and Richardson, 1991) as diagnostic tools (Rolfson et al., 2006), because the EFS is recognized as a valid and reliable measurement tool for identification of frailty (Dent et al., 2017a) and is widely recommended in clinical guidelines (Dent et al., 2017b).

Using the EFS as a guide, our study identified 15 blood metabolites involved in antioxidation, cognition, and mobility as frailty markers (Table 1 and Figure 4) (Kameda et al., 2020), while studies based on the Fried CHS reported blood metabolites related mainly to physical or sarcopenic frailty (Marron et al., 2019).

First, among 15 frailty markers, seven compounds that decreased in frailty are associated with antioxidative defense: acetyl-carnosine, ergothioneine (ET), S-methyl-ET, trimethyl-histidine, OA, 2-ketobutyrate, and urate (Figure 4). Trimethyl-histidine and S-methyl-ET are involved in ET synthesis (20). 2-ketobutyrate is a precursor of OA. Thus, the ergothioneine and OA pathways are greatly affected in frailty. Second, we observed

that 5 amino acids (methionine, proline, tryptophan, isoleucine, and leucine) decreased significantly in frail subjects. Among these five amino acids, methionine, proline, and tryptophan, have been reported as radical scavengers *in vitro* (Marcuse, 1960) (Meucci and Mele, 1997). Thus, our whole blood metabolome for frailty revealed antioxidant enrichment in cellular components. Consistently, recent findings of longitudinal studies support our notion that diminished antioxidative defenses are heavily involved in pathogenesis of frailty (Marron et al., 2020). It is noteworthy that most cognitive markers and some hypomobility markers overlap with frailty markers, supporting the notion that frailty is an integrated spectrum of age-related disorders (Figure 4). On the other hand, only acetyl-carnosine overlapped in aging and cognitive markers, indicating the gap between physiological and pathological aging in cognition.

Interestingly, metabolites affected in frailty largely overlap with metabolites that decrease during aging (acetyl-carnosine, OA, 1,5-AG, isoleucine, and leucine) and compounds that increase during fasting (2-ketobutyrate, OA, isoleucine, leucine, urate and ET), indicating an intriguing metabolic link between frailty and human aging (Table 1 and Figure 4). Various antioxidative metabolites are conspicuously included among frailty and aging markers, suggesting that one of the key stressors to which frail elderly are vulnerable is oxidative stress. In this context, increased oxidative defense during fasting may effectively moderate aging or aging-relevant disorders.

Summary

Diversity of aging has a significant impact not only on clinical and basic research, but also on society and economies, and facilitates structural changes in entire societies. The answer to the fundamental question, "What is aging?", is not yet fully known.

Accumulating evidence suggests that defining "aging" only on the basis of calendar age does not explain the entire situation.

Recent advance in ageing research identified mechanisms and biomarkers for biological aging. The research on cellular senescence suggests the significance of telomere length, p16, p53, inflammatory cytokines and others. On the other hand, the aging research on metabolic aspects, including metabolomics, disclosed the metabolites closely involved in aging and its relevant diseases.

Interestingly, some of the metabolites described in this review are known to interact with the signal molecules (Figure 2). NAD^+ , decreased in elderly, activates Sirtuin, while citrulline, accumulated in elderly, stimulates TOR kinase. 3-HB inhibits histone deacetylases, followed by FOXO activation. Thus, metabolites might affect aging or aging-related diseases by modulating signal modules. As one hint of human aging research, results of whole blood metabolomics help to better understand biological age, especially given the variable nature of aging.

Fig. 1

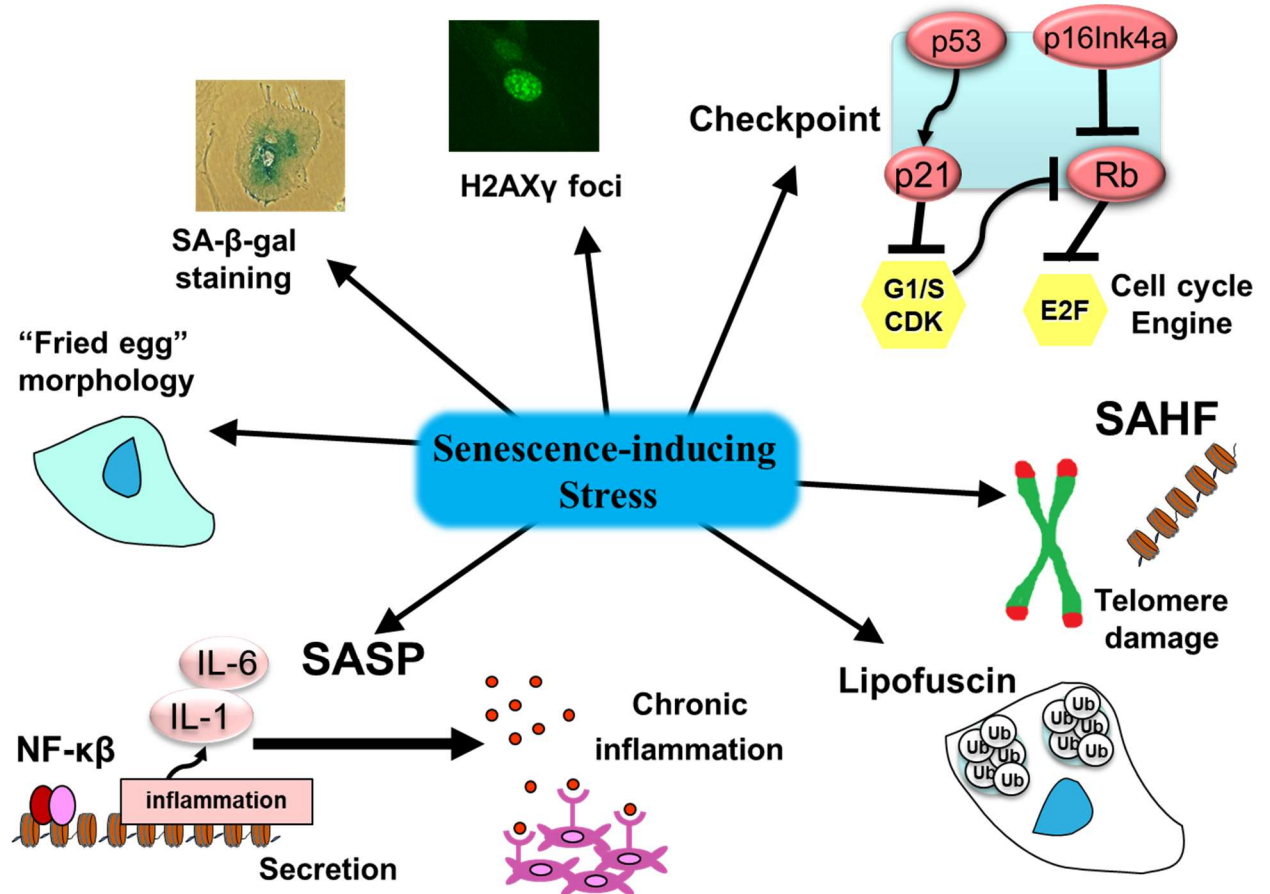


Fig.1 Hallmarks of stress-induced senescence (SIS)

Senescence-inducing stress provokes several features of cellular senescence; “Fried egg” morphology, positive staining of SA-β-gal, H2AX γ foci formation, senescence-associated heterochromatin foci (SAHF) and others. Cyclin-dependent kinases (CDKs) drive progression of the cell cycle, while CDK inhibitors, checkpoint genes, exert an opposite effect. As senescent cells suffer permanent cell cycle arrest, p21 and p16Ink4a, senescence markers, accumulate. Moreover, activation of transcription factor NF- κ B in senescent cells induces inflammation-related genes. Such inflammatory cytokines are secreted from senescent cells (senescence associated secretory phenotype; SASP), which aggravates chronic inflammation in surrounding tissues.

Figure 2.

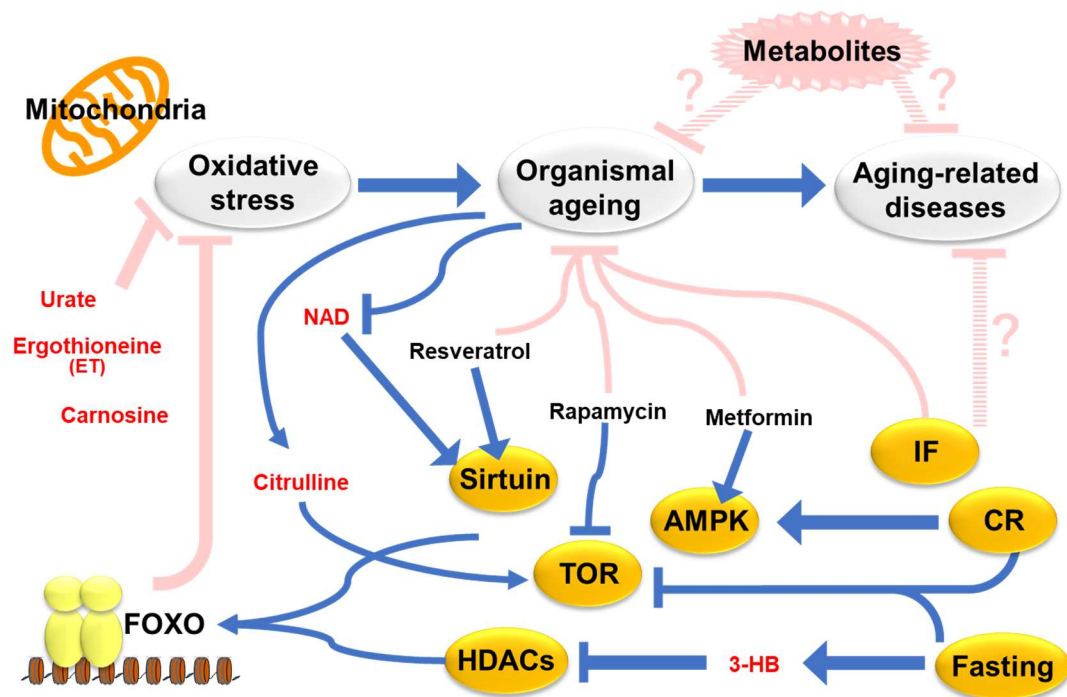


Figure 2. The aging hypothesis relative to metabolism.

Earlier, “the oxidative damage hypothesis” or “mitochondrial aging hypothesis” was proposed by Harman, as oxidative stress reduces organismal lifespan. Calorie restriction (CR) or intermittent fasting (IF) effectively extends lifespans of experimental models. During CR, several signal modules, including sirtuin, Tor kinase, and AMPK, are activated or inactivated. Consistently, chemicals or compounds mimicking CR conditions increase longevity (resveratrol for sirtuin, rapamycin for Tor kinase, and metformin for AMPK). One of the targets of these signals is FOXO transcriptional factor, which activates radical scavengers. However, little is known about the effect of CR or fasting on human longevity. Some of the metabolites described in this review (red color) are known to interact with the signal molecules or to serve as antioxidants. HDACs; histone deacetylase.

Table 1.

Metabolites	Levels in elderly	Levels in frailty	Levels in fasting	Role in blood
1,5-anhydroglucitol (1,5-AG)	↓	↓		Antioxidant
acetyl-carnosine	↓	↓		Antioxidant
carnosine	↓		↑	Antioxidant
ophthalmic acid (OA)	↓	↓	↑	Antioxidant
leucine	↓	↓	↑	Muscle maintenance
isoleucine	↓	↓	↑	Muscle maintenance
NAD ⁺	↓			Redox homeostasis
NADP ⁺	↓			Redox homeostasis
UDP-acetyl-glucosamine	↓			Sugar nucleotide
2-ketobutyrate		↓	↑	Butyrate
urate		↓	↑	Antioxidant
Ergothioneine (ET)		↓	↑	Antioxidant
trimethyl-histidine		↓		Antioxidant
s-methyl-ET		↓		Antioxidant
tryptophan		↓		Amino acid
methionine		↓		Amino acid
Proline		↓		Amino acid
citrulline	↑			Urea cycle
pantothenate	↑		↑	Precursor of CoA
dimethyl-guanosine	↑			Urine compound
N-acetyl-arginine	↑			Urea cycle
N6-acetyl-lysine	↑			Acetylated amino acid
UDP-glucuronate		↑		Sugar derivative
creatine		↑		Energy storage

Table 1. Fourteen metabolites for aging overlap with those for fasting and frailty.

Whole blood metabolomics reported 14 metabolites (red) as aging markers. The upper panel (blue box) shows metabolites that decrease in the elderly or frailty, while the lower panel (grey boxes) list metabolites that increase in those. Please notice that several compounds for aging or frailty overlap with markers which increase in fasting. Properties of metabolites are added.

Figure 3.

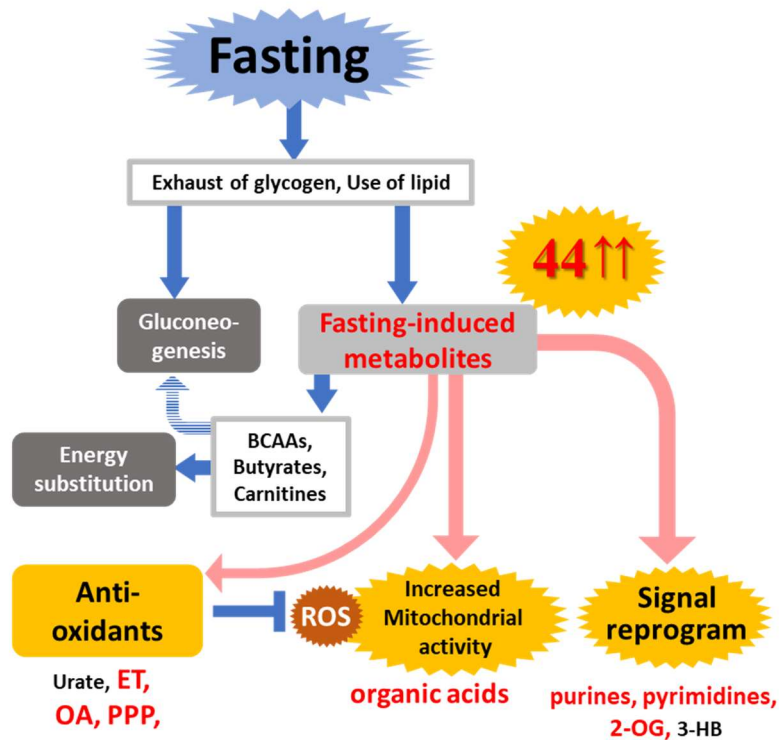


Figure 3. 44 metabolites that increase during fasting include antioxidants, organic acids and signaling-related compounds.

Non-targeted comprehensive metabolomics of whole blood detected increases of one-third (44) of metabolites identified during 58 hr of fasting. In addition to metabolites for energy production, antioxidative metabolites were identified as fasting markers, which may combat oxidative stress resulting from enhanced mitochondrial activity. Moreover, signaling metabolites would contribute for remodeling of metabolic homeostasis during fasting. See the text for details. Abbreviations: ET; ergothioneine, OA; ophthalmic acid, PPP; pentose phosphate pathway, 3-HB; 3-hydroxybutyrate and 2-OG; 2-oxoglutarate.

Figure 4.

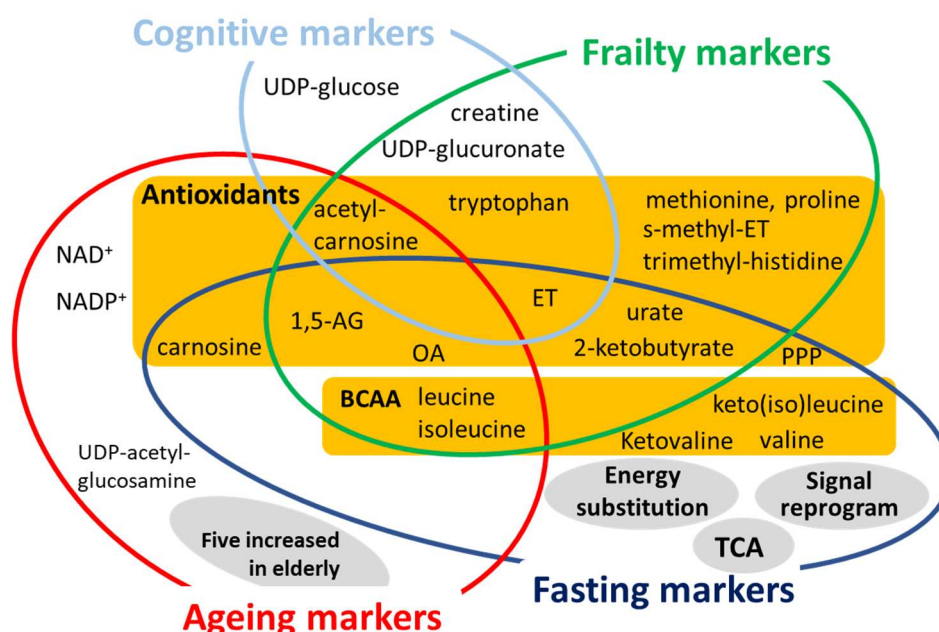


Figure 4. Summary of metabolites for aging, fasting, frailty, and cognitive impairment.

Overlapping, but distinct markers for aging, fasting and frailty are identified by non-targeted, comprehensive metabolomic analysis of human whole blood. Various antioxidative metabolites are included in the list of aging and fasting markers. Please notice that four metabolites decreased in elderly (OA, carnosine, leucine, and isoleucine) were increased during fasting, indicating the possible anti-aging effect of fasting.

Among fifteen frailty markers, eleven anti-oxidative metabolites are decreased, indicating the involvement of antioxidative defense in the pathogenesis of frailty. Among fourteen aging markers, five metabolites (acetyl-carnosine, 1,5-AG, OA, leucine, and isoleucine) overlapped with frailty markers. Among six cognitive markers, five compounds (acetyl-carnosine, 1,5-AG, OA, UDP-glucuronate and creatine) overlapped with frailty markers, reflecting the cognitive aspects of frailty. Thus, cognitive markers are much involved in frailty markers, while one compound (acetyl-carnosine) are listed as overlapping both in aging and cognitive markers.

BCAA; branched chain amino acid, ET; ergothioneine, 1,5-AG; 1,5-anhydroglucitol (1,5-AG), OA; ophthalmic acid, PPP; pentose phosphate pathway.

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