FEBS Letters

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REVIEW

Decline of ergothioneine in frailty and cognition impairment

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(Received 18 November 2021, revised 19 January 2022, accepted 20 January 2022, available online 3 February 2022)

doi:10.1002/1873-3468.14299

Edited by Barry Halliwell

Ergothioneine is a well-known antioxidant that is abundant in both human red blood cells and in fission yeast responding to nutritional stress. In frail elderly people, whose ageing organs undergo functional decline, there is a correlation between ergothioneine levels and cognitive, but not skeletal muscle decline. In patients suffering from dementia, including Alzheimer's disease with hippocampal atrophy, deteriorating cognitive ability is correlated with declining ergothioneine levels. S-methyl-ergothioneine, trimethyl-histidine and three other trimethyl-ammonium compounds also decrease sharply in dementia, whereas compounds such as indoxyl-sulfate and quinolinic acid increase, possibly exacerbating the disease. Using these opposing dementia markers, not only diagnosis, but also therapeutic interventions to mitigate cognitive decline may now become possible.

Keywords: antioxidant; dementia; ergothioneine; frailty; metabolomics; sarcopenia; starvation; whole blood

Due to ageing of the global population, ageing-related diseases are increasing, including diabetes, atherosclerosis, hypertension, osteoporosis, dementia, cancer and others [1]. Because 'ageing' is a complex multi-faceted process, it is characterised by great individual variability [2,3]. In countries with a high proportion of elderly people, a greater variety of ageing and ageing-related diseases is observed. While the number of healthy elderly people is increasing, the number of bedridden patients and people who need long-term nursing care is increasing as well [1,4]. Patients with ageing-related diseases, such as dementia, frailty and sarcopenia, also require intensive nursing care and family support.

Dementia is a form of cognitive impairment that develops into an irreversible cognitive decline [5,6]. In its initial stage, it is easily confused with forgetfulness due to ageing. But later, in addition to core symptoms of memory impairment or disorientation, patients are frequently afflicted with behavioural and psychological symptoms of dementia, such as delusions, hallucinations, wandering, aggressiveness, sleep disorders, resistance to nursing care and depression, resulting in severely impaired daily life activities [7]. On the other hand, frailty is defined as a condition in which vulnerability to stress increases due to a decreased physiological reserve capacity [8]. Frailty comprises hypomobility, impaired cognition and social disturbance, while sarcopenic patients show reduced volume and strength of skeletal muscles due to muscle ageing [9,10]. Both frailty and sarcopenia result in life dysfunction and

Abbreviations
CR, calorie restriction; CV, coefficient of variation; EFS, Edmonton Frailty Scale; ERG, ergothioneine; IF, intermittent fasting; LC-MS, liquid chromatography-mass spectrometry; MMSE, mini mental state examination; MoCA-J, the Japanese version of the Montreal Cognitive Assessment; OA, ophthalmic acid; OCTN, organic cation transporter; PPP, pentose phosphate pathway; RBCs, red blood cells; ROS, reactive oxygen species; Se, selenium; S-methyl-ERG, S-methyl-ergothioneine; WBCs, white blood cells.

nursing care, condemning affected individuals to a bedridden state and death [11,12].

Ergothioneine (ERG), a water-soluble amino acid derivative, was discovered in 1909, when it was isolated from *Claviceps purpurea*, the fungal parasite that causes ergot in rye [13,14]. ERG is found in many organisms, although animals and plants cannot synthesise it themselves [15,16]; indeed, ERG is mainly produced by fungi, yeasts, bacteria and a few other organisms [17–19]. ERG has excellent heat and pH stability and its antioxidative capacity against hydroxyl radicals and peroxynitrite *in vitro* is about three times and about 30 times higher than that of glutathione, another antioxidant respectively [20]. Because vertebrates cannot synthesise ERG, they extract it from food and store it in the brain, liver, kidneys, red blood cells (RBCs), skin, etc. [21–23].

We have established a metabolomic approach using whole blood, which enables us to comprehensively analyse metabolite profiles in cellular and non-cellular components of human blood [24,25]. This whole blood metabolome identified several markers for ageing, fasting, frailty, sarcopenia and dementia. Among them, we noticed a decline in ERG levels in the blood of patients suffering from frailty and dementia, but not from sarcopenia [26–28]. As glucose starvation leads to ERG upregulation in both in human blood and yeast, the antioxidant properties of ERG may protect individuals from ageing-relevant dysfunctions [29,30]. Here we review recent findings on ERG in relation to frailty and dementia, including Alzheimer's disease.

Whole blood, urinary and salivary metabolomes

Metabolic activity *in vivo* is affected by a subject's genetic background, environmental factors, lifestyle, habits, health conditions, etc. During these activities, cells and tissues generate metabolites, a portion of which are excreted into or generated in body fluids (blood, urine, saliva, cerebrospinal fluid, sweat, tears, faeces, etc.) [31]. These metabolites closely reflect organismal health, physiological homeostatic responses, disease states, etc. [32]. Thus, metabolites are good disease indices.

Improvements in analytical instruments and high-volume data processing techniques have accumulated evidence that information on health, ageing and disease status can be interpreted using quantitative data on metabolites in various body fluids. Metabolomics is an efficient tool to evaluate metabolite profiles, to discover and decipher highly integrated, but complex biological processes [32–34]. As blood is constantly circulating throughout the body, it reflects the metabolic status of the individual. Human blood comprises cellular and non-cellular components, RBCs, white blood cells (WBCs), platelets and plasma. Many studies on human blood have been conducted on plasma or serum, the non-cellular component [35,36], partly due to the difficulty in handling unstable cellular metabolites [37]. However, as about half of the metabolites in whole blood are derived from RBCs, we established a metabolomic approach to analyse whole blood, plasma and RBCs. The whole blood metabolome includes about 130 metabolites that are implicated in energy production, DNA, RNA, protein and lipid metabolism, mitochondrial respiration, redox homeostasis and so on.

The whole blood metabolome provides valuable information about individuals and diseases. Based on the coefficient of variation (CV: SD divided by the mean), metabolites detected by whole-blood metabolomics are classified into two subgroups: less or highly variable (CV < 0.4 or CV > 0.4) [25]. The former comprises many vitally essential metabolites (ATP, glutathione, phospho-sugars, etc.), while the latter include dietary compounds such as caffeine, carnosine, ERG, 4-aminobenzoate and others. ERG is a sulfur-containing Nα,Nα,Nα-trimethyl-L-histidine-derived metabolite that is synthesised by various species of bacteria and fungi [17,18,38]. ERG is generated from trimethyl-histidine, known as hercynine, followed by its conversion into S-methyl-ergothioneine (S-methyl-ERG). ERG functions as an antioxidant by scavenging free radicals and chelating transition metals [39]. Selenoneine, the selenium (Se)-bound form of ERG, is enriched in tuna and other fish, exhibiting higher antioxidative capacity [40], whose status in whole blood metabolome is much unknown. Several antioxidants, for example, ERG and acetyl carnosine, are enriched in the RBC fraction [25,26,30].

Alternatively, other biofluids, such as urine and saliva, can easily be acquired noninvasively and are suitable for daily observation. Production and excretion of urine are fine-tuned *in vivo*. As urine contains not only waste products or toxins, but also various nutrients, their filtration and reabsorption are tightly regulated in kidney. Accordingly, urine has been broadly utilised for diagnosis of renal dysfunction in kidney diseases [41]. Saliva is secreted mainly from salivary glands. It also contains metabolites such as sugars, amino acids, antioxidants and high-energy compounds [42,43]. Daily salivary secretion accounts for 0.75–1.5 L, similar to the quantity of urine, suggesting that both urine and saliva can provide useful information on physiological and pathophysiological conditions [44]. In addition to basic metabolites synthesised in the body, various food-derived metabolites are also detected in urine [45] and saliva [46] as well as in blood [25]. Three ERG-related compounds (ERG,

S-methyl-ERG and hercynine) are also detected in samples of human blood, urine or saliva. ERG is abundant in RBCs but is hardly detected in urine and saliva [25,45,46]. It would be worthy to evaluate the levels of salivary and urinary ERG-related compounds in age-related disease, dementia and frailty, in the near future. The abundance of S-methyl-ERG, like ERG, is much lower in urine and saliva than in blood. However, trimethyl-histidine is more abundant in urine than in blood or saliva (T. Teruya and M. Yanagida, unpublished data).

Starvation-induced upregulation of ergothioneine in yeast cells and human blood

Oxidative stress, damage to large molecules caused by free radicals and reactive oxygen species (ROS), is well known as a major cause of cellular dysfunction and death, and has been implicated in ageing and ageing-relevant diseases [47–49]. Ectopic expression of 'radical scavengers' in experimental models exerts beneficial effects during disease states. In addition, genetic manipulation to extend the life span is accompanied with increased antioxidants, for example, glutathione [50,51]. When calorie intake is reduced by 20%–30%, life span is extended by 20% or more in many model organisms, accompanied by reduced oxidative stress [52]. This is partly due to activation of FOXO transcription factor during calorie limitation, which upregulates a group of radical scavenger genes [53]. Alternatively, small-molecule antioxidants containing sulfur or selenium can ameliorate oxidative damage [54]. However, for the purpose of treatment, calorie restriction (CR) or intermittent fasting (IF) is applicable only to human obesity and diabetes. Fasting studies in model organisms would give us alternative hints.

The fission yeast, *Schizosaccharomyces pombe* (*S. pombe*), is an excellent model eukaryote in research for various biological processes, such as proliferation, meiosis, cytoskeletal organisation, metabolism, and chromosome/DNA biology [55–60]. Like human cells, *S. pombe* is equipped with mitochondria, lysosome-like vacuoles, peroxisomes, lipid droplets, endosomes and endoplasmic reticulum, which perform important functions in cellular metabolism [61–63]. While excess calories or glucose accelerates ageing in *S. pombe* [64], responses to limited nutrients are distinct. When nitrogen is absent, cells arrest completely after two rounds of cell division without cell growth [65,66]. The resulting small, round G0 cells remain quiescent and metabolically viable for months, as they possess the ability to reuse intracellular nitrogen. Under glucose fasting, however, *S. pombe* immediately stops cell division and loses viability within 32 h. These glucose-fasting arrested cells are rod-shaped and arrested at G2 phase of cell cycle with postreplicative DNA [29]. Thus, the study of starvation in fission yeast would give us mechanistic insights in its metabolic mechanism. We performed non-targeted metabolomics in *S. pombe* under starved conditions. We observed that both glucose and nitrogen starvation provoke drastic increases of ERG in *S. pombe* [29,67] (Fig. 1). Ablation of the $egt1^{+}$ gene, the main ERG biosynthetic enzyme, abolished the boost of ERG in *S. pombe* [68].

Next, we performed non-targeted comprehensive liquid chromatography-mass spectrometry (LC-MS) analysis of whole blood during 58 h fasting by four young, non-obese volunteers [30]. Notably, 44 of 120 metabolites increased 1.5- to 60-fold during this period, suggesting that fasting increases the activity of diverse metabolic pathways. In addition to established fasting markers, such as butyrates, acylcarnitines and branched-chain amino acids, several TCA cycle-related compounds (cis-aconitate, malate, 2-oxoglutarate and succinate) and coenzymes (nicotinamide and pantothenate, a precursor for acetyl-CoA) also increased, reflecting enhanced mitochondrial activity in tissues during fasting. Besides increased metabolites for energy production, previously unappreciated impacts of prolonged fasting were disclosed. Increases of several antioxidants (carnosine, ERG, urate and xanthine), antioxidative marker [ophthalmic acid (OA)] and pentose phosphate pathway (PPP) metabolites were observed. The transporter for ERG, organic cation transporter (OCTN)-1. Interestingly, it was reported that mRNA for OCTN-2, a homologue of OCTN-1, was upregulated in rats under fasting. However, little is known about the activity of human OCTN-1 under fasting [69]. Previously it was observed that ERG levels ($n = 4$) are constant on a daily basis (fig. S1A in Chaleckis et al. [25]) (its CV on a daily basis is very low, 0.05). Thus, ERG levels are upregulated not by daily basis, but by longer fasting. As our study was performed in limited numbers ($n = 4$), it would be worthy to perform fasting experiment in larger samples in the future. These findings imply that boost of ERG level may be also adaptive response to fasting stress in both humans and fission yeast, which was not expected previously, implicating that ERG may also exert beneficial effect on ageing-relevant events (Fig. 1).

Ergothioneine declines in frailty and dementia, but not in sarcopenia

Various ageing-related diseases share some clinical features, such as frailty, dementia and sarcopenia, with

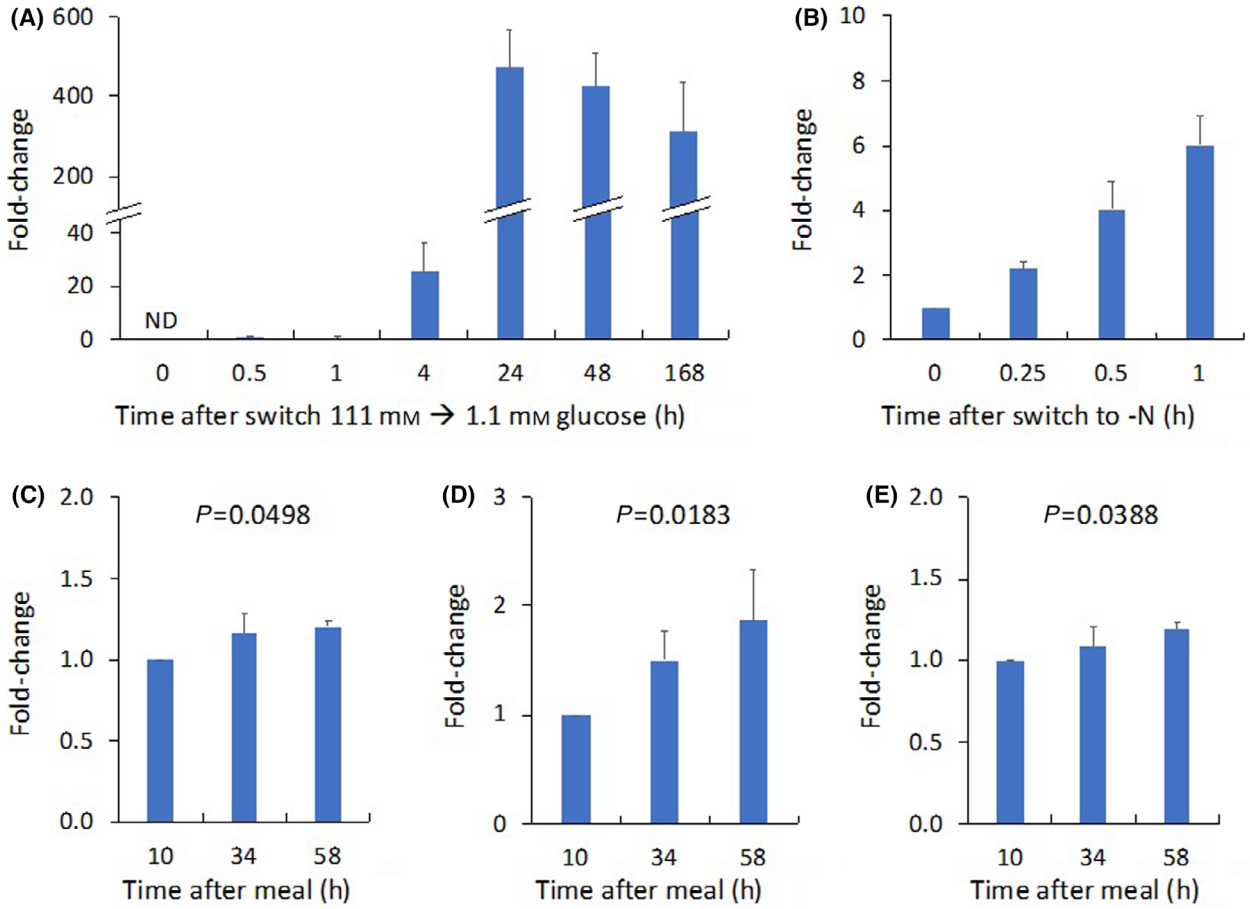

Fig. 1. Increased ergothioneine yeast cells upon starvation. Time course of changes in ergothioneine levels, evaluated by LC-MS during starvation in *S. pombe* (A, B) and human blood (C–E). *S. pombe* was grown in medium with low glucose (1.1 mM) (A) or without nitrogen (−N) (B). (C–E) Four human volunteers fasted for 58 h. Ergothioneine was detected in whole blood (C), plasma (D) and RBCs (E) under prolonged fasting. *P*-values were calculated with the Friedman test.

increased needs for life support [70,71]. We performed whole blood metabolomics in relation to these three diseases to clarify the metabolic basis of their pathophysiology [26–28].

Non-targeted metabolomics of 19 elderly subjects (average 84.2 ± 6.9 years; 80.5 ± 4.7 years for frail subjects vs. 88.2 ± 6.8 years for non-frail subjects) identified 15 frailty markers. Strikingly, these 15 markers include seven metabolites related to antioxidation that decrease in frailty (acetyl-carnosine, ERG, S-methyl- ERG, trimethyl-histidine, OA, 2-ketobutyrate and urate) and three amino acids with radical scavenger properties (methionine, proline and tryptophan; Fig. 2). Recent longitudinal studies have also suggested the involvement of antioxidants in frailty [72]. Three ERG-relevant metabolites (ERG, S-methyl- ERG and trimethyl-histidine) decline and the metabolic or uptake pathway of ERG is much affected in frailty (Fig. 2).

As frail elderly people commonly manifest complex clinical symptoms, including cognitive dysfunction, hypomobility and impaired daily activity, we also evaluated cognitive function using the Japanese version of the Montreal Cognitive Assessment (MoCA-J) and sarcopenia in these same populations [26,27]. Six cognitive markers were identified, five of which are also frailty markers (acetyl-carnosine, ERG, tryptophan, creatine and UDP-glucuronate) (Fig. 2). On the other hand, 22 sarcopenia markers were identified in blood. Most of the metabolites that decreased in blood of sarcopenia are uraemic compounds that increase in kidney dysfunction, including TCA cycle, urea cycle, nitrogen and methylated metabolites. Thus, sarcopenia markers imply a close link between muscle and kidney function. However, little is known on sarcopenia markers in urine. Notably, the 22 sarcopenia markers are distinct from the 15 frailty markers in the blood of same

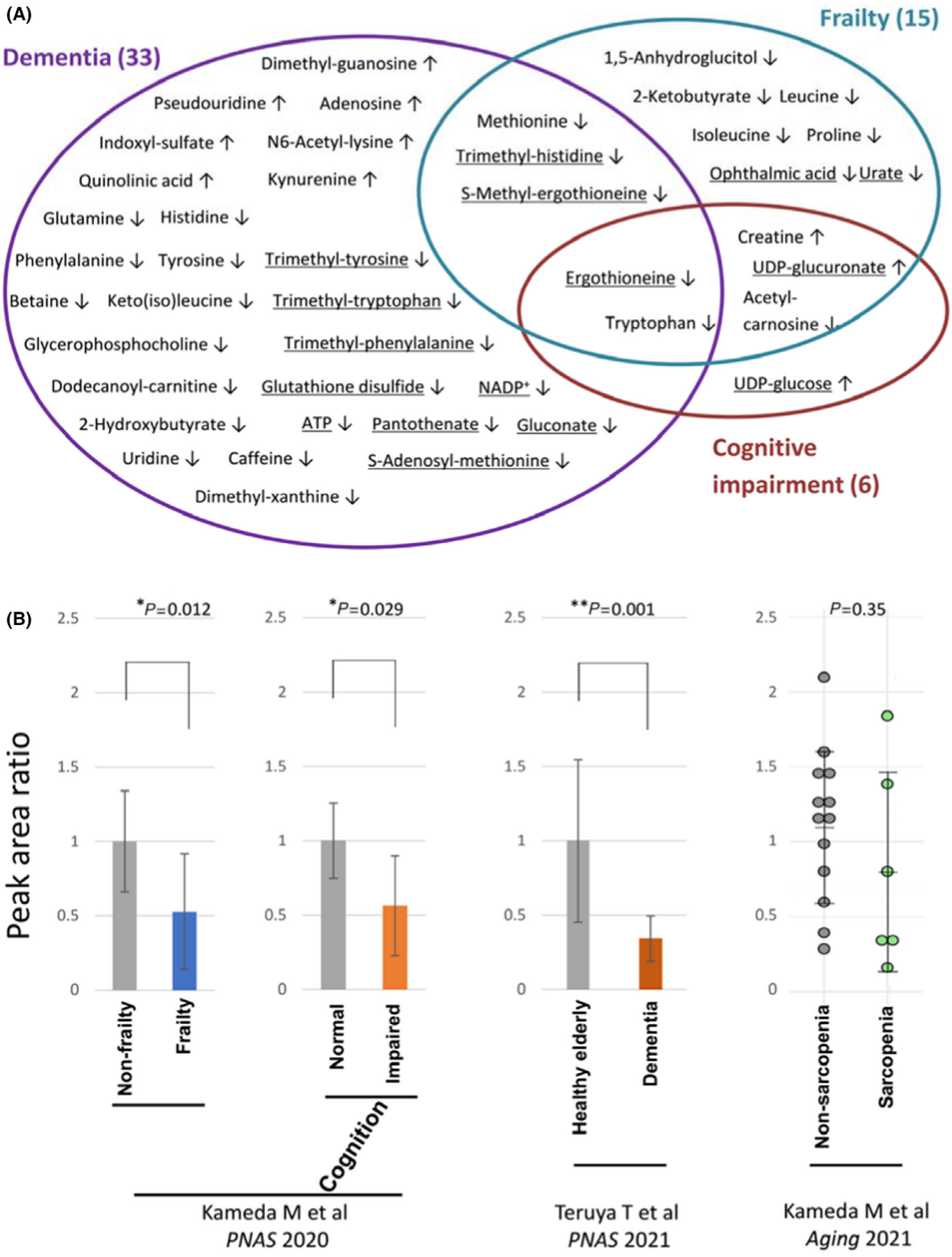

Fig. 2. Ergothioneine in frailty and dementia. (A) Summary of biomarkers identified with whole blood metabolomics in frailty, dementia and cognitive impairment. Arrows indicate increased or decreased metabolites. Note that ergothioneine is a biomarker for all three disease states. (B) The ergothioneine level in whole blood is compared in the indicated disease states. The relative ratios of the peak areas are shown, after normalisation against controls. In the same populations, frailty, cognitive impairment and sarcopenia were diagnosed using criteria in the EFS (Edmonton Frailty Scale), the MoCA-J and the AWGS 2014 (Asian Working Group for Sarcopenia) [26,27]. Dementia was diagnosed according to DSM-IV [28].

subjects, although sarcopenia overlaps clinically with physical frailty. Thus, ERG in blood of sarcopenia is comparable to that in controls. It is possible that ERG transporter OCTN-1 might be affected in these specific diseased states, whose significance in the pathogenesis is still unknown. Further study is required to acquire its mechanistic insights in the future.

Moreover, we quantified metabolites in whole blood of dementia, the majority of which were diagnosed as Alzheimer patients, using non-targeted LC-MS [28]. Thirty-three dementia markers are classified into five groups. Seven metabolites, such as possible neurotoxins increased [73,74], including quinolinic acid, kynurenine and indoxyl-sulfate, while the remaining 26 dementia markers decreased. Six metabolites in this subgroup, enriched in RBCs, contain trimethylated ammonium moieties, ERG derivatives (ERG, S-Methyl-ERG and trimethyl-histidine), trimethyl-tryptophan, trimethyl-tyrosine and trimethyl-phenylalanine (Fig. 2). Pearson's correlation analysis showed that ERG, S-methyl-ERG and trimethyl-histidine were inversely correlated with indoxyl-sulfate, quinolinic acid and kynurenine ($-0.66 < r < -0.42$). Cheah et al. [75] showed that in whole blood of subjects with Mild Cognitive Impairment, ERG was reduced by 0.65-fold compared to its levels in healthy individuals. ERG in frailty was 0.53-fold lower than in non-frail subjects, while that in dementia was 0.41-fold lower than in healthy elderly. The reduction of S-methyl-ERG in frailty or dementia was more remarkable 0.48 and 0.20 fold respectively, compared to healthy counterparts, while changes of trimethyl-histidine in frailty and dementia were comparable to those of ERG. A recent large-scale study of metabolomics ($n = 496$) also identified ERG as a dementia marker [76]. We also investigated the correlation between ERG levels in whole blood and the degree of frailty (Edmonton Frailty Scale, EFS score) or cognitive function (mini mental state examination, MMSE score) in dementia patients. The EFS showed a moderate correlation with ERG ($r = -0.45$, $P = 0.053$) and S-methyl-ERG ($r = -0.51$, $P = 0.026$) [26]. On the other hand, the MMSE showed a significant correlation between ERG ($r = 0.64$, $P = 0.018$) and S-methyl-ERG ($r = 0.62$, $P = 0.024$; calculated based on the raw data [28]). The decrease of ERG, S-methyl-ERG and hercynine in a concerted manner suggests that the intake of ERG might be affected in frailty or dementia. Collectively, ERG, a potent antioxidant, is significantly decreased in dementia, cognitive impairment and frailty, but not in sarcopenia (Fig. 2).

Conclusions and perspectives

In addition to whole blood metabolomics, we analysed both the urine and saliva metabolomics to comprehensively understand ageing-relevant diseases. Our studies of these three types of biofluids showed that ERG levels have no significant age-related differences. Previous studies have suggested that ERG exerts antioxidative and anti-inflammatory effects that are involved in several human diseases, for example, rheumatoid arthritis. We noticed that ERG is increased in human blood and yeast under glucose starvation, implying a protective role for ERG under CR. As fission yeast can produce ERG, it is a useful model organism for investigating the biochemical function of ERG. Although frailty comprises both cognitive impairment and hypomobility, ERG is identified as a biomarker for frailty and dementia, but not for sarcopenia, at least according to our whole blood metabolomics. These findings suggest the significance of ERG in ageing-related diseases: as oxidative damage accelerates organismal ageing and ageing-relevant disorders [77], it is possible that a decline in a radical scavenger such as ERG would promote the progression of frailty, dementia and ageing-related events. Several reports support the notion that ERG supplementation alleviates cognitive impairment and tissue oxidative damage in experimental animal models [78]. Thus, ERG treatment is a potential therapeutic approach for frailty and dementia.

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