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Review Title: Protein, Metabolism and Ageing

Author names: George A. Soultoukis[1] & Linda Partridge[1,2]

Affiliations:
1) Max Planck Institute for Biology of Ageing, Department Biological Mechanisms of Ageing, Joseph-Stelzmann-Str. 9b, Cologne, 50931, Germany
2) Institute of Healthy Ageing, Department of Genetics, Evolution & Environment, University College London, Gower Street, London, WC1E 6BT, United Kingdom

Emails:
Partridge@age.mpg.de
GSoultoukis@age.mpg.de

Corresponding author:
Linda Partridge

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Abstract (word limit: 150, word count: 148)

Dietary restriction (DR), a moderate reduction in food intake, improves health during ageing and extends lifespan across multiple species. Specific nutrients, rather than overall calories, mediate the effects of DR, with protein and specific amino acids playing a key role. Modulations of single dietary amino acids affect traits including growth, reproduction, physiology, health, and longevity in animals. Epidemiological data in humans also link the quality and quantity of dietary proteins to long term health. Intricate nutrient-sensing pathways fine-tune the metabolic responses to dietary amino acids in a highly conserved manner. In turn, these metabolic responses can affect the onset of insulin resistance, obesity, neurodegenerative disease, and other age-related diseases. In this review we discuss how amino acid requirements are shaped, how ingested amino acids regulate a spectrum of homeostatic processes, and finally we highlight the unique opportunity of using related nutritional strategies to improve human health during ageing.

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1. Introduction

I. Diet and health

Obesity and its associated metabolic diseases are a global health problem, linked to reduced life expectancy. Both quantity and quality of food intake are clearly important in the development of obesity, with excess fat (1) and carbohydrate intake detrimental to health and lifespan in flies (2), mice (3), and humans (4). Clearly diets that promote obesity should be avoided, as should those that induce nutritional deficiencies. But what dietary compositions best promote health, and why? Does the optimal balance of macronutrients vary with age, gender, genotype or disease state? Defining the macronutrient composition of a healthy diet, and identifying the molecular and physiological mechanisms by which it promotes health, are important challenges. In this review, we focus particularly on the roles of dietary protein.

II. Dietary restriction

A nutritional intervention that has clear health benefits is dietary restriction (DR), a moderate reduction in food intake that protects against multiple ageing-related diseases and impairments, and extends lifespan in most animals tested. The severity of DR can range from ~10 to ~50% of ad libitum intake levels, and the lifespan increase can be as modest as a few percent or as high as three-fold (5) . In rodents and primates, DR protects against ageing-related loss of function and disease, including cardiovascular disease, obesity, multiple cancers, neurodegeneration, nephropathy, loss of sensory, motor and immune function, and diabetes (5-6) . Short-term DR in humans also benefits glucose and energy homeostasis, increasing insulin sensitivity and reducing body fat (5) . However, DR is not a practical intervention for most humans because it is difficult to implement and sustain. Moreover, DR can decrease wound healing capacity and increase susceptibility to viral infections (5). Thus an important aim is to identify the nutrients that mediate the health benefits of DR. Understanding the physiological and molecular mechanisms by which these key nutrients exert their effects may pave the way to DR-mimicking diets, as well as pharmacological interventions to improve health during ageing with minimal side-effects.

III. Dietary protein and amino acids

Recent findings have increasingly pointed to a causal role of the protein component of the diet in promoting the health and lifespan benefits of DR. In the fruit fly *Drosophila*, restriction of dietary yeast, the fly's usual protein source, but much less so of carbohydrate or total calories, extends lifespan (7), an effect attributable to the amino acids (AAs) (8) and the protein-to-carbohydrate ratio of the diet (2). In mice (3) and rats (9), reducing dietary protein, thereby decreasing the diet's protein-to-carbohydrate ratio, also increases healthspan and lifespan. The beneficial effects of protein restriction outweigh those of carbohydrate or fat restriction (3,10-11). Indeed, dietary carbohydrates and fats are largely interchangeable without detrimental effects in many species (1,12). Moreover, specific AAs or their ratio can determine health and ageing, because reduced intake of methionine in *Drosophila* (8),

and of methionine or tryptophan in rodents (13-14) results in improved health during ageing and increased lifespan. Additionally, increases in circulating branched-chain AAs (BCAAs) stimulate the target of rapamycin (TOR) and IGF/insulin signaling (IIS) pathways in rodents (3, 15), which may be detrimental for health, since suppression of TOR and IIS signaling is often beneficial for a healthy lifespan (5). The mechanisms by which individual dietary amino acids affect metabolism and health are starting to be understood, and are revealing potential targets for improvement of organismal health during ageing.

2. Dietary restriction, health, and ageing

I. Protective effects of dietary restriction

The physiological, metabolic, and molecular changes through which DR extends healthspan and lifespan are becoming clearer, although a complete account is lacking for any organism. Reduction in nutrient intake triggers modulations in the activity of nutrient-sensing pathways, which stimulate protective mechanisms over most aspects of health during ageing. From flies to humans, the DR response is highly conserved and involves an extensive array of protective metabolic changes that include an increase in stress resistance, detoxification capacity, and genome stability, and promotion of proteostasis and energy homeostasis (5,106). The IIS and TOR pathways are also conserved in humans, and so are their responses to DR (5). Long-term and short-term DR trials in humans result in marked reductions in IIS and TOR signaling, decreasing multiple risk factors such as obesity, insulin resistance, and cardiovascular disease (16). These evolutionarily conserved responses to DR present an opportunity for significant health-promoting applications in human nutrition (11). Apart from its effects on energy homeostasis, DR also reduces cancer propensity. A reduction in tumour incidence (lymphomas, pituitary, and thyroid neoplasms) accompanies DR treatment in mice (6). Furthermore, primate studies also implicate dietary protein in health during ageing. Two recent Rhesus monkey lifespan trials found conflicting results, with one (WNPRC), but not the other (NIA), reporting a lifespan extension as a response to DR (11), although both found multiple improvements in health in the DR animals. Among several experimental differences between the two trials, differences in the amount and type of dietary protein were prominent (5,11). In humans too, DR reduces IGF-I and decreases the risk of cancer (16). However, DR in humans only lowers circulating IGF-I if protein intake is also restricted (5). Human trials also highlight a role for protein quality, because diets containing low, plant-based AAs promote multiple aspects of healthspan (17). Such findings emphasize a difference between protein sources, for example plant versus animal protein, discussed further below. Moreover, recent work suggests that increased activity of the transsulfuration pathway is required for the extension of lifespan by DR in *Drosophila* (18). Indeed, hydrogen sulfide production by the transsulfuration pathway is associated with extension of lifespan by DR in yeast, flies, and mice (19).

There is thus an emerging role of dietary protein, and of specific AAs, in modulating the health benefits of DR in humans, suggesting the potential of interventions in protein intake to improve human health.

II. Protein content and AA imbalance

In agreement to what is observed in DR animals, restriction of dietary protein or AAs reduces wound healing capacity and increases susceptibility to viral infections (20). Also in accord with what is seen in DR animals, protein-restricted mice have protected cognitive function and live longer (3,21). However, in contrast to DR animals, protein-restricted mice can show an increase in body fat and insulin resistance (3). Decreasing dietary protein also increases body fat in humans (22), but a high intake of dietary protein and AAs promotes insulin resistance and adversely perturbs glucose homeostasis (23). In consequence, only 10-15% of energy intake as protein is recommended for humans (30), although for weight loss management the absolute amount of protein consumption is of greater importance than the percentage of energy (22).

The effective protein uptake depends on the efficiency of a protein's usage. This is the result of the combined effects of (i) how much protein is ingested and metabolized, (ii) its essential to non-essential AA ratio (EAA:NEAA), and (iii) its precise AA composition (24). Adequate protein intakes can be achieved with lesser amounts of high quality protein than of low quality protein, where quality is determined by its efficiency for anabolic traits. The effects of a dietary AA imbalance are more severe when overall AA intake is low (25), as AA imbalances further decrease AA usage. A low EAA:NEAA can also be inefficient for anabolic traits at low AA intakes. Therefore, the protein content and effective macronutrient ratio of protein to carbohydrates and fats greatly depends on the AA proportions of the ingested protein. Consequently, the AA proportions of a diet are critical for health, both through effects on total protein usage, and through mechanisms mediated by specific AAs. We highlight the metabolic fate of AAs, from ingestion to effects upon metabolism and health, in the following sections.

III. Multivariate complexity of defining a dietary AA-imbalance

Identifying a diet with a healthy mixture of nutrients is complicated by the multivariate nature of diets, which poses a challenge for experimentation, and by the synergistic effects of multiple essential macro- and micro-nutrients (26). Accordingly, complex interactions between individual AAs render phenotypic responses to different AA ratios hard to interpret. Moreover, an interaction of AAs with other nutrients such as vitamins can also modulate metabolism (27), which further increases the complexity of nutritional space and confounds biological interpretations. Consequently, defining a balanced AA intake is a challenge.

To simplify the nutritional landscape, recent methods dissect nutritional interactions and their physiological effects in multidimensional space (2,26). Such a representation of nutritional space, called the geometric framework, can better describe the responses of metabolic, lifespan, and other traits, and can reconcile apparently contradicting results (2,26). Multi-dimensional approaches are desirable but sometimes impractical, and progress can also be impeded due to the lack of standardized methods. In flies, the recent

development of holidic diets enables the accurate analysis of the effects of single dietary AAs (46), and such tools would benefit work in other model organisms such as mice and rats. This kind of experimental standardization will aid the systematic analysis of multivariate AA interactions and their effects on health and ageing.

It is difficult to discern the physiometabolic effects of subtle AA imbalances that occur under conditions of normal nutrition, which is usually characterized by the intake of varied dietary protein sources. Even in laboratory model organisms, with carefully controlled conditions using chemically defined diets, problems can persist. Autoclaving and irradiation, both common sterilization steps in laboratory rodent food preparation, can degrade certain AAs including lysine, methionine, and cysteine, as well as vitamins like A and B_1 (27). In everyday human nutrition, food processing such as cooking may alter the AA contents of a protein source. Food texture can also have dramatic effects upon metabolism, as soft foods increase nutrient efficiency and adiposity. In humans, ageing also leads to anorexia and weight loss, largely due to the progressive functional decline of the digestive system (138), which also likely results in deceased AA absorption. All these factors render practical diet design challenging.

IV. Anabolic traits and their experimental assessment

Although health and lifespan responses to AA sources are sometimes evaluated, experimentally the balance of an AA source is typically defined by its ability to maximize production traits (27).

With regards to the growth effect of single EAA limitations in vertebrates, the principle of the minimum is typically applied. The principle states that when all essential nutrients required for growth are abundant in a diet except for one, the limiting essential nutrient, incremental additions of this limiting essential nutrient only will increase growth (28). For EAAs, this has been repeatedly demonstrated experimentally in rodents (24). In addition, the principle is coupled to the law of diminishing returns, according to which each succeeding increment of the limiting essential nutrient will produce a smaller increment of growth than the preceding increment (28,29). However, the nature of the link between anabolic traits and long-term health is complex and is discussed below with respect to protein and AA intakes.

V. Link between anabolic traits and long-term health?

Growth: Both DR and protein restriction can suppress growth and extend lifespan in rodents (3,5,24). The developmental theory of ageing holds that a prolonged lifespan is caused by retarded development, and this notion was quickly adopted as an explanation of the lifespan response to DR (31). In flies and mice, the reduced growth observed upon DR or protein restriction respectively seems to be effected by a reduced cell number, implicating the suppression of IIS (32,33). Life-extending tryptophan or methionine restrictions also reduce growth in mice and rats via reduction in circulating IGF-I (13,14). Reduced IGF-I signaling modulates the negative correlation between body size and lifespan in mice (34) and dogs (35). Several rodent

studies show a negative correlation between body size and longevity in both genders (34,36) , as do several genetic models of extended longevity under reduced IIS (37). All these observations together suggest an inverse correlation between growth in body size and lifespan.

However, growth depression is not a prerequisite for lifespan extension. Body growth has been uncoupled from longevity both in mice and in flies (37). Thus, manipulations of growth signaling can extend longevity with no effect on body growth. Moreover, although anabolic traits are often used in the nutritional evaluation of a dietary protein, such traits are not reliable predictors of health during ageing. In rodents, some AA imbalances that do not cause growth depression can be detrimental for health and cause fatty infiltration of the liver (24). Therefore, reduction in growth signals can cause growth suppression and lifespan extension, but the former is not a prerequisite for the latter. From this perspective it remains possible that specific dietary AA intakes could optimize anabolic traits and lifespan, avoiding trade-offs between them.

Reproduction: Fecundity depends on nutrient utilization, and animal models are used to evaluate the link between fecundity and lifespan. In flies, DR reduces fecundity and increases lifespan (38). Fecund females allocate much of their ingested nutrients to reproductive processes, a proportion diminished in DR flies (39). In rodents, some long-lived models show a marked reproductive capacity reduction (38). Apart from extending rodent lifespan, protein restriction also suppresses fecundity (27). Reproductive output is also negatively associated with lifespan in dogs (40) and humans (41). However, in several fly models lifespan extension is not characterized by a lower reproductive output (38). Supplementing methionine in a methionine-restricted fly diet can rescue fecundity with no lifespan shortening (8), indicating that dietary modulation of AAs can promote longevity without impairing fecundity. Therefore, suppression of reproduction may not be indispensible for lifespan extension, and the design of diets that optimizes both traits is possible.

3. Amino acids: from ingestion to regulation of metabolism and health

3.1. Absorption and systemic availability of amino acids

I. Dietary protein and amino acid absorption

The intake of AAs is achieved through the consumption of either whole protein or free AAs in the diet. Following their ingestion, the identity and amount of the AAs that become available to cells and tissues depends on AA absorption by epithelial enterocytes. The digestibility of whole dietary proteins is confounded by numerous factors (42), which makes it difficult to establish the identity and amount of bioavailable AAs after the ingestion of whole protein foods (43). Yet, whether derived from digested peptides or free AA diets, AAs show substrate antagonism and other physicochemical properties that can complicate estimations of their availability (42-44). In contrast to whole protein diets, free AA diets avoid many of the confounding factors influencing whole protein digestion, and are more readily absorbable (45-46). Moreover, in

contrast to oligopeptide transporters, characterization of the free AA transporters in the human intestinal epithelium is comprehensive (44). Therefore, free AA diets are more suitable for the assessment of post-absorptive effects of dietary AAs upon metabolism, health, and ageing.

II. Dietary AAs and the microbiota

The uptake of AAs is influenced by gut bacteria, and this can greatly affect the response to a dietary protein or AA source. Instead of epithelial enterocytes, free AAs in the gut's lumen may first encounter gut microbes. Gut bacteria are both consumers and producers of AAs, but the net exchanges between host and microbiota of specific AAs, and the factors that influence this exchange, are not yet fully understood (47-48). Nevertheless, many AA exchanges between the host and the microbiota have been characterized. Gut bacteria synthesize all essential AAs (EAAs), and contribute up to 10% of mammalian plasma metabolites, including the EAAs tryptophan, phenylalanine, and lysine (48-49). Although such contributions for other EAAs are not yet described, they are likely to occur. In addition, during the host-colon nitrogen cycle, microbes further contribute towards AA re-absorption by the host (48). However, when rodents with a gut microbiota are fed single EAA-deficient diets, their health quickly deteriorates, which suggests that bacterial EAA contributions are modest (24). The effects of the deficiency also depend on the identity of the AA deprived (24), and on how much of each EAA gut bacteria can provide to the host. However, ruminants with a rumen microbial load that is able to synthesize all EAAs, still require an ample dietary AA supply to achieve high levels of growth or milk production (50). Therefore, the contribution of EAAs from gut bacteria to the host appears limited. In contrast, the microbiota consumes substantial amounts of AAs, with up to 50% of fecal nitrogen being of bacterial origin (1,4). However, more work is needed to establish how much of each ingested AA can be used by gut bacteria (47). Taken together these observations demonstrate that the gut microbiota can shape AA availability to the host. This can be especially important in low protein diets, because small changes in AA availability can have a proportionally greater effect on the available AA profile. In turn, this can also influence the useable dietary protein and the macronutrient ratio, which shapes health and ageing.

Changes in the microbiota are also associated with the risk for obesity, diabetes, and heart disease, and in mice DR enriches microbiota phylotypes associated with increased longevity (51). Gut bacteria adapt to ingested nutrients and can shift focus from dietary carbohydrate to dietary AA metabolism (52), while they also benefit from ample dietary fibre, which increases fecal nitrogen and decreases net AA uptake by the host (47). By adapting to macronutrients and metabolizing fibre, gut bacteria can stimulate the secretion of intestinal growth factors or satiety hormones (53,54). Apart from the health risks of a chronically over-stimulated growth axis, such interactions also complicate estimations of efficiency of dietary AA sources that rely on the evaluation of anabolic traits (growth) or behavioural traits (food intake). Also, the metabolism of AAs by gut bacteria may be influenced by circadian rhythms, or the host's age and immune status. Due to such complications, quantifying the net AA exchanges between host and microbiota

is easier in animals with a small number of gut bacteria species and chemically defined diets, such as fruit flies (46,53). Understanding of the factors affecting AA usage by gut bacteria, the mechanisms by which gut microbes influence AA availability to the host and the health consequences, will be aided by approaches in which the amount or composition of the microbiome is experimentally manipulated.

III. The splanchnic bed and systemic AA availability

The amount of each AA that becomes systemically available is critical for metabolism, affecting health through AA-sensing mechanisms discussed in the following sections. However, this amount greatly depends on how many AAs are metabolized immediately after absorption in the gut. Once absorbed by enterocytes, free AAs enter into the splanchnic bed (SB), which comprises the gut, liver, spleen, and pancreas, where free AAs can be metabolized. In general, up to a third of all dietary AAs are metabolized by the SB (47), greatly shaping the AA profile that reaches circulation to become systemically available. Metabolism of AAs in the SB also depends on AA identity. Despite arterial supply of AAs, enterocytes greatly rely on dietary AAs (55), and a low AA intake may contribute to enteral atrophy. Enteral usage of threonine is particularly high, presumably for the synthesis of threonine-rich mucins (47,55). In the liver, methionine enters many transulfation, transmethylation, and folate metabolism reactions (55). Glutamate, valine, isoleucine, leucine, and phenylalanine are also largely used by the SB. For all these AAs, an estimated 35%-100% of dietary intake is used by the SB, never reaching systemic circulation (47,55). In contrast, arginine, alanine, tyrosine, and proline undergo minimal usage by the SB (55). An AA's conformation can also determine its SB usage. In flies, mice, and rats, D- and L-methionine are highly bioavailable, while in humans D-methionine is only ~30% bioactive (24,56-57). In contrast, all other AAs are fully usable only in the L- form across the four species (24,56). Thus, SB metabolism of free AAs substantially affects their systemic availability depending on the individual AA's identity.

Following their passage through the SB, free AAs pass into circulation, to become part of the free AA pool. Free AAs represent a very small fraction of a body's total AA contents, but are metabolically significant as they form the systemically available AA profile. Indeed, free AAs are associated with lifespan across species. In flies low levels of glutamine, lysine, and alanine are linked to extended longevity (58). In mice, circulating metabolites including glutamine, methionine and proline decrease with age (59). This decrease is countered by acute DR, which increases circulating methionine, glutamine, alanine, and valine indicating a shift towards gluconeogenesis and energy conservation (60). However, an opposite metabolic shift has been suggested in dogs, where lower levels of isoleucine, leucine, phenylalanine, and valine are associated with the health benefits induced by DR (61). The same association has been made in humans too, where a plasma decrease in isoleucine, leucine, valine, lysine, phenylalanine, and histidine was linked to a reduced carbohydrate metabolism and an increased AA catabolism (62). Moreover, depleted levels of circulating methionine and BCAAs have been observed in long-lived IIS mutant mice (63). Finally, in mice elevated circulating BCAAs stimulate their catabolism in the liver (15). Thus, it is

currently difficult to interpret the mechanisms involved in plasma AA changes with age or with DR, or the consequences of these changes for health. Some possible mechanisms linking such free AA modulations to lifespan are discussed in more detail in following sections.

From circulation, free AAs can enter interstitial fluids and cells, to become part of tissue intracellular pools. Although the circulated free AA pool is available to all tissues reached by circulation, cell-specific AA availability depends on AA transporters whose abundance can vary between cell types (64-65). Abundance of transporters is well characterized for enterocytes, hepatocytes, pancreocytes, nephrocytes, and in the brain, as is substrate antagonism between AAs for transporters (44,65-66). Thus, despite equilibrium between circulatory and intracellular pools for most free AAs, substantial differences in concentrations between the two pools are seen in some cases. In humans, glycine, glutamate, and glutamine are 10-50 times more concentrated in intracellular pools (4). And it is also noteworthy that the free AAs in a tissue do not match the AA composition of the tissue's proteome. In rat muscle, compared to protein-bound AAs, there are depleted levels of phenylalanine, methionine, and BCAAs (4). Therefore, circulating, intracellular, and protein-bound AA profiles differ significantly, but circulating and intracellular AAs fluctuate more dynamically and play a prominent metabolic role.

The dynamics between transporter abundance and tissue-specific AA availability need more clarification, as do the effects of bidirectional transport between specific AAs, such as glutamine and leucine (155) upon AA tissue specific AA availability. Nonetheless, some physiological effects of AA antagonisms are clear. Antagonisms between the BCAAs can result in growth depression upon supplementation of one of these three AAs in the diet (24). Similarly, antagonisms between lysine and arginine can suppress growth upon addition of lysine or arginine only in the diet (24). Excess leucine or methionine deppress rat growth independently of food intake, with excess leucine increasing the growth requirement for tryptophan (24). However, there is a lack of long-term studies, and the effects of an AA imbalance-induced decrease in growth signaling upon health and lifespan await further study.

IV. Metabolic fate of ingested amino acids

The metabolic fate of intracellular AAs is important in determining the effects of AA intake upon health and ageing. An outline of AA metabolism is given in Figure 1. Once in the splanchnic bed, free AAs can be used for protein biosynthesis (e.g. in liver or intestinal muscle cells), or can be broken down to their carbon skeleton and amine groups. Amine groups are typically excreted, but carbon skeletons can have a diverse fate. They can be used in the biosynthesis of acetyl-coenzyme A (acetyl-CoA) or acetoacetyl-CoA, the main precursors of fatty acids, which are in turn stored as triacylglycerides (TAGs) in adipose tissue. Alternatively carbon skeletons can be used to synthesize pyruvate and oxaloacetate, the precursors of glucose (stored as glycogen) fueling the tricarboxylic acid (TCA) cycle. Finally catabolism of the carbon skeleton can also be used for cellular respiration and energy production in the form of ATP. Those AAs not metabolized in the splanchnic bed can enter circulation, from where they can be absorbed by cells and tissues.

Consequently, the proportion of AAs in the diet can affect many of these metabolic pathways. This aspect is discussed separately for AA limitations and excesses in the following sections.

3.2. Detection of AA limitations

The sensing of both circulating and intracellular free AAs occurs through various mechanisms, at both the cellular and systemic levels. These two modes of AA-sensing determine many of the metabolic and physiological responses to fluctuations in AA availability.

I. GCN2-dependent detection of AA limitation

A metabolic response to limited AAs can only occur after their limitation is detected. In flies, nutrient perception involves chemosensory sensila, enteroendocrine, and gustatory signals (67). The consequences of the fly's nutrient sensing can be uncoupled from its actual food intake, because stimulating odorant receptors can reverse the benefits of DR upon lifespan independently of food or protein intake (68). The fly's selection of an AA source is partly mediated by the serine/threonine-protein kinase general control nonderepressible 2 (GCN2) acting in dopaminergic neurons in the brain (69). The mammalian central chemosensor detecting decreased circulating essential AAs is found in the brain's anterior prepiryform cortex (APC) (70). Here, low levels of EAAs stimulate GCN2, which suppresses anabolism and promotes catabolism through the AA response (AAR) pathway (70,72,87) (Figure 2). This GCN2 activation is independent of the AA's identity, because GCN2 senses the AA deficiency by binding non-specifically to any uncharged transfer RNA (70,73). However, branched chain AAs, and in particular leucine, the most abundantly used AA in mammalian proteomes, appear to play a predominant role (74). Upon binding an uncharged tRNA, GCN2 changes its conformation to promote inhibitory phosphorylation of its primary downstream translation activator, the eukaryotic initiator factor 2 alpha (eIF2α) (70). This leads to global down-regulation of transcription and translation through changes in mRNA levels or mRNA stabilization, growth arrest and reductions in lipid and carbohydrate anabolism, activation of AA transporters (e.g. asparaginase synthetase ASNS), and changes in neuronal glutamatergic activity, intracellular calcium and GABAergic signaling (64,70,72,75). A common downstream effector of GCN2 activation upon methionine or leucine restriction is fibroblast growth factor 21 (FGF21), which represses liver fatty acid synthesis and increases fatty acid mobilization (13,76).

Although GCN2 is a key modulator of the systemic response to decreased levels of circulating AAs, it is also expressed across tissues and may also act in a cell-autonomous manner (70). In rodents, three isoforms of GCN2 are found: α, β, and γ. The α and γ isoforms have no functional GI (GCN2/Impact) domain to bind GCN2 to its activator GCN1, and are expressed tissue-specifically, while β has a functional GI domain and is expressed similarly across tissues (73). However, mice lacking GCN2 specifically in the brain fail to show the normal aversive behaviour towards AA-imbalanced diets. AA sensing in the APC thus overrides peripheral GCN2 activity with regards to feeding behaviour (72). Therefore, low circulating AAs result in stimulation of the AAR pathway through the activation of GCN2, which orchestrates cell-

autonomous and non-cell autonomous effects to promote catabolism, suppress anabolism, and thereby induce a metabolic maintenance mode.

II. GCN2-independent detection of AA limitation

Although much evidence supports a role for GCN2 in the sensing of ingested AAs, some studies cast doubt over its significance in the physiological and behavioural responses to AA-deficient foods. Recently, GCN2 has been shown to have no effect on the detection of AA-deficient diets in mice (145). In addition, sensing of AAs such as alanine or glycine in hypothalamic neurons also occurs via excitatory signals that are modulated within seconds from the moment an AA is supplied (146). Such response mechanisms are GCN2-independent, as transcriptional changes modulated by GCN2 would likely require a longer time period. Indeed, some preliminary findings in GCN2 knockout mice indicate that the response to methionine restriction is not dependent on GCN2 (13). Therefore, given the central role of the hypothalamus in modulating feeding behavior, obesity, and metabolism (152), it will be interesting to see how different ingested AAs give rise to an organism's hypothalamic and consequent metabolic response to ingested AAs.

Dietary AA restriction also regulates gene expression via multiple GCN2-independent pathways, including transcriptional (e.g. Cxcl10) or post-transcriptional (e.g. Dusp16) responses (72,77). Although the molecular mechanisms behind the activation of such responses by low AAs are not known, such changes are able to increase catabolic processes and metabolic efficiency independently of the GCN2-mediated AAR response. For example, efficiency of AA uptake is increased by up-regulation of asparagine synthetase ASNS, or of plasma membrane AA transporters such as the neutral AA transporters SNAT2 and LAT-1 and the cationic AA transporter CAT-1 (64,72,78). Other GCN2-independent responses can include transcription factor adjustments (ATF2-5, C/EBP and other ATF/CREB TFs), and changes in ribosomal proteins that affect translation (64,72,78). Although GCN2 is the only kinase exclusively responsive to AA deprivation, phosphorylation of eIF2α can also be effected by other kinases including heme-regulated inhibitor kinase (HRI), double-stranded RNA-activated protein kinase (PKR), and PKR-like endoplasmic reticulum-resident kinase (PERK), and considerable overlap has emerged in the activation of downstream effectors between PERK and GCN2 upon methionine restriction in mouse liver (75). Moreover, internal ribosomal entry sites (IRESs), such as that in the CAT-1 mRNA, allow preferential translation by phosphorylated eIF2α, and the role of such IRESs in PERK or GCN2 activation also requires further characterization (75). Another GCN2-independent mechanism may involve the AMP-activated protein kinase AMPK, which senses low-energy states by detecting high AMP levels (79). AMPK functions both cell-autonomously and non-cell-autonomously and is also activated upon low AA status (71,79). Importantly, increased AMPK activation extends worm and fly lifespan (79).

The dynamics of GCN2-independent responses can vary. As mentioned, sensing of supplied AAs by hypothalamic neurons can occur within seconds, thereby comprising a rapid response (146). In contrast, other modulators can vary across a wide range of time. For ASNS, a translational surge upon AA

limitation is followed by a more sustained transcriptional activation (64). Moreover, plasma AA responses to dietary AA limitations indicate that the limited AA drops in the plasma initially, but after several days its levels are restored (80). As a response to an AA-imbalanced diet, growth and food intake depression also subside after several days (81), perhaps through reconstitution of hormonal homeostasis (discussed below). In contrast, short-term responses relying on GCN2 do not require hormonal adjustments (70). Thus, although several GCN2-independent responses to limited AAs have emerged, further clarification of the molecular mechanisms and characterization of the short- versus long-term responses is needed.

III. Effects of AA limitations on ageing

All limitations or excesses of dietary AAs are sensed in a way that modulates anabolic and catabolic processes and, ultimately, homeostasis. Suppression of anabolism and growth signaling can extend lifespan and is induced by AA limitations. The identity of essential AAs is conserved between rodents and humans, and human cell culture work on AA limitations shows consistent results to murine cell systems implying conserved molecular mechanisms (135). In rodents, methionine and tryptophan limitations suppress anabolism and translation, and promote catabolic processes (13). Glucose, insulin, thyroid hormones, and IGF-I levels are also reduced in methionine-restricted mice. Yet, generally, low levels of circulating AAs reduce IGF-I function largely independently of their identity (83). Lack of the AA building blocks for anabolic traits also results in induction of apoptosis by IGF-I, which activates the apoptosis inducer CHOP (64). Stimulation of apoptosis aids the recycling of molecular building blocks, including AAs. Therefore, AA limitations deplete growth signaling and can thus induce a maintenance mode that benefits long-term health.

By increasing catabolism, methionine or tryptophan restrictions also reduce fat storage in rodents (13,14). However, although across multiple organisms and humans DR results in leanness or rescue from obesity to confer multiple metabolic advantages that favour longevity, the role of fat loss per se in promoting health is not clear. For instance, diets low in protein increase adiposity in mice because of increased food intake, but these mice are as healthy as DR mice (2,3). Additionally, the ability of animals to maintain their adiposity despite DR appears to mediate the beneficial effects of DR (84). The decline of mTOR expression with age in rat white adipose tissue is also prevented by DR (85). Therefore, as the role of fat deposition in DR is unclear, and given that different types of fat affect health differently, the role of fat deposition in mediating the health benefits of protein or single AA restriction requires further investigation.

IV. TOR-dependent detection of AA abundance

In mammals, the cell-autonomous AA response upon excess of AAs relies primarily on the mammalian target of rapamycin (mTOR), which occurs in two complexes, mTORC1 (rapamycin/nutrient sensitive) and mTORC2 (rapamycin/nutrient insensitive). Absence of AAs results in the TORC1-inhibitory recruitment of the tuberous sclerosis protein TSC2 onto the

lysosomal membrane (149). Mechanisms of activation of mTORC1 in the presence of AAs are shown in Figure 3. Sensing involves a complex interplay between numerous molecules recruited in or around the lysosome, which is a key site of AA recycling, and intracellular/intravacuolar AA sensing (74,86). Intracellular AAs prevent the inhibitory association of Sestrins with the GATOR2 complex (87)(150). This lowers GATOR1's GAP activity upon Rag A/B, which is bound to Rag C/D and is important for the activation and translocation of TORC1 onto the lysosomal membrane (87). Intracellular AAs are taken into the lysosome by transporters such as SLC38A9, which has a particularly high affinity for arginine (88). This transport induces conformational changes in the endolysosomal V-ATPase, which dissociates from the Ragulator/Rag complex (88). The Ragulator then enables the activation of Rag A/B through its guanine nucleotide exchange factor activity (88). Intracellular AAs can also promote the GTPase activity of the folliculin complex FLCN/FNIP (89), which results in the RagC/D complex being loaded with GDP, and stimulates Arf1 and Rab5, which are involved in intracellular trafficking inducing TORC1 activation (90).

The identity of the AA also determines how it is sensed by TORC1. Leucine activates Rag A/B through Sestrin 2 (150), glutamine sensing involves Arf1 and the V-ATPase, but not Rag A/B, and arginine sensing involves SLC38A9 (91). Other factors involved in TORC1 activation by AAs may involve the kinase Vps34 and SH3BP4 (86). Importantly, inhibition of mammalian TORC1 activation extends lifespan and, although activated TOR is critical in regulating adiposity by inducing lipid synthesis (86), insulin resistance is effected primarily by mTORC2 (92). The interplay between TOR and AMPK in sensing AAs also requires further characterization. Inhibition of rat muscle mTORC1 with rapamycin has no effect on AMPK, but activation of AMPK suppresses mTOR signaling and insulin resistance (93). Accordingly, reduced AMPK activity precedes mTOR activation by glucose or leucine, leading to insulin resistance (93).

As discussed for GCN2, not all AAs stimulate mTORC1 equally. In rodents leucine is a particularly strong activator (74). It has been reported that intracellular leucine is uniquely sensed by leucyl-tRNA synthetase (LeuRS), which activates the mTORC1 complex (Figure 3) (94). Nonetheless, it is now known that Sestrin2 possesses a leucine pocket to bind to, and sense, cytoplasmic leucine levels (151). Some obese animal models deplete their circulating glucogenic AAs, leaving higher circulating sulphur (95) and BCAAs including leucine (15), which can result in chronic TOR activation. In contrast, a low protein diet decreases circulating BCAAs and mTOR activation (3) and sensitizes animals to AA imbalances (24). Importantly, TOR activation can stimulate the secretion of hunger or satiety hormones in the GI tract and brain, such as ghrelin and leptin, respectively (23), and recent evidence suggests TOR is a mediator of the enteroendocrine hormonal responses to dietary proteins and AAs (96). Therefore a regularly high dietary intake of AAs induces chronic mTOR activation, which is detrimental to health and lifespan, whereas AA imbalances or limitations can inhibit TOR. However, TOR activation with respect to single AA modulations requires further elucidation both in invertebrates and vertebrates.

V. TOR-independent detection of AA abundance

Multicellular organisms have both intracellular and extracellular AA sensors, as well as neural sensors that respond to the intake of nutrients. Although TORC1 senses endocellular AAs and can stimulate satiety signals, in the GI tract extracellular AA sensors also play a prominent role. At least some mammalian G-protein-coupled receptors (GPCRs) are transceptors. Transceptors are transmembane transporters of nutrients, including AAs, that also act as receptors involved in inducing endocellular signaling. In mammals, GPCR receptors of the T1R family are activated mostly by L-AAs in the digestive tract (100), and even modulate TOR signaling independently of intracellular AA levels (65). Transmembrane GPCRs in enteroendocrine cells can stimulate the release of appetite-regulating incretins or decretins (98-99). Specifically, high luminal AA concentrations increase secretion of satiety incretin hormone GLP-1 by enterocytes (101). Additionally, in response to food or AA intake, density-, stretch-, and other chemo-receptors in the GI tract release neural signals of satiety to the CNS (54,97). Cholecystokinin (CCK) is secreted in response to luminal AAs, and CCK receptors stimulate vagal afferent signals to the nucleus of the tractus solitaries (NTS) of the brainstem, which relays signals to the hypothalamus (97). Other secreted peptides by the GI tract or along the splanchnic bed include leptin, insulin, and peptide YY (PYY), which suppress appetite in response to bulk food intake or protein intake, while ghrelin increases it (97) (Figure 4). Furthermore, recent evidence strongly supports unique ingested amino acid-specific signaling to the CNS, involving vagal afferents and the area postrema (148). All the above responses have some degree of conservation between invertebrates and vertebrates as similar mechanisms are involved in the fly's intestinal nutrient sensing (67). Thus, neural and hormonal modulations in the gut can function independently of intacellular AA sensing by TOR. In this way, ingested AAs trigger the release of hormones to regulate homeostatic processes in the whole organism (Figure 4).

VI. Responses to AA surpluses that affect physiology and ageing

High protein diets increase satiety and decrease food intake in many organisms, including flies (26), mice (2), and humans (22), an effect referred to as 'protein leverage' (2), with the main driver of appetite a target protein intake. Therefore the reduced obesity and insulin resistance of animals and humans fed a high protein diet ad libitum can be explained by their decreased food intake (23). However, dietary AA-induced chronic stimulation of the IIS/TOR pathways is detrimental for health (3,23,71,86,102). In yeast and worms, AA restrictions can inhibit TOR and extend lifespan (5,103,104). Inhibition of TOR by rapamycin or of S6 kinase (S6K), a downstream effector of TOR, also extends fly and rodent lifespan (102). Another main effector of mTOR is the translation repressor 4E-BP, which is activated upon TOR inhibition by DR in flies (102) or methionine-restriction in rodents (105). In humans, high levels of protein or AA intake also result in TOR activation (23) and insulin secretion (22), while excess acidifying AAs or sulphur AAs also raise blood pressure (22). With ageing, mTOR activity in mouse hypothalamic neurons increases, silencing anorexic neurons and contributing to age-related obesity (106). Moreover, TOR function can affect diverse systemic processes,

including cell and tissue growth signaling, immune function, proteostasis, neurodegeneration and cognitive function, tissue and stem cell physiology, and others (86,102). Thus, activation of TOR by high AA intakes can in the long-term be detrimental for health, promoting age-related disease such as neurodegeneration (107).

Although AAs promote growth signaling and TOR, excess AAs can also suppress growth, and therefore growth signaling, through antagonistic interactions. Mechanistically, this may occur if an AA is ingested in amounts that saturate specific AA transporters due to the AA's higher abundance, substrate affinity, or kinetics. In this case, it is possible that in some tissues intracellular levels of out-competed AAs become limited, thereby triggering the AAR to inhibit anabolic processes. Specific examples of moderate additions of AAs inhibiting growth in rodents were discussed earlier. Unpublished data in our laboratory also indicate that moderate additions of AAs can inhibit growth or growth signaling in both flies and mice. Importantly, such inhibitions of growth signaling may also affect health and longevity. In worms, addition of some AAs extends lifespan significantly through TOR inhibition (103). However, the identity of the AA is important, as addition of some AAs in the worm's diet had no effect, or even decreased lifespan drastically (103). In rats, an excess of threonine can be well tolerated but a similar excess of tyrosine can cause pathological lesions (24). Therefore the identity of the AA ingested in excess determines its effects upon health. More life-long studies will further clarify these interactions upon long-term health and ageing.

VII. Convergence of AA sensing pathways

There are many interactions between the multiple nutrient and AA sensing pathways discussed above (77). Phosphorylation of some translation initiation factors by TOR changes their conformation to allow accessibility by other kinases or phosphatases (including GCN2 downstream effectors) (108). Protein synthesis inhibition by GCN2/eIF2α stimulation occurs in conjunction with mTOR inhibition, and some cancer drugs deplete circulating AAs and trigger GCN2 to decrease mTORC1 signaling (109). Indeed, upon deprivation of AAs GCN2 induces the expression of Sestrin 2, thereby blocking the activation of mTORC1 (154). In yeast, the AAR pathway is most responsive when mTORC1 is inhibited by rapamycin (73). In worms, there is also a convergence of GCN2 and TOR upon AA limitation towards inhibition of global translation and down-regulation of FOXO transcription factors (110). Stimulation of FOXO transcription factors modulates the life-extending effect of IIS downregulation across species (5,106). Therefore, the orchestration of these two nutrient sensing pathways (TOR and GCN2) modulates AA sensing, although a detailed characterization of this interaction, especially with respect to individual AAs, remains to be established.

VIII. Food aversion, protein leverage, and growth signaling

Because imbalanced protein sources prevent the usage of excess and therefore total AAs, adequate protein intakes can be achieved with lesser amounts of high quality protein than of low quality protein. In order to achieve the target AA intake as driven by protein leverage, a less usable protein will therefore be consumed in greater amounts than a highly usable protein. For

instance, whey promotes growth more than does casein, and also induces a higher satiating effect in humans (111-112). However, imbalanced AA sources can result in deficiencies for specific essential AAs, and so animals must also have protective aversive responses to direct them to alternative, balanced AA sources (25). Thus the motive for increasing the intake of an imbalanced AA diet to achieve a target protein consumption may conflict with the motive to avoid a detrimentally imbalanced AA intake. The thresholds distinguishing between such conflicting motives are unclear, as is the impact of the imbalanced AA's identity on such effects.

Rodents are more sensitive to limited than they are to excess AAs. Very small AA limitations are detectable by rats, representing a 0.009% w/w change in the diet (70). Such limitations are not reflected in the plasma, but are seen in the APC region within 15 minutes of feeding on the imbalanced diet, resulting in loss of appetite (70). In contrast, growth suppression is detectable upon changes that represent >0.1% w/w of the limiting AA in the diet (24). In addition, ad libitum fed rodents on severely AA-limited diets decrease their food intake and growth but, if the animals are made to eat equal amounts, growth returns to normal (24). Therefore, appetite is more malleable in response to ingested AAs than is growth signaling. Moreover, responses to ingested AAs also depend on the identity of the imbalanced AA. Restriction of specific AAs (lysine, threonine, or isoleucine) alters food preference but not food intake in rats (70) . In mice, excess consumption of some AAs (e.g. methionine, tryptophan) suppresses food intake and growth more than does excess intake of others (e.g. threonine) (24). Therefore, the response to an imbalanced AA ingestion depends on the AA identity and on the physiological (e.g. growth) or behavioural (e.g. appetite, food choice) trait assessed. Further understanding of these aspects and interactions will be important in elucidating how AA modulations regulate metabolism and ageing, and in designing nutritional applications for humans.

IX. Distinct bioenergetic and metabolic roles of amino acids

Because of their different molecular structures, free AAs are broken down through distinct biochemical reactions. According to their catabolism, AAs can be glucogenic (all AAs except lysine and leucine), leading to the generation of glucose, or ketogenic (lysine, leucine), resulting in ketone bodies, although some AAs can be both (isoleucine, threonine, phenylalanine, tyrosine, and tryptophan). Glucose and ketones are the body's main energy sources, and cellular energy production from AA catabolism can represent 10-15% of total energy production (22). Importantly, the energy density of glucose is typically lower than that of ketones. Moreover, the energy expenditure for the metabolism of different AAs varies. Glutamate is the most energetically efficient AA (120), which may explain the central role of glutamate in providing TCA cycle precursors (66). Each AA also has a different metabolic efficiency for anaplerotic reactions, i.e. reactions that produce TCA cycle intermediates from precursors including AAs. Therefore, the metabolism of specific AA can uniquely affect energy homeostasis, which may impact on ageing. In worms, dietary supplementation with the ketogenic beta-hydroxybutyrate (103), the ketone derivative α-ketoglutate (119), or with several TCA cycle metabolites extends lifespan (103). This longevity gain in worms is thought to be mediated

by anaplerotic reactions (103,119). The energy sensor AMPK senses and modulates the metabolic and energy homeostasis changes of these long-lived worms, and the DAF-16/FOXO pathway is also activated by higher levels of TCA cycle intermediates (103).

The ketogenic or glucogenic potential of ingested AAs may also affect long-term health and ageing in rodents and humans. In mice, highly ketogenic diets reduce the catabolism of ketogenic AAs to prevent further ketogenesis, but do not alter lifespan (13). However, a modest increase in the intake of ketogenic compounds may be beneficial for mouse lifespan. Increasing the intake of the ketogenic AA leucine contributes to the mouse lifespan extension by BCAA supplementation (113), while in a mouse cancer model two different ketogenic compounds, butanediol and ketone ester, significantly increased survival independently of DR (114). In humans, ketogenic or leucine-supplemented diets may decrease food intake, adiposity, insulin resistance, sarcopenia, and cognitive deterioration with age (115-118). However, the TCA cycle is amphibolic, i.e. it is both anaplerotic and cataplerotic. This makes it difficult to quantify its bioenergetic modulations upon intake of different AAs. Therefore ketogenesis, TCA metabolite levels, and energy flux are coordinated by different AA ingestions to induce health and longevity gains across species. However, more investigations are needed to further elucidate how AA catabolism impacts on health and ageing through such modulations.

X. Health biomarkers of specific AA-imbalances

The AA profile of a dietary protein is generally the primary determinant of the protein's nutritional value. Several studies have identified effects of different dietary proteins with distinct AA profiles upon health and ageing. Soy and whey proteins improve a range of health markers and longevity, including increased insulin sensitivity and reduced adiposity (Table 1). In contrast, milk or casein proteins increase circulating IGF-I, insulin, and satiety hormones compared to other protein sources, and such chronic IIS over-stimulation can be detrimental for ageing (Table 1). The molecular mechanisms mediating the effects of such different protein sources implicate their AA contents. Soy (Figure 5a) and whey (Figure 5b) proteins are low in methionine and tryptophan content (1,121), while casein has a higher methionine content than soy protein (Figure 5c). Tryptophan (122) and methionine (13) promote growth hormone (GH) secretion, so diets with lower levels of these AAs decrease IGF-I, thereby promoting long-term health (121). Whey protein is also high in the BCAAs leucine and isoleucine (Figure 5b), which may explain its growth-promoting and appetite-suppressing effects in animals (112), and its prevention of muscle loss in older humans (117). Egg protein is a high-quality protein for growth (112), but is not necessarily optimal for long-term health as it causes high postprandial levels of circulating glucose accompanied by a low appetite suppression effect (111). Therefore specific protein sources with distinct AA profiles can down-regulate IIS and increase healthspan and longevity.

Other endocrine modulations involve thyroid hormones, with soy protein lowering parathyroid (PTH) hormone secretion (Table 1), which in humans is linked to BMI and mortality, at least under some pathological conditions.

Along with growth hormones, secretion of thyroid hormones is also reduced by DR (6) and tryptophan restriction (14). In mice, increased plasma levels of BCAAs are associated with decreased lifespan (3), but high dietary BCAAs have also extended lifespan presumably through different protective mechanisms (113) that require more detailed investigation. In humans, increased plasma BCAAs are linked to insulin resistance and type 2 diabetes (123).

Fish protein is also linked to human health benefits, including increased insulin sensitivity and reduced circulating low-density lipoproteins (Table 1) (22-23). A comparison of the AA content in >10 fish species shows that the two most limiting AAs in fish are tryptophan and methionine (Figure 5d), with cysteine as the most limiting non-EAA. Some long-lived human populations, like Okinawans, Sardinians, or Ikarians (124) are located in areas were fish is a predominant protein source (125-126). Thus it is tempting to draw a link between the reduced sulphur AAs and tryptophan and the insulin sensitivity and lifespan-extension observed in these populations.

In humans, several cohort studies show that high intakes of animal protein, which is typically methionine-rich, are positively associated with chronic and age-related disease, and this association is abolished when the dietary protein source is plant-based (11). However, the age of an individual also determines the health response to the ingested protein (11), as discussed below.

The bioenergetics of different AA sources may also contribute to health effects. The metabolic efficiency of different dietary proteins integrates their AA composition and the energy used in the catabolism of each AA to produce one ATP molecule (120). A comparison of the metabolic efficiency of different dietary proteins shows that proteins linked to beneficial effects for healthspan and lifespan in animals and humans (Table 1) tend to have an essential AA profile that has a higher metabolic efficiency (Figure 6a). The calculated % energy efficiency is lower for lactalbumin, egg, and casein, and higher for soy and fish proteins. Therefore it is possible, although not yet established, that a link between metabolic efficiency of AA catabolic reactions and health exists.

Interestingly, in the two recent DR primate studies, the WNPRC diet had higher contents of tryptophan and BCAAs (lactalbumin) than the NIA diet (fish-soybean-wheat-corn-alfalfa) (Figure 6b). Milk and dairy proteins such as lactalbumin can induce TOR activation and insulin resistance in humans (23,122). Thus, AA intake differences between the two studies could contribute to differences in mortality and cancer incidence, as a higher intake of BCAAs and tryptophan could have lead to a chronically higher TOR/IIS stimulation (5).

In conclusion, a number of observations suggests an important role of dietary AA intake upon health and ageing in humans.

4. Optimal amino acid intake

I. Variation in requirement for amino acids.
The AA needs of individuals, populations, and species, are influenced dynamically by internal state and environmental factors. The main sources leading to variation in AA requirements are discussed here.

Although not much studied beyond inborn errors of metabolism, genetic background across and within species impacts greatly on the response to AA consumption. Inter-strain variability for the requirement of some AAs (e.g. glycine) has been shown to be significant in Drosophila (147). In rodents and humans, genetic background profoundly affects body size, AA requirements, and food intake (4,27). Wild strains of worms and flies live longer upon DR (127), and mice of different strains respond diversely to a single DR regime (128). The genetic background may also contribute to differences in the DR response observed in the recent primate studies (5). Single DR regimes do not indicate the response of a mouse strain across different restriction levels (26), but do suggest that genetic constitution profoundly impacts on the response to reduced intake of nutrients including AAs. Some recent approaches assess single nucleotide polymorphisms (SNPs) to identify specific genes and to explain how genetic variation in inbred mouse populations determines traits of interest (129). Similar approaches could be employed to evaluate the role of genetic determinants in the DR response, both in rodents and in humans. Such information will inform our understanding of how natural genetic variation predisposes the DR response both in model organisms and in humans, and even aid the design of individualized nutritional interventions.

All growing or reproducing mammals, including humans, have higher AA requirements than adults (27,30). Consequently young children are more susceptible to protein malnutrition and related diseases such as Kwashiorkor. For laboratory animals there is a clear distinction between diets optimized for breeding or growth stages versus diets optimized for long-term maintenance (27). Adjusting the dietary protein supply to match AA requirements with age promotes health and longevity. Providing high dietary protein to young animals and lower to mature ones extends lifespan in rats (9,130) and mice (6). In rodents, protein absorption reduces with age as older rats show a decreased ability to digest proteins and AAs (131). Moreover, mature rodents fail to show some of the adverse effects of ingesting AA-imbalanced diets (24-25). Accordingly, BCAA stimulation of the IIS/TOR pathways is greater in younger, not older, animals (78). Therefore it is not surprising that early onset DR extends rodent lifespan significantly (132), but late onset DR is less effective (6). The protein source during early life also impacts on health during ageing. Although milk protein can chronically over-stimulate IIS and contribute to insulin resistance (Table 1), restriction of milk-protein during weaning only can significantly increase mouse lifespan (133). Requirements for AAs may also change qualitatively as an animal physically matures. Some evidence in mice suggest subtle changes in the body's AA composition with development (134). In humans, a low protein intake appears to benefit groups of 50-65 years of age, but may be detrimental when applied to older ages (11). Therefore, it is clear that age and life stage can affect both the requirement for AAs, and the response to AA intakes.

The EAA requirements of individual cells or tissues can vary depending on tissue-specific AA metabolism. For example, enterocytes secrete threonine-rich proteins, so require a higher threonine intake than other cells (47,55). Hepatocytes require high levels of methionine to serve many transulfation, transmethylation, and folate metabolism reactions (55). The type and abundance of AA transporters also determines which AAs enter readily into which cells. These aspects require further study in conjunction with more systematic analysis of tissue-specific usage of individual AAs.

In humans, gender defines AA requirements as males require more AAs than non-pregnant females, which reflects body size differences to an extent (4,30). Beyond inborn errors of metabolism, the maintenance of health requires adequate AA supply, as multiple immunological processes depend on AAs, and an imbalanced AA intake can suppress the immune system (20). Indeed, the efficiency of the immune response declines and the susceptibility to infections increases upon low AA intake (4). Thus, it is possible that infectious or disease conditions that increase the function of immunological processes may raise dietary AA requirements (20). In the future, more work is required to understand how specific disease states increase the requirement for specific AAs.

In addition, healthy physical activity increases the metabolic rate and promotes protein degradation, AA oxidation, and depression of protein synthesis in humans, thereby increasing AA requirements (4). The metabolic rate of individuals can also be modulated by environmental conditions, as lower temperatures can increase the metabolic rate in endothermic animals (4). Similarly, seasonal increases in day cycle duration can promote physical activity, thereby increasing the metabolic rate particularly at younger ages (4). Such increases in metabolic rate also translate in increases in AA requirements.

In summary, numerous findings from different branches of nutritional research clearly indicate that both environmental and internal state factors must be considered when estimating of AA requirements.

5. Conclusions

Identifying beneficial AA intakes can lead to improvements in human nutrition. In human populations, health benefits for older age groups mirror most mortality gains, and late life dietary interventions based on AA intake are beneficial (11). As AA intakes are critical to the DR response, dietary AAs provide a powerful intervention strategy for human health. Indeed, recent evidence shows that a fasting mimicking diet based on a limited plant-based AA intake benefits human health (17). Such dietary manipulations comprise a drug-free way of intervening towards healthy ageing. Moreover, nutritional efficiency can have diverse applications within our societies, as it can help to end starvation, to devise tools against obesity and disease, to enhance produce yield in the food industry, and to assist patients in numerous clinical

applications including cancer.

FIGURE AND CAPTIONS (CAPTIONS WORD COUNT: 492)

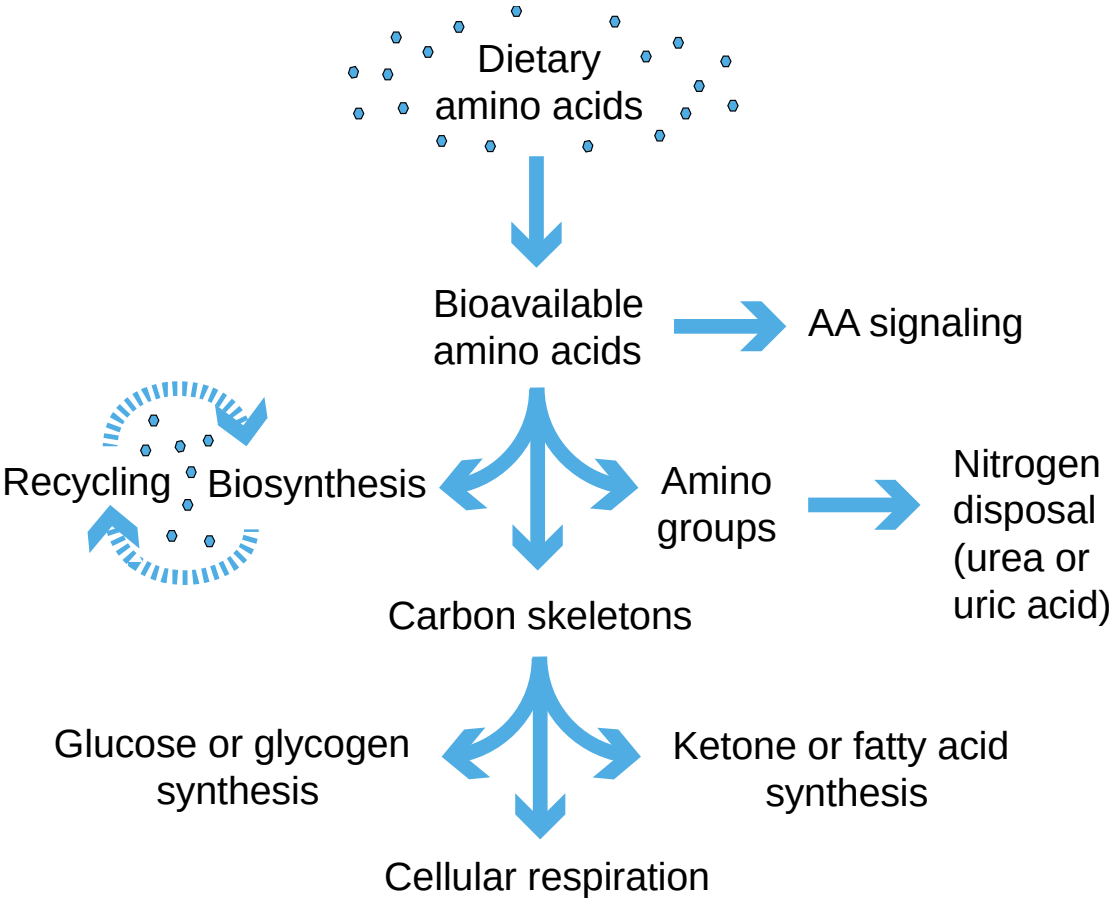

Figure 1

The metabolic fate of ingested amino acids. Modified from Berg et al. 2007 (144)

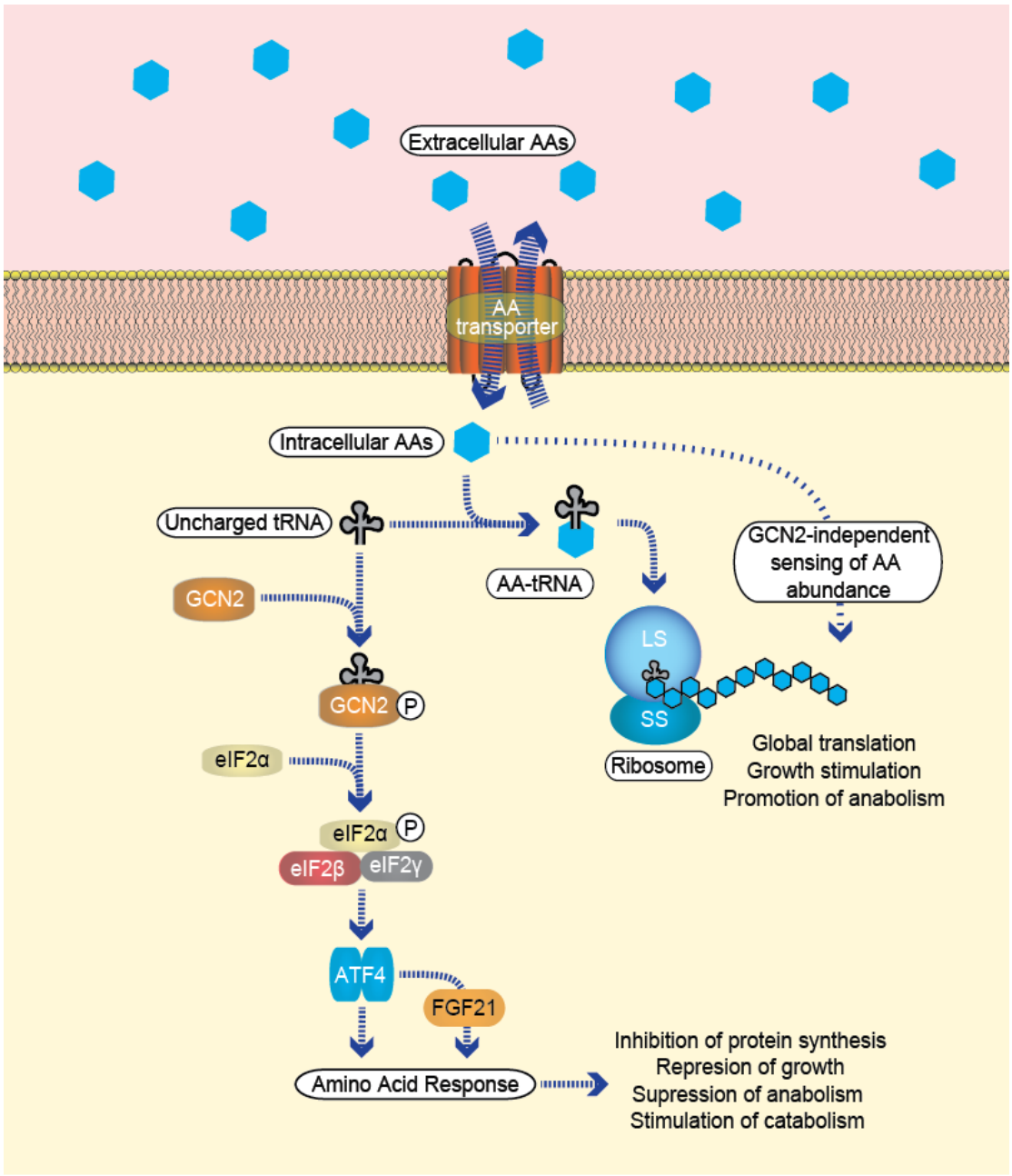

Figure 2

GCN2-dependent sensing of AA limitations. Extracellular amino acids (AAs) can activate their cognate tRNA, which is now available for ribosomal protein synthesis (LS=large subunit, SS=small subunit). In contrast, uncharged tRNAs bind to and activate by phosphorylation GCN2, which in turn phosphorylates eIF2α. This activates the eIF2 complex to stimulate ATF4,

which induces FGF21 to trigger the amino acid response (AAR), which inhibits anabolic processes, and promotes catabolism (see main text).

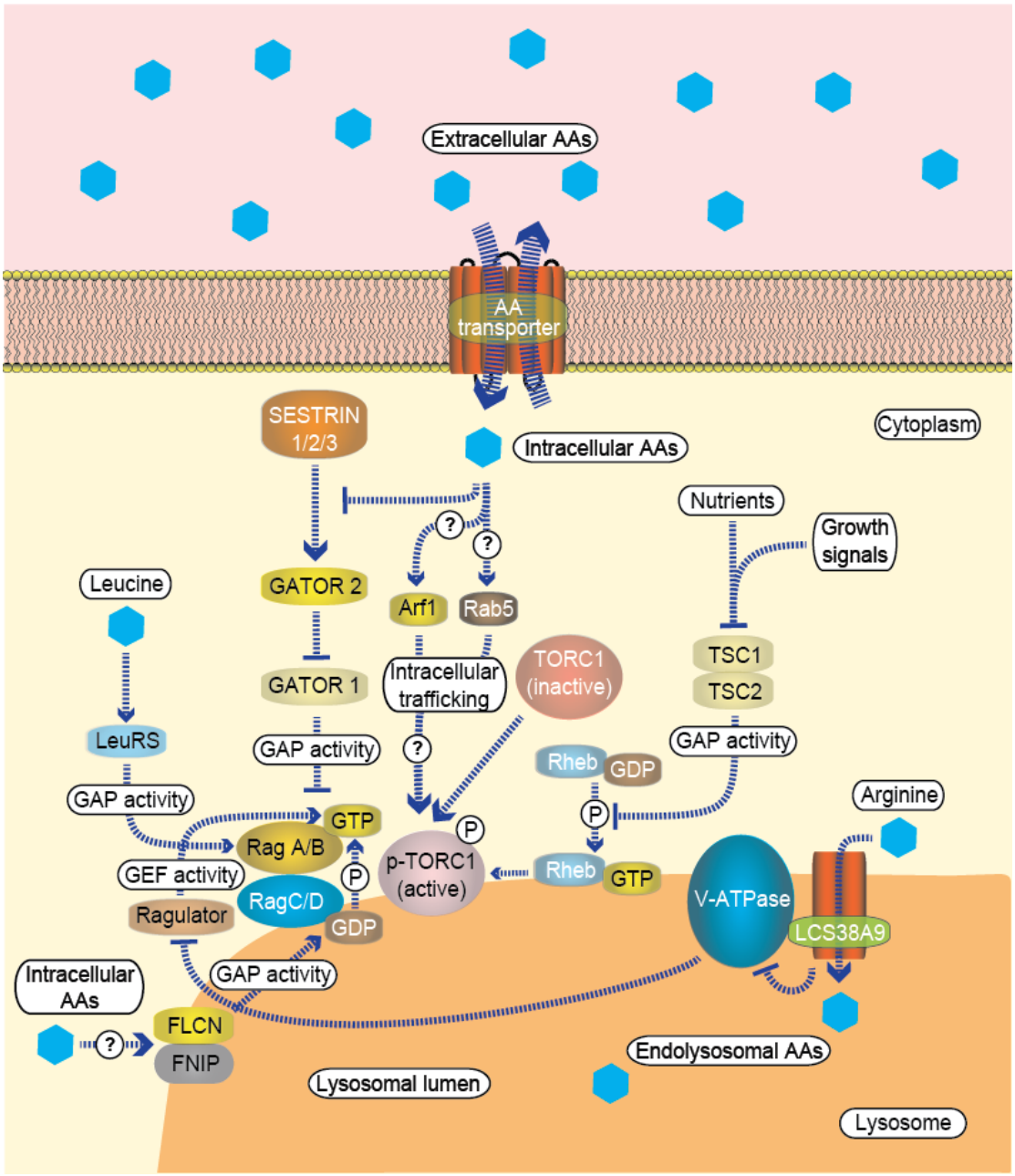

Figure 3

TOR-dependent sensing of AAs. Intracellular AAs activate TORC1 through multiple TOR-associated factors (see main text for details). GAP - GTPase-activating protein; GEF - guanine nucleotide exchange factor (GEF)

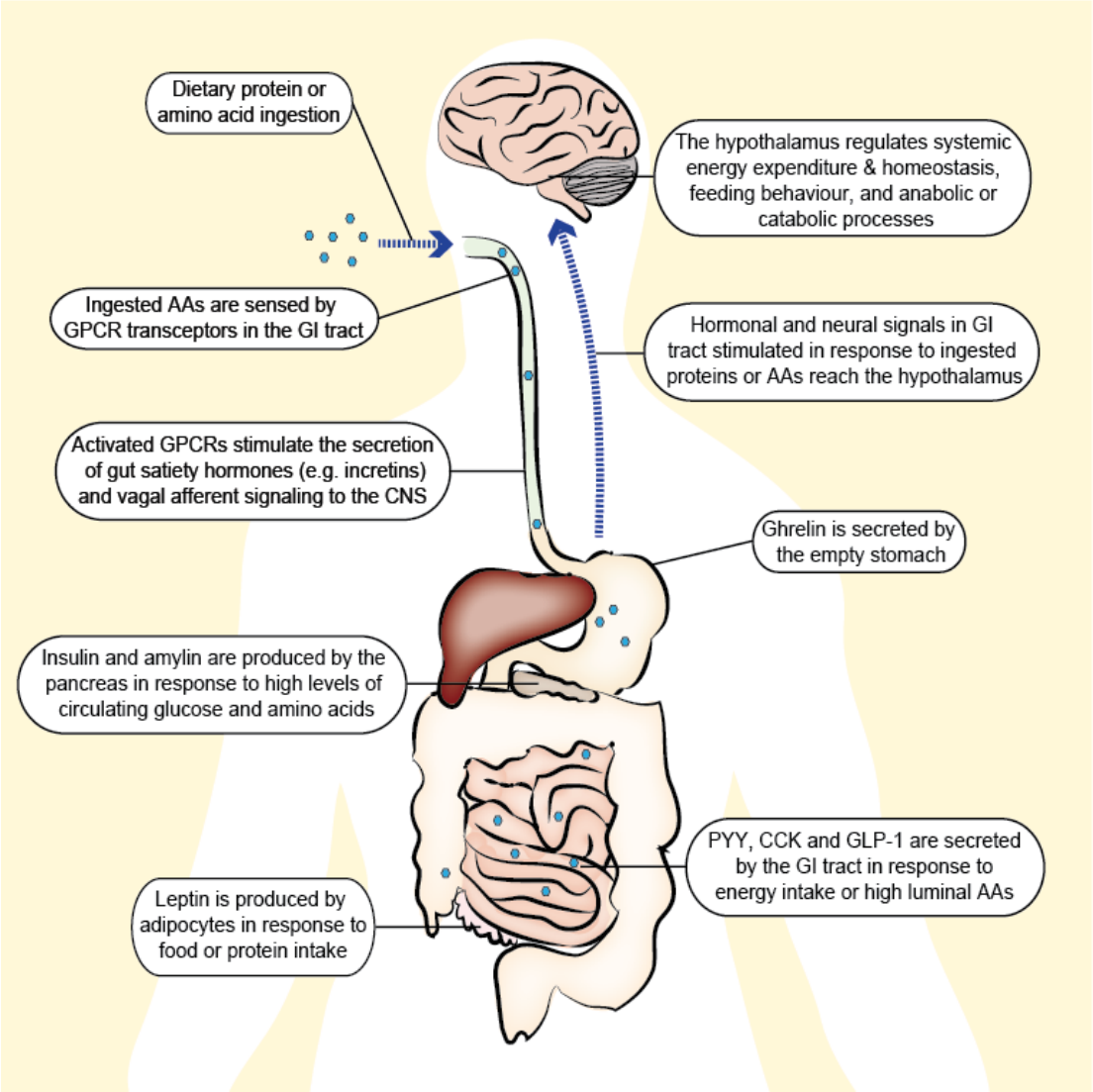

Figure 4

Sensing of dietary proteins and AAs along the gastrointestinal (GI) tract. Ingested AAs activate transceptors (e.g. GPCRs) that relay hormonal and neural signals of satiety to the brain. Protein or amino acid intake can also stimulate the secretion of a range of appetite suppressors including PYY, CCK, and GLP-1 (see main text). Such hormonal signals are targeted to the hypothalamus, which regulates a range of systemic and metabolic processes that are associated with homeostasis and health during ageing.

Species examined	Physiological effects observed	Measured effect on health and/or lifespan	Reference(s)*
Homo sapiens	Increase in circulating IGF levels, mTORC1 activation, increase in circulating AAs and in growth rate, reduced insulin sensitivity, changes in calcium signaling	Negative (milk and dairy protein)	Crowe et al. 2009, Melnik et al. 2013, Tucker et al. 2015
Homo sapiens	Reduced plasma lipids and adipocity, reduced prostate and breast cancer, and improved insulin and glucose homeostasis	Positive (soy)	Tremblay et al. 2007
Homo sapiens	Compared to animal meat, soy protein decreased protein synthesis, protein oxidation, energy expenditure, and thermogenesis.	n.d.	Westerterp-Plantenga et al. 2009
Homo sapiens	Whey increased circulating GLP-1 levels, thereby increasing satiety	n.d.	Westerterp-Plantenga et al. 2009
Ratus norvegicus	Soy reduced serum parathyroid hormone and nephropathy	Positive (soy)	Kalu et al. 1988
Homo sapiens	Fish protein increases insulin sensitivity, reduces plasma LD and increases HDLs	Positive (fish)	Tremblay et al. 2007
Ratus norvegicus	Rats fed whey protein had reduced plasma and liver cholesterols	Positive (whey)	Zhang et al. 1993
Mus musculus	Mean lifespan increase, increased liver and heart glutathione levels, increased insulin sensitivity	Positive (whey)	Bounous et al. 1989, Shertzer 2011
Homo sapiens	Reduction in adipocity, increase in muscle protein, protection from high blood pressure	Positive (whey)	Coker et al. 2012, Jakubowicz 2013
Ratus norvegicus	Casein increased circulating IGF levels	Negative (casein)	Noguchi 2000
Homo sapiens	Plant proteins decrease circulating IGF-I levels compared to animal proteins, and increase the IGF-I inhibitor IGFBP-3	Negative (plant and soy protein)	McCarty et al. 2009, O'Neill 2010
Homo sapiens	Whey increased satiety, decreased post-prandial levels of circulating glucose, and decreased subsequent food intake compared to egg and turkey proteins	Positive (whey)	Pal 2010

Table 1

List of findings relating dietary protein sources to healthspan and lifespan, including effects upon the IIS and TOR pathways, and on circulating metabolites linked to health-related parameters.

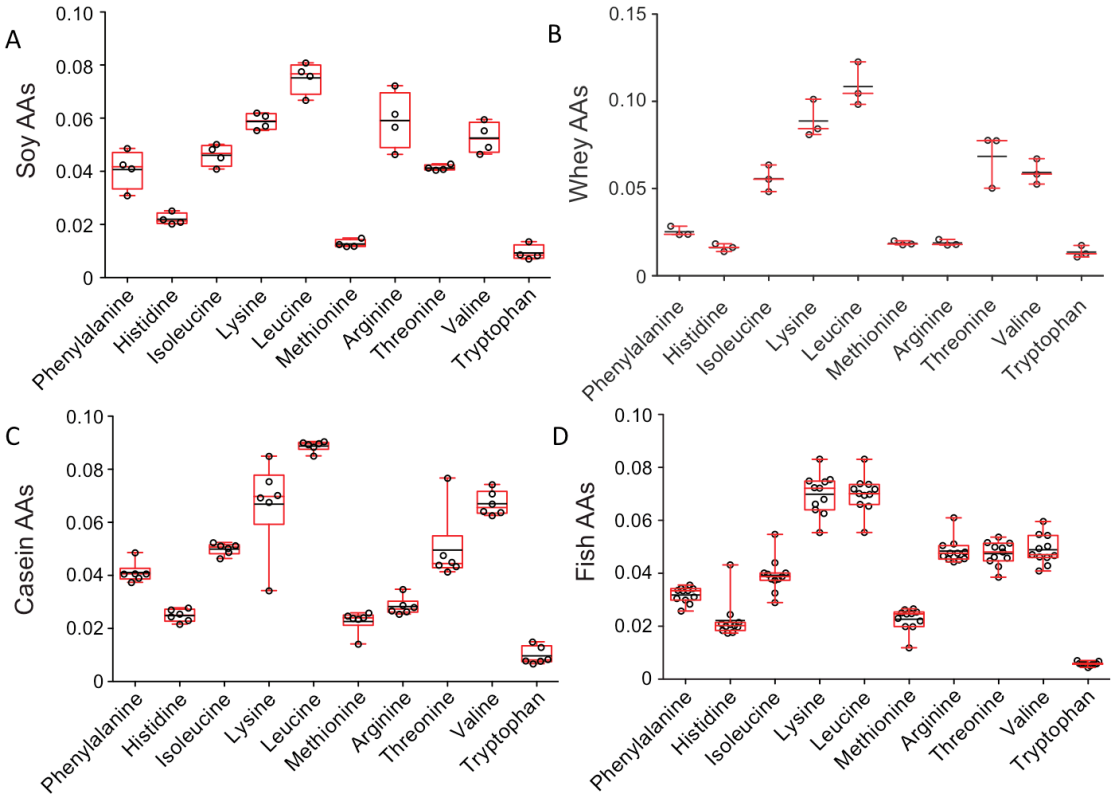

Figure 5

A) Four published soy EAA profiles show considerable batch-to-batch variability and low levels of tryptophan and methionine. Molar proportions are shown.

B) The essential AA proportional representation in three published whey profiles. Five of the essential AAs, including methionine and tryptophan, are particularly low, but leucine is higher than in soy, fish, or casein proteins.

C) Six bovine casein EAA profiles indicate differences between casein sources within the same species (*Bos taurus*), low contents of tryptophan, and higher levels of methionine compared to whey or soy proteins.
D) An analysis of the AA profile of ten teleost fish species indicates limitations in tryptophan, histidine, and methionine. However, due to the severe limitation in cysteine, the limitation of methionine likely surpasses that of histidine, making methionine the second most limiting AA. Also, a lower leucine level is seen compared to soy, whey, or casein proteins.

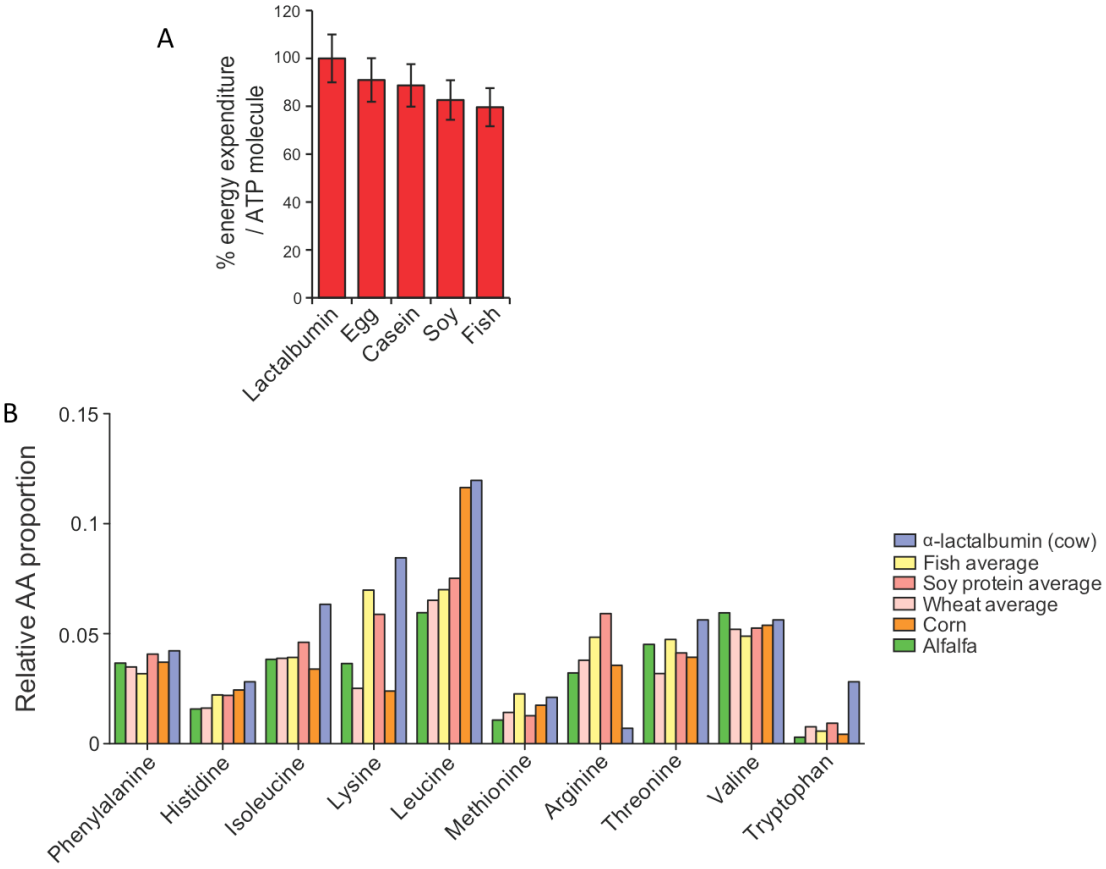

Figure 6

A) The calculated % energy expenditure for the production of one ATP molecule based on non-integral P/O ratios (Milgen 2002), shown for the essential AA composition of five common types of dietary protein: lactalbumin, egg, casein, soy, and fish. Differences are due to the range of carbon chain and cofactors that result from essential AA catabolism. Some protein sources associated with health benefits (Table 1) appear to have a proportionally higher metabolic efficiency than proteins associated with detrimental effects.

B) Comparison of the mean EAA content (from published data) of the six protein sources used in the two primate studies (see main text for discussion). Lactalbumin has a particularly high content in tryptophan and isoleucine, as well as leucine.

Summary points list (8)

1. Even with adequate intake of macronutrients, the protein and amino acid content of the diet are critical for health during ageing

2. An imbalanced supply of amino acids occurs when the requirement for dietary amino acids, usually determined by their effects on anabolic traits, is not matched by their intake

3. This requirement is affected by multiple factors including genetic diversity, gender, age, and health status

4. Amino acid absorption and availability is determined by the gut microbiota, the amino acid's identity, and first pass metabolism

5. Ingested and systemically available AAs are sensed by various mechanisms, involving TOR, GCN2, GPCRs, and other sensors

6. Excess intakes of amino acids can over-stimulate growth signaling, which can be chronically detrimental and decrease longevity

7. Limiting or imbalanced intakes of amino acids can down-regulate growth signaling, inducing a maintenance mode

8. Experimental animal models can inform human nutrition, increasing our understanding of how AA intakes affect human health and ageing

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Abbreviations-Acronyms

1. DR – dietary restriction
2. AA – amino acid
3. EAA – essential AA
4. NEAA – non EAA
5. BCAA – branched-chain AA
6. IIS – insulin and insulin-like growth factor 1 (IGF1) signalling
7. mTOR – mammalian target of rapamycin
8. SB – splanchnic bed
9. TCA cycle - tricarboxylic acid cycle
10. TAGs – triacylglycerides
11. ATF – activating transcription factor
12. GCN2 - general control nonderepressible 2
13. AMPK - denosine monophosphate-activated protein kinase
14. AAR – amino acid response
15. ASNS – asparagine synthetase
16. CNS - central nervous system
17. GAP activity - GTPase-activating protein (GAP) activity
18. GEF activity - guanine nucleotide exchange factor (GEF) activity
19. GPCR - G-protein-coupled receptor
20. GLP-1 - Glucagon-like peptide-1
21. PYY - peptide YY
22. CCK – Cholecystokinin
23. FOXO - transcription factor Forkhead box O
24. BMI – body mass index

Key terms – definitions (9)
(<20 words / definition)

1. Macronutrient ratio: the relative proportions of protein, carbohydrates, and fats in a diet

2. Amino acid bioavailabilty: the fraction of an absorbed amino acid that reaches systemic circulation

3. Dietary amino acid imbalance: when the intake of amino acids does not match the AA requirements of an organism for a specific trait

4. Dietary amino acid limitation: when an AA intake is below the organism's requirement for a given trait

5. Dietary amino acid excess: when the AA intake exceeds the organism's requirement for a given trait

6. Nutrient sensing: a collection of systemic, neural and cell autonomous signals elicited in response to nutrient and/or energy intake

7. Transceptors: transmembane transporters of nutrients, including amino acids, that can also act as receptors, therefore having signaling capacity

8. Energy homeostasis: the set of balancing adjustments aiming at metabolic equilibrium

9. Amino acid bioavailabilty: the fraction of an ingested amino acid that can be metabolized

Future issues list (8)

1. A full characterization of the AA transporters across tissues, e.g. along the brain-blood-barrier, to further inform how the CNS senses each AA limitation

2. Further elucidation of the interactions of transceptors and intracellular AA sensors across tissues

3. Full description of the interactions between systemic (e.g. hormonal) and local (e.g. GCN2, TOR) responses to ingested AAs

4. Characterization of the metabolic role of circulating AAs, and of the energy efficiency and metabolic flux of different AA sources

5. Further clarification of TOR activation by specific-AAs to include all essential and non-essential AAs

6. Detailed quantification of uncharged tRNA abundance and consequent GCN2 activation, both with free AA diets and protein diets

7. Elucidation of TOR-independent and GCN2-independent sensing of AAs

8. Clarification of the role of IIS and TOR over-stimulation in health deterioration and age-related pathology

Annotated references
(<15 words per annotation)

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