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Low Protein Intake is Associated with a Major Reduction in IGF-1, Cancer, and Overall Mortality in the 65 and Younger but Not Older Population

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Summary

Mice and humans with Growth Hormone Receptor/IGF-1 deficiencies display major reductions in age-related diseases. Because protein restriction reduces GHR-IGF-1 activity, we examined links between protein intake and mortality. Respondents (n=6,381) aged 50–65 reporting high protein intake had a 75% increase in overall mortality and a 4-fold increase in cancer and diabetes

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Competing interests

V.D.L has equity interest in L-Nutra, a company that develops medical food. The other authors declare that they have no conflicts of interest.

mortality during an 18 year follow up period. These associations were either abolished or attenuated if the source of proteins was plant-based. Conversely, in respondents over age 65, high protein intake was associated with reduced cancer and overall mortality. Mouse studies confirmed the effect of high protein intake and the GHR-IGF-1 axis on the incidence and progression of breast and melanoma tumors, and also the detrimental effects of a low protein diet in the very old. These results suggest that low protein intake during middle age followed by moderate protein consumption in old subjects may optimize healthspan and longevity.

Introduction

Caloric restriction (CR) without malnutrition has been consistently shown to increase longevity in a number of animal models, including yeast, *C. elegans*, and mice (Fontana et al., 2010). However, the effect of CR on the lifespan of non-human primates remains controversial, and may be heavily influenced by dietary composition (Cava and Fontana, 2013; Colman et al., 2009; Fontana and Klein, 2007; Mattison et al., 2012; Mercken et al., 2013; Stein et al., 2012). The lifespan extension associated with CR in model organisms is believed to operate through its effects on GH, GHR, leading to subsequent deficiencies in IGF-1 and insulin levels and signaling (Bartke et al., 2001; Bellush et al., 2000; Fontana et al., 2010; Hauck et al., 2002; Wei et al., 2009). The effect of the insulin/IGF-1 pathway on longevity was first described in *C. elegans*, by showing that mutations in the insulin/IGF-1 receptor or in the downstream age-1 gene, caused a several fold increase in lifespan (Johnson, 1990; Kenyon et al., 1993; Kenyon, 2010). Other studies revealed that mutations in orthologs of genes functioning in insulin/IGF-1 signaling, including Tor-S6K and Ras-cAMP-PKA, promoted aging in multiple model organisms, thus providing evidence for the conserved regulation of aging by pro-growth nutrient signaling genes (Fabrizio et al., 2001; Guarente and Kenyon, 2000; Kapahi and Zid, 2004; Kenyon, 2005, 2011; Longo, 1999; Tatar et al., 2001). Not surprisingly, in mice, growth hormone receptor deficiency (GHRD) or growth hormone deficiency (GHD), both of which display low levels of IGF-1 and insulin, cause the strongest lifespan extension but also reduction of age-related pathologies including cancer and insulin resistance/diabetes ((Brown-Borg and Bartke, 2012; Brown-Borg et al., 1996; Masternak and Bartke, 2012).

Recently, we showed that humans with growth hormone receptor deficiency (GHRD), also exhibiting major deficiencies in serum IGF-1 and insulin levels, displayed no cancer mortality or diabetes. Despite having a higher prevalence of obesity, combined deaths from cardiac disease and stroke in this group were similar to those in their relatives (Guevara-Aguirre et al., 2011). Similar protection from cancer was also reported in a study that surveyed 230 GHRDs (Steuerman et al., 2011).

Protein restriction or restriction of particular amino acids, such as methionine and tryptophan, may explain part of the effects of calorie restriction and GHRD mutations on longevity and disease risk, since protein restriction is sufficient to reduce IGF-1 levels, and can reduce cancer incidence or increase longevity in model organisms independently of calorie intake (Ayala et al., 2007; Fontana et al., 2013; Fontana et al., 2008; Gallinetti et al., 2013; Horakova et al., 1988; Hursting et al., 2007; Leto et al., 1976; Mair et al., 2005;

Pamplona and Barja, 2006; Peng et al., 2012; Ross, 1961; Sanz et al., 2006; Smith et al., 1995; Youngman, 1993).

Here, we combined an epidemiological study of 6,381 US men and women aged 50 and above from NHANES III, the only nationally-representative dietary survey in the U.S., with mouse and cellular studies to understand the link between the level and source of proteins and amino acids, aging, diseases, and mortality.

Results

Human Population

The study population included 6,381 adults ages 50 and over from NHANES III, a nationally representative, cross-sectional study. Our analytic sample had a mean age of 65 years and is representative of the U.S. population in ethnicity, education, and health characteristics (Table S1).

On average, subjects consumed 1,823 calories, of which the majority came from carbohydrates (51%), followed by fat (33%), and protein (16%)—with 11% from animal protein. The percent of calorie intake from protein was used to categorize subjects into a high protein group (20% or more of calories from proteins), a moderate protein group (10–19% of calories from proteins), and a low protein group (less than 10% of calories from proteins).

Mortality follow-up was available for all NHANES III participants through linkage with the National Death Index up until 2006 (DHHS, 2001). This provided the timing and cause of death. The follow up period for mortality covered 83,308 total person-years over 18 years, with 40% overall mortality, 19% cardiovascular disease (CVD) mortality, 10% cancer mortality, and about 1% diabetes-caused mortality.

Association between Protein and Mortality

Using Cox Proportional Hazard models we found that high and moderate protein consumption were positively associated with diabetes-related mortality, but not associated with all-cause, CVD, or cancer mortality when subjects at all the ages above 50 were considered. Results showed that both the moderate and high protein intake groups had higher risks of diabetes mortality, compared to participants in the low protein group. Although taken together these results indicate that moderate to high protein intake promotes diabetes mortality, larger studies are necessary to test this possibility further. An alternative explanation for the elevated diabetes mortality in the higher protein group is the possibility that, following a diabetes diagnosis, some individuals may switch to a diet comprised of higher protein, lower fat, and low carbohydrate intake. To test this, we examined the association between protein intake and diabetes mortality in participants who had no prevalence of diabetes at baseline (Table S7).

Among subjects with no diabetes at baseline those in the high protein group had a 73-fold increase in risk (HR: 73.52; 95% CI: 4.47–1209.7), while those in the moderate protein category had an almost 23-fold increase in the risk of diabetes mortality (HR: 22.93; 95%

CI: 1.31–400.7). We underline that our hazard ratios and confidence intervals may be inflated due to our sample size and the extremely low incidence of diabetes mortality in the low protein group. Overall, there were only 21 diabetes deaths among persons without diabetes at baseline—only 1 of which was from the low protein group. Nevertheless, despite the small sample size, our results still show strong significant associations between increased protein intake and diabetes-related mortality.

To determine whether the association between protein and mortality differed for middle-aged and older adults, Cox Proportional Hazard models were rerun testing for an interaction between protein consumption and age. Significant interactions were found for both all-cause and cancer mortality, indicating that the low protein diet was beneficial in mid-life; however, its benefits declined with age (Fig. 1). Based on these results, we stratified the population into two age groups—those ages 50–65 (n=3,039), and those ages 66+ (n=3,342) and reexamined relationships between protein and cause-specific mortality. Among those aged 50–65, higher protein levels were linked to significantly increased risks of all-cause and cancer mortality (Table 1). In this age range, subjects in the high protein group had a 74% increase in their relative risk of all-cause mortality (HR: 1.74; 95% CI: 1.02–2.97), and were more than 4-times as likely to die of cancer (HR: 4.33; 95% CI: 1.96–9.56) when compared to those in the low protein group. None of these associations were significantly affected by controlling for percent calories from total fat or for percent calories from total carbohydrates. However, when the percent calories from animal protein was controlled for, the association between total protein and all-cause and cancer mortality was eliminated or significantly reduced, respectively, suggesting animal protein mediates a significant portion of these relationships. When we controlled for the effect of plant-based protein, there was no change in the association between protein intake and mortality, indicating that high levels of animal proteins promote mortality and not that plant-based proteins have a protective effect (Table S5).

Compared to subjects reporting a low protein diet, subjects who consumed moderate levels of protein also had a 3-fold higher cancer mortality (HR: 3.06; 95% CI: 1.49–6.25), that was not accounted for by either percent calories from fat or percent calories from carbohydrates, but was marginally reduced when controlling for percent calories from animal protein (HR: 2.71; 95% CI: 1.24–5.91), although the size of the effect was not as large as for those in the high protein group. Taken together, these results indicate that respondents aged 50–65 consuming moderate to high levels of animal protein display a major increase in the risks for overall and cancer mortality, however, the risks may be somewhat decreased if protein does not come from an animal source. Similar results were obtained if the population 45–65 was considered, although few deaths occurred in the 45–50 group (data not shown).

In contrast to the findings above, among respondents who were 66 years of age and over at baseline, higher protein levels were associated with the opposite effect on overall and cancer mortality but a similar effect on diabetes mortality (Table 1). When compared to those with low protein consumption, subjects who consumed high amounts of protein had a 28% reduction in all-cause mortality (HR: 0.72; 95% CI: 0.55–0.94), while subjects who consumed moderate amounts of protein displayed a 21% reduction in all-cause mortality (HR: 0.78; 95% CI: 0.62–0.99). Furthermore, this was not affected by percent calories from fat, from carbohydrates, or from animal protein. Subjects with high protein consumption also had a 60% reduction in cancer mortality (HR: 0.40; 95% CI: 0.23–0.71) compared to those with low protein diets, which was also not affected when controlling for other nutrient intake or protein source.

The Influence of IGF-1 on the Association between Protein and Mortality

Adjusted mean IGF-1 levels were positively associated with protein consumption for both age groups (Fig. 2). Because IGF-1 was only available for a randomly selected subsample (n=2,253) we reexamined the age-specific associations between protein and cause-specific mortality in this sample and found them to be similar to what was seen in the full sample; although, with somewhat larger effect sizes (Table S3). Next we examined whether IGF-1 acted as a moderator or mediator in the association between protein and mortality. We found that while IGF-1 did not account for the association between protein consumption and mortality (Table S3), it was an important moderator of the association—as indicated by the statistically significant interactions between protein and IGF-1 level (Table S4).

From these models, predicted hazard ratios by IGF-1 and protein group were calculated (Fig. S2). Results showed that for every 10 ng/ml increase in IGF-1 the mortality risk of cancer among subjects ages 50–65 increases for the high protein *vs* the low protein group by an additional 9% ($HR_{\text{high protein x IGF-1}}$: 1.09; 95% CI: 1.01–1.17). In contrast, among older subjects (66+ years), when comparing those in the low protein group, subjects with high or moderate protein diets had a reduced risk of CVD mortality if IGF-1 was also low; however, no benefits were found with increased IGF-1.

Protein Intake, IGF-1, and Cancer in Mice

To verify causation and understand the mechanism that may link proteins to cancer and overall mortality, we studied the effect of a range of protein intake (4–18%) similar to that of subjects in the NHANES III study, on the levels of circulating IGF-1, cancer incidence and progression in mice. 18-week-old male C57BL/6 mice were fed continuously for 39 days with experimental, isocaloric diets designed to provide either a high (18%) or a low (4–7%) amount of calories derived from protein, without imposing CR or causing malnutrition (Fig. S1A,B).

To understand how the different levels of protein and IGF-1 may affect the ability of a newly formed tumor to survive and grow after one week on diets containing different protein levels, both groups were implanted subcutaneously with 20,000 syngeneic, murine melanoma cells (B16). Tumor measurements began 15 days post implantation and after 22 days on the diets, at which point incidence was found to be 100% for the high protein level (18%) group but 80% for the low protein level (4%) group (Fig. 3A). At day 25, incidence rose to 90% in the low protein group, and remained there until the end of the experiment (Fig. 3A). From day 22 until the end of the experiment tumor size was significantly smaller in the group consuming lower amount of protein indicating a much slower tumor progression. At day 39 the mean tumor size was 78% larger in the high compared to the low protein group (day 36 P=0.0001; day 39 P<0.0001)(Fig. 3B). To test the hypothesis that GHR signaling may be involved in this effect of protein levels, blood samples were obtained and analyzed at day 16 to determine the levels of IGF-1 and the IGF-1 inhibitory protein, IGFBP-1 (Wolpin et al., 2007). Serum IGF-1 was 35% lower (*P*=0.0004) in the low protein group when compared to animals fed the high protein diet (Fig. 3C). Conversely, serum IGFBP-1 was 136 % higher (*P*=0.003) in the low protein group compared to the high protein group (Fig. 3D).

To test further the hypothesis that the GHR-IGF-1 axis promotes cancer progression, we implanted subcutaneous melanoma (B16) into GHR/IGF-1 deficient GHRKO mice and their respective age- and sex-matched littermate controls (18-week-old male C57BL/6 mice). Tumor measurements began 10 days post implantation and continued until day 18. The data shows that tumor progression is strongly inhibited in the GHRKO mice when compared to progression in the control group (Fig. 3E, S1L; *P*<0.01).

We also tested the effect of protein intake on a breast cancer incidence, and progression in a mouse model. 12-week-old female BALB/c mice were placed under the same dietary regimen as described for C57BL/6 mice, except that the mice had to be switched from a 4% to a 7% kcal from protein diet within the first week in order to prevent weight loss (Fig. S1E,F). After a week of feeding on these diets mice were implanted subcutaneously with 20,000 cells of syngeneic, metastatic, murine breast cancer (4T1), and 15 days later animals were assessed for tumors. On day 18 post-implantation (day 25 on the diet) tumor incidence was 100% in the high protein (18%) group but only 70% in the low protein (7%) group. The incidence in the low protein group rose to 80% at day 39 where it remained until the end of the experiment (Fig. 3F). A 45% smaller mean tumor size was also observed in the low protein group compared to the high protein group at the end of the experiment at day 53 (*P*=0.0038)(Fig. 3G). As for C57BL/6 mice, IGF-1 was measured after 16 days from the switch to different protein levels. In the low protein intake group, IGF-1 levels were reduced by 30% compared to those in the high level group (P<0.0001) (Fig. 3H). Additionally, a low protein intake also caused an IGFBP-1 increase of 84% (*P*=0.001)(Fig. 3I), similar to what was observed in the C57BL/6 genetic background (Fig. 3D). Analogously, when soy protein intake was reduced from high levels to low levels we observed a 30 % decrease in IGF-1 (p<0.0001) (Fig. 3J) and a 140% increase in IGFBP-1 (p<0.0001)(Fig. 3K). Although there was a trend for an effect of substituting the same level of animal protein with plant protein on IGF-1 and IGFBP1, the differences were not significant. These data suggest that lower protein intake may play a role in decreasing cancer incidence and/or progression in part by decreasing IGF-1 and increasing the IGF-1 inhibitor IGFBP-1. Additional studies on various types of animal *vs* plant based proteins are necessary to determine their effect on IGF-1 and IGFBP-1.

Low Protein Intake and Weight Maintenance in Old Mice

Based on the observed opposite effects of a low protein diet in subjects 50–65 year old versus those 65 and older and on the major drop in BMI and IGF-1 levels after age 65, we hypothesized that older subjects on a low protein diet may become malnourished and unable to absorb or process a sufficient level of amino acids. To test this possibility in mice, we fed young mice (18-week-old) and old mice (24-month-old) with isocaloric diets containing either 18% or 4% animal protein. A very low protein diet was purposely selected to reveal any sensitivity to protein restriction in an old organism. Whereas old mice maintained on a high protein diet for 30 days gained weight, old but not young mice on a low protein diet lost 10% of their weight by day 15 (Fig. 4A,B) in agreement with the effect of aging on turning the beneficial effects of protein restriction on mortality into negative effects.

Cellular Studies

To test the hypothesis that there is a fundamental link between the level of amino acids and lifespan, the impact of the presence of specific concentrations of amino acids on yeast growth and development was assessed by survival and mutation rate assays. A wild type DBY746 *S. cerevisiae* strain was grown in the presence of half (0.5X), standard (1X), and double (2X) amino acid concentration with all other nutrients maintained constant. Survival was measured at days 1, 3, 5, and 8. No survival differences were observed during days 1 and 3. At day 5, the 2 highest amino acid concentrations showed a trend for increased mortality, which resulted in a 10-fold decrease in surviving cells by day 8 (Fig. 3L).

In order to assess the relationship between amino acids, aging, and age-dependent DNA damage we used aging *S. cerevisiae* to measure spontaneous mutation rate (Madia et al., 2007). The mutation rate was 3- and 4-fold higher in 5 day old but not young cells exposed to 1X and 2X amino acid levels, respectively, compared to cells exposed to a 0.5 X amino acid concentration (Fig. 3M). These results indicate that even in unicellular organisms, amino acids promote cellular aging and age-dependent genomic instability.

To further discern the pathways involved in promoting age-dependent genomic instability we measured the induction of stress responsive genes regulated by the Ras-PKA-Msn2-4 and Tor-Sch9-Gis1 pathways in the presence or absence of amino acids. For cells grown in control media containing only Trp, Leu, and His (essential for growth in this strain) the presence of all amino acids in the media reduced the induction of stress resistance transcription factors Msn2/4 (STRE) and Gis1 (PDS), indicating that the addition of amino acids was sufficient to inhibit cellular protection (Fig. 3N).

The Tor-Sch9 pathway extends longevity but also promotes DNA mutations (Madia et al., 2009; Wei et al., 2008). To determine whether Ras-cAMP-PKA signaling also regulates age-dependent genomic instability we studied *ras2* deficient mutants. We confirmed that *ras2*Δ mutants are long-lived (Fig. S1H) but also show that inactivation of Ras signaling attenuated age- and oxidative stress-dependent genomic instability (Fig. S1I, S1J, 3O, S1K). Together, these results indicate a mechanism where amino acids are able to affect mutation frequency and thus genomic instability, at least in part, by activation of the Tor-Sch9 and Ras/PKA pathways and decreased stress resistance (Fig. 3P).

Discussion

Here, using a major nationally-representative study of nutrition in the United States population, our results show that among those ages 50 and above, the level of protein intake is associated with increased risk of diabetes mortality, but not associated with differences in all-cause, cancer, or CVD mortality. Nevertheless, we found an age interaction for the association between protein consumption and mortality, with subjects ages 50–65 years, but also ages 45–65, potentially experiencing benefits from low protein intake and subjects ages 66+ reporting a low protein diet experiencing detriments—at least for overall mortality and cancer. This may explain why the strong association between protein intake, IGF-1, diseases, and mortality reported here has been poorly understood and controversial (Saydah et al., 2007). Furthermore, among 2253 subjects the risks of all-cause and cancer mortality for those with high protein intake compared to the low protein intake group were increased even further for those who also had high levels of IGF-1. This is in agreement with previous studies associating IGF-1 levels to various types of cancer (Giovannucci et al., 2003; Guevara-Aguirre et al., 2011; Pollak et al., 2004).

Notably, our results showed that the proportion of proteins derived from animal sources accounted for a significant proportion of the association between overall protein intake and all-cause and cancer mortality. These results are in agreement with recent findings on the association between red meat consumption and death from all-cause, CVD, and cancer (Fung et al., 2010; Pan et al., 2012). Previous studies in the U.S. have found that a low-carbohydrate diet is associated with an increase in overall mortality and showed that when such a diet is from animal-based products, the risk of overall, as well as CVD and cancer mortality, is increased even further (Fung et al., 2010; Lagiou et al., 2007). Our study indicates that high levels of animal proteins promoting increases in IGF-1 and possibly insulin, and aging may be the major promoter of mortality for people age 45–65 in the 18 years following the survey assessing protein intake. Notably, by then, the cohort that was 65 at the time of the interview would be 83 years old, underlining that the high protein intake may promote mortality in subjects that are older than 65.

Our results from yeast and mice may also explain at least part of the fundamental connection between proteins, cancer and overall mortality by providing a link between amino acids, stress resistance, DNA damage, and cancer incidence/progression. In mice, the changes caused by reduced protein levels had an effect potent enough to prevent the establishment of 10–30% of tumors, even when 20,000 tumor cells were already present at a subcutaneous site. Furthermore, the progression of both melanoma and breast cancer was strongly inhibited by the low protein diet indicating that low protein diets may have applications in both cancer prevention and treatment, in agreement with previous studies (Fontana et al., 2006; Fontana et al., 2008; McCarty, 2011; Youngman, 1993).

Although protein intake is associated with increased mortality for adults who were middle-aged at baseline, there was also evidence that a low protein diet may be hazardous for older adults. Both high and moderate protein intake in the elderly were associated with major improvements compared to the low protein group, suggesting that protein intake representing at least 10% of the calories consumed may be necessary after age 65 or possibly 75 to reduce age-dependent weight loss and prevent an excessive loss of IGF-1 and of other important factors. In fact, previous studies have noted that an increased protein intake and the resulting increase in IGF-1 may prove beneficial in older adults (Heaney et al., 1999) and the dramatic switch from the protective to the detrimental effect of the low protein diet coincides with a time at which weight begins to decline. Based on previous longitudinal studies, weight tends to increase up until age 50–60, at which point it becomes stable before beginning to decline steadily by an average of 0.5% per year for those over age

65 (Villareal et al., 2005; Wallace et al., 1995). We speculate that frail subjects who have lost a significant percentage of their body weight and have a low BMI may be more susceptible to protein malnourishment. It is also possible that other factors such as inflammation or genetic factors may contribute to the sensitivity to protein restriction in elderly subjects, in agreement with our mouse studies.

Although other studies have noted age-associated declines of nutrient absorption in rodents related to changes in the pH microclimate, impaired adaptive response in the aged gut, and changes in the morphology of the intestine, there is still no clear association between morphological and absorptive changes in ageing (Chen et al., 1990; Woudstra and Thomson, 2002). In humans, some studies have shown that dietary protein digestion and absorption kinetics are not impaired *in vivo* in healthy, elderly men, however, these studies have also reported increased splanchnic extraction of AAs which might result in decreased availability to peripheral tissues, and speculate that in the case of low protein intake or increased protein requirement the limited systemic availability of dietary amino acids may contribute to decreased muscle protein synthesis (Boirie et al., 1997; Koopman et al., 2009). Furthermore, in humans factors like poor dentition, medication, and psychosocial issues also play a significant role on rates of malnourishment. (Woudstra and Thomson, 2002)

IGF-1 has also been previously shown to decrease at older ages (Iranmanesh et al., 1991) possibly increasing the risk of frailty (Lamberts et al., 1997), and mortality (Cappola et al., 2003). Thus our findings may explain the controversy related to IGF-1 and mortality indicating that a minimum levels of proteins and possibly IGF-1 is important in the elderly or that low circulating IGF-1 reflects a state of malnourishment frailty and/or morbidity (Maggio et al., 2007). In fact, inflammation and other disorders are known to decrease IGF-1 levels, raising the possibility that the low protein and low IGF-1 group may contain a significant number of both malnourished and frail individuals having or in the process of developing major diseases (Fontana et al., 2012).

There are some limitations to our study, which should be acknowledged. First, the use of a single 24-hour dietary recall followed by up to 18-years of mortality assessment has the potential of misclassifying dietary practice if the 24-hour period was not a normal day. However, 93% of our sample reported that the 24-hour period represented a normal day. We also include this variable as a control in our analysis. Furthermore, the 24-hour dietary recall has been shown to be a very valid approach to identify the “usual diet” of subjects (Blanton et al., 2006; Conway et al., 2004; Coulston and Boushey, 2008; Prentice et al., 2011). While we must admit that the lack of longitudinal data on dietary consumption is a potential limitation of our study, study of dietary consistency over six years among older people revealed little change over time in dietary habits (Garry et al., 1989). Another study looking at dietary habits over twenty years showed that while energy intake decreased for protein, fat, and carbohydrates as people aged, the decreases were equal across the three types (Flynn et al., 1992).

Another limitation of our study is that classification of respondents into protein groups, and then stratifying the sample for analysis, produced relatively small sample sizes, especially for analyses involving diabetes mortality among persons without diabetes at baseline or participants in the IGF-1 subsample. As a result, our Hazard Ratios and 95% confidence intervals may be much larger than what would have been seen with a larger sample size. Nevertheless, one would expect a small sample size to decrease our power and make it harder to detect associations. Therefore, our ability to detect significance indicates that the associations between protein and mortality are robust. Furthermore, the lower limits of the 95% confidence intervals from our mortality analyses were well above 1.0, signifying that the increased risk is probably large. Finally, given these limitations, our study was strengthened by its use of reliable cause-specific mortality data, as well as its inclusion of a large nationally-representative sample—a feature often missing from the previous literature.

Overall, our human and animal studies indicate that a low protein diet during middle age is likely to be useful for the prevention of cancer, overall mortality, and possibly diabetes through a process that may involve, at least in part, regulation of circulating IGF-1 and insulin levels. In agreement with other epidemiological and animal studies (Estruch et al., 2013; Linos and Willett, 2007; Michaud et al., 2001; Willett, 2006) our findings suggest that a diet, in which plant-based nutrients represent the majority of the food intake, is likely to maximize health benefits in all age groups. However, we propose that up to age 65 and possibly 75, depending on health status, the 0.7 to 0.8 grams of proteins/kg of body weigh/day reported by the Food and Nutrition Board of the Institute of Medicine, currently viewed as a minimum requirement, should be recommended instead of the 1–1.3 g grams of proteins/kg of body weigh/day consumed by adults ages 19–70 (Fulgoni, 2008). We also propose that at older ages, it may be important to avoid low protein intake and gradually adopt a moderate to high protein possibly mostly plant based consumption to allow the maintenance of a healthy weight and protection from frailty (Bartali et al., 2006; Ferrucci et al., 2003; Kobayashi et al., 2013).

Experimental Procedures

Nutrient Intake for Human Data

Nutrient intake data is based on reports of food and beverage intake during a 24-hour period. Data were collected via an automated, microcomputer-based coding system, with information on over eighty nutrients. There are several advantages to using this method for collecting dietary data. Given that the time elapsing between consumption and recall is short, participants are typically able to recall more information. Also, unlike reporting methods, 24-hour dietary recall relies on data collection after consumption, reducing the potential for assessment to alter dietary behaviors (Coulston and Boushey, 2008). Furthermore, 24-hour recalls have been shown to be stronger estimates of total energy and protein consumption compared to the commonly used food frequency questionnaires (Prentice et al., 2011) and have also been shown to be a more valid measure of total energy and nutrient intake than both the Block food-frequency questionnaire, and the National Cancer Institute’s Diet History Questionnaire (Blanton et al., 2006). Finally, this approach has also been found to accurately assess energy, protein, fat, and carbohydrate intake, regardless of body mass index (Conway et al., 2004).

Epidemiological Mortality Follow-up

Mortality data were available from the National Death Index. Information for 113 potential underlying causes of death (UCOD-113) was used to determine all-cause mortality, cardiovascular mortality, cancer mortality, and diabetes mortality.

Statistical Analysis for Human Data

Cox Proportional Hazard Models were used to estimate the association between intake of calories from protein on subsequent all-cause, CVD, cancer, and diabetes mortality—with the latter three run using competing risks structures. Next we tested the interaction between age and protein consumption on the association with mortality. Based on these results, we categorized subjects into two age groups (50–65 years and 66+ years), which were used in the remainder of the analyses. Age-Stratified Proportional Hazard Models were used to estimate the association of percent calories from protein with mortality within the two age groups, and examine whether the relationship was influenced by percent of calories from fat, percent of calories from carbohydrates, or animal protein. Hazard models were re-estimated for the IGF-1 subsample to determine whether including IGF-1 changed the association between protein intake and mortality. Finally, proportional hazard models were used to examine the interaction between protein and IGF-1, and used to calculate predicted hazard ratios for each protein group at various IGF-1 levels, to determine whether protein intake differentially impacts mortality depending on levels of IGF-1. All analyses were run using sample weights, accounting for sampling design, and controlling for age, race/ethnicity, education, sex, disease status, smoking, dietary changes, and total calorie consumption.

Cancer models in Mice

All animal experiments were performed according to procedures approved by USC's Institutional Animal Care and Use Committee. To establish a subcutaneous cancer mouse model, we injected 18-week-old, male C57BL/6 mice as well as 10-month-old GHRKO mice, age-matched littermate control mice, and wild type littermates with B16 melanoma cells, and 12-week-old, female BALB/c with 4T1 breast cancer cells. Before injection, cells in log phase of growth were harvested and suspended in serum-free, high glucose Dulbecco's modified Eagle's medium (DMEM) at 2×10^5 cells or 2×10^6, and 100 ul (2×10^4 cells per C57BL/6 or BALB/c mouse; 2×10^5 cells per GHRKO mouse) was subsequently injected subcutaneously in the lower back. All mice were shaved before subcutaneous tumor injection. Tumor incidence was determined by palpation of the injected area and tumor size was measured using a digital Vernier caliper starting 10–15 days post implantation. The experiments for C57BL/6 and BALB/c ended at different time points based on USC IACUC approved humane endpoint criteria for tumor size and ulceration. GHRKO (C57BL/6 background) mice were kindly provided by J.J. Kopchick (Ohio University, Athens).

Further materials and methods can be found in supplemental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. High protein intake linked to increased cancer, diabetes, and overall mortality.
2. High IGF-1 levels increased the relationship between mortality and high protein.
3. Higher protein diet may be protective for older adults.
4. Low protein intake in mice resulted in reduced IGF-1 and tumor progression.

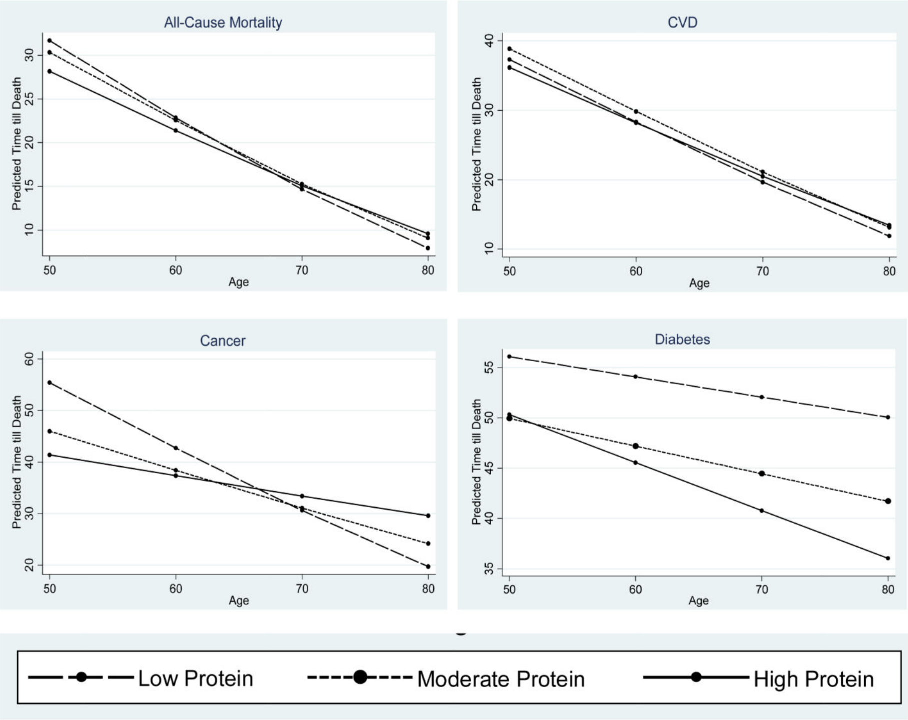

Figure 1.

Using Cox Proportional Hazard Models, statistically significant ($p<.05$) interactions between age and protein group were found for all-cause and cancer mortality. Based on these models, predicted remaining life expectancy was calculated for each protein group by age at baseline. Based on results, low protein appears to have a protective effect against all-cause and cancer mortality prior to age 66, at which point it becomes detrimental. No significant interactions were found for cardiovascular disease (CVD) and diabetes mortality.

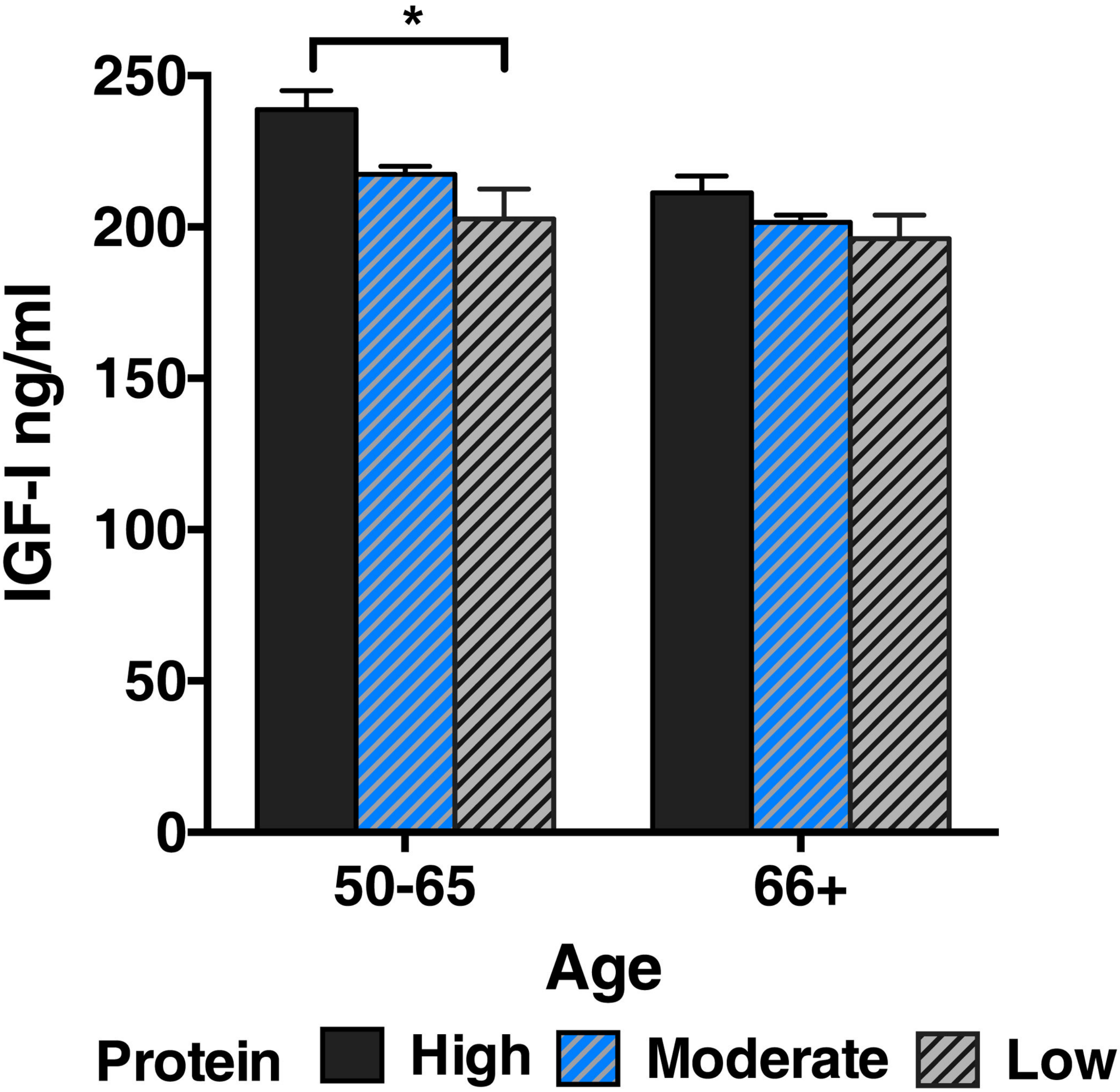

Figure 2.

Serum IGF-1 levels in respondents 50–65 and 66+ reporting low, moderate, or high protein intake. IGF-1 in respondents 50–65 is significantly lower among those with low protein intake when compared to high (*P*=0.004). At age 66+ the difference between high and low intake becomes marginally significant (*P*=0.101). The cohort for which IGF-1 levels were calculated includes 2253 subjects. Of those ages 50–65 (n=1,125), 89 were in the low protein category, 854 were in the moderate protein category, and 182 were in the high protein category. Of those ages 66+ (n=1,128), 80 were in the low protein category, 867 were in the moderate protein category, and 181 were in the high protein category. Data points represent the mean ± SEM. $^*P<0.01$.

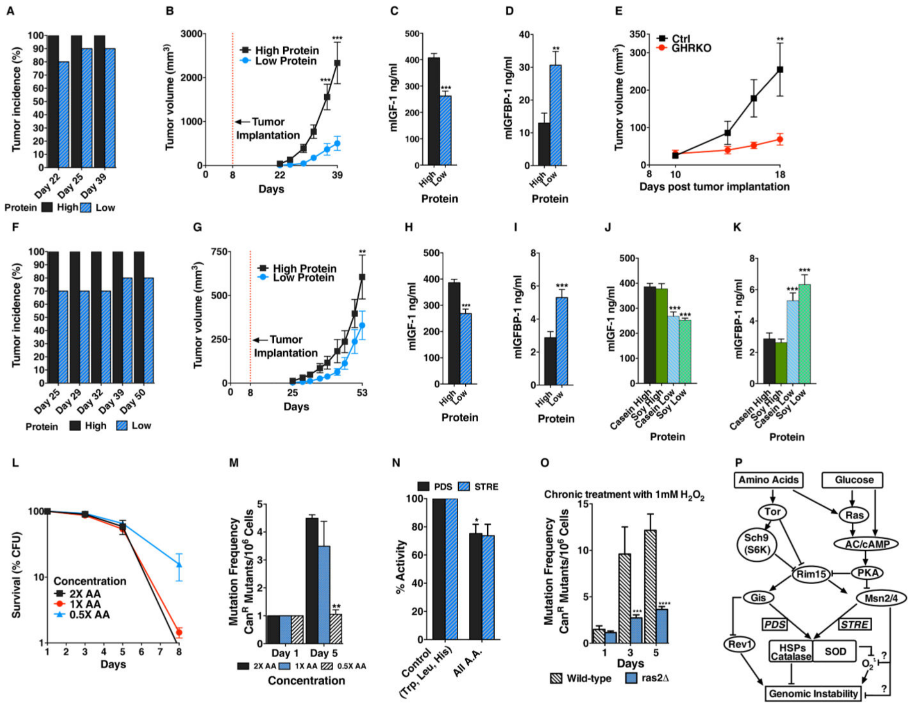

Figure 3.

(A) Tumor incidence in 18-week-old male C57BL/6 mice implanted with 20,000 melanoma (B16) cells, and fed either a high protein (18%; n=10) or low protein (4%; n=10) diet. **(B)** B16 Tumor volume progression in (18wk) C57BL/6 male mice fed either a high protein (n=10) or low protein (n=10) diet. **(C)** IGF-1 at day 16 in (18wk) male C57BL/6 mice fed either a high protein (n=5) or low protein (n=5) diet. **(D)** IGFBP-1 at day 16 in male (18wk) C57BL/6 mice fed either a high protein (n=10) or low protein (n=10) diet. **(E)** B16 melanomatumor progression in 10-month old female GHRKO mice (n=5) *vs* age-matched littermate controls (Ctrl; n=7). **(F)** Tumor incidence in 12-week-old female BALB/c mice implanted with 20,000 cell breast cancer (4T1) fed either a high protein (18%; n=10) or low protein (7%; n=10) diet. **(G)** 4T1 breast cancer progression in female (12wk) BALB/c mice fed either a high protein (n=10) or low protein (n=10)diet. **(H)** IGF-1 at day 16 in female (12wk) BALB/c mice fed either a high animal protein (n=5) or low animal protein (n=5) diet. **(I)** IGFBP-1 at day 16 in female (12wk) BALB/c mice fed either a high animal protein (n=10) or low animal protein (n=10) diet. **(J)** IGF-1 at day 16 in female (12wk) BALB/c mice fed either a high soy protein (n=5) or low soy protein (n=5) diet. **(K)** IGFBP-1 at day 16 in female (12wk) BALB/c mice fed either high soy protein (n=10) or low soy protein (n=10) diet. **(L)** Survival and **(M)** DNA mutation frequency of yeast exposed to a 0.5x, 1x, or 2x concentration of a standard amino acid mix. **(N)** PDS and STRE activity in yeast grown in media containing only Trp, Leu. and His compared to those grown in the presence of all AA. (**O)** Ras2 deletion protects against oxidative stress-induced genomic instability measured as DNA mutation frequency (Can^r) in wild-type (DBY746) and *ras2*Δ mutants chronically exposed to 1mM H_2O_2. **(P)** A model for the effect of amino acids on aging and genomic instability in *S. cerevisiae*. Amino acids activate the Tor-Sch9 and Ras-cAMP-PKA pathway also activated by glucose and promote age- and oxidative stress-dependent genomic instability in part via reduced activity of Gis1 and Msn2/4. In all graphs, data points represent the mean of the biological replicates ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

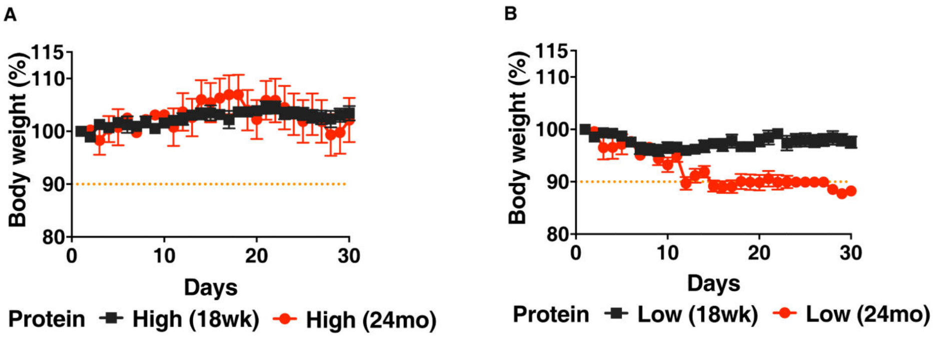

Figure 4.

Effect of protein intake on body weight in young and old mice. **(A)** Young (18-week-old) (n=10) and old (24-month-old) (n=6) C57BL/6 mice fed a high (18%) protein diet. **(B)** Young (18-week-old) (n=10) and old (24-month-old) (n=6) C57BL/6 mice fed a low (4%) protein diet.

Table 1

Associations between Mortality and Protein intake

	Hazard Ration ***(95% CI)***							
	Ages 50–65 (N=3,039)				**Ages 66+ (N=3,342)**			
	Model 1	**Model 2**	**Model 3**	**Model4**	**Model 1**	**Model 2**	**Model 3**	**Model4**
All-Cause Mortality								
Moderate Protein (n=4,798)	1.34 *(0.81–2.22)*	1.37 *(0.82–2.27)*	1.35 *(0.80–2.29)*	1.15 *(0.67–1.96)*	0.79 *(0.62–0.99)*	0.79 *(0.62–0.99)*	0.79 *(0.62–0.99)*	0.79 *(0.61–1.01)*
High Protein (n=1,146)	1.74 *(1.02–2.97)*	1.77 *(1.03–3.03)*	1.74 *(0.99–3.05)*	1.18 *(0.60–2.31)*	0.72 *(0.55–0.94)*	0.73 *(0.56–0.95)*	0.72 *(0.55–0.94)*	0.72 *(0.50–1.02)*
% kcal Fat		0.99 *(0.98–1.01)*				1.00 *(0.99–1.01)*		
% kcal Carbs			1.00 *(0.99–1.01)*				1.00 *(0.99–1.00)*	
% kcal Animal Protein				1.03 *(1.00–1.06)*				1.00 *(0.98–1.02)*
CVD Mortality								
Moderate Protein (n=4,798)	0.79 *(0.40–1.54)*	0.83 *(0.43–1.60)*	0.81 *(0.41–1.62)*	0.61 *(0.29–1.29)*	0.80 *(0.57–1.12)*	0.80 *(0.57–1.12)*	0.80 *(0.57–1.12)*	0.80 *(0.56–1.14)*
High Protein (n=1,146)	1.03 *(0.51–2.09)*	1.08 *(0.54–2.15)*	1.10 *(0.52–2.31)*	0.55 *(0.19–1.62)*	0.78 *(0.54–1.14)*	0.79 *(0.54–1.15)*	0.78 *(0.53–1.15)*	0.77 *(0.48–1.25)*
% kcal Fat		0.99 *(0.97–1.01)*				1.00 *(0.99–1.01)*		
% kcal Carbs			1.00 *(0.99–1.02)*				1.00 *(0.99–1.01)*	
% kcal Animal Protein				1.04 *(0.99–1.11)*				1.00 *(0.98–1.02)*
Cancer Mortality								
Moderate Protein (n=4,798)	3.06 *(1.49–6.25)*	3.13 *(1.52–6.44)*	3.56 *(1.65–7.65)*	2.71 *(1.24–5.91)*	0.67 *(0.43–1.06)*	0.67 *(0.43–1.06)*	0.67 *(0.42–1.05)*	0.66 *(0.40–1.07)*
High Protein (n=1,146)	4.33 *(1.96–9.56)*	4.42 *(2.01–9.74)*	4.98 *(2.13–11.66)*	3.19 *(1.21–8.35)*	0.40 *(0.23–0.71)*	0.41 *(0.23–0.73)*	0.39 *(0.22–0.69)*	0.38 *(0.17–0.82)*
% kcal Fat		0.99 *(0.98–1.01)*				1.02 *(1.01–1.03)*		
% kcal Carbs			1.00 *(0.98–1.01)*				1.00 *(0.99–1.01)*	
% kcal Animal Protein				1.02 *(0.97–1.07)*				1.00 *(0.97–1.04)*
Diabetes Mortality								
Moderate Protein (n=4,798)	3.43 *(0.69–17.02)*	3.36 *(0.67– 16.96)*	3.41 *(0.67–17.36)*	2.99 *(0.58–15.31)*	5.38 *(0.95–30.49)*	5.05 *(0.93–27.34)*	4.93 *(0.89–27.35)*	6.20 *(0.35–37.01)*
High Protein (n=1,146)	3.93 *(0.73–21.07)*	3.88 *(0.71–21.17)*	3.90 *(0.67–22.84)*	2.77 *(0.24–31.73)*	10.64 *(1.85–61.31)*	10.42 *(1.88–57.87)*	9.07 *(1.49–55.30)*	15.16 *(1.93–118.9)*
% kcal Fat		1.01 *(0.97–1.05)*						
% kcal Carbs			1.00 (0.96–1.04)					
% kcal Animal Protein				1.02 *(0.92–1.14)*				

Reference=Low Protein (n=437 in both age groups)

Model 1: (Baseline Model) Adjusted for age, sex, race/ethnicity, education, waist circumference, smoking, chronic conditions (diabetes, cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories.

Model 2: Adjusted for Covariates and % kcals from Total Fat

Model 3: Adjusted for Covariates and % kcals from Total Carbohydrates

Model 4: Adjusted for Covariates and % kcals from Animal Protein