An increased need for dietary cysteine in support of glutathione synthesis may underlie the increased risk for mortality associated with low protein intake in the elderly

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Abstract Restricted dietary intakes of protein or essential amino acids tend to slow aging and boost lifespan in rodents, presumably because they downregulate IGF-I/Akt/mTORC1 signaling that acts as a pacesetter for aging and promotes cancer induction. A recent analysis of the National Health and Nutrition Examination Survey (NHANES) III cohort has revealed that relatively low protein intakes in mid-life (under 10 % of calories) are indeed associated with decreased subsequent risk for mortality. However, in those over 65 at baseline, such low protein intakes were associated with increased risk for mortality. This finding accords well with other epidemiology correlating relatively high protein intakes with lower risk for loss of lean mass and bone density in the elderly. Increased efficiency of protein translation reflecting increased leucine intake and consequent greater mTORC1 activity may play a role in this effect; however, at present there is little solid evidence that leucine supplementation provides important long-term benefits to the elderly. Aside from its potential pro-anabolic impact, higher dietary protein intakes may protect the elderly in another way—by providing increased amino acid substrate for synthesis of key

protective factors. There is growing evidence, in both rodents and humans, that glutathione synthesis declines with increasing age, likely reflecting diminished function of Nrf2-dependent inductive mechanisms that boost expression of glutamate cysteine ligase (GCL), rate-limiting for glutathione synthesis. Intracellular glutathione blunts the negative impact of reactive oxygen species (ROS) on cell health and functions both by acting as an oxidant scavenger and by opposing the pro-inflammatory influence of hydrogen peroxide on cell signaling. Fortunately, since GCL's K_m for cysteine is close to intracellular cysteine levels, increased intakes of cysteine—achieved from whole proteins or via supplementation with *N*-acetylcysteine (NAC)—can achieve a compensatory increase in glutathione synthesis, such that more youthful tissue levels of this compound can be restored. Supplementation with phase 2 inducers—such as lipoic acid—can likewise increase glutathione levels by promoting increased GCL expression. In aging humans and/or rodents, NAC supplementation has exerted favorable effects on vascular health, muscle strength, bone density, cell-mediated immunity, markers of systemic inflammation, preservation of cognitive function, progression of neurodegeneration, and the clinical course of influenza—effects which could be expected to lessen mortality and stave off frailty. Hence, greater cysteine availability may explain much of the favorable impact of higher protein intakes on mortality and frailty risk in the elderly, and joint supplementation with NAC and lipoic acid could be notably protective in the elderly, particularly in those who follow plant-based diets relatively low in protein. It is less clear whether the

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lower arginine intake associated with low-protein diets has an adverse impact on vascular health.

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Low-protein diets—protective in mid-life, detrimental in the elderly

A recent analysis of the National Health and Nutrition Examination Survey (NHANES) III prospective cohort study, focusing on the association between protein intake at baseline and subsequent health outcomes in subjects over 50, found that a relatively low protein intake (under 10 % of calories) in subjects between 50 and 65 correlated with lower overall mortality, including decreased mortality from cancer and diabetes (Levine et al. 2014). In contrast, among subjects over 65 at baseline, such a low protein intake was associated with increased risk for overall and cancer-linked mortality. This latter finding is concordant with considerable evidence that the elderly may have an increased requirement for dietary protein—perhaps even higher than current recommendations (Raffi et al. 2014)—and that increased protein intakes in the elderly tend to be associated with improved health outcomes (Bauer et al. 2013).

The favorable impact of mid-life protein restriction might be anticipated, given that diets restricted in proteins or essential amino acids, consumed in unrestricted amounts, have been shown to boost mean and maximal lifespans in rodents (Mirzaei et al. 2014; Sanchez-Roman and Barja 2013). A recent study evaluating the impact of ad libitum diets of widely varying macronutrient composition on longevity in mice found that the highest mean and maximal lifespan was seen in mice consuming a diet providing 5 % protein and 75 % carbohydrates (Solon-Biet et al. 2014). Protein restriction downregulates hepatic production of IGF-I (Fontana et al. 2008)—an effect traceable to activation of the kinase GCN2 by a detected deficiency of one or more essential amino acids (Gallinetti et al. 2013; McCarty 2014)—and also suppresses mTORC1 activity, owing in part to decreased availability of leucine and branched-chain amino acids (Gallinetti et al. 2013). As a result, the IGF-I/Akt/mTORC1 signaling pathway—known to be a key driver of the aging process (Bartke 2008)—is downregulated, “cell cleansing” autophagy (Cuervo 2008) is upregulated, and translation of most proteins is slowed.

These effects are similar to those evoked by calorie restriction and by germline mutations that compromise GH/IGF-I signaling—which typically slow aging, prevent cancer, and boost maximal lifespan (Bartke 2008; Brown-Borg et al. 2009; Anisimov and Bartke 2013).

In light of these aging-retardant, pro-longevity effects of protein-restricted diets observed in rodents, why then do low protein intakes (as a fraction of total calories) predict increased mortality risk in aging humans? One possibility is that, at a time in life when sarcopenia, osteoporosis, and frailty pose a growing risk to health and survival, the anti-anabolic effects of protein restriction become counterproductive. In a prospective study targeting older adults, those whose baseline energy-adjusted protein intake was in the top quintile lost 40 % less lean mass during 3-year follow-up than those whose protein intake had been in the bottom quintile (Houston et al. 2008). Other studies have correlated higher protein intakes in the elderly with higher bone mass, or, prospectively, less loss of bone mass and lower fracture risk (Kerstetter et al. 2000; Rapuri et al. 2003; Hannan et al. 2000; Misra et al. 2011). There is a growing consensus among gerontologists that protein requirements are higher in elderly people than in younger people (Bauer et al. 2013).

Although IGF-I activity appears to play a pacesetter role in the aging process, and is sometimes referred to as a universal cancer promoter, it does however have a direct favorable impact on vascular endothelial function and endothelial precursor cells, and its dietary downregulation might therefore have a countervailing negative impact on vascular health (Perticone et al. 2008; Csiszar et al. 2008; Conti et al. 2011; Devin and Young 2008)—an effect which might have a greater impact on health in aging humans than in rodents. It seems unlikely, however, that IGF-I’s impact on the vasculature played an important role in the lower mortality associated with higher protein intakes among the elderly in the NHANES III analysis, as, among those over 65, plasma IGF-I levels did not differ significantly as a function of protein intake (Levine et al. 2014). However, IGF-I did moderate the association between protein intake and cardiovascular risk—for those over 65, a higher protein intake predicted reduced cardiovascular risk only in those whose IGF-I levels were relatively low. Hence, an aging vasculature experiencing low IGF-I activity may derive the greatest benefit from increased dietary protein.

The essential amino acid restriction associated with vegan diets of modest protein content does

downregulate IGF-I levels (Fontana et al. 2008). Hence, elderly vegans, in whom IGF-I tend to be low, might achieve some vascular protection by consuming more protein (albeit most life-long vegans have low LDL levels and are at low risk for atherosclerosis (Resnicow et al. 1991)). Laron dwarves, whose livers fail to express growth hormone receptors, and consequently have very low circulating IGF-I levels, appear to enjoy virtual immunity from cancer and diabetes, but their lifespans are not notably longer owing to a fairly high risk for vascular disorders (Shevah and Laron 2007; Steuerman et al. 2011).

Dietary leucine promotes anabolism by stimulating mTORC1 activity

Dietary intake of leucine, via modulation of mTORC1 activity, a key determinant of the efficiency of protein translation, seems likely to be a mediator of the anabolic effects of increased protein intake in the elderly. The ability of a protein bolus to acutely activate muscle protein synthesis has been traced to the leucine content of the protein, likely reflecting leucine's ability to boost the activity of mTORC1, a key determinant of the efficiency of protein translation (Rieu et al. 2006). Elderly people require a higher dose of leucine to achieve this benefit (Katsanos et al. 2005; Katsanos et al. 2006). Nevertheless, there appears to be little evidence at present that long-term leucine supplementation can favorably impact lean mass in the elderly (Balage and Dardevet 2010).

Protein ingested soon after a resistance training session amplifies the resulting increase in muscle protein synthesis; the anabolic signal imparted by the exercise session is potentiated by leucine-mediated mTORC1 activation (Esmarck et al. 2001; Breen and Phillips 2012; Burd et al. 2012; Volek et al. 2013). Hence, postexercise supplementation with 2–3 g of leucine (or ingestion of an equivalent amount from leucine-rich protein, such as whey), along with some carbohydrate to further enhance mTORC1 activity (via insulin), may have potential as an adjuvant to resistance training for prevention of sarcopenia. Long-term studies evaluating this strategy are needed.

It should be borne in mind that an upregulation of mTORC1 activity is not necessarily innocuous. The cancer preventive effects of metformin, rapamycin, adiponectin, and vegan diets are at least partially traceable to inhibition of this activity (McCarty 2011).

Administration of the mTORC1 inhibitor rapamycin to mice beginning late in life (600 days) extends both mean and maximal lifespan; mice genetically deficient in mTOR activity (25 % of normal) enjoy a 20 % increase in mean lifespan (Harrison et al. 2009; Wu et al. 2013). The late-life failure of autophagy would be exacerbated by mTORC1 activation (Cuervo 2008). High-leucine diets can accelerate the growth of some cancers (Liu et al. 2014).

The leucine metabolite beta-hydroxy-beta-methylbutyrate (HMB) exerts a modest anabolic effect on skeletal muscle, but perhaps more importantly has anti-catabolic activity. When administered orally, it induces a modest and transient activation of mTORC1 in skeletal muscle; its anti-catabolic activity appears to be of more significance and may reflect downregulation of proteasomal activity (Wilkinson et al. 2013; Holecek et al. 2009). The clinical utility of supplemental HMB for preserving muscle mass has been demonstrated in cancer cachexia and bed rest atrophy; studies of HMB supplementation in the healthy elderly, with or without concurrent exercise training, suggest that it may favorably modulate the lean-to-fat ratio, though some studies are difficult to interpret owing to concurrent use of supplemental arginine and lysine (Fitschen et al. 2013; Deutz et al. 2013a; Deutz et al. 2013b). In rats, supplementation with HMB beginning at 86 weeks of age prevents any loss of muscle fiber dimensions and prevented an increase in ubiquitin-dependent protein degradation (Fitschen et al. 2013). HMB slows the proliferation of Walker 256 cancer in rats, owing to downregulation of the constitutively high NF-kappaB activity in this cancer; this contrasts with the cancer-stimulatory potential of leucine (Caperuto et al. 2007; Kuczera et al. 2012). Small reductions in LDL cholesterol and systolic blood pressure have been seen in placebo-controlled clinical studies with HMB (Nissen et al. 2000). Supplementation with this well-tolerated natural factor might be considered as an alternative to leucine administration for prevention of sarcopenia; conceivably, it might aid retention of muscle mass while minimally impacting IGF-I/Akt/mTORC1 signaling.

We are faced with an intriguing question to which we do not yet have a clear answer: In humans, is there an inflection point beyond age 65 at which downregulation of aging-linked anabolic pathways becomes counterproductive and increased IGF-I/mTORC1 activity promotes increased survival? Might such an inflection point exist in rodents? That question at least could be addressed experimentally.

A role for catabolic tissue loss in increased mortality risk during old age is suggested by epidemiological studies concluding that, in the elderly, moderately elevated BMI tends to be associated with improved survival (Janssen 2007; Auyeung et al. 2010; Winter et al. 2014). Yet analyses of the Adventist Health Study and Adventist Mortality Study, in which BMI was monitored serially, indicated that, among non-smoking men who maintain a relatively stable BMI during their mature years, mortality risk increases directly with BMI—as one might expect from the health consequences of excess abdominal adiposity (Singh et al. 1999; Singh et al. 2011). Furthermore, among the elderly, waist circumference correlates directly with mortality risk except for the very leanest subjects (de Hollander et al. 2012). Hence, the greater mortality risk associated with lower BMI in the elderly noted in many studies may reflect higher mortality in people who have lost lean mass, often for no clear reason. This weight loss might be reflective of circumstances that contribute to the increased mortality—low-grade inflammation, occult cancer, lack of exercise, depression—but loss of lean mass and accompanying frailty may of themselves increase risk. As noted, loss of lean mass tends to be blunted in those with relatively high protein intakes.

Glutathione levels decline during aging

Nonetheless, it remains uncertain whether the lesser mortality associated with higher protein intakes among elderly subjects in the NHANES III study is primarily reflective of higher anabolic activity. An alternative (or additional) possibility is that much of the benefit is conferred by certain specific amino acids which serve as precursors to physiological factors—such as glutathione or nitric oxide—that may be particularly protective for the elderly. Cysteine availability is rate-limiting for glutathione synthesis (Richman and Meister 1975; Suh et al. 2004a). In this regard, there is considerable evidence that glutathione synthesis and glutathione tissue levels tend to decline in rodents and humans as they age (Suh et al. 2004a; Rebrin and Sohal 2008; Rebrin et al. 2007; Droge et al. 2006; Sekhar et al. 2011; Wang et al. 2003; Lean et al. 2005). This appears to reflect, in turn, an age-related decline in expression and function of the Nrf2 transcription factor (Suh et al. 2004a; Suh et al. 2004b; Ungvari et al. 2011; Shih and Yen 2007). Nrf2 promotes transcription of a wide array of enzymes

which combats oxidative stress and aids the detoxification of mutagenic electrophiles; among these is the rate-limiting enzyme for glutathione synthesis, glutamate cysteine lyase (GCL) (Wild et al. 1999; Lee and Surh 2005). Why Nrf2 expression declines with age remains mysterious; a concurrent upregulation of its functional antagonist Keap1 may also contribute to a loss of Nrf2 activity in the elderly (Ungvari et al. 2011). The age-related decline in GCL activity may also reflect a structural alteration in this enzyme, as there is one report that the affinity of this enzyme for its rate-limiting substrate cysteine declines in aging rat liver (Suh et al. 2004a).

Strategies for boosting glutathione synthesis

Fortunately, there are simple practical strategies which can compensate for the age-related decline in GCL activity. The K_m for GCL's rate-limiting substrate cysteine is close to usual intracellular levels of this amino acid; it follows that an increase in dietary cysteine intake can be expected to boost glutathione synthesis, particularly when cellular glutathione levels are relatively low (Richman and Meister 1975; Suh et al. 2004a). (Glutathione interacts with GCL allosterically to inhibit its activity (Richman and Meister 1975)). An efficient way to achieve this, without increasing protein intake per se, is to supplement with *N*-acetylcysteine (NAC), a well-absorbed and chemically stable compound that is rapidly cleaved to liberate free cysteine soon after it is absorbed (Atkuri et al. 2007; Dodd et al. 2008). (Cysteine per se is too unstable to be employed as a concentrated supplement, and the cystine which it readily gives rise to is poorly absorbed.) The ability of supplemental NAC to boost tissue glutathione levels in rodents and humans is well documented.

Moreover, the expression of GCL can be enhanced by administration of phase 2 inducer chemicals—compounds which disrupt the inhibitory association of Nrf2 with Keap1 (Wild et al. 1999; Lee and Surh 2005). Some of these—such as lipoic acid—also boost translation of Nrf2 messenger RNA via an internal ribosome entry sequence (IRES) (Shay et al. 2012). Hence, phase 2 inducers typically increase intracellular glutathione synthesis by increasing GCL transcription and expression. They may also increase this synthesis by promoting intracellular uptake of cysteine in the brain via induction of a transporter that carries cysteine (Suh et al. 2004a). The impact of the phase 2 inducer lipoic acid

has received particular attention in this regard. (Suh et al. 2004a; Suh et al. 2004b; Flier et al. 2002; Jia et al. 2008). Arguably, concurrent administration of lipoic acid and NAC should be a highly effective strategy for amplifying deficient glutathione levels that has the ancillary benefit of increasing the expression of a number of other antioxidant and cytoprotective proteins whose transcription is promoted by Nrf2 (Lee and Surh 2005).

Crucial antioxidant roles for glutathione

The key importance of intracellular glutathione, typically present in low millimolar concentrations, reflects two phenomena. Glutathione is a highly effective oxidant scavenger and hence can prevent structural damage to DNA, proteins, and membrane lipids in oxidatively stressed tissues. But it also functions as an antagonist of the pro-inflammatory/pro-apoptotic effects of the ubiquitous cell metabolite hydrogen peroxide. By oxidizing susceptible sulfhydryl groups in proteins to sulfenic acids—which are labile, and often subsequently undergo rapid intramolecular or extramolecular reactions to form disulfides or sulfenyl amides, or are irreversibly oxidized to sulfinic acid—hydrogen peroxide can modulate the function of key signaling proteins, often in ways that promote inflammation, cell proliferation, or apoptosis (Tanner et al. 2011; Bindoli and Rigobello 2013; Lo and Carroll 2013). Glutathione opposes this effect, first, by functioning as a reducing substrate for glutathione peroxidase, which converts hydrogen peroxide to water (Arthur 2000). Second, via direct reactions with sulfenic acid, or via the catalytic impact of glutaredoxin, which likewise employs glutathione as a reducing substrate, glutathione can restore sulfenic acids to their native sulfhydryl form (Dickinson and Forman 2002; Shelton et al. 2005; Parsons and Gates 2013). These protective mechanisms are evidently compromised when cellular levels of glutathione decline during the aging process.

Supplemental NAC in aging humans and rodents provides versatile health benefits

The effects of NAC administration in elderly humans or aging rodents have been reported in several studies. Perhaps the most intriguing of these was a multicenter controlled clinical trial in which people over 65,

suffering from various non-respiratory chronic disorders, were randomized to receive 1.2 g NAC daily or placebo for 6 months during the flu season (De et al. 1997). The impact of this supplementation on symptoms stemming from influenza infection, the frequency of seroconversion to the influenza virus then extant and the efficacy of cell-mediated delayed hypersensitivity reactions, was then assessed. Although NAC supplementation was not found to modify the frequency of influenza infection, as determined by seroconversion, it markedly decreased the severity of symptoms associated with such infections. Hence, among those taking NAC, only 25 % of the flu-infected subjects became symptomatic, 79 % of those subjects in the placebo group did. Days spent bedridden owing to flu infection were markedly lower in the NAC-treated group. Those receiving NAC also showed a progressive rise in cell-mediated delayed hypersensitivity, as assessed by skin tests; no such change was observed in the placebo group. Given the substantial morbidity and mortality associated with flu infection in the elderly, it is rather perplexing that, 17 years after the publication of this study, no clinical attempt to replicate its findings has appeared.

The findings of this study may reflect the fact that the symptoms of influenza are often attributable to an over-exuberant immune response evoked by infection, rather than from the direct cytopathic impact of the virus on lung epithelium (McCarty et al. 2010). With respect to the impact of NAC on cell-mediated immunity—the efficiency of which typically declines with aging—there is evidence that glutathione is crucially important to efficient function of dendritic cells, the central coordinators of cell-mediated immunity (Kim et al. 2007). Moreover, administration of NAC to aging mice has been shown to restore the delayed-type hypersensitivity to more youthful levels, in precise parallel to the findings of the Italian clinical trial with NAC (Kim et al. 2008).

Sekhar and colleagues measured erythrocyte glutathione levels in eight elderly subjects (aged 60–75 years) and in eight younger subjects (aged 30–40 years); they were 46 % lower in the older group (Sekhar et al. 2011). Erythrocyte cysteine levels in the elderly subjects were more moderately depressed—by 24 %. Plasma markers of oxidative stress were higher in the elderly subjects. The elderly group was then supplemented with 100 mg/kg/day of both NAC and glycine for 2 weeks. This led to a 95 % increase in their erythrocyte glutathione levels, with the plasma markers of oxidative stress reverting to levels indistinguishable from those seen in

the younger subjects. It is questionable whether the glycine supplementation contributed to the impact of supplementation on glutathione level, as glycine availability is not thought to be rate-limiting for glutathione synthesis.

Decreased expression of Nrf2 and of Nrf2 target genes has been demonstrated in the vascular system of aging rats (Ungvari et al. 2011). This suggests that NAC supplementation might favorably influence vascular function in aging humans. A cross-over placebo-controlled study in elderly hypertensives (average age 69) treated with ACE inhibitors found that supplementing NAC at 600 mg three times daily amplified the blood pressure control achieved; 24-h blood pressure averaged 9 mmHg (systolic) and 6 mmHg (diastolic) lower when NAC was being administered—a very substantial effect (Barrios et al. 2002). Another controlled trial evaluated a combination of 1200 mg daily of cysteine and 1200 mg arginine, given for 6 months, to type 2 diabetic (median age 64) (Martina et al. 2008). As compared to response in the placebo group, the patients receiving this joint supplementation achieved reductions in systolic, diastolic, and mean blood pressure, various markers of endothelial activation, and plasma C-reactive protein, as well as an improvement in endothelium-dependent vasodilation.

Droge and colleagues have pioneered the notion that aging may be “a cysteine deficiency syndrome” and have conducted several clinical trials in elderly subjects to document this (Droge et al. 2006; Hauer et al. 2003; Droge 2005). They report that NAC supplementation (600 mg t.i.d.) increased muscle strength, raised serum albumin, and lowered serum TNF- α . They provocatively hypothesize that “practically everyone experiences sooner or later an ageing-related deficit in the body cysteine and glutathione reservoirs that warrants cysteine supplementation” (Droge 2005).

Reactive oxygen species play a key role in the induction of osteoclast differentiation and in osteolytic activity; hydrogen peroxide has been shown to drive the increase in osteolysis associated with ovariectomy in rats (Lean et al. 2005). The increase in osteolysis consequent to ovariectomy in rats is associated with a decline in bone marrow glutathione content (Lean et al. 2003). Administration of NAC to rats prevented ovariectomy-induced bone loss—whereas administration of an agent that inhibits glutathione synthesis promoted bone loss in intact rats (Lean et al. 2003). In a small, randomized, placebo-controlled clinical trial, 21 early postmenopausal patients were all placed on calcium/vitamin D supplementation, and in addition

received 2 g NAC daily or matched placebo for 3 months (Sanders et al. 2007). Whereas a serum marker of bone resorption (C-telopeptide) fell slightly in both groups after a month, it continued to fall over the next 2 months in the NAC-supplemented subjects—but not those receiving placebo throughout the trial; after 3 months, this marker was 15 % reduced from baseline in the NAC group, as opposed to a 5.6 % reduction in the placebo group. While this differential did not achieve statistical significance, the authors recommended that an adequately powered controlled trial be conducted to better assess NAC’s anti-osteoporotic potential. (Seven years later, this trial is still awaited.)

Since lipoic acid can boost glutathione levels via phase 2 induction, it is not surprising that lipoic acid has shown a favorable impact on maintenance of bone density in ovariectomized rats; this agent also opposes osteoclastogenesis in vitro (Kim et al. 2006; Polat et al. 2013). In 50 osteopenic postmenopausal women, randomized to receive an antioxidant regimen including lipoic acid + calcium/vitamin D or calcium/vitamin D alone, bone mass was significantly higher after 1 year in the patients receiving the antioxidants (Mainini et al. 2012).

In aging mice, the content of reduced glutathione in the brain’s cortex (hippocampus) and striatum is about 20–25 % lower than in young mice, whereas the ratio of reduced to oxidized glutathione is about 50 % as high—indicative of a major increase in brain oxidative stress during aging (Rebrin et al. 2007). Beginning at 18 months of age, rats were treated with a daily antioxidant cocktail—providing NAC, lipoic acid, and vitamin E; others were employed as controls (Thakurta et al. 2014). After 4–6 months of such supplementation, the spatial learning and memory of these rats was assessed with a standard T-maze procedure; young rats (4–6 months old) were also studied in this way. As compared to the performance of the young rats, the aging controls performed notably more poorly in acquisition and retention; however, in the aging rats who had received the antioxidants, this deficit in performance was attenuated significantly by about 50 %.

Oxidative stress is believed to play a key pathogenic role in certain neurodegenerative disorders linked to aging such as Parkinson’s and Alzheimer’s disease. It is reasonable to suspect that an age-related decline in brain glutathione and Nrf2 could exacerbate their progression. Indeed, NAC has shown efficacy in rodent models of these disorders (Offen et al. 1996; Martinez 2000; Chen et al. 2007; Clark et al. 2010; Berman et al.

2011; Martinez-Banaclocha 2012; Smeyne and Smeyne 2013; Moreira et al. 2007; Huang et al. 2010; Pocernich and Butterfield 2012; Hsiao et al. 2012; Farr et al. 2003). NAC is also beneficial in a mouse model of ALS (Andreassen et al. 2000). In a 6-month controlled clinical trial of NAC supplementation in Alzheimer's patients, there was a trend for measures of cognitive function to decline less rapidly on all tests employed, albeit significant differences were seen for only a subset of the cognitive tasks (Adair et al. 2001).

One study has examined the impact of NAC supplementation (5 or 10 g of NAC per liter of drinking water) on median and maximal lifespan in mice (Flurkey et al. 2010). In males, but not in females, both of these parameters were extended significantly by NAC supplementation. In light of the failure of females to respond in this study, it is pertinent to note a report concluding that tissue glutathione levels tend to fall more during aging in male than in female mice; estrogen administration increases the hepatic expression of GCL in both male and female mice (Wang et al. 2003). This inductive effect of estrogen on GCL expression appears to be tissue-specific; nonetheless, it would be of interest to explore whether the impact of estrogen on glutathione metabolism plays some mediating role in the higher average and maximal lifespans of females in humans and certain other species.

Erythrocyte glutathione levels have been assessed as a marker for health status in the elderly. In a small cross-sectional study, higher glutathione levels were associated with fewer numbers of illnesses and higher self-rated health status; glutathione level explained 24 % of the variance in an index of morbidity (Julius et al. 1994).

In aggregate, current evidence strongly suggests that an increased intake of cysteine from dietary protein and/or NAC supplementation could notably downregulate the pathogenic impact of reactive oxygen species (ROS) in the elderly by boosting glutathione levels, potentially exerting a positive impact on vascular health, immune capacities, bone density, cognitive function, progression of neurodegeneration, metabolism of mutagens and toxins, and systemic inflammation. Almost certainly, low cysteine intake contributes importantly to the increased mortality observed in elderly subjects eating low-protein diets. Complementing NAC supplementation with an effective phase 2 inducer such as lipoic acid could be expected to further enhance tissue levels of glutathione, while restoring expression of a range of antioxidant enzymes to more youthful levels. And by

supplementing with NAC, it should be feasible to achieve these benefits without increasing IGF-I or mTORC1 activities.

A note on arginine

Dietary arginine can act as a precursor for nitric oxide. Could increased arginine intakes associated with a higher protein diet provide protection by improving the function of nitric oxide synthase (NOS) in dysfunctional vascular endothelium (eNOS)?

Ambient variations in the arginine content of natural diets may have a relatively small impact on plasma and tissue levels of arginine because much arginine is synthesized endogenously, and a high proportion of ingested arginine (~60 %) is rapidly degraded by arginase activity in the intestinal mucosa and liver (Mariotti et al. 2013). Indeed, the latter factor explains why citrulline supplementation is more effective than arginine supplementation for raising plasma arginine levels (Schwedhelm et al. 2008). Studies of arginine supplementation which have demonstrated physiological effects have typically employed 6 g daily or more (Boger 2008)—a higher amount than that provided by average diets. A 6-month study evaluating 3 g of supplemental arginine daily—an amount comparable to the differential arginine content between high protein and low protein diets—in patients with peripheral artery disease, failed to observe any favorable clinical impact on symptoms or markers of NO activity (Wilson et al. 2007).

A further concern is that, whereas arginine's favorable impact on eNOS activity presumably can exert a positive impact on progression of atherosclerosis and endothelial function, increased arginine levels might also increase nitric oxide—or superoxide—production by the inducible nitric oxide synthase (iNOS) (Xia et al. 2010). In patients with advanced atheroma, this might have a destabilizing impact on plaque, inasmuch as increased expression of arginase I is reported to have a stabilizing impact in that regard (Wang et al. 2014; Teupser et al. 2006; Wang et al. 2011). Conceivably, this might help to explain why a controlled trial of arginine supplementation (9 g daily) large and long enough (6 months) to report hard endpoints, enrolling patients who had recently suffered a myocardial infarct, observed six deaths in the treatment group, versus zero in those receiving placebo ($P = 0.01$); as a result, the trial was terminated sooner than planned (Boger 2008;

Schulman et al. 2006). On the other hand, another randomized controlled trial in post-MI patients, again using 9 g of arginine daily, but of only 1-month duration, found a non-significant trend toward protection with arginine (Bednarz et al. 2005). A meta-analysis which incorporated these two trials found no impact of arginine on mortality risk (RR = 0.93; 95 % CI 0.74–1.17) (Sun et al. 2009).

Given that there is still no clear evidence that modest, dietary range increases in arginine intake can favorably influence vascular health outcomes—and the possibility that boosting iNOS activity could exert a countervailing negative effect in this regard—it seems doubtful that decreased arginine intake contributes importantly to the increased mortality observed in elderly people consuming low-protein diets. However, further studies evaluating the impact of high-dose citrulline supplementation in elderly subjects with vascular disease appear warranted.

Curiously, there is evidence that dietary citrulline exerts an anabolic effect on skeletal muscle in aging rats, while concurrently reducing fat mass (Moinard et al. 2015). This anabolic effect is clearest in the context of underfeeding and reflects an increased rate of synthesis of muscle myofibrillar proteins and glycolytic enzymes (Osowska et al. 2006; Faure et al. 2013). Data are inconsistent as to whether muscle-specific activation of mTORC1 activity plays a role in this effect; it is also unknown whether this effect is mediated by arginine or NO (Moinard et al. 2015; Cynober et al. 2013). In healthy human volunteers fed a low-protein diet, postabsorptive administration of citrulline, in comparison to equal intakes of a mixture of non-essential amino acids, increases the fractional synthesis rate of mixed muscle protein (Jourdan et al. 2015). Whether citrulline supplementation can aid control of sarcopenia in humans remains to be determined, and, as citrulline is not a constituent of proteins, this phenomenon cannot explain the favorable impact of higher protein intake on mortality in the elderly. Nonetheless, the impact of long-term supplemental citrulline on body composition in the elderly merits evaluation.

Summing up

Despite the fact that ample intakes of high-quality protein increase the activity of pro-anabolic signaling pathways that drive the aging process and boost cancer risk—likely explaining why low-protein diets are emerging as beneficial in rodent studies and in middle-

aged humans—a recent analysis of the NHANES III cohort reveals that low-protein intakes may be associated with increased mortality in the elderly. Since other epidemiological studies have correlated increased dietary protein with favorable anabolic effects in the elderly—including better preservation of lean mass and bone mass—it is conceivable that there is an inflection point during human aging when an increase in IGF-I and mTORC1 activities becomes of net benefit to survival, owing to better prevention of frailty. Nonetheless, this possibility remains quite speculative; whether such an inflection point exists in rodents could be examined experimentally. As dietary leucine is a key determinant of mTORC1 activity, further studies evaluating supplemental leucine in the elderly, particularly in the context of resistance training, could prove illuminating; so far, there is little evidence that long-term leucine supplementation benefits the elderly.

On the other hand, there is quite compelling evidence that a low-protein diet could amplify the contribution of oxidative stress to numerous age-related disorders by exacerbating the decline in glutathione synthesis that accompanies aging. Supplementation intended to boost glutathione synthesis in aging humans or rodents—employing NAC and/or the phase 2 inducer lipoic acid—reveals that restoring tissue glutathione to more youthful levels can have a favorable impact on vascular health, bone density, muscle strength, systemic inflammation, preservation of cognitive function, risk for neurodegeneration, and immune capacities. Such supplementation would presumably be most beneficial in elderly humans whose dietary protein intake is relatively low—as it often is in people who follow plant-based diets—and would not entail increases in IGF-I or mTORC1 activities. Whether low-protein diets might worsen health outcomes owing to suboptimal arginine intakes is at present much more speculative.

Conflict of interests Mark McCarty owns a small nutraceutical company, one of whose products contains *N*-acetylcysteine; James DiNicolantonio has no conflicts of interest.

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