DIETARY PROTEIN AND BONE HEALTH: HARMONIZING CONFLICTING THEORIES

BY

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DISSERTATION

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ABSTRACT

No clear consensus on the role of higher protein intakes has emerged, in spite of many decades of research. Protein unambiguously increases urinary calcium losses, which is completely attributable to the dietary acid load imposed by metabolism of sulfur containing amino acids into acid equivalents. Although alternate dietary sources of fixed acid cause demineralization of bone and apparent osteoporosis, this effect has not been consistently observed for protein, suggesting opposing, beneficial effects on bone. Specifically, protein may improve bone health through improving calcium absorption, increasing total circulating insulin-like growth factor 1 or by improving lean body mass which in turn increases bone growth. Although the notion of competing positive and negative pathways has been articulated theoretically, statistical mediation models of this "dual pathways" relationship have not been employed to quantify these relationships.

In a cross-sectional investigation of postmenopausal women, protein intake is positively related to bone mineral density of the lumbar spine following adjustment for an accompanying negative effect mediated by the sulfur containing fraction of protein. In growing rats, an analogous and complementary pattern emerged: A negative association of protein intake with bone strength was suppressed by an opposing, positive effect of protein mediated by insulin-like growth factor 1. A second animal study assessed the influence of protein source on bone strength and bone mineral content of growing rats consuming isoenergetic, isonitrogenous diets. The influence of protein source was completely mediated by the corresponding changes in lean body mass.

A randomized, controlled trial indicated a higher protein diet preserved bone density during weight loss compared to a conventional, MyPyramid based diet; however the protein diet also contained more calcium. A mathematical model of calcium availability in this trial suggested that this additional dietary calcium was not sufficient to explain differences in calcium accrual between groups unless calcium availability was also improved in the higher protein diet. Also, within this study, urine calcium (a surrogate of the diet acid load) exhibited a negative association with bone density change, in spite of its positive association with protein intake.

The striking consistency of a dual pathway model across populations and experimental models lends credence to the notion that dietary protein may hold a positive or negative effect on bone health, depending on other factors in the diet. Specifically, we find support that the sulfur containing amino acid induced dietary acid load holds negative effects that may be opposed by positive influences of insulin-like growth factor 1, calcium availability or lean body mass. On average, this effect is probably null or too small to be of clinical importance.

The effect may be of public health relevance, however, if the diet can be manipulated in order to uncouple positive and negative pathways. If correct, the dual pathway model predicts higher protein intakes will have modest benefits on bone health in the context of adequate calcium intake, selection of protein sources lower in sulfur amino acids or ample intake of fruits and vegetables to buffer the dietary acid load.

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Chapter 1

Introduction

Despite decades of research no clear consensus has emerged on the influence of dietary protein on bone health. Early research revealed calcium loss in urine increases in proportion to protein intake. The source of this calcium, however, was unclear. Over time, a theoretical model emerged positing that a chronic, low-grade acidosis imposed by metabolism of sulfur amino acids causes demineralization of bone tissue. This model has been supported indirectly by work in animals and isolated tissues; however demineralization of bone has not been consistently established in humans in relation to protein intake.

More recently it has been determined that calcium becomes more bioavailable at higher levels of protein in the diet. Mechanisms for this effect are unknown, but may relate to homeostatic compensation for calcium lost in urine, or regulation of endocrine pathways by circulating amino acids. In particular, higher protein intakes may suppress circulation of bioactive parathyroid hormone and calcitriol, and augment circulation of insulin-like growth factor 1, each with well established influences on bone metabolism. Emergent is a concept that protein may have a two-faced influence on bone health, acting simultaneously through bone-building and bone-destructive pathways.

The complexities of apparently contradictory influences of protein on bone health have spurred many studies, yielding wildly variable data and leaving the protein-bone question murky. Current reviews and meta-analyses determine higher protein intakes impart a modest benefit to bone. This summary statement, although accurate on average, masks the convoluted state of clinical and epidemiological research identifying occasionally negative, commonly null and often positive results.

Throughout this dissertation, I will defend the position that ambiguity in the available data reflect, in part, failure of current theoretical (and statistical) models to account for the duality of protein's influence on bone. I will promote a model in which the sum total effect of dietary protein intake arises from the balance of competing positive and negative paths. Although slightly more complex than the hypotheses tested in previous research, this model is unique in its ability to bring clarity to an otherwise chaotic body of research.

Throughout this dissertation I will compare, unless otherwise indicated, intakes of protein that are adequate according to current recommendations to higher levels of protein intake. Frank protein insufficiency is well documented to be damaging to bone, and levels below 0.8 g protein / kg body weight daily are not at issue here.

I will present data from epidemiological, animal and clinical investigations supporting the premise that dietary protein has a predictable influence on bone health provided that both sides of the dual influence are accounted for. Limitations of these studies will be examined, and the strength of the model weighed.

If correct, the implication of the dual influence model is that positive and negative pathways may be manipulated in order to shift the balance toward a (probably modest) benefit to bone health. Specifically, if correct, we would predict that protein will be more beneficial where calcium intakes are sufficient to replace urinary losses, where intakes of alkalizing nutrients (readily available in fruits and vegetables) neutralize the dietary acid load associated with protein, or where protein source is naturally low in acid producing amino acids or naturally rich in amino acids promoting growth pathways. In addition, this model implies that higher protein diets, though much criticized in the past for a theoretical harm to bone, are unlikely to have a substantially detrimental effect in free-living populations.

Chapter 2

Dietary protein and bone health: Harmonizing conflicting theories

Introduction

Argument regarding the relationship between dietary protein and bone health has unrolled over more than fifty years. Animal, epidemiological and clinical work have corroborated that high protein intakes can impose a small but chronic metabolic acid load (1-3), and that this acid load may cause bone loss (4-6). Indeed, urinary calcium losses are unequivocally induced by high protein intakes (7). In contest, equally diverse research paradigms support that dietary protein increases calcium absorption or bioavailability (8-13), casting the net effect on the calcium economy into doubt. Furthermore, trials have persuasively argued that increased protein may initiate bone anabolism mediated by the protein-sensitive insulin like growth factor 1 (IGF-1, 14-16).

In the midst of so many valid mechanisms for dietary protein to either enhance or degrade bone health, a recent meta-analysis (17) observed no overall effect in observational studies, excepting a modest benefit to bone mineral density (BMD) of the lumbar spine. Even this effect was so small as to be of questionable clinical importance, casting doubt as to whether higher protein intakes influence bone at all.

A newer emerging concept has been that protein does indeed influence bone through simultaneous beneficial and detrimental pathways (18). Specifically, it is thought that a negative influence of protein's diet acid load is opposed by increased calcium absorption and anabolic influence of IGF-1 (19). The implication of this dual-pathway model is that the net influence on bone may be positive, negative or null, depending on additional dietary

considerations (19-21). The dual-pathway model is attractive in its capacity to harmonize apparently conflicting data, providing a sound theoretical basis to reconcile divergent views.

In this review, we will summarize the history and current state of the literature of dietary protein in urinary calcium loss, intestinal availability, dietary acid load and bone turnover. We will attempt to illustrate that no one of these factors is sufficient to explain the complex relationship of dietary protein and bone health. Throughout this review, we will discuss the influence of protein intakes *above* the current RDA of 0.8 g protein / kg body weight. That protein intakes *below* this level cause a protein deficient state that is harmful to bone is plainly apparent (*see* 22 *for a review*), and outside of our present scope.

If the competing pathways model is correct, we might predict that additional manipulations of the diet, such as providing ample calcium or acid-neutralizing foods such as fruits and vegetables, may "tip the scales" (23) to a favorable role of protein in bone health.

Protein and Urine Calcium

Protein causes urine calcium loss

In 1920 HC Sherman, an important figure in early mineral balance research, published the observation that adding meat to the diet of a healthy young male increased the amount of calcium intake required to maintain equivalence of calcium in and outputs (24). Subsequently a consistent elevation of urinary calcium in response to changes in protein intake has been found across many human studies. A meta-analysis by Kerstetter *et al.* (7) produced this equation, which explains 49% of the variation in urinary calcium across 26 studies:

$$\textit{Urine Ca, mmol/d} = (3.208e\text{-}2) * \textit{protein, g/d} + 1.501 \qquad \textit{eq. 1}$$

This equation would predict that a 40 g/d increase in protein intake (roughly equivalent to a typical American woman switching from a conventional to a South Beach diet) would translate to an additional 50 mg of calcium lost in urine daily. Though small, the accumulation of 50 mg/d calcium losses over decades could indeed cause clinically significant osteoporosis if this calcium is depleted from roughly 1 kg of skeletal calcium in adult humans (25).

Dietary acid causes urine calcium loss

Views on the source of protein induced urinary calcium loss have changed over time. Acid-base balance studies by Lemann *et al.* in the 60s (26) demonstrated that supplementation of up to 280 mmol/d ammonium chloride is only partially buffered by declining serum bicarbonate and renal net acid excretion, leaving a residual "acid gap" that must be accounted for by some alternative buffer source in the body. In subsequent research those authors found this acid gap corresponded to an increase in urine calcium losses and, ostensibly, negative calcium balance (27). Furthermore, calcium losses were only partially recovered following correction of acidosis with supplemented bicarbonate.

The idea emerged that release of buffer from bone tissue, including bicarbonate and phosphate, would accompany the release of calcium observed in the urine, and that exchange of acid for this buffer would account for the missing acid (28-30). The "acid-ash" hypothesis (28) posited that this buffering action of bone mineral would lead to bone wasting over time, contributing to the development of osteoporosis. Acid ash refers to acids remaining after combustion of food, which correspond to a fixed acid load in the diet that must be compensated by metabolic, rather than respiratory systems (25). Correction of metabolic acidosis using supplemental base reduced urine calcium loss to normal levels (31). Since

then, the observation that bicarbonate or citrate supplementation reduces urine calcium has been replicated in many randomized trials (*e.g.*, 32-36), including over a time span up to 36 mo (37).

Dietary protein increases the dietary acid load

Dietary protein is a major contributor to the acid ash of the diet (38). In humans this is completely attributable to metabolism of sulfur containing amino acids (SAA) to sulfuric acid (39). Indeed, renal Net Acid Excretion (NAE), a measure of renal excretion of acid equivalents (measured as total urine NH_4^+ - HCO_3^- + titratable acid, 40) varies in proportion to SAA in the diet (41-44). Schuette *et al.* (45) report that SAA adequately explained the complete difference in NAE in response to protein source. The importance of SAA in the dietary acid load caused protein, and more specifically protein sources high in SAA to be viewed as potentially promoting the gradual loss of bone mineral and development of osteoporosis over time (25).

Frassetto *et al.* (45) developed prediction equations for NAE based on analysis of their own data in addition to meta analysis of available, sufficiently detailed reports, concluding that across 20 diets and 141 subjects, NAE increases one mEq for each additional 0.91 g protein consumed on average, when holding potassium constant (r^2=0.67):

$$NAE = 0.91(Pro, g)\ 2\ 0.57(K, mEq) + 21 \qquad eq.\ 2$$

The range of NAE occurring in the 20 diets studied by Frassetto was approximately 15mEq/d to 115mEq/d. Accordingly, an increase of mixed protein intake from 60 to 100g/d, as described before, would be expected to increase the NAE by roughly 36.4 mEq/d, or over 1/3 of the complete range of NAE encountered across from the low to high extremes of realistic diets.

The acid load of protein explains protein-induced urine calcium loss

A 1980 study in rats compared the effect of various protein sources on urine calcium, observing that the SAA or acidogenic fraction accounted for the effect (46). To verify, those authors fed sulfate independently of protein and observed increased urine calcium consistent with that induced by SAA. The following year two clinical studies reported that feeding isolated SAA increased urine calcium (47), and that the change in urine calcium with protein correlated with the change in NAE (48). Another trial in premature infants found urine calcium increased with the addition of cysteine to total parenteral nutrition solution (49). More recently it has been shown that a mineral water rich in sulfate increased urine calcium relative to milk, in spite of a similar calcium content (50).

Recently, a meta analysis by Fenton (51) illustrated that across 25 clinical trials meeting selection criteria, 86% of the average change in urine calcium may be explained by the experimentally manipulated change in NAE:

$$\textit{Urine Ca change, mmol} = 0.28884 + 0.027118(\textit{NAE change, mEq}) \qquad \textit{eq. 3}$$

We performed a secondary analysis of the data summarized by Fenton (excluding two extreme values), testing whether the slope of urine calcium change regressed onto NAE differed between protein and non-protein sources of acid. We found no difference in the slope of protein vs. other acid sources ($p=0.37$), suggesting that the rise in urine calcium observed with increasing dietary protein can be completely explained by the dietary acid load of the protein.

Reinforcing this idea, we compared the value of urine calcium predicted from protein intake (using the meta analysis of Kerstetter, 50 mg/d with a 40g/d increase in protein, eq. 1) with that predicted from the combination of protein-induced increase in NAE and NAE-

induced urine calcium (using eq. 2 from Frassetto and eq. 3 from Fenton, respectively). Again, for a realistic 40g increase in protein intake, the combination of eq.s 2 and 3 would predict an increase in NAE of 36.4 mEq and a corresponding increase in urine Ca of 55 mg, well within confidence limits of the 50 mg predicted by Kerstetter. Although the comparable outcomes of these analyses do not definitively rule out other mechanisms, they suggest that urine calcium loss in response to protein intake can be completely explained by the acid load of the protein.

Mechanisms for Urine calcium Loss

The acid ash hypothesis posits that the well documented increase in urine calcium with additional dietary fixed acid or equivalent protein is the result of bone demineralization. However many studies have tested the possibility of a concomitant rise in intestinal calcium absorption, with discordant results. Additionally, newer research is beginning to cast light on plausible mechanisms for protein modified calcium handling in the kidney.

Renal mechanisms

Increased protein intake reduces the reabsorption of calcium in the kidney (52-55), based on the difference between observed calcium excretion and that expected given glomerular filtration rate (GFR) and plasma calcium. An older microperfusion study in dogs found that experimentally induced acidosis led to reduced reabsorption of calcium in the distal tubule of the nephron (52). Correction of acidosis with supplemental base (NaHCO3) recovered calcium reabsorption. This was true in normal and parathyroidectomized animals, indicating the effect was independent of the PTH axis. A 2006 study in mice (56) found induced acidosis decreased expression of the calcium transporter TRPV5 in the distal tubule, and that acidosis did not modify urine calcium excretion in mice lacking the TRPV5 gene. A

subsequent study demonstrates in vitro that physiological acidosis impairs calcium uptake by TRPV5. Although the regulation of this gene is not completely understood, it is reasonable at this time to suspect that the acid load accompanying dietary protein, as evidenced by increasing NAE, reduces the amount and/or activity of this calcium reabsorbing gene in the distal nephron, causing extra calcium to be lost in the urine.

Several studies have also observed an increase in GFR in response to increased dietary protein (45; 57; 58), which also might account for additional calcium filtration and excretion. One randomized trial reported GFR was not increased by equivalent amounts of SAA (47). The mechanisms and implications of GFR in response to protein intake are not completely understood, but it is possible that some effect of protein on urine calcium exists independently of the protein acid load. It has also been recently proposed that the calcium sensing receptor CaR, which is involved in renal acid-base regulation (59) and calcium reabsorption (60), is regulated by aromatic amino acids (61) and may provide another link between protein intake and urinary calcium loss, independent of acid base status. However these mechanisms are not well understood, particularly in vivo.

Clinical investigations suggest higher protein intakes increase urine calcium by a similar amount irrespective of calcium intake (62). Jajoo *et al.* (63) observed a paradoxical increase in urine calcium with protein even in the face of increasing parathyroid hormone (that is, urine calcium increased when statistically holding PTH constant). This suggests protein causes an obligate calcium loss that is not simply a byproduct of other perturbations of calcium balance. If correct, this would suggest that urine calcium losses with protein may cause negative calcium balance unless compensated by increased calcium intake or absorption.

Although increased urine calcium has been associated with osteoporosis (64) and bone loss (19), the absence of predictable loss of bone mass with higher protein intakes (17) implies that calcium lost in the urine is otherwise recovered.

Protein and Calcium Absorption

Results are mixed on the clinical effect of protein on calcium absorption

Over time several randomized studies have noted improvements in calcium absorption with higher protein intakes(57; 62; 65-67), however several other studies have seen no such effect (68-71). This discrepancy has been attributed to inadequacies in older methods of tracing calcium (8; 72) compared to modern dual stable isotope methods, though even this approach has not observed an effect in every study (71). A well controlled animal study by Bell *et al.* in 1975 (10) showed that excretion of radiolabeled ^{45}Ca in the feces declined as its excretion rose in the urine in response to increasing protein intake. Net calcium balance was unaffected, except in rats consuming 10% energy from protein, which was concluded to be deficient for bone maintenance.

Comparable observations were made in later rat studies (11; 12) and Whiting and Draper confirmed no benefit or harm to bone mass or composition after 10 months of a diet providing 35% energy as protein (soy and lactalbumin). A recent study by Gaffney-Stromberg *et al.* (73) studied vesicles developed from the intestinal brush border mucosa of rats fed 5, 20 and 40% energy as protein, observing an increased Vmax, but no change in Km of calcium uptake. These changes explained a 14.4% improvement in calcium absorption in high vs. low protein animals, which more than compensated for protein induced urine losses.

Protein effects on calcium absorption may be independent of PTH and vitamin D

Kerstetter *et al.* have demonstrated in humans that protein intakes below (42; 74; 75) 0.9g/kg reduce calcium absorption, but elevate PTH and calcitriol, which normally increase calcium absorption. This counterintuitive result stresses that increases in absorption with protein intake are not likely to be mediated by canonical PTH and vitamin D pathways. Consistent with this, these same studies identified no changes in PTH or calcitriol between protein intakes of 1.0g/kg and 2.1g/kg, despite improvements in calcium absorption. Similarly, other randomized trials have not observed differences in calcitriol (48) or PTH (53; 62; 70) with higher compared to normal protein intakes. Conigrave (76) has shown in vitro that amino acid regulation of the calcium sensing receptor (CaR) suppresses the secretion of PTH from parathyroid cells. This would explain the results of Kerstetter, but it is not clear whether this mechanism has clinical relevance at *high* compared to *normal* intakes. Taken together, the presently available randomized trials suggest PTH is not involved beyond a threshold similar to the current RDA for protein.

Geibel (77) reviews new knowledge that the CaR may also regulate calcium absorption in the intestine, potentially explaining the effect of dietary protein on calcium absorption. Dawson-Hughes *et al.* (78) did not directly measure absorption, but observe no changes in bone turnover in spite of increased urinary calcium in patients randomized to a five-fold increase in aromatic amino acids, the most potent regulating amino acids of the CaR, relative to a similar increase in branched chain amino acids. Those authors speculate that calcium absorption may have increased, since urine calcium losses did not appear to originate from bone resorption.

In contrast, we estimate using food composition tables that the aromatic, aliphatic and polar amino acids shown to most potently regulate the CaR (79) are present in similar or slightly higher amounts in soy compared to whey, casein and beef protein, and that aromatic amino acids alone are 20-40% higher in soy than these other proteins. If these amino acids are indeed responsible for clinically observed improvements in calcium absorption, it is curious that soy protein appears to not elevate absorption to the degree of other protein sources, if at all (42; 43). Note that this analysis does not account for digestibility or kinetics of amino acid absorption.

Protein effects on absorption may be masked by alternate homeostatic mechanisms

It is also possible that protein effects on absorption, including real effects of aromatic amino acids in soy, may be masked by other nutrients regulating the calcium balance. A recent study by Hunt *et al.* (62) observed that in subjects consuming 675 mg calcium daily, 20% energy as protein increased urine calcium but also increased calcium absorption (29.5% *vs.* 26.0% at lower protein), such that net calcium balance was only slightly negative. In contrast, high protein did not alter calcium absorption in subjects consuming 1510 mg calcium daily (18.0%).

These data might be explained by a consistent increase in calcium absorption induced by protein, combined with a linear decrease in calcium absorption fraction in response to total calcium intake as has been described previously (80). Specifically, if dietary calcium is sufficiently high to meet requirement, any additional absorption as a result of protein intake may be compensated by alternate homeostatic mechanisms. This paradigm would also account for the increase in PTH and calcitriol observed at low protein intakes (42; 74; 75), as alternate homeostatic mechanisms intervene to compensate for the lost efficiency of

absorption related to protein. Of course, these considerations are only speculative, and further understanding of the mechanisms of protein's role in absorption is necessary.

In spite of mixed results, it appears likely at this time that calcium absorption is indeed dependent on dietary protein, but that this effect may be masked in particular scenarios by interactions with other nutrients or by effects specific to protein source.

Protein and Bone

Bone cells are regulated by acid-base balance

In vitro work on the putative buffering of metabolic acid by bone tissue has verified that bicarbonate, phosphate, sodium and potassium on the bone surface can be exchanged for acid (30; 81; 82), which reasonably accounts for the acid gap described by Lemann (26; 27). In the last decade it has been increasingly appreciated that calcium efflux from bone in response to acid may not be a passive physiochemical process (83), but the result of physiological regulation of bone cells by acid base balance (4). Both osteoblasts and osteoclasts are responsive in vitro to changes in pH. The regulatory range is inverse but overlapping, such that at pH 6.9, osteoclasts are maximally active (84), but osteoblasts are strongly inhibited (85), whereas at pH 7.4, osteoclasts are 'turned off' and osteoblasts demonstrate high expression and activity of alkaline phosphatase (85). These changes appear to occur through well developed cell signaling cascades which are not fully understood, but include PGE2 stimulation of RANKL (86-88). These effects are potentiated by TNFα (89) and nuclear factor NFATc1 (90). Furthermore, H^+ sensing receptors have been identified on the surface of osteoclasts (4). These novel pathways may also explain an older observation that acid moderates the influence of PTH on calcium efflux from bone in culture (91).

The relevance of these pathways to the in vivo system is unclear. The intricacy of the emerging signaling cascade as well as the striking inverse correspondence between acid regulation of osteoclasts and osteoblasts imply these systems evolved to fill a defined physiological role. Arnett (4; 84) has described acid-base regulation of bone cells as a "fail-safe" mechanism, ensuring that adequate buffer could be mobilized from bone to buffer an otherwise uncompensated acid load. This line of reasoning has been criticized (*e.g.*, 22) on the grounds that a dietary acid load would hardly drive the pH of extracellular fluid below the well-buffered set-point of 7.4. Indeed, while an extreme dietary acid load can measurably depress the pH of the blood in animals (83; 92-94) and humans (95-97), a change greater than 0.014 on the pH scale was not observed in our review of the literature (96).

In spite of the small effect of diet acid load on blood pH, this effect may still be clinically meaningful. Arnett (84) notes that changes in pH on the order of 0.05 can double resorption pit formation. As previously mentioned, very small changes in net calcium flux from bone may contribute to gradual demineralization over decades, though it is clearly imprudent to extrapolate quantitative clinical estimates from studies in culture. Osteoclasts are reportedly most sensitive to pH changes near the middle of the sensitive range, around pH 7.1 (84), which is not unrealistic for the layer of extracellular fluid bathing cells at the low end of the pH gradient in interstitial fluid, based on study in skin tissue (98).

In rats, NH_4^+ loading causes an increase in serum ionized calcium, even in parathyroidectomized animals (99). This rise in serum calcium was blunted with pharmacological inhibition of cell mediated bone resorption (using colchicine or calcitonin), supporting the notion that bone matrix is actively degraded in response to an acid load. A randomized trial by Osther (100) also observed increasing serum ionized calcium in response

to acute acid loading. This effect has been reported in one trial of high protein (*e.g.*, 22); however most studies have not seen a change in serum calcium with high protein intakes (53; 66; 101).

Effect of diet acid load on bone health

In animals, it has been reasonably consistent that feeding acid ash damages bone over time (93; 102; 103), though not in all studies (104). Barzel (105) reviewed that chronic NH_4Cl supplementation in rats invokes a, "non-hormonal, slow but progressive and unrelenting mobilization of bone," and that the end result is, "indistinguishable in all parameters measured from human osteoporosis." Lemann (106) observed that NH_4Cl administration in humans increases hydroxyproline, a marker of bone resorption, at a change in NAE that could reasonably be induced by a dietary acid load. A case study from 1982 describes clinical improvement in osteomalacia secondary to chronic metabolic acidosis following administration of alkaline supplement. Similarly, a case series (107) reported clinically subnormal bone formation rate and bone mineral density in 10 patients with distal renal tubular acidosis. These bone abnormalities were corrected following one year of therapy with potassium citrate.

Epidemiology supports the notion that a high diet acid load negatively influences bone. The estimated net endogenous acid production (estimated NEAP) was developed by Frassetto *et al.* (38) to predict the change in NAE from the combination of protein and potassium intakes, with potassium serving as a surrogate for associated alkaline ash components in the diet. Another estimation of the diet acid load, the Potential Renal Acid Load (PRAL), was developed by Remer and Manz (1) using intakes of protein, phosphorus and negative coefficients (representing more alkaline contribution) for potassium,

magnesium and calcium (108). The estimated NEAP and PRAL are adversely correlated with bone density (109; 110) and broadband ultrasound attenuation (BUA) of the heel (111; 112), as well as bone turnover markers (109; 110), though not in all studies (113).

Both of these dietary acid load estimations include total protein intake. Two studies accounting for the diet acid load as well as the independent effect of protein within the same statistical model have observed an adverse effect of acid on bone mass that is opposed by a beneficial effect of total protein intake (19; 114). Corresponding to this evidence, Whiting and Draper (46) reported that feeding of SAA to rats induced osteopenia, while feeding of protein containing equivalent amounts of SAA had no such effect. In light of these observations, it seems that the total effect of protein on bone may conceivably be positive, neutral or negative, depending on the relative contribution of the protein to the diet acid load compared to an alternate, beneficial pathway.

Effect of protein on bone density

High protein intakes have been shown to inversely predict bone mass in animal (115), epidemiological (116) and clinical (117) investigations; however positive associations have been much more frequently reported in cross sectional studies (118-124). Hannan (125) reported that lower protein intakes predicted bone loss longitudinally; however in this and some of the cross sectional investigations cited above, it is ambiguous whether benefits of additional protein correspond intrinsically to higher protein intakes, or to prevention of a protein deficiency. Cohort studies have not consistently observed a noticeable effect of protein intake on change in bone density over time (123; 126; 127), suggesting that if protein indeed causes the changes observed cross-sectionally, these benefits are likely to accrue very gradually over the lifespan.

The division into animal and vegetable protein sources has been made as a crude index of the acid load of protein. Weikert (128) reports in a large sample (n=8,178) that while vegetable protein positively predicts BUA of the heel, animal protein is inversely associated. Similarly, Beasley (126) reports in young women that low intake of vegetable protein corresponds to lower bone density of the spine, while no association is observed for animal or total protein. In contrast, Promislow reports positive associations of bone density with animal protein, and negative associations with vegetable protein.

While it can generally be said that meat protein is higher in SAA than protein from plant sources, Massey (129) identifies that the variation in acid load within a food group can vary as much or more than variation between food groups. For example, egg protein provides 79.6 mEq sulfate per 100g protein, chicken provides 65.0 and milk provides 54.8. For plant proteins, soy provides 39.8 while corn provides 61.4 and white rice provides 68.0. Additionally, cereals contain other nutrients which increase the dietary acid load (1) while vegetables are rich in organic bases (23). Animal / vegetable protein ratios may conflate the beneficial effects of a diet rich in vegetables in general with the effect of vegetable specific proteins, and also the effects of relatively low acid animal foods like milk with those of high acid proteins like pork. A high ratio may also represent an overall diet that does not contain adequate fruits and vegetables. Because of this potential bias, we discourage the use of animal / vegetable protein ratios in favor of investigation of more specific, individual food groups or nutrients.

Possible interaction of protein with calcium intakes

Strong contradiction of the hypothesis that animal proteins are generally harmful comes from observations that dairy products improve bone accrual to a greater extent than

equivalent supplementation of calcium and vitamin D in growing children (130) and postmenopausal women (131). The possibility of a beneficial interaction of protein with calcium has been emphasized by Dawson-Hughes *et al.* (132). In a nested cohort investigation of 342 men and women randomized to calcium and vitamin D supplementation, they report increasing bone density over time among patients in the highest tertile of protein intake, but no such association in patients randomized to placebo. Vatanparast *et al.* (133) reported a positive correlation between protein intake and bone accrual over ~ 11.4 y in growing adolescents, and an augmentation of that benefit when calcium intake exceeded 1 g/d.

Protein intake and bone turnover

The effects of higher protein intakes on bone turnover markers are more equivocal. Allen *et al.* (70) reported no change in urine hydroxyproline, a product excreted in the urine in proportion to bone degradation, in response to formula diets containing 12 and 36 g nitrogen. Roughead also found no effect on bone metabolism markers in response to 12 vs. 20% energy from protein, with meat accounting for the difference. Dawson-Hughes (78) also failed to show any effect of AAA compared to BCAA on turnover markers.

Hunt (62) reports a beneficial uncoupling of turnover given 20% vs. 10% energy as protein. Specifically, protein reduced deoxypyridinoline (a marker of bone degradation) without a concomitant decrease in bone specific alkaline phosphatase or osteocalcin (markers of osteoblast activity). In contrast, Kerstetter (134) found that relative to 0.7g/kg protein, 2.1 g/kg increased N-telopeptide (another marker of bone degradation) and decreased bone specific alkaline phosphatase. Reddy (135) observed that a low carbohydrate, high protein

diet reduced osteocalcin without affecting N-telopeptide, bone specific alkaline phosphatase or deoxypyridinoline, suggesting possible harm to bone metabolism.

Several trials have reported markers of bone formation or resorption, but not both together, making them difficult to interpret. Bone turnover markers are normally well coupled, moving up or down in tandem (136), such that an increase in bone resorption is ambiguous unless bone formation is also known. That is, it cannot be determined whether bone formation increased to a greater degree than resorption, suggesting a net benefit to bone, or to a lesser degree, suggesting net harm to bone, or to a similar degree, suggesting bone turnover remains tightly coupled.

It has been shown that increased turnover can predict risk of fracture even when formation and resorption remain tightly coupled (reviewed in 136); however it is not always so. Turnover is related to fracture risk along a J shaped curve (136), such that low and high levels may be detrimental. For example, within the present review, protein or energy deficiency cause a depression of both formation and resorption markers, which may remain well coupled (*e.g.*,137; 138). Despite decreased turnover, the long term impact of such deficiency is known to be harmful (22; 139-141). Conversely, increases in non-extreme exercise can elevate formation and resorption in tandem, yet produce improvements in bone health over time (*e.g.*, 142; 143). Accordingly, the long term impact on bone health of a well-coupled increase in turnover depends on the underlying cause of turnover changes. The long term consequence of protein induced turnover changes are not yet understood, and probably differ when comparing high to adequate vs. adequate to deficient intakes.

Protein intake and fracture

Fracture rates have been reported to increase (144-147) and decrease (139; 148; 149) with dietary protein. Available studies have generally been limited by a small number of incident fractures. One of the largest available studies (n=85,900 women over 12 y) observed no effect of protein on hip fracture, but even this tremendous sample size yielded only 234 incident hip fractures. The same study did observe a linear increase in wrist fracture with protein intake, with a 22% increase in risk at intakes > 95g/d compared to <68g/d. This effect was attributable to animal, and not vegetable protein, consistent with the acid-ash hypothesis. Darling (17) summarized the available data for hip fracture, finding no effect of protein intake in a meta-analysis weighted by sample size. This was true for total, animal and vegetable protein, however only 3 studies were included in each analysis.

Heaney and Rafferty have recently discussed the standard of "preponderance of evidence" in drawing conclusions from conflicting reports (150). They illustrate that because most studies are not sufficiently powered to reduce the type II error risk (failing to reject a null hypothesis that is false) to the same level as the 5% standard for type I error risk, any one study is more likely to incorrectly report no effect than to incorrectly declare statistically significant results. In this context, they advise that a mixture of positive and null study results be cautiously interpreted as evidence for a probable true effect. Aggregate data for urine calcium, calcium absorption and bone density are amenable to this standard, as studies have found significant effects in a consistent direction as often or more often than not. However data for bone turnover and fracture rates in response to protein intakes are more puzzling, since both positive *and* negative results have been reported as frequently as has no association.

A Dual-Pathways Model of Protein and Bone Health

It is curious that fracture rates may be influenced in both directions. As discussed by Darling *et al.* (17), publication bias may cause a polarization of available literature on these outcomes, causing significant findings in both directions to be over-reported with respect to null results. Alternatively, as introduced previously, we (19) and others (21; 151; 152) have supported a competing pathway model, in which dietary protein may cause an increase or decrease in bone health, depending on the availability of calcium and alkali ash in the diet. The cause of purported negative effects is clear as delineated by the acid-ash hypothesis. The pathway for positive effect may be mediated by increases in calcium absorption above urinary losses. IGF-1 has also been proposed as a mediator of beneficial influences of protein.

Insulin-like growth factor 1

Insulin-like growth factor 1 (IGF-1) has been shown to hold predictive value in osteoporosis in older individuals (153) and in bone accrual in young males (154). It has been linked to multiple pathways in bone cell regulation, and administration of IGF-1 ex vivo stimulates a general increase in bone growth (155). Dietary protein is known to modify both IGF-1 and some of its binding proteins (156); it has therefore been an attractive candidate as a mediator of protein's influence on bone (22; 151; 157; 158).

Schurch *et al.* (159) showed that supplementation of protein reduced bone loss in the femur in patients with recent hip fracture. These benefits were accompanied by and attributed to simultaneous increases in circulating IGF-1. This group reported recently (160) a similar response to protein supplementation in a comparable population, with measurable increases in IGF-1 within 7 days of initial supplementation. These changes, if generalizable, could

certainly explain benefits of protein to bone health. These patients were initially somewhat protein deficient, however, and the notion that IGF-1 can be elevated by increasing protein beyond the threshold of adequate intake levels is not uniformly supported (161; 162).

Three randomized trials to implicate protein intakes above the RDA as relevant to IGF-1 regulation. Ballard (163) randomized young adults to 70 g supplemental carbohydrate or 42 g supplemental protein plus 28 g carbohydrate during an exercise intervention. The additional protein was observed to increase IGF-1, though protein intake appeared adequate in both groups. The previously reviewed study of protein intake at low and high calcium intakes by Hunt (62) discovered improved IGF-1 with protein, independently of calcium intake. Two studies by Dawson-Hughes *et al.* (14; 78) shed additional light. The first shows increased IGF-1 and decrease in a bone resorption marker with 1.6 in lieu of 0.8 g/kg protein. The second shows an increase in IGF-1 following a large increase in isolated aromatic amino acids, while no such increase is observed with branched chain amino acid supplementation.

IGF-1 may also link protein nutrition to muscle mass (156). It has been proposed elsewhere (164) that documented increases in lean body mass in response to the dietary protein level (165; 166) may connect protein intake and bone health. Increased mechanical loading of bone by an increased muscle mass would be expected to promote bone mass and strength (167).

Alternative suppression of the diet acid load

An almost overwhelming amount of support exists for the notion that increasing sources of alkali ash in the diet is beneficial to bone (63; 168-174). The primary difficulty in the epidemiology in this area has been attributing benefits specifically to suppression of the dietary acid load, as opposed to the additive or interactive influence of the many nutrients

provided by enrichment of fruits and vegetables in the diet. Zerwekh (115) illustrated in rats the ability of supplemental base, potassium citrate, to ameliorate the osteomalacia induced by a high casein diet, while KCl had no such effect. Additionally, a remarkable number of randomized trials of supplemental sources of base have fortified this concept over the past few years, relying mostly on bone turnover markers for shorter term inference about bone metabolism.

Comparisons of salts of sodium and / or potassium with bicarbonate, citrate and / or chloride indicate that supplemental base, not potassium or other components, favorably uncouples turnover, suppressing resorption without reducing formation (35; 36; 175; 176). This effect is also achieved with alkaline, compared to relatively more acidogenic mineral water (177). Furthermore, a longer term study demonstrated that potassium citrate, and not potassium chloride, improves bone density of the lumbar spine, femoral neck and total hip (178).

Taken together, the epidemiology of fruits and vegetables and clinical investigations of supplemental base offer solid documentation that positive changes to bone health are achievable through a diet rich in fruits and vegetables, with essentially no risk, but rather many additional health benefits (23).

Methodological Issues

Accounting for simultaneous, opposing effects of protein and the diet acid load

In light of the evidence reviewed herein, we propose that experiments or statistical models that fail to account for both proposed positive and proposed negative effects of protein may fail to observe real effects in both directions. Where positive and negative effects arise from the same independent variable, the total effect will be biased toward zero

unless mediators of opposing effects are accounted for (179). In epidemiological study, opposing effects may be investigated using meditational or path analysis (180). For example, the effect of total protein on bone health may be modeled with and without adjustment for sulfur amino acids and/or IGF-1. The change in the main effect of protein before and after adjustment represents the portion of protein's influence that is attributable to the mediating factor (180). In experiments, appropriate controls should be incorporated to isolate potentially conflicting effects.

Estimation of the diet acid load in bone studies

Fenton *et al.* (181) performed a meta-analysis of the influence of phosphate intake on calcium balance, finding no net effect across 12 studies of acceptable methodological quality. Those authors argue this result refutes the acid ash hypothesis, since phosphate is included in those dietary components though to influence NAE (1). It has been known for many years, however, that the role of phosphorus in calcium balance is more complex than predicted based only on its role in the diet acid load (182; 183). In 1981 Linkswiler (184) reviewed consistent evidence that phosphorus intake depressed urine calcium loss. Heaney *et al.* have since repeatedly shown (185-187) that phosphorus also increases fecal calcium loss, specifically endogenous calcium loss through secretions into the intestine, such that any gain at the kidney is lost in the intestine.

These unique effects of phosphorus are not observed with other dietary acid ash components, and may have an opposite or no effect on bone in spite of its role in predicting NAE. This does not refute the negative role of an acidic diet in bone health, but rather calls into question the utility of NAE prediction equations that include phosphorus or other nutrients with multiple influences on bone. In a cross sectional study of postmenopausal

women (19) we observed a negative influence of diet acid load from SAA on bone density of the spine after adjusting for total dietary protein. We observed no similar association with the NEAP or PRAL, which would theoretically also represent the diet acid load. The PRAL (1) accurately predicts change in NAE induced by diet, but includes phosphorus, magnesium, calcium and total protein in its estimation. Each of these nutrients appears to have unique, non-acid related roles in bone health (168; 169; 188; 189). Similarly, estimated NEAP (38) accurately predicts NAE, but does not account for possible positive roles of protein independently of the acid load from dietary protein (44). For general prediction of NAE, these equations are superior to the use of SAA alone; however, when the effect of the diet acid load on bone health is specifically investigated, it seems prudent to test individual, rather than aggregate effects of SAA, total protein, potassium (as a surrogate for organic base) and minerals which may influence bone health through alternate pathways.

Discerning high from non-deficient protein intakes

The importance of dose cannot be overemphasized in this research. In many trials, it has been ambiguous whether purported protein effects are due to benefits of higher protein intakes, or due to correction of a frank protein deficiency. In future trials, it would be very helpful to compare 3, rather than 2 levels of intake where possible: one level that is marginally deficient, one level sufficiently high as to preclude frank deficiency and one that is higher to explore the impact of protein above adequate levels. For example, the intakes 0.7g/kg, 1.0g/kg and 2.1g/kg have been used by Kerstetter (57; 75; 134), and have resulted in more decisive interpretation. Where 3 parallel arms are not feasible, care should be taken to unambiguously compare high to adequate, or adequate to deficient protein intakes, as the

comparison between high and deficient intakes is not useful in evaluating the possibility of harm of higher protein intakes to bone.

Similarly, in epidemiological studies, it would be preferable to compare subgroups of the population divided according to comparable 'adequate' and 'high' thresholds, rather than comparing tertiles within a population that may be generally protein deficient. For clarity of interpretation, protein intakes should be reported as both absolute gram quantities and as intakes normalized to body size (*e.g.*, g/kg body weight), as reporting only % energy as protein leaves the sufficiency of protein intake uncertain.

Measuring and reporting bone turnover

Where turnover markers are reported, bone formation and degradation markers should always be reported in tandem. Several forms of reporting an "uncoupling index" have been described (*e.g.*,190) and are helpful in evaluating the likely net effect of changes in turnover to bone health. We recommend reporting uncoupling as the ratio of percent change from baseline for formation and resorption markers:

$$Uncoupling\,Ratio = \frac{(end\ \ formation - baseline\ formation)}{baseline\ formation} \Big/ \frac{(end\ resorption - baseline\,resorption)}{baseline\,resorption} \qquad eq.\ 4$$

A value > 1 would suggest net formation. The ratio of percent change is likely to best account for the high degree of inter-individual variation in turnover markers.

The use of ratios in prediction equations

Ratios of nutrients have often been reported where nutrient interactions are thought to exist, for example, between animal and vegetable protein or between protein and calcium. Statisticians have warned for decades of the problems of including ratios in statistical prediction equations (191-193), including difficulties in interpretation, violation of

distribution assumptions, spurious associations between ratios with the same denominator and inaccuracy in individual variable coefficients (although the predictive accuracy of the complete model is not affected). Although it may be appropriate to compare calculated ratios across groups (for example, the uncoupling ratio described above was greater in population A than in population B), it is generally preferable to form prediction equations using main effects and an interaction term. A model including interactions should always include corresponding main effects, unless compelling theoretical reasons exist for excluding them (194). For example, applying such a model for the possible interaction of protein and calcium:

$$BMD = B_0 + B_1(protein) + B_2(calcium) + B_3(protein*calcium) \qquad eq.\ 5$$

This model avoids the disadvantages outlined above, but still provides all the information afforded by a ratio and is more easily interpreted. See (194) for a non-technical discussion of dealing with interaction in regression.

Conclusions

Our review finds ample evidence that dietary protein may hold both positive and negative effects on bone. In many scenarios, these opposing effects may cancel one another out, explaining the lack of a result in the meta analysis by Darling (17). It is feasible, though requires additional study, that the combination of moderate increases in protein intake with ample dietary calcium, alkalizing nutrients such as fruits and vegetables or alkaline mineral waters may uncouple positive and negative effects. This may permit the benefits of dietary protein to bone to be enjoyed without contradictory adverse effects.

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Chapter 3

A positive association of dietary protein with lumbar spine bone mineral density is suppressed by a negative association of protein sulfur

Abstract

Dietary protein is theorized to hold both anabolic effects on bone and demineralizing effects mediated by the diet acid load of sulfate derived from methionine and cysteine. The relative importance of these effects is unknown but relevant to osteoporosis prevention. Post-menopausal women (n=161, mean±SD 67.9±6.0 y) were assessed for areal bone mineral density (aBMD) of lumbar spine (LS) and total hip (TH) using dual X-ray absorptiometry, and dietary intakes of protein, sulfur-containing amino acids and minerals using a USDA multiple-pass 24 h recall. The acidifying influence of the diet was estimated using the ratio of protein / potassium intake, the potential renal acid load (PRAL) and intake of sulfate equivalents from protein. aBMD was regressed onto protein intake, then protein controlled for estimated dietary acid load. A step-down procedure assessed potential confounding influences (weight, age, physical activity and calcium and vitamin D intakes). Protein alone did not predict LS aBMD (P=0.81); however, after accounting for a negative effect of sulfate (ß=-0.28, P<0.01), the direct effect of protein intake was positive (ß=0.22, P=0.04). At the TH protein intake predicted aBMD (ß=0.18, *P*=0.03), but R^2 did not improve with adjustment for sulfate (*P*=0.83). PRAL and the protein / potassium ratio were not significant predictors of aBMD. Results suggest that protein intake is positively associated with aBMD, but benefit at the LS is offset by a negative impact of the protein sulfur acid load. If validated experimentally, these findings harmonize conflicting theories on the role of dietary protein in bone health.

Introduction

Recent literature reflects discordant views on the role of dietary protein in bone health (1). Protein appears to hold an anabolic influence on bone, mediated by bone-active hormones, particularly insulin-like growth factor-1 (1), and may increase calcium absorption (2). Conversely sulfate equivalents derived from methionine and cysteine metabolism are exchanged in the kidney for acid equivalents (3); such a dietary acid load has been demonstrated to cause bone demineralization in animals (4;5) and is associated with reduced bone mineral mass in humans (6;7).

It has been proposed that bone demineralization is promoted by a mild but chronic dietary acid load characteristic of the Western diet (7). This acid load can be characterized by nutrient intake as the estimated net endogenous acid production (NEAP), calculated using a ratio of protein to potassium intake (8;9), or using a function of protein, calcium, potassium, magnesium and phosphorus intake known as the potential renal acid load (PRAL) (3;10). These methods assume the sulfur content of protein is a fixed ratio; however, it is acknowledged that the estimation of actual sulfur intakes improves estimations of dietary acid load (11;12), as actual methionine and cysteine contents vary according to protein source. Currently nutrient databases are available which account for variation in sulfur-containing amino acids (13).

The primary aim of this study was to elucidate the role of dietary protein in bone health status as estimated by dual X-ray absorptiometry (DXA) measures of areal bone mineral density (aBMD). Diet record analysis was used to estimate intakes of total protein and sulfate from amino acids for calculation of the dietary acid load. We anticipated that although dietary protein would be positively related to aBMD, this relationship would be

suppressed by a negative association of aBMD with the dietary acid load related to protein intake.

Methods

Participants

Our sample consisted of 161 post-menopausal women (mean±SD 67.9±6.0 y) from Champaign County, IL participating in an ongoing study of the relation between physical activity, gait ability and self-efficacy. Subjects were recruited using local media advertising, churches, senior centers and health facilities. Women with neurological illness or severe orthopedic or cognitive limitations preventing physical testing for the broader study were excluded. Cross-sectional data were used for the present analysis. Study participants provided written, informed consent; all study procedures met ethical standards of and were approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign.

Dual x-ray absorptiometry

For bone measures, women changed into medical "scrubs" or wore light-weight clothing and removed all jewelry and other clothing except underwear. Lumbar spine (LS) and total hip (TH) aBMD were measured by DXA using a Hologic QDR 4500A bone densitometer (software version 11.2, Bedford, Massachusetts). LS scans included lumbar vertebrae L1-L4. Short and long-term accuracy of the densitometer were verified by scanning a manufacturer's hydroxyapatite spine phantom of a known density. All DXA scans were performed by an Illinois state licensed x-ray technologist and analyzed by the same investigator trained in scan analysis by Hologic, Inc. (EM Evans). Precision for DXA BMD

measures of interest are 1 – 1.5% in our laboratory with CV% calculated from duplicate scans of both young adults and postmenopausal women.

Dietary intake and estimation of acid load

Intake was assessed using the USDA multiple-pass 24 h dietary recall method (14;15). Participants completed an interview with researchers to screen for missed foods, portion size clarification, and recall completeness. Diet records were analyzed for total energy, protein, methionine, cystine and micronutrients of interest for calculation estimated NEAP: potassium, calcium, magnesium and phosphorus. Nutrient analysis was performed using Nutritionist Pro version 2.3.1 (First Data Bank, San Bruno, CA).

Protein sulfur load was calculated as mEq/d using intakes of methionine and cystine divided by their molecular weights, as described by Frassetto *et al.* (8).

$$Sulfur(mEq) = 2 \cdot \left(\frac{methionine(mg)}{149.2} + 2 \cdot \frac{cystine(mg)}{240.3} \right) \qquad eq.\ 1$$

The PRAL of the diet was estimated according to the method of Remer, Dimitriou and Manz (3; 10):

$$PRAL = 0.49 \cdot protein(g) + 0.037 \cdot /P(mg) - 0.021 \cdot K(mg) - 0.02 \cdot Mg(mg) - 0.013 \cdot Ca(mg) \qquad eq.\ 2$$

The protein / potassium ratio estimation of NEAP was calculated according to the method of Frasetto *et al.* (8):

$$NEAP = 54.5 \cdot \frac{protein(g/d)}{potassium(mg/d)} - 10.2 \qquad eq.\ 3$$

Statistical analyses

Distributions were assessed for normality and outliers using the Shapiro-Wilk statistic in conjunction with box plot outlier labeling (16). Correlations for energy intake, protein,

protein sulfur, minerals of interest, vitamin D, aBMD and body composition (weight, fat mass and lean mass) were calculated for descriptive purposes.

Statistical tests followed the prescriptions of MacKinnon, Krull and Lockwood (17) for modeling suppression and mediation effects. Mediation effects, more commonly described as intermediate endpoints in epidemiology, describe a situation where all or part of the influence of X on Y is transmitted through a third, intermediate variable. Modeling these effects is similar statistically, but different conceptually from confounding variables, in which a spurious association between two variables is explained by a third, non-intermediate variable which presumably causes both X and Y. A suppressor effect is a special case of an intermediate endpoint in which X has a direct association with Y and an indirect association, transmitted through a third variable, which is opposite in sign to the direct effect (17). For example, our present hypothesis posits that protein intake will be positively associated with aBMD (the direct effect), but that a third, intermediate variable, the acid load associated with protein intake, will be negatively associated with aBMD, thereby *suppressing* the association of protein intake with aBMD unless the model is statistically controlled for the intermediate variable.

Sulfate, PRAL and the protein / potassium calculation were entered individually as the second block of a regression equation containing dietary protein to predict aBMD of the LS and TH. The change in R^2 was observed between first and second blocks, indicating the improvement in the model conferred by the inclusion of respective estimates of diet acid load. A step-down regression procedure was then applied to examine the potentially confounding influences of body weight, age, physical activity and calcium as well as vitamin D intakes. Because the interaction of dietary protein and calcium intakes have been reported

to impact bone health (1; 18), a protein by calcium interaction term was also tested for statistical control. At each step, covariates yielding a *P* value >0.1 were removed from the model. Covariates were tested before and after the addition of sulfate to the model to ascertain any confounding influences. The variance inflation factor was observed as a test of multicollinearity.

Where change in R^2 was significant, unique protein effects and sulfur-mediated protein effects were estimated and tested for significance. The "Indirect" mediation macro for SPSS by Preacher and Hayes (19) provided bootstrapped variance estimates for the indirect or sulfur-mediated effect using 10,000 resamples with replacement. To describe the individual contributions of protein and related sulfur intakes, scatter plots and least squares regression lines were produced using sample Z-scores (difference between observation and sample mean divided by SD) of aBMD and protein adjusted for sulfur, then of aBMD and sulfur, adjusted for protein (Figure 1). Additionally mean LS aBMD was compared for subjects split across the median for both total protein and sulfur from protein (Figure 2). SPSS 14.0 was used in all analysis.

Results

Among participants, 128 (80%) were taking supplemental calcium (defined as a daily supplement containing at least 100 mg calcium), 34 (20%) were taking a prescribed osteoporosis medication, 50 (31%) were taking hormone replacement therapy and an additional 64 (40%) had taken hormone replacement therapy in the past. One participant completed the LS scan, but not the TH scan, reducing the sample size for TH tests to 160. Parametric and robust descriptive statistics are presented in Table 1. Spearman's correlations of aBMD with protein, energy and estimates of the diet acid load are presented in Table 2.

Protein, sulfur, PRAL, and the protein / potassium index of NEAP were positively correlated with one another (all $P<0.01$), but of the dietary variables only sulfur exhibited a significant, negative correlation with LS aBMD.

At the lumbar spine, the step-down procedure eliminated, in order, vitamin D ($P=0.83$), calcium x protein interaction ($P=0.77$), energy/wk expended in physical activity ($P=0.55$), age ($P=0.63$) and calcium ($P=0.10$), leaving only weight as a covariate in the final model ($P<0.01$). In the first block, protein held no association with LS aBMD (P=0.81). Neither the addition of PRAL ($P=0.66$) nor estimated NEAP using the protein / potassium ratio (P=0.97) significantly improved the model fit; however, adding sulfate demonstrated a negative association of sulfur from amino acids with LS aBMD, and a positive association of protein with LS aBMD (Table 3).

The standardized coefficient for sulfate regressed onto protein intake was 0.69 ($P<0.01$). The standardized indirect coefficient of protein, or that portion of protein's total predictive influence that is mediated by its sulfur content, was -0.19 (95% CI, -0.35, -0.04), opposite in sign and similar in magnitude to the estimated ß for sulfur-controlled protein, 0.21 (Table 3). Despite correlations between protein and acid-load estimators, the largest Variance Inflation Factor observed was 1.9 (for protein and sulfur), well below the recommended threshold of 10 for detecting problematic multicollinearity (20). The relationships of LS aBMD with protein and LS aBMD with sulfur were linear within the range of reported protein and sulfur intakes (Figure 1).

At the total hip, step-down regression removed energy expended in physical activity weekly ($P=0.95$) vitamin D ($P=0.33$) and the calcium · protein interaction ($P=0.26$) terms, but retained age ($P<0.01$), weight ($P<0.01$) and calcium intake ($P=0.02$) as covariates.

Protein was a significant (*P*=0.03), positive (ß=0.18) predictor of TH aBMD; however, no improvement in R^2 was observed with the addition of PRAL (*P*=0.87), estimated NEAP using the protein / potassium ratio (*P*=0.29) or sulfate (*P*=0.83) to the model. The largest Variance Inflation Factor observed was 2.1.

Discussion

Our findings suggest that, within the range of intake reported in our sample, increasing dietary protein is beneficial to aBMD of the lumbar spine and total hip of postmenopausal women, but that this benefit is suppressed at the lumbar spine by the dietary acid load associated with sulfur containing amino acids. Neither the PRAL nor estimated NEAP using the protein / potassium ratio contributed to the prediction of aBMD at either site. The observed regression coefficients are small but clinically meaningful. A participant consuming mean levels of sulfate, but protein at +1 SD would be predicted to have 3.2% (95% CI: 0.12 to 6.4) additional LS aBMD above the sample mean. Conversely a subject consuming mean protein but +1 SD sulfate would be predicted to have 4.3% (-7.4 to -1.2) *lower* LS aBMD. Although further research is necessary to validate these cross-sectional data, the observed differences support hypotheses that improving intake of low-sulfate protein sources, or alternatively improving protein intake with a corresponding reduction in the dietary acid load may be beneficial in osteoporosis prevention.

Protein deficiency is detrimental to bone health (21). Observational studies of bone health tend to promote a positive view of protein intakes above 0.8 $g.kg^{-1}.d^{-1}$ (22-26), though not all studies are favorable (27-29). It has long been established that increasing protein intake elevates calcium losses in urine; in many studies fecal calcium measures indicated no apparent compensation in calcium absorption, promoting the view that urinary calcium must

reflect mineral lost from bone (30). More recently, however, Kerstetter *et al.* (2) have demonstrated not only increased calcium absorption, but a reduction in urinary calcium of bone origin in subjects consuming 2.1 $g.kg^{-1}.d^{-1}$ protein compared to 1.0 $g.kg^{-1}.d^{-1}$ in a kinetic study using dual stable calcium isotopes. In the same study, no net difference in bone mineral mass were observed between levels of protein intake, however there was a trend toward reduced bone turnover.

In light of the influence of protein on urinary calcium excretion, Dawson-Hughes (1; 31) suggests that increased protein intake is beneficial provided that calcium intake is sufficient to support bone growth and urinary losses. Skov *et al.* (32) and unpublished data in our lab (in review) demonstrate benefits of protein above current recommended levels to bone mineral mass, in the presence of adequate calcium, during weight loss. In the present study the interaction of calcium and protein intake was not significant.

Proposed mechanisms whereby dietary protein may enhance bone health include providing substrate for collagen deposition and increasing circulating levels of insulin-like growth factor-1, a known growth factor for bone (1). A recent prospective study by Alexy *et al.* (33) demonstrated improved bone health in youth with increasing protein intake. Conversely, bone status was negatively associated with the diet acid load, estimated by the PRAL, which includes estimation of the acidifying effect of dietary protein. The authors noted that this index of dietary acid load does not directly assess methionine and cysteine content of individual proteins, but assumes a fixed proportion of sulfur to protein. The young, growing population is distinct in several important ways from the older population of the present study, but the results illustrate a similar concept: protein is positively associated with bone mineral mass, but also contributes to an acid load with negative ramifications for bone.

Total protein and protein sulfur are highly and intuitively correlated, but the actual ratio depends on protein source. Methionine is an essential amino acid, and deficiency causes adverse health outcomes. However, our results suggest that the addition of lower sulfate protein to a diet that is already adequate in all essential amino acids may be beneficial in osteoporosis prevention; further research is necessary to test this hypothesis. Soy is implicated as a protein source with a low sulfur to protein ratio (13), estimated by Massey (34) at 39.8 mEq sulfur / 100 g protein, compared to a mid-range 54.8 mEq / 100 g protein for milk and 59.4 mEq / 100 g protein for beef, and a higher 73.0 mEq / 100 g protein for pork and 82.2 mEq / 100 g protein for oatmeal. Experiments have tested the impact of soy protein, with and without isoflavones, on calcium balance with mixed results (35-38). Alternatively increased protein from all sources in connection with enhanced intake of alkalizing nutrients may be beneficial. It has been shown that a more alkaline diet is associated with improved bone density (39-43), and that supplementation of potassium bicarbonate or potassium citrate attenuates bone turnover (44;45). Jajoo *et al.* (46) demonstrated that replacing some cereal, an acidifying diet component, with more base-producing fruits and vegetables resulted in reduced levels of parathyroid hormone, bone resorption and calcium excretion relative to controls.

It is conceptually important that the positive association in this sample between protein and LS aBMD, controlling for sulfur, was almost perfectly negated by the negative association of sulfate equivalents from methionine and cysteine and aBMD. This suppressor effect indicates that any study evaluating the association between protein intake and bone mineral status without controlling for actual sulfur content of protein may observe no significant correlation, despite real positive *and* negative protein effects. A neutral combined

influence of protein and sulfur on aBMD prediction would mask true effects in both studies hypothesizing detrimental acid load effects to bone, and studies hypothesizing beneficial effects of dietary protein. This suppressor effect might explain the discordance of reports concerning the impact of dietary protein or acid load on bone health. Likewise it is possible that uncontrolled influences of the total dietary acid load might explain conflicting reports. If true, this would suggest that future studies of associations between protein intake and bone density must account for the dietary acid load in order to produce unbiased results.

Our results suggest that direct estimation of sulfur from amino acids may perform better than the protein / potassium ratio or PRAL in studies of acid-base balance and bone mineral mass. These constructs were developed and validated for the estimation of the acidifying influence of dietary intake (3; 8-10). However because each estimates the acidifying effect of protein as a fixed ratio of total protein intake, they may not be well suited to the investigation of bone, where the negative acid effect seems to be opposed by an anabolic protein effect. Indeed our results suggest that the negative and positive effects of protein may neatly cancel one another out at the LS, leaving the estimated influence *on bone* of total protein, as used in the protein / potassium ratio and PRAL, neutral in spite of otherwise reliable estimations of dietary acid load. The PRAL is also a function of calcium and phosphorus intake, which have well established influences on the calcium economy (47). These factors may further confound the influence of the PRAL on bone. In light of these complex associations, it may be advisable to use direct intakes of protein, amino-acid sulfur (the acidifying agent of protein), and other relevant minerals such as potassium in lieu of estimated NEAP when calcium balance or bone mineral mass are investigated.

It is not clear why this suppressive association of sulfur intake would describe aBMD at the lumbar spine and not at the total hip, although differences in the response of these two sites are commonly reported (48-50). The change in R^2 at the LS indicated a small likelihood of a type I error, or inappropriate rejection of the null hypothesis that sulfur intake does not predict aBMD. Conversely, a post-hoc power analysis of the regression at the hip was performed, and indicated a power of 0.74 to detect an improvement in R^2 as large as that observed at the spine. Though it is not entirely improbable that our regression failed to detect a true difference at the hip, it is very possible that a real difference between protein associations at the hip and spine explain divergent findings. Differences in observations between these measurement sites may relate to differing levels of trabecular compared to cortical bone content; specifically, the ratio of trabecular to cortical bone is greater at the spine than at the hip. It has been theorized that trabecular bone, being more metabolically active, may be more sensitive to dietary intervention, at least in the short term (51;52). However a post-hoc analysis of the trochanter sub region, with a greater proportion of trabecular bone, did not mimic the findings at the LS in this sample (data not shown). Alternatively it is possible that the weight-bearing load at the hip during ambulation may blunt the effects of dietary factors.

This study is not without limitations; the constraints of cross-sectional data are recognized. This research is performed in the context of a broader body of literature suggesting causal connections between protein intake and bone mineral mass (31;32), sulfate intake and acid-base balance (53;54) and acid base balance and bone demineralization (4;5;6;44;55). The results are theoretically consistent and harmonize apparently conflicting

research; however, this observational study offers no basis for new causal inference and is best interpreted as a rationale for additional investigation.

In conclusion, protein intake is positively associated with aBMD in postmenopausal women, but this association is suppressed by a negative association of sulfur from amino acids at the lumbar spine. This observation may reconcile reports of positive impacts of dietary protein on bone health with reports of a negative impact of the acid load from sulfur-containing amino acids. At the total hip dietary protein intake is positively associated with aBMD, and may not exhibit the same negative association with sulfur from protein as observed at the spine. These results highlight the need to evaluate actual sulfur contents of varying dietary protein sources rather than assuming a fixed ratio of sulfur to protein. Future research in this line of inquiry should evaluate the role of dietary protein in preserving bone health in populations at higher risk for fracture such as the elderly.

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Table 3.1. Descriptive statistics, 161 postmenopausal women.

	Mean	SD	Median	IQR[1]
Age, y	68	6.0	66	63 – 72
Weight, kg	73.1	15.1	70.7	62.7 – 80.7
BMI, kg/m²	28.2	5.3	27.7	24.4 – 31.4
kJ/d	7063	2335	6749	5402 - 8548
Protein, g/d	74.7	25.0	76.9	54.7 - 88.9
calcium, mg/d	840	34.5	781	513 - 1079
Vitamin D, mg/d	3.36	2.73	2.85	1.08 - 4.90
PRAL[2], mEq/d	2.4	16.6	1.9	-9.9 - 13.3
Est. NEAP[3]	49.2	22.2	45.0	35.2 - 57.6
Protein Sulfur[4], mEq/d	28.4	15.6	27.4	17.3 – 38.6
LS aBMD[5], g/cm^2	0.99	0.15	0.98	0.88 - 1.08
TH aBMD, g/cm^2	0.88	0.13	0.87	0.78 - 0.97

[1] Inter-quartile range. [2] Potential Renal Acid Load (3,10), calculated according to formula 2. [3] Estimated Net Endogenous Acid Production (8), calculated using the ratio of protein to potassium intake, according to formula 3. [4] Sulfur content of reported dietary protein sources, calculated according to formula 1. [5] Areal Bone Mineral Density.

Table 3.2. Correlations of dietary intakes, diet acid load estimations and areal bone mineral density (aBMD) of the lumbar spine (LS) and total hip (TH) in postmenopausal women.

	Protein	Energy	PRAL[1]	NEAP[2]	Sulfur	K^+	LS BMD
Energy, kJ	0.59						
P	<0.01						
PRAL[1]	0.43	0.05					
P	<0.01	0.56					
Est. NEAP[2]	0.41	0.02	0.88				
P	<0.01	0.82	<0.01				
Sulfur[3]	0.67	0.33	0.33	0.27			
P	<0.01	<0.01	<0.01	0.03			
K^+	0.59	0.56	-0.33	-0.41	0.47		
P	<0.01	<0.01	<0.01	<0.01	<0.01		
LS aBMD[4]	-0.01	0.10	-0.03	-0.03	-0.17	-.04	
P	0.94	0.19	0.75	0.74	0.03	0.66	
TH aBMD	0.08	0.08	0.12	0.08	0.04	-.04	0.59
P	0.30	0.32	0.12	0.30	0.64	0.63	<0.01

[1] Potential Renal Acid Load (3,10), calculated according to formula 2. [2] Estimated Net Endogenous Acid Production (8), calculated using the ratio of protein to potassium intake according to formula 3. [3] Sulfur content of reported dietary protein sources, calculated according to formula 1. [4] Areal bone mineral density, g/cm^2. Values are Spearman's Rho and associated *P* values, n=161.

Table 3.3. Regression of lumbar spine areal bone mineral density (aBMD, g/cm²) on protein and sulfur from protein, controlled for body weight[1].

	B	SE	ß	*P*
Entered in separate regression models:				
Protein	$1.11e^{-4}$	$4.65e^{-4}$	0.02	0.81
Sulfur	$-1.35e^{-3}$	$7.39e^{-4}$	-0.14	0.07
Entered simultaneously, i.e., mutually adjusted:				
Protein	$1.35e^{-3}$	$6.30e^{-4}$	0.22	0.04
Sulfur	$-2.82e^{-3}$	$1.01e^{-3}$	-0.28	<0.01

[1] Estimated using ordinary multiple regression. Final model controlling for weight was determined using a step-down procedure removing age, physical activity, calcium and vitamin D intakes and an interaction of dietary protein with calcium. n=161 postmenopausal women.

Figure 3.1. Scatterplot with least squares regression line and mean 95% CI of lumbar spine areal bone mineral density (aBMD, g/cm^2) with protein, adjusted for associated sulfur intake (A) and with sulfur from protein, adjusted for total protein intake (B); n=161 postmenopausal women. Values are sample Z-scores, adjusted by ordinary multiple regression. Regressions were also controlled for body weight. For aBMD on protein, ß=0.21 (*P*=0.04). For aBMD on sulfur, ß=-0.28 (*P*<0.01).

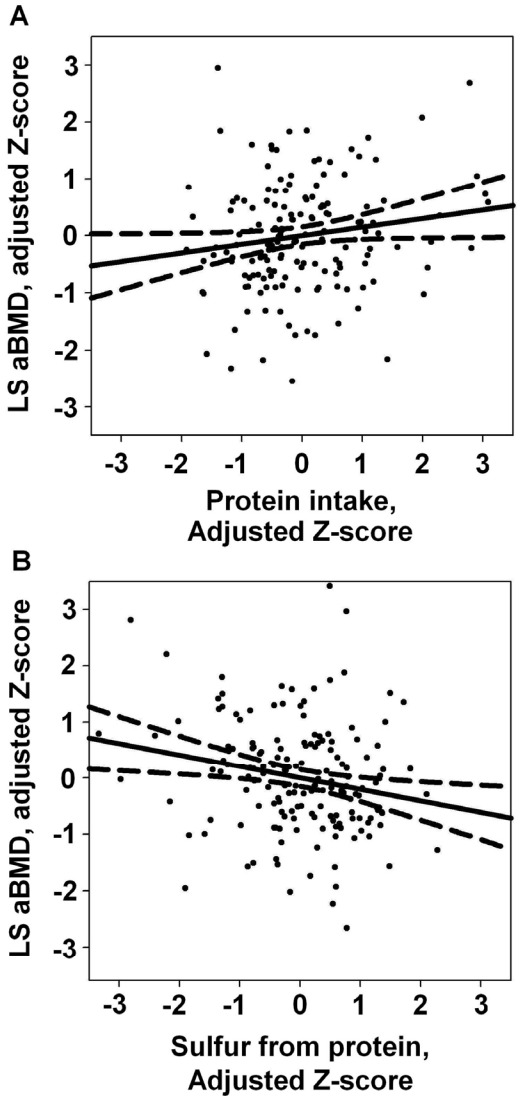
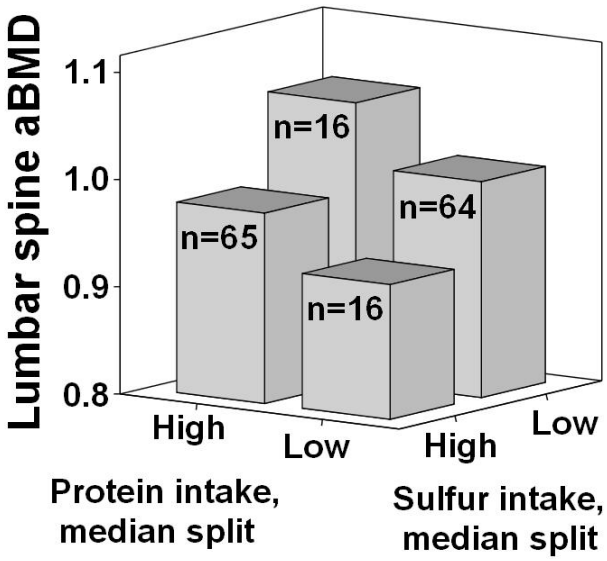


Figure 3.2. Descriptive representation of median lumbar spine areal bone mineral density (aBMD) in 161 postmenopausal women with intakes above (high) and below (low) median intakes of protein and sulfur from protein.



Chapter 4

Dietary protein holds IGF-1 mediated positive effects, as well as competing negative effects on bone health in rats

Abstract

The effect of high protein intakes on bone health is controversial, with both positive and negative effects reported. Human studies are clouded by correlations of protein, calcium and other factors.

Sprague-Dawley rats (n=48) aged 52d were randomized to 10 wk diets: high protein (HP): 35% energy as casein, 40% as carbohydrate; normal protein (NP): 15% casein and 60% carbohydrate. All other nutrients were matched. Measures included serum IGF-1 by RIA, mechanical strength of the femur midshaft by 3-point bend test, bone density by dual x-ray absorptiometry and trabecular microarchitecture by micro-computed tomography.

Energy intakes did not differ. No net differences in bone outcomes were observed between diet groups, except trabecular spacing (HP: 234±8, NP: 227±8 μm, p=0.023). However after covarying for IGF-1 in a mediational model, the high protein diet had a negative direct effect (standardized regression coefficient, -0.39 for ultimate stress, -0.45 for Young's Modulus, -0.32 for Modulus of Toughness) that was opposed and negated by positive indirect effects (0.34 for ultimate stress, 0.35 for Young's Modulus and 0.36 for Modulus of Toughness) mediated by IGF- 1 (all $p < 0.05$). Opposing effects were roughly equal in magnitude, producing no net effect on bone strength.

Results are consistent with the hypothesis that protein has both positive effects, mediated by IGF- 1, and negative effects on bone health, but that the average total effect of a higher protein diet is null.

Introduction

Influences of protein intake on bone health have been historically controversial. Although the negative effect of protein deficiency on bone health is practically indisputable (1-3), many studies and reviews have supported both bone building (e.g., 1; 4) and bone degrading effects (e.g., 5) of high compared to normal protein intakes. A recent meta-analysis (6) found a modest benefit, but in the context of highly variable results across studies.

This variability of results may reflect a dual-nature of dietary protein's influence on bone health. Above threshold adequate protein intakes determined by nitrogen balance (7), dietary protein imposes a metabolic acid load(8) and causes loss of calcium into urine (9). Alternatively, these higher protein intakes also increase intestinal calcium absorption (10) and increase circulating insulin-like growth factor (IGF) 1 (11; 12), a hormone regulating bone turnover and anabolism. Several authors including our lab have suggested that the net effect of dietary protein on bone health may depend on the relative balance of apparent bone-building and bone-degrading influences (13-15).

Specifically we observed that a positive association of protein intake with bone density of the lumbar spine was observed only after statistical adjustment for the negative association of the sulfur amino acid fraction of dietary protein in a sample of postmenopausal women (13). The positive and negative pathways were approximately equal in magnitude and opposite in direction, and so tended to negate one another when only the total association

of protein with bone density was observed. This type of association has been characterized statistically as a 'suppressor effect' (16), and appropriate statistical models have been developed for addressing this pattern of competing positive and negative pathways (17; 18). We concluded that investigations may miss real, simultaneous positive and negative effects of dietary protein unless statistical or experimental models address both the proposed positive and negative pathways (13). A similar pattern was observed in adolescents by Alexy *et al.* (14), where an otherwise positive effect of dietary protein was offset by a negative association of the dietary acid load.

These epidemiological studies are limited in part by the complex nutrient interactions of a free-living diet. The present study seeks to clarify this theoretical dual relationship of dietary protein with bone health using an animal model and highly controlled diets, matched in all nutrients except the ratio of protein to carbohydrate. In order to evaluate the proposed competing positive and negative pathways, we measure IGF-1 and attempt to quantify how much, if any of the influence of dietary protein is mediated by IGF-1 levels.

Methods

Experimental model

Forty-eight female Sprague-Dawley rats (45 d old, body weight 135 ± 4.8 g, Harlan-Tekland, Indianapolis, IN) were trained to consume a modified AIN-93 semipurified diet (19) as three meals per day, consisting of 3g of feed as "breakfast" (0700-0720 h) and unrestricted access to food at 1300-1400 h and 1800-1900 h. The baseline diet consisted of 15% protein (casein), 12% lipid (soybean oil) and 63% carbohydrate (40% cornstarch, 10% sucrose and 13% maltodextrin) by weight. Each kg of diet also provided 0.014 g t-Butylhydroquinone, 2.5 g choline, 50 g cellulose, 2.36 g cystine, 35 g of AIN93G mineral

mixture and 10 g AIN93G vitamin mixture. Water was provided ad libitum. Rats were individually housed in wire-bottom cages at 24°C in a 12 h light-dark cycle.

At 52 d of age, rats were randomly assigned to continue the baseline, control diet or to an isoenergetic experimental diet, matched in composition of all nutrients excepting the ratio of protein to carbohydrate. Specifically, the experimental diet replaced 21% of the diet's weight from carbohydrate with an equal weight of casein. Accordingly, randomly assigned diets provided 60% energy as carbohydrate, 15% as protein and 25% as fat (CARB diet), compared to 40% carbohydrate, 35% protein and 25% fat (PRO diet).

Percent body fat was measured at 0 and 10 wks using small animal body composition settings using a Hologic QDR 4500A dual x-ray absorptiometer (Bedford MA). After 10 wk, animals were sacrificed, and left femurs were excised and stored in saline soaked gauze at -20°C, a mode of storage demonstrated to not alter bone mechanical properties (20; 21). The University of Illinois Institutional Animal Care and Use Committee approved this protocol.

Bone mineralization

Femora were thawed over 4 hours to room temperature and scanned with small animal bone settings using a Hologic QDR 4500A dual x-ray absorptiometer (Software version 11.2, Bedford MA) to measure areal bone mineral density (BMD, g/cm^2) of the total femur.

Bone strength

Following absorptiometry, femora were loaded to failure in a 3 point bend test using an Instron MINI 44 machine (Grove City, PA). Force was applied at the mid shaft along the anteroposterior axis. Strain rate was 0.6 mm/min, with a loading span of 15 mm. Following

fracture, digital calipers were used to measure the thickness of the cortical shell at 4 points, the midpoints of the quadrants created by intersection of the anteroposterior and mediolateral axes in cross section (see 22). Width of the shaft along anteroposterior and mediolateral axes were also measured. Cross-sectional moment of inertia (*I*) was approximated using an elliptical model as in Turner *et al.* (22):

$$I = (\pi / 64)[ab^3 - (a - 2t)(b - 2t)^3] \quad \textit{eq. 1}$$

where *a* is the mediolateral width, *b* is the anteroposterior width and *t* is the average cortical thickness from the four quadrants.

Force-displacement data from the bend apparatus were processed using custom programming by MP Thorpe in Matlab version 7.6.0. Ultimate force (*Fu*) is represented by the maximum force observed during bending, just prior to failure (complete breaking) of the bone. Stiffness (*S*) is the amount of force generated per unit of displacement prior to yield (partial breaking or loss of structural integrity of the bone), or the slope of the force-displacement curve over the initial, non damaging region of the curve. Work to failure (*U*) is the energy absorbed by bone prior to complete fracture, or the area under the force-displacement curve. Work to failure is frequently accepted as the single best indicator of overall bone strength (20; 22; 23).

Intrinsic biomechanical properties, or adjustments to the parameters described above to account for the physical dimensions of the bone, were derived using the following calculations, as in Turner *et al.* (22):

$$\textit{Ultimate stress, } \sigma_u = F_u\left(\frac{Lb}{8I}\right) \quad \textit{eq. 2}$$

$$\textit{Young's Modulus, } E = S\left(\frac{L^3}{48I}\right) \quad \textit{eq. 3}$$

$$\textit{Modulus of Toughness, } u_T = U\left(\frac{3b^2}{4LI}\right) \quad \textit{eq. 4}$$

where again, I is the cross-sectional moment of inertia, b is the anteroposterior width and L is the loading span of the bend test (15 mm in this protocol).

After 3 point bend test to failure, the fractured, proximal portion of each femur was mounted vertically in a chuck and load was applied at 0.6 mm/min at the superior femoral head along an axis parallel to the shaft of the femur until failure of the femoral neck. The resulting force displacement curves were processed for ultimate force, stiffness and work to failure; however because the femoral neck is an irregular structure, no attempt was made to calculate intrinsic mechanical properties (22).

Bone structure

In order to quantify the trabecular architecture of the bone, the distal portion of each fractured femur was scanned in a Skyscan 1172 micro computed tomographer using software version 1.5. A 1 mm Al filter was used to reduce beam hardening artifact through cortical bone. X-rays were applied at 74 kV source voltage and 100 μA current. Reconstructed image sets of 15.89 μm^3 voxels were analyzed using Skyscan's CTAn software and built-in trabecular analysis algorithms. The region of interest included 15 slices distal to the distal-most point of the epiphyseal plate. Boundaries for the first and last slice this region were hand traced by the same investigator for all samples, and interpolated to the intervening slices. Intervening slices were then examined and adjusted as necessary such that analyzed regions included all and only trabecular bone. Voxels were segmented into bone or non-bone using a uniform grayscale value for all image sets. Analysis yielded mathematical estimates of average trabecular thickness (Tb.Th), number (Tb.N) and spacing (Tb.Sp) (24).

Insulin-like growth factor 1

IGF-1 was assayed using competitive binding of a radioactive ligand, [^{125}I]-IGF-1, to polyclonal anti-human IGF-1 antibody (National Hormone and Pituitary Program, NIDDK, Torrance, CA). Concentrations were determined using a gamma counter (Packard Instruments, Meriden, CT) against a standard curve prepared from known concentrations of recombinant human IGF-1 (25).

Statistical analysis

Ordinary least squares regression models were fit to quantify differences between PRO and CARB animals, using a dummy-code for diet assignment. Following initial tests, regression models were run again using IGF-1 concentration as a covariate in order to test its mediation of protein effects. The original estimate of the effect of diet on a bone outcome represents the total effect of the diet, whereas the change in this estimate after covarying for IGF-1 represents the portion of the effect of protein that is mediated, or explained by the effect of protein on IGF-1 (16; 26). This second estimate is also called the indirect effect, meaning the effect of protein that is indirectly channeled through the influence of IGF-1. Because estimates of the indirect effect do not have a single characteristic sampling distribution (17; 27), bootstrapping methods were employed to provide accurate confidence intervals (28) using the SPSS 'INDIRECT' mediation macro by Preacher and Hayes (29). For all statistics, $\alpha = 0.05$.

Results

No differences in total food intake between diet groups were observed. No baseline differences in body weight were observed, but PRO rats were 4.3% heavier than CARB rats

at 10 wk ($p<0.05$). Percent body fat did not differ according to diet. We observed no total or net effect of diet on any outcome (Table 1); however when applying mediation models, we observed significant suppressor effects on several outcomes, with a negative direct effect of protein negating a positive indirect effect of protein mediated by IGF-1 concentrations (Table 2).

Specifically, IGF-1 concentrations were 75 μg/L (95% CI: 35,115) higher in PRO animals. Within mediation models, 1 SD increase in IGF-1 predicted an increase of 0.59 SD increase in ultimate stress, 0.60 SD in Young's Modulus and 0.61 SD in Modulus of Toughness in 3 point bend testing; as well as a 0.58 SD increase in the CSMI (all $p <0.05$, see Table 2 for variance). Although not significant, values for femoral neck mechanical strength and trabecular thickness followed a comparable trend. No apparent influence of IGF-1 was observed for BMD, trabecular number or trabecular spacing.

Within the mediation model, these positive effects of protein mediated by IGF-1 were negated by inverse associations between protein intake and bone outcomes in the covariance that was not explained by IGF-1 (Table 2; Figures 2-3). The protein diet exerted a significant, positive effect on trabecular spacing (lower values are desirable to prevent fracture); this effect was not negated by IGF-1. No significant interactions of IGF-1 with diet were observed for any outcome (all $p>0.2$).

Discussion

We observed both positive and negative associations of the protein diet with bone outcomes when employing a mediational model. Mediational modeling is appropriate when multiple pathways are thought to exist between independent and dependent variables (16). With regards to bone health, multiple investigators have noted that higher protein appears to

hold a dual influence (15; 30; 31). In our prior, human study (13), we observed a positive association of protein with bone health after accounting for a negative, indirect effect of protein mediated by the sulfur amino acid content of protein. The mechanistic source of the positive effect was unexplained. In contrast, the present study demonstrated a marked negative influence of protein after accounting for a positive effect mediated by protein induced elevations in IGF-1. Although we are naturally reluctant to compare human epidemiology and experimental animal data too freely, it is striking that together these studies support the theory other authors have proposed, that the dietary acid load of protein and protein induced IGF-1 exert opposing effects on bone health (15; 30; 31).

The competing direct and indirect (IGF-1 mediated) pathways characterize a special case of statistical mediation known as a suppressor effect. As the name implies, a suppressor effect denotes direct and indirect pathways which cancel, or suppress one another. Suppressor effects are mathematically similar, but conceptually distinct from confounding effects: Although both may bias the estimate of the direct effect of the independent variable, a confounder is a third variable which would cause both protein intake and bone outcomes, resulting in a non-causal association between them. In contrast, the proposed suppressor effect is caused by protein intake and in turn causes a change in bone outcomes (16). Accordingly, while a confounder obscures the true relationship between independent and dependent variables, a suppressor is part of the true relationship and speaks to mechanism of the influence of protein.

Mediation and suppressor effects should also not be confused with statistical interactions. An interaction in this case would imply that the relationship of IGF-1 with bone outcomes differs between diets. As shown in Figures 2 and 3, no such interaction was

observed here and the influence of IGF-1 is parallel in PRO and CARB groups. In the observed mediational model, the influences of diet and IGF-1 are independent of one another and additive, such that CARB animals expressing high levels of IGF-1 have the highest bone strength, while PRO animals expressing low levels of IGF-1 have the poorest bone strength. However, because additional dietary protein itself increased IGF-1 levels, these extreme cases are relatively less common in our sample. Indeed, only 3 PRO animals were observed to have IGF-1 levels below the median for the sample, as a result of a well documented IGF-1 response to increased dietary protein (32; 33). For this reason, the total effect of protein on bone health is approximately zero, implying that protein induced increases in IGF-1 completely compensate for any negative effect of increased protein.

Our results are consistent with prior research and reviews suggesting that dietary protein imposes a mild, chronic metabolic acid load (34-38), that such a mild, chronic acid load can adversely affect calcium balance and bone metabolism (34; 39-43), but that actual bone outcomes in response to protein intake are stable or slightly improved (6; 35; 37; 44-46). These data also support the proposition that IGF-1 explains or mediates some part of observed benefits of protein on bone outcomes (12; 32; 44; 47; 48). The practical implications are that a higher protein intake may benefit bone health if measures are taken to counter or reduce the associated metabolic acid load. Again, this is consistent with existing theory and evidence (15; 49-51). Indeed, Zerwekh *et al.* observed that apparently deleterious effects of a protein-induced acid load on bone were compensated by coadministration of alkali therapy in the form of potassium citrate (51). Also, if the suppressor effect observed in this study is generalizable, such an effect may mask statistical detection of positive and

negative pathways in studies that do not statistically or experimentally isolate proposed mediators of the influence of protein on bone.

Because IGF-1 levels were not experimentally manipulated, but allowed to respond naturally to diet, we cannot strictly conclude that elevated IGF-1 caused improvements in bone health. It is possible that some confounding factor increases the propensity of IGF-1 to respond to protein as well as bone strength. We consider this possibility unlikely in light of the well established role of IGF-1 in bone growth and turnover. A second limitation is that this experiment was not designed to evaluate the influence of IGF binding proteins, which may be regulated by protein intake (33) and modify effects on bone health (52).

In conclusion, we observed a null total effect of a higher protein relative to a normal protein diet on bone health in rats. Mediation analysis indicated that a positive, indirect effect of dietary protein, mediated by increasing circulating levels of IGF-1, cancels out an otherwise negative influence of high protein through an untested mechanism. The results are consistent with a competing pathways model of protein and bone health, and may clarify why, as observed by Tucker (35), "The role of protein [in bone health] appears to be complex and is probably dependent on the presence of other nutrients available in a mixed diet."

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Table 4.1. Insulin-like growth factor 1 (IGF-1), bone mineral density (BMD), cross sectional moment of inertia (CSMI) and femur mechanical strength and structure in rats fed normal protein (NP) or higher protein (HP) diet.

Mean ± SD	NP	HP	p
IGF-1, µg/L	190 ± 39	266 ± 61	0.001
BMD, mg/cm²	210 ± 10	206 ± 9	0.362
CSMI,	4.04 ± 0.57	3.98 ± 0.52	0.775
3 point bend test of the femoral midshaft			
Ultimate Stress, MPa	2.58 ± 0.62	2.54 ± 0.58	0.829
Young's Modulus, GPa	8.06 ± 1.58	7.79 ± 1.58	0.653
Modulus of Toughness, MJ/m³	18.3 ± 6	18.7 ± 6.9	0.860
Femoral neck bend test			
Ultimate Load, N	91.9 ± 15.8	82 ± 9.7	0.060
Work to Failure, mJ	42.2 ± 16.3	35.4 ± 7.8	0.184
Micro computed tomography, trabecular morphometry			
Thickness, µm	119 ± 9	117 ± 7	0.439
Number, mm^{-1}	2.96 ± 0.21	2.87 ± 0.21	0.258
Spacing, µm	227 ± 8	234 ± 8	0.023

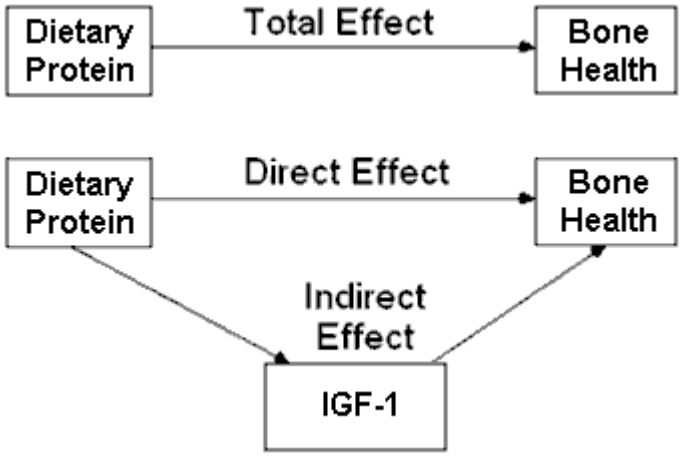
Table 4.2. Mediation model parameters for the direct and indirect (IGF-1 mediated) associations of diet (higher protein vs. normal protein) with bone health parameters. Values are regression coefficients (95% CI) [a].

Outcome	Direct diet effect [b]		Indirect (IGF-1 mediated) effect [c]	
BMD, mg/cm²	0.24	(-0.66, 0.33)	0.01	(-0.34, 0.34)
CSMI	0.24	(-0.90, 0.09)	0.34 [d]	(0.11, 0.70)
3 point bend test of the femoral midshaft				
Ultimate Stress, MPa	0.24	(-0.89, 0.11)	0.35 [d]	(0.11, 0.72)
Young's Modulus, GPa	0.24	(-0.95, 0.05)	0.35 [d]	(0.09, 0.71)
Toughness, MJ/m³	0.22	(-0.77, 0.12)	0.36 [d]	(0.10, 0.73)
Femoral neck bend test				
Ultimate Load, N	0.19	(-0.73, 0.03)	0.04	(-0.20, 0.27)
Work to Failure, mJ	0.17 [d]	(-0.70, 0.004)	0.14	(-0.04, 0.35)
Micro computed tomography, trabecular morphometry				
Number, mm-1	0.26	(-0.83, 0.24)	0.05	(-0.38, 0.38)
Thickness, μm	0.24	(-0.91, 0.08)	0.26	(-0.02, 0.60)
Spacing, μm	0.23 [d]	(0.07, 1.02)	-0.10	(-0.33, 0.23)

[a] Mediation analysis performed using INDIRECT macro for SPSS by Peacher & Hayes (29). The total or net effect of higher protein diet membership is the sum of direct and indirect effects, and in these data, were not significant except for trabecular spacing (see Table 1). [b] Coefficents reflect the predicted standardized change in outcome for animals fed high protein, vs. normal protein diet. [c] Standardized coefficients reflect predicted change in

outcome for each 1 SD increase in circulating IGF-1 (SD = 64 μg/L) . [d] Statistically significant ($p<0.05$).

Figure 4.1. A mediational model of the influence of protein on bone health [a].



[a] Mediation models are analyzed by testing the effect of x on y with and without covarying for the proposed mediator, m. The total effect is the influence of protein on bone outcomes without regard to IGF-1. After covarying for IGF-1, the direct effect is the effect of protein on bone outcomes, statistically adjusted for IGF-1. The indirect effect is the part of the influence of protein on bone that is mediated by IGF-1, and is represented by the product of the effect of protein on bone with the effect of IGF-1 on bone (26). A suppressor effect is a special case of mediation, where the direct and indirect effect are opposite in direction, and therefore negate or 'suppress' one another.

Figure 4.2. Femur shaft strength is poor in PRO fed animals with IGF-1 levels below the median, relative to PRO fed animals with IGF-1 levels above the median and relative to CARB fed animals.

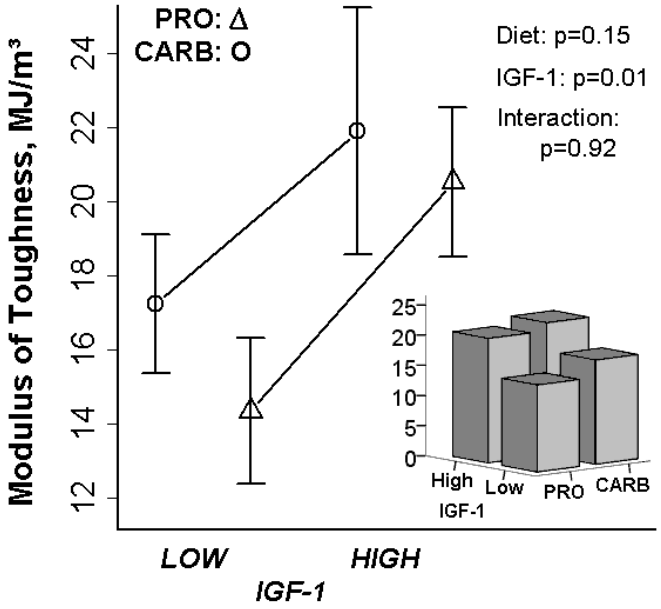
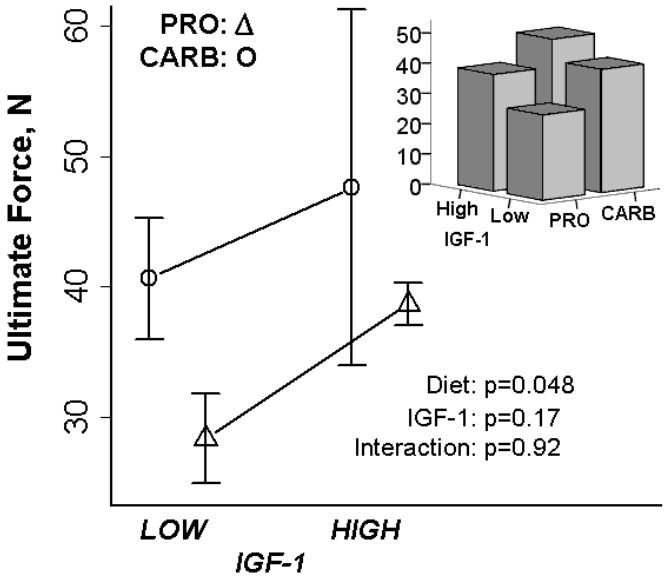


Figure 4.3. Femur neck strength is poor in PRO fed animals with IGF-1 levels below the median, relative to PRO fed animals with IGF-1 levels above the median and relative to CARB fed animals.



Chapter 5

Lean body mass mediates the influence of protein source on bone health

Abstract

Studies of protein and bone health report variable results. Differential effects of protein sources may explain incongruities. In particular, sulfur amino acids may impose a bone-demineralizing acid load while aromatic amino acids may enhance calcium balance. Protein may also improve body composition, which in turn positively influences bone health.

We randomized 52, 52 d old rats to semi-purified, isonitrogenous diets (16% energy as protein, 54% carb) with protein isolate from soy, egg white + lysine, wheat or whey. Total energy was restricted to 80% of ad libitum intake. Animals were sacrificed at 11 wk, and femurs excised for 3 point bend tests of mechanical strength, dual x-ray absorptiometry (DXA) and micro-computed tomography (μCT).

In spite of 20% energy restriction, weight gain was approximately normal for all animals. Greater force was required to break the femur midshaft with whey (119±10 N) compared to soy (110±9 N, $p=0.02$). Bone mineral mass was also greater given whey (480±25 mg) vs. egg (466±14 mg) and soy (456±27 mg). Differences in bone strength and mass dissolved after statistically adjusting for differences in lean body mass between groups, suggesting lean body mass was the principle mediator of protein effects on bone parameters.

Lean body mass completely mediated the influence of protein source on bone health in rats during modest energy restriction, independently of total protein and energy intake.

Introduction

Dietary protein may influence bone health through various pathways (1). The acid load of protein varies in proportion to the amount of sulfur containing amino acids (SAA), which differ twofold across various common dietary protein sources (2). This SAA induced acid load elevates urinary calcium (3; 4), which may reflect bone demineralization in response to mild acidosis (5; 6). Alternatively, it has been shown that dietary protein increases calcium absorption (7; 8), and this effect may also vary in proportion to specific source-dependent amino acids (9; 10). Finally, we have previously shown that protein sources rich in leucine positively regulate lean body mass (11), which is consistently associated with bone health, perhaps causally through increased loading of bone tissue (12).

The net effect of dietary protein on bone may be positive (1; 13) or negative (14). This discrepancy may be attributable to the protein source (1), including the specific fractions of SAA, leucine or the cluster of amino acids thought to regulate the calcium sensing receptor, CaR (15). In this context, the present study evaluates the influence of protein isolates from wheat, soy, egg and whey on the mineral mass, mechanical strength and trabecular microarchitecture of bone in rats. In light of prior studies (4), we anticipate that bone parameters will correspond inversely with the SAA content of the protein source.

Methods

Fifty-two male Sprague-Dawley rats (52d old, 275±10g, Harlan-Tekland) were randomly assigned to a semi-purified, isonitrogenous diet providing protein isolate from wheat, soy, egg or whey. Diets provided 16% energy as protein and 54% as carbohydrate (Tables 1 and 2). Diets were based on the AIN-93 rodent diet (16). Animals were fed 80% of

their ad libitum food intake (80% of 18g/d) as 4 g between 0700 and 0720, 4 g between 1300 and 1400 and 6 g between 1800 and 1900. This schedule was developed to mimic human meal patterns (17). Mild energy restriction (80% of average ad libitum intake) was employed to ensure similar total food intakes across animals. Water was given ad libitum and animals were housed individually at 24°C in wire-bottom cages over a 12 h light-dark cycle.

Following 11 weeks on the experimental diets lean body mass was measured via Hologic QDR 4500A dual x-ray absorptiometer (Bedford, MA) using small animal body composition scans. Animals were then sacrificed and excised femurs were stored in saline soaked gauze at -20°C. Storage in this manner has been shown not to affect material properties of rodent femora (18). Oversight and approval for this protocol was provided by the University of Illinois Institutional Animal Care and Use Committee.

Bone densitometry and mechanical strength testing

Right femora were thawed to room temperature and scanned using small animal bone settings on a Hologic QDR 4500A bone densitometer (version 11.2). Femurs were then tested to failure in 3 point bending at the mid shaft in an anteroposterior axis using an Instron MINI 44 (Grove City, PA) bend apparatus. Loading span was 20 mm and load rate was 0.6 mm/min. Average cortical thickness at the plane of fracture was estimated using by digital calipers at 4 sites around the cortical rim, and the cross-sectional moment of inertia (I) was approximated using the formula described by Turner (19) for approximating *I* of an ellipse:

$$I = (\pi / 64)[ab^3 - (a - 2t)(b - 2t)^3] \quad \textit{eq. 1}$$

a is the mediolateral width and b is the anteroposterior width of the bone at the cross section of fracture, and t is the average cortical thickness.

Ultimate force (*Fu*) or maximum force observed during loading, just prior to complete fracture; stiffness (*S*) or the slope of the force displacement curve prior to bone yielding; and the work to failure (*U*) or area under the force displacement curve were derived from the force displacement curve using Matlab version 7.6.0. Physical dimensions of the bone were then used to calculate intrinsic parameters as follows (19):

$$\text{Ultimate stress, } \sigma_u = F_u\left(\frac{Lb}{8I}\right) \quad \textit{eq. 2}$$

$$\text{Intrinsic stiffness (Young's Modulus), } E = S\left(\frac{L^3}{48I}\right) \quad \textit{eq. 3}$$

$$\text{Modulus of Toughness, } u_T = U\left(\frac{3b^2}{4LI}\right) \quad \textit{eq. 4}$$

The intact proximal half of the femur following 3 point bend testing was mounted vertically using a chuck and the head of the femur was loaded to failure of the femoral neck, again at a rate of 0.6 mm/in, along a parallel axis to the femur shaft. F_u, S and U were derived from the resulting force displacement curves, however no intrinsic mechanical properties were calculated due to the irregular shape of the femoral neck.

Micro computed tomography

The distal half of the broken femur was scanned with a Skyscan 1172 (Skyscan, Aartselaar, Belgium) micro computed tomographer (μCT) with acquisition software version

1.5. A 1 mm aluminum filter was employed to reduce beam hardening artifact of the final images. Five frame averaging was used to reduce noise. Scans were performed using 74 kV source volage and 100 mA current. Reconstruction of projections was performed with NRecon software (Skyscan), for a resulting voxel size of 16 μm3. The volume of interest was defined in CTAn software (Skyscan) by hand tracing the first and last slices of the range within 0.79 mm proximal to the proximal-most point of the growth plate. The volume of interest between the first and last slice were initially interpolated from these tracings, and then corrected as necessary such that analyzed bone within each slice consisted of only the trabecular bone space inside the cortical rim. Within the volume of interest images were binarized, leaving every voxel defined as bone or non-bone using an intensity threshold of 55 on a 256 intensity scale. CTAn software calculated volume of bone, % bone tissue within the region of interest and the average number, spacing and thickness of trabeculae within this volume.

Statistics

In order to allow comparisons of protein source on bone outcomes while covarying for combinations of other body composition variables, we analyzed outcomes using regression analysis and dummy coded protein source assignments for protein source (20). Dummy coded regression coefficients reflect the difference between their respective protein sources and the grand sample mean. Where covariates are included, dummy coded coefficients represent the model predicted effect of the protein source, holding the covariate constant. This approach allowed for flexible modeling of the data, such that main effects of protein source, correlations with body composition and the potential role of body

composition as a mediator of protein's effect on bone health could be tested through the same underlying model (21).

Results

In spite of modest weight restriction, rats followed an approximately normal growth curve over 11 week; however differences in weight emerged by endpoint testing (Table 3). Results for bone outcomes are also presented in Table 3. We observed improvements in bone strength and bone mineral content in whey animals, and slight decreases in soy fed animals. Other protein groups did not differ. Wheat protein additionally improved some measures of trabecular structure, but these changes did not confer an improvement in bone strength.

Protein source increased weight gain relative to soy, and also similarly increased lean body mass in the whey group (Table 3). Statistical models investigating the role of body composition on bone outcomes indicated that weight, lean body mass and percent body fat were predictive of bone outcomes (Table 4). Note that weight, body fat and lean body mass are never reported in the same model, due to the high degree of mutual information in these variables ($R=0.69$ for weight and lean body mass, 0.52 for weight and fat mass, all $p<0.05$). Overall, lean body mass was observed to have the strongest positive effect on bone health, with weight (which was predominantly lean mass) also exhibiting positive effects. Body fat was negatively related, though the association was weak.

A mediation analysis was performed to assess the degree to which body composition explained the observed changes to strength and mineralization. Upon adjusting the effects of protein source on bone outcomes for the concurrent effects on lean body mass, the significant effects of whey and soy were abolished. This result indicates that in this experiment, the

effect of whey relative to soy protein on bone health was completely mediated by body composition (21).

Discussion

Whey protein increased bone strength and mass, and soy protein reduced these parameters; however these differences appear to be completely attributable to changes in body composition, especially lean body mass. No changes were observed in intrinsic bone properties, which is consistent with the finding that body size accounts for increases in bone strength. Similarly, bones were increased in mass and area, but not density. Wheat protein conferred some benefit to trabecular architecture, although these were not associated with improved bone strength in this group. Our results do not support the theory that the acid load imposed by sulfur containing amino acids negatively affects bone health. They are also inconsistent with the theory that aromatic amino acids improve calcium balance or bone metabolism. It is possible, but not knowable, that effects of these amino acids were masked by more prominent regulation by body composition. The most important implication of these data is that changes in body composition mediate the influence of protein source on bone.

Our results are analogous to those of Pye et al (22), who studied rats consuming 15 or 35% of energy from protein, with egg albumen and skim milk powder constituting the difference, over a time span up to 17 mo. They report a benefit to weight adjusted regional bone mineral content but no effect of protein on bone turnover, mechanical strength or trabecular architecture. In addition they observed a dramatic reduction in body weight and particularly fat mass with an increase in lean mass in response to higher protein intake. In light of the correspondence of bone strength with body mass, it is noticeable that the higher

protein intake preserved bone strength in the face of decreased weight in that study. Although levels or protein intake were not compared in the present study, we likewise observe that effects of protein source on bone health in rats can be explained in no small part by changes in body composition.

The element of energy restriction in this study is important to bear in mind. Intakes at 80% of ad libitum amounts were sufficient to promote growth while allowing assurance of equal total calorie and protein intakes across groups, but it is possible that energy restriction moderates the influence of protein source. Mardon *et al.* studied the influence of protein and energy restriction in rats. They report expected adverse effects of 40% energy restriction on bone that is not compensated by normalization of protein intake or by substitution of whey protein for casein (23). In another study, however, they report that supplementation of protein intake was able to recover part of the calcium accrual otherwise lost with energy restriction in growing rats (13). In both of these studies, levels of insulin-like growth factor 1 (IGF-1) were reduced with protein and/or energy restriction, which may mediate some part of the detrimental changes to bone (24; 25).

Dawson-Hughes *et al.* (9) reported that aromatic amino acids (AAA) increased IGF-1 relative to branched chain amino acids administered as supplemental isolates in humans, suggesting a possible role for protein sources differing in the AAA load in regulation of bone growth. Conigrave (15) reviews preliminary evidence that AAA may influence multiple aspects of bone health as potentiators of the calcium sensing receptor, CaR. Our data do not support this, as AAA was unable to explain the observed changes; however the five-fold increase in AAA intake in that investigation may also have been larger than may be reasonably achieved through typical selection of protein sources.

Whiting and Draper (26) showed that rats fed sulfur amino acids (SAA) exhibited bone damage over time, while no such effect was observed in rats consuming protein with equivalent amounts of SAA. They also showed SAA to be primarily responsible for the well documented increase in urine calcium in response to protein intake (27). These observations supported the concept that the dietary acid load associated with sulfur amino acids (28) is damaging to bone (29-31). It is indeed known that SAA are responsible for perturbations in acid-base balance (32; 33) and that a dietary acid load is damaging to bone (34-36). We have previously proposed that protein intakes may be beneficial to bone in the context of a diet that is adequate in calcium (37) or relatively low in SAA (1). However, as with AAA, the present data offer no support for the role of SAA in mediating protein influences.

It has alternatively been proposed that dietary protein above adequate thresholds increases lean body mass (38; 39), and that lean body mass in turn improves bone health (40). Frost (12) has suggested that muscle mass, moreso than body weight per se, is responsible for the mechanical loading of bone and its remodeling to meet that demand.

Limitations of this study include a relatively short duration in terms of bone adaptation, and the absence of plasma indices of bone health and the high variability observed in some measures of bone fragility and trabecular structure, which may have made some true treatment effects more difficult to detect. Energy restriction was both limiting in that we cannot necessarily generalize our result to ad libitum intakes, and a strength in that it ensured that observed differences in bone and body composition were consequences of protein source, and not total protein or energy intake.

In summary, we do not observe effects of protein source on bone in proportion to the fraction of SAA or AAA; however these effects may have been masked by a more important

influence of lean body mass. Improvements in lean body mass with whey compared to soy protein during modest restriction of energy completely explains an increase in bone mass and strength. Again, we suggest that body composition mediates the relationships between dietary protein and bone health. Accordingly, body composition should be accounted for in studies of protein and bone.

Chapter 5 References

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Table 5.1. Composition of animal diets, g nutrient/kg feed [1]

Component	Wheat Diet	Soy Diet	Egg Diet	Whey Diet
Vital Wheat Gluten	190.2	0.0	0.0	0.0
Soy Protein Isolate	0.0	185.3	0.0	0.0
Egg White Solids	0.0	0.0	195.6	0.0
Whey Protein Isolate	0.0	0.0	0.0	188.8
L-Lysine	10.1	0.0	0.0	0.0
Cornstarch	316.7	331.7	321.4	328.2
Maltodextrin	134.1	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5	101.5
Soybean Oil	140.9	140.9	140.9	140.9
Cellulose (Fiber)	53.7	53.7	53.7	53.7
AIN-93 Mineral Mix	37.6	37.6	37.6	37.6
AIN-93 Vitamin Mix	10.7	10.7	10.7	10.7
Choline Bitautrate	2.7	2.7	2.7	2.7
Biotin (mg/kg)	0.0	0.0	16.0	0.0

[1] Table prepared by Layne Norton. Not yet published, used with permission.

Table 5.2. Amino acid content of diets, g/kg diet, and NRC requirements[1,2,3]

Amino Acid	Wheat Diet	Soy Diet	Egg Diet	Whey Diet	NRC Requirement[1]
Phenyalanine/Tyrosine	11.5	17.0	16.8	10.7	1.9
Histidine	3.1	4.2	3.9	3.4	0.8
Isoleucine	5.1	8.1	9.0	10.5	3.1
Leucine	11.5	13.6	14.9	18.5	1.8
Lysine	15.4	10.7	11.0	15.4	1.1
Methionine/Cysteine	6.5	4.4	13.9	7.6	2.3
Threonine	4.4	6.5	7.6	10.9	1.8
Tryptophan	2.2	2.0	2.7	2.7	0.5
Valine	7.6	8.0	11.5	10.2	2.3

[1] Table prepared by Layne Norton. Not yet published, used with permission.

[2] Table 2-2 from Nutrient Requirements of Laboratory Animals Fourth Revised Edition (41).

[3] Requirements estimated for a 300 g rat in weight maintenance.

Table 5.3. Body composition, bone mineralization, bone biomechanical properties and trabecular architecture, mean ± SD, according to protein source.

	Soy	Whey	Wheat	Egg
Body composition, scales and dual x-ray absorptiometry				
Weight, g	368±7 [a]	382±11	392±9 [a]	378±10
Fat Mass, g	37.4±9.2	36.9±10.6	48.5±7.8 [a]	36.8±5.1 [b]
Lean mass, g	330±8 [a]	345±11	344±11	341±9
Body Fat, %	10.2±2.4	9.6±2.7	12.3±2 [a]	9.7±1.3 [b]
Bone mineralization, dual x-ray absorptiometry				
Mineral Content, g	0.46±0.03 [a]	0.49±0.03 [a]	0.48±0.03	0.47±0.01
Density, g/cm²	0.26±0.01 [b]	0.26±0.01	0.26±0.01	0.26±0.01
Area, cm²	1.78±0.07 [a]	1.85±0.07 [a]	1.82±0.06	1.8±0.04
Trabecular architecture, micro computed tomography				
Bone volume, %	9.6±2.6	9.1±1.5	10.9±3.1 [a]	8.2±2.1 [b]
Thickness, µm	91±4	91±3	93±6	89±5 [b]
Spacing	462±48	473±37	446±52 [b]	482±30
Number	1.04±0.25e-3	1.00±0.15e-3	1.17±0.28e-3[a]	0.92±0.21e-3[b]

Table 5.3 (cont.)

	Soy	Whey	Wheat	Egg
Bone mechanical strength, femoral neck and 3 point bend test				
Neck Fu, N	82±7	82±11	82±9	80±8
Neck U, mJ	33±9	36±13	34±10	34±8
Neck S, N/mm	192±30	166±36 [b]	193±32	173±30
Fu, N	110±9 [a]	119±11 [a]	114±10	115±5
U, mJ	56±9	60±16	60±8	60±8
S, N/mm	226±15	238±14 [a]	226±24	228±17
Ultimate Stress, MPa	154±11	157±10	158±9	159±11
E, mJ	6.67±0.44	6.63±0.56	6.65±0.54	6.65±0.75
Ut, MJ/m^3	48.5±12.6	56.2±21.9	50.2±11.6	51.4±7.4

[a] Mean differs from grand sample mean ($p<0.05$) based on dummy coded regression analysis, prior to adjustment for covariates. [b] Mean exhibits a non-significant trend ($p<0.1$) for difference from the grand sample mean.

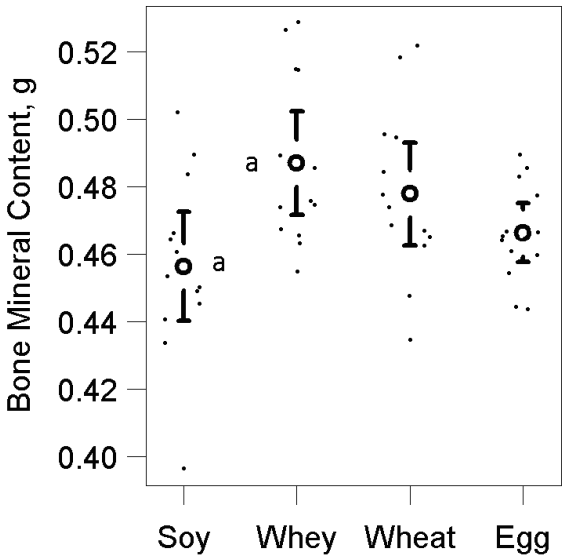
Table 5.4. Unstandardized (B) and standardized (ß) regression coefficients for the prediction of ultimate force (N change per g change in weight, lean body mass or fat mass) and bone mineral content (mg change per g change in weight).

	Ultimate Force			Bone Mineral Content		
Independent Variable	**B±SE**	**ß**	**p**	**B±SE**	**ß**	**p**
Weight	0.21±0.1	0.29	0.04	0.85±0.27	0.42	0.00
LBM	0.31±0.11	0.37	0.01	1.48±0.26	0.65	0.00
Fatmass	-0.05±0.14	-0.05	0.74	-0.55±0.39	-0.20	0.16
LBM, covaried for weight	0.27±0.15	0.33	0.09	1.57±0.36	0.68	0.00
Fat mass, covaried for weight	-0.27±0.15	-0.27	0.09	-1.57±0.36	-0.58	0.00

Table 5.5. Effect of protein source on ultimate force (N) and bone mineral content (mg) before and after adjustment for body composition. Standardized (B) and unstandardized (ß) regression coefficient.

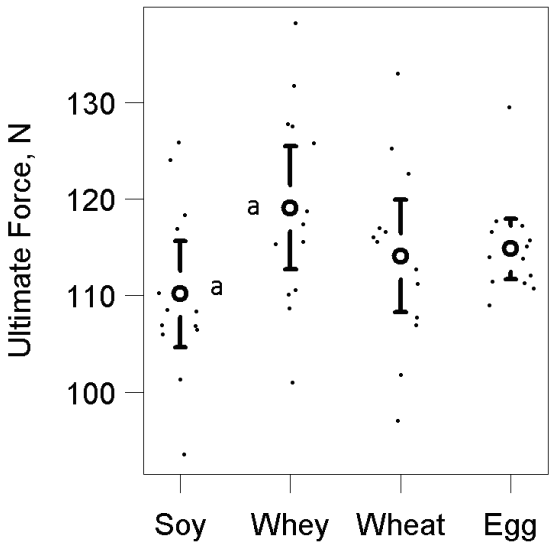
	Soy			Whey		
	B±SE	**ß**	**p**	**B±SE**	**ß**	**p**
Bone Mineral Content, change in mg, adjusted for body composition						
Effect of Protein Source	-20.6±7.7	-0.35	0.010	20.1±7.8	0.34	0.013
Weight adjusted	-11.3±9.3	-0.19	0.229	19.9±7.5	0.33	0.011
Lean body mass adjusted	-3.3±7.6	-0.06	0.668	13.3±6.7	0.22	0.052
Ultimate Force, change in N, adjusted for body composition						
Protein Source	-5.8±2.8	-0.28	0.046	6±2.838	0.29	0.038
Weight adjusted	-4.1±3.5	-0.20	0.242	5.7±2.893	0.27	0.056
Lean body mass adjusted	-3.1±3.3	-0.15	0.340	4.5±2.926	0.21	0.132

Figure 5.1. Bone mineral content in response to protein source.



Means and 95% confidence intervals are depicted, with small dots representing individual data points. [a] Group differs from grand sample mean, $p<0.05$, based on dummy coded multiple regression. These effects are abolished after statistically adjusting for the positive effect of weight.

Figure 5.2. Ultimate force, or force at breaking, in response to protein source.



Means and 95% confidence intervals are depicted, with small dots representing individual data points. [a] Group differs from grand sample mean, $p<0.05$, based on dummy coded multiple regression. These effects are abolished after statistically adjusting for the positive effect of lean body mass.

Chapter 6

A diet high in protein, dairy and calcium attenuates bone loss over 12 months of weight loss and maintenance

Abstract

Weight loss causes bone mineral loss. Higher protein diets remain criticized for further potential harmful bone effects, including elevated urinary calcium, but may promote bone health if protein sources include dairy. Overweight middle-aged subjects (n=130, 59 males) were randomized to a diet providing 1.4 g/(kg·d) protein and 3 daily servings of dairy (PRO) or 0.8 g/(kg·d) protein and 2 daily servings of dairy (CARB) for 4 mo weight loss plus 8 mo weight maintenance. Diets prescribed 6,276 kJ/d for females and 7,113 kJ/d for males. Bone mineral content (BMC) and density (BMD) for whole body (WB), lumbar spine (LS) and total hip (TH) were measured using dual X-ray absorptiometry (DXA), and dietary intake using 3-d weighed food records. Urinary calcium was measured using 24-hour collection at 0 and 8 mo for a subsample (n=42). Participants lost body weight (mean, 95% CI) of 8.2% (7.5-8.9) at 4 mo, 10.6% (9.5-11.8) at 8 mo and 10.5% (8.9-12.0) at 12 mo without differences between groups at any time (P=0.64). At 12 mo, PRO BMD was higher by 1.6% (0.3-3.0) at WB, 2.1% (0.6-3.7) at LS and 1.4% (0.2-2.5) at TH compared to CARB. PRO calcium intake was higher (PRO: 1140±58 mg/d, CARB: 766±46, P<0.01), as was urinary calcium (PRO:163±15 mg/d, CARB:100±9.2, P<0.01). A reduced energy diet supplying 1.4 g/(kg·d) protein and 3 dairy servings increased urinary calcium excretion but provided improved calcium intake and attenuated bone loss over 4 mo of weight loss and 8 additional mo of weight maintenance.

Introduction

Weight loss has well-established, favorable effects on metabolic disease risk such as type 2 diabetes mellitus and cardiovascular disease in overweight populations (1); however, weight loss also promotes loss of bone mass and increases fracture risk (2-4). As an aging population confronts concurrent threats of obesity and osteoporosis, diets that promote weight loss while maintaining bone mineral are of special interest.

Higher protein weight loss diets have received attention due to purported improvements in adherence and body composition, including enhanced loss of fat mass and preservation of lean mass (5-7). Recent prospective and clinical trials suggest that higher protein diets, if accompanied by adequate calcium, enhance bone health (8-11). This remains controversial in light of longstanding theory and evidence that increasing protein intakes promote calciuria (12). Increased urinary calcium with greater protein intake is traditionally considered to reflect bone demineralization; however, Kerstetter *et al.* (13) have shown that additional dietary protein promotes intestinal calcium absorption and reduces the fraction of urinary calcium of bone origin. Dawson-Hughes (14) has proposed that the net effect of dietary protein on bone mineral status depends on dietary availability of calcium.

In light of these observations, we propose that a diet utilizing dairy foods as a source of both protein and calcium will preserve bone mineral density (BMD) and content (BMC) relative to a conventional higher carbohydrate weight loss diet. We have adopted a free living, freely selected diet approach in which patients are educated on selection of food groups rather than directly controlling intakes of individual nutrients. This design is oriented to prove effectiveness, rather than efficacy. That is, our design will not resolve the independent or interactive effects of dietary protein and calcium on bone health during

weight loss, however the design is more generalizable to clinical practice than highly controlled feeding studies, and more directly answers the question most relevant to the clinical practitioner: "What is the effect on bone health of prescribing a higher protein weight loss diet emphasizing dairy?"

The primary aim of this study was to compare bone mineral content (BMC) and density (BMD) during 4 mo of active weight loss and 8 additional mo of weight maintenance in free-living participants on a diet providing 1.4 g/(kg·d) protein and 3 dairy servings (PRO) compared to an isocaloric, conventional weight loss diet providing 0.8 g/(kg·d) protein (CARB). Based on previous work we anticipated increased protein and calcium intakes, elevated urinary calcium excretion and attenuated bone loss in PRO compared to CARB participants.

Subjects and Methods

One-hundred-thirty subjects (59 males) aged 30 and 65 y (mean ± SD: 45.6 ± 8.9 y) were recruited to a 12 month randomized, parallel-arm, multi-center (Illinois and Pennsylvania sites) weight loss trial. Exclusion criteria were as follows: BMI below 26 kg/m^2, body weight exceeding 140 kg (due to constraints of the DXA scanning bed), smoking and conditions or medications affecting study outcomes, including cancer, heart disease, diabetes mellitus, renal disease or insufficiency, major weight change in the prior 6 mo or use of bone active medications or supplements, oral steroids or anti-depressants. Participants were blocked into groups of similar age and BMI, then blocks were randomly divided into PRO or CARB diets. Institutional Review Boards of the University of Illinois at Urbana-Champaign and The Pennsylvania State University approved all methods; subjects provided written informed consent.

Treatments

The PRO diet prescribed 1.4 g/(kg·d) protein, 3 daily servings of dairy and roughly 30, 40 and 30% of energy respectively from protein, carbohydrate, and fat (ratio of carbohydrate to protein < 1.5). The CARB diet prescribed 0.8 g/(kg·d) protein, 2 daily servings of dairy and roughly 15, 55 and 30% of daily energy respectively from protein, carbohydrate, and fat (ratio of carbohydrates to protein > 3.5). Both diets prescribed 6,276 kJ/d for females and 7,113 kJ/d for males, and equal total fat (~ 57 g/d) and fiber (~ 17 g/d). Subjects were asked not to take any dietary supplements during the study.

Research dietitians instructed participants on portion sizes and emphasized the Food Guide Pyramid (15) for the CARB group, and replacement of starchy staples (breads, rice, pasta, cereals) with meats, eggs and dairy for the PRO group. Participants were provided 2-week cycling menu plans. Each diet prescribed micronutrient intakes meeting Recommended Dietary Allowances (RDAs) (16), fat intake meeting guidelines by the American Heart Association (17), and 5 servings of vegetables and 2-3 servings of fruit daily. Participants reported 1 hr weekly for support, questions and answers, review of diet records and compliance, submission of 3-day weighed food records, and body weight measurement. Mean intakes at 0, 4, 8 and 12 mo were calculated with Nutritionist Pro software (First DataBank Inc., San Bruno, CA), and averages were used for analysis of dietary compliance and nutrient intakes. Participants were encouraged to spend at least 30 min walking 5 d weekly according to NIH Guidelines for Weight Management (1). Activity was monitored using daily logs and 3 d/mo using armband accelerometers (BodyMedia, Cincinnati, OH).

Bone and body composition

Whole body (WB), lumbar spine (LS) and total hip (TH) DXA scans were performed at 0, 4, 8 and 12 mo (Illinois: Hologic QDR 4500A, software version 11.1:3; Penn State: Hologic QDR 4500W, software version 12.5). Scans for a given individual were analyzed by the same technician at each site using standard manufacturer guidelines. The same array scan mode was used for all central measure scans. DXA machines were calibrated daily using manufacturer phantoms. Analyzed LS data included L1–L4. Volunteers wore light, metal-free, cotton clothing. CVs for DXA outcomes of interest are 1.0 to 2.0%.

Urinary calcium measures

Twenty-four hour urine samples were collected at baseline and at 8 mo from Illinois participants. Forty two Illinois participants remained in the study at 8 months, providing complete urinary calcium data. Ten ml aliquots of mixed collections were diluted and tested by atomic flame absorption spectroscopy, using a Perkin-Elmer 306 atomic absorption spectrophotometer to determine calcium concentration.

Statistical analysis

SPSS version 14 (SPSS, Inc., Chicago, IL) provided all statistical analyses. Normality assumptions were tested by the Shapiro-Wilk statistic. Reported values are mean ± SEM for normally distributed variables, and median (inter-quartile range, IQR) for non-normally distributed variables. Baseline characteristics, weight change and intakes are compared using ANOVA. Linear mixed models with random slopes and time of measurement as a repeated effect were applied to BMC and BMD at the three measurement sites (WB, LS, TH) in intent-to-treat fashion, adjusted for baseline values and gender, site of study participation and two- and three- way interactions of gender and study site with diet and time. We also tested

tertiles of age among female participants to indirectly control for menopausal status. Dietary treatment effects on urinary calcium measures at 8 mo were analyzed using ANCOVA, controlling for baseline urinary calcium and gender. ANCOVA was also employed to assess whether elevated urinary calcium excretion was related to BMC within dietary treatment groups. Additional details of the statistical analysis may be found in the supplemental material.

Results

At baseline PRO females were lower in WB BMD compared to CARB females (Table 1) and CARB males reported smaller energy and macronutrient intakes compared to PRO males (Table 2, Supplemental Table 1). Subjects were otherwise similar across groups. One extreme, lone outlier (sample Z score < -3.5 or > 3.5 without skew) was identified in both BMC and BMD at the WB and TH, and two extreme outliers at the LS. Examination revealed no clear reason for their departure from predictions; they were excluded from analysis. In the PRO group, 12, 6 and 5 participants withdrew from the intervention at 4, 8 and 12 mo; in the CARB group, 14, 14 and 7 participants withdrew.

Participants lost body weight, mean (95% CI) of 8.2% (7.5-8.9) at 4 mo and 10.5% (8.9-12.0) at 12 mo (no differences between diets, P=0.64; Supplemental Table 2). By 4 mo, energy intake was centered approximately at prescribed levels, and protein and calcium intakes diverged according to diet as prescribed (diet x time interaction $P<0.05$; Table 2). Energy intake did not differ by diet through the intervention (Table 2). At 4 mo protein intake was 1.37±0.04 g/(kg·d) or 29±0.6 percent of energy in the PRO group and 0.82±0.03 g/(kg·d) or 18±0.3 percent of energy in the CARB group, indicating compliance to the prescribed dietary treatments. Protein intake at 12 mo was comparable (Table 2). By 12 mo

Fat intake was mildly increased compared to 4 mo irrespective of diet group ($P<0.05$) (Supplemental Table 2).

As expected, increased protein in PRO participants replaced predominantly carbohydrate, although PRO participants also consumed slightly more fat (Supplemental Table 2). Calcium intake increased in the PRO group and declined in the CARB group with energy restriction (Table 2). PRO participants consumed (mean ± SEM) 387 ± 72 mg more calcium daily than CARB participants at 4 mo and 261 ± 81.6 at 12 mo (both $P<0.01$). While PRO calcium intakes met the RDA, calcium intake provided by the CARB diet was inadequate for female participants (Table 2). Because protein and calcium intakes increased or decreased together according to diet assignment, the ratio of protein:calcium intakes did not differ by diets or time (all $P>0.05$, Supplemental Table 2). Mean servings of dairy were as prescribed: 2 servings per day in CARB and 3 in PRO participants. Subjects reported no intake of supplements, as prescribed. Food records estimated PRO participants consumed 169±16 IU vitamin D, 34±23 IU more than CARB participants ($P=0.15$); however our nutrient database was not complete for all foods with respect to vitamin D, and actual content may vary considerably from label values (18;19), making the accuracy of these estimates questionable. Physical activity was similar between groups ($P>0.10$). BMD at the WB, LS and TH was superior over the course of the study in PRO participants (Figure 1). BMC was also superior in PRO participants ($P<0.05$, Supplemental Table 3).

Treatment differences were similar across gender and its two- and three-way interactions with diet and time for all bone outcomes (all $P>0.05$). Gender differences in BMD could be predicted by pre-intervention status, therefore gender effects were not significant after controlling for baseline values ($P>0.10$), indicating males and females were

similarly affected by the diets. Similarly, tests of age group (<40, 40-50, >50) and its interactions with diet and time in females were not significant, indirectly suggesting menopausal status did not moderate response.

Urinary calcium declined by month 8 in the CARB group, but was maintained on the PRO diet (Figure 2). Adjusting for baseline BMC, diet group and gender, urinary calcium levels predicted decreased WB BMC (ß=-0.38, *P*=0.032), indicating that a portion of variation in urinary calcium that was not explained by protein intake was negatively associated with WB BMC. This effect was not observed for LS BMC (*P*=0.65) or TH BMC (*P*=0.59).

Discussion

After a combined 12 mo of weight loss (4 mo) and maintenance (8 mo), a weight loss diet prescribing 1.4 g/(kg·d) protein and 3 daily servings of dairy provided more calcium and preserved bone mineral relative to an isocaloric control diet prescribing 0.8 g/(kg·d) protein and 2 daily servings of dairy. Targeting lean protein sources including low fat dairy improved calcium intake in free-living individuals under reduced energy intake conditions, while a conventional, high carbohydrate diet provided inadequate calcium in females, consistent with other reports (20-21). These data indicate that a higher protein diet specifically emphasizing dairy preserves bone loss compared to an isoenergetic higher carbohydrate diet proportioned according to the Food Guide Pyramid. The present data do not permit the resolution of independent or interactive effects of protein and other dairy components such as calcium and vitamin D, but support the effectiveness of a high protein, high dairy diet for protecting bone health during weight loss in free living patients.

Shapses and Riedt (4) review reports of bone mineral loss with weight loss, and summarize that 10% weight loss would be expected to produce 1-2% bone loss at various sites. Loss of BMD with weight loss is thought to occur due to changes in the weight-bearing load. estrogen status, circulating leptin and reduced calcium intake with energy restriction. Skov *et al.* (8) reported that a higher protein diet conferred greater weight loss but similar bone loss, or an improved bone loss to weight loss ratio over 6 mo in adults. As in the present study, Skov *et al.* reported adequate calcium intakes on the higher protein diet, but low calcium intakes among higher carbohydrate dieters.

The influence of protein intake on calcium balance and bone health remains controversial. Higher protein intakes consistently increase urinary calcium excretion (22). Early research found no connection between calcium absorption and protein intake (23-26), suggesting extra urinary calcium must reflect loss from bone (12). Specifically it is thought that bone is demineralized to buffer acid reabsorbed during renal handling of sulfate metabolites of dietary methionine and cysteine (27). More recently Kerstetter *et al.* (28-30) have reported increased calcium absorption with higher protein intakes using dual stable calcium isotopes and calcium- controlled interventions; however a similar study did not find elevated absorption based on protein intake (31). Roughead *et al.* (32) report no differences but a trend toward higher calcium retention in a group consuming increased protein from whole food (meat) sources. Critically, no differences in bone metabolism markers were observed. Another study demonstrated an *increase* in the fraction of urinary calcium of *dietary* origin during a diet providing 2.1 g/(kg·d) protein compared to a control diet providing 1.0 g/(kg·d) (13), suggesting additional excreted calcium did not originate in bone, but rather from improved intestinal absorption.

Dawson-Hughes *et al.* (33) reported a calcium, protein interaction in participants randomized to calcium supplementation or placebo and divided into tertiles of freely selected protein intake. Elderly participants consuming more protein and calcium gained bone mineral relative to those at lower intakes of either nutrient. Bowen, Noakes and Clifton (11) measured bone resorption and formation markers across 12 wk of weight loss on a mixed protein or dairy protein weight-loss diet, concluding that the calcium-rich, dairy based diet reduced bone turnover compared to a diet rich in protein but poorer in calcium. In the present study, ample calcium was supplied as a natural consequence of emphasizing lean protein sources, including dairy.

Median daily urinary calcium was 167 mg for PRO and 98 mg for CARB dieters. On average, PRO males consumed approximately 400 mg and females 300 mg more calcium daily than CARB participants. Accordingly, assuming a conservative 20-25% calcium absorption, additional available calcium would approximately compensate or exceed urinary losses. We have no data to account for endogenous fecal calcium losses, which may also be influenced by protein intake (34), nor can we report actual calcium absorption. However if absorption increases with protein as reported by Kerstetter *et al.* (13; 28-30), gains in available calcium could substantially outweigh urinary losses, supporting bone mineralization. Our data support this speculation.

Our study is not without limitations. Compliance assessment relies on 3 d weighed food records. Available nutrient databases are incomplete with respect to vitamin D, making inference concerning this nutrient difficult. DXA measurement is influenced by changes in tissue thickness, an effect that is not well quantified (35;36). Accordingly changes over time within diet groups should be interpreted with care; however the relative impact of the PRO

vs. CARB diet is interpretable, as groups did not differ in loss of weight, fat or body thickness. All measures demonstrated a marked benefit of the PRO compared to the CARB diet, on the order of 1 to 3%. The independent effects of increased protein and calcium on bone health are not known given the present design. Although limiting internal validity (i.e. isolation of protein and calcium effects), the design maximizes external validity, demonstrating that a high dairy, high protein diet will protect, not harm bone relative to a conventional weight loss diet in free living patients.

In conclusion, a higher protein weight loss diet emphasizing dairy as a lean protein source naturally improved calcium intake and preserved bone mineral during weight loss relative to a conventional higher carbohydrate diet in this free living population. Though not observed in the present sample, it is likely that such a diet would improve vitamin D intake for some patients due to fortification of this nutrient in dairy, conferring further bone benefits. These data are consistent with current literature indicating dietary protein may have direct benefits to bone health, provided calcium intake is adequate (8-10;33). Obesity and osteoporosis are major public health concerns, and are at odds with one another: to treat the first is often inviting to the other. A dietary regimen that protects bone mineral while promoting fat loss is of high clinical value.

Chapter 6 Supplement: Additional details of statistical analysis

The mixed model approach provides more accurate confidence intervals in the face of study attrition compared to last value carried forward and list wise deletion approaches (37). An autoregressive (AR1) covariance structure was applied to the repeated measure, selected based on Schwarz's Bayesian Criterion from a number of commonly used structures.

Following the primary analysis, each model was re-tested covarying for calcium intake, with missing values imputed by the expectation maximization (EM) algorithm. Though linear mixed models perform well in analyses with missing data for dependent variables (37), missing values for covariates may introduce excessive variability into parameter estimates (38). Accordingly dietary calcium was tested as a covariate, with missing values (due to attrition) imputed using an expectation maximization (EM) procedure. For purposes of imputation calcium intake was normalized using a square root transformation. A single, influential outlier in the resulting, otherwise normal distribution was winsorized to the 99^{th} percentile. In simulation studies the EM results in valid mean estimates but artificially narrow standard errors (39). This would increase the risk of an inappropriate finding of significance (a type I error); however in our analysis the calcium covariate was not a significant contributor to the model, precluding the possibility of a type I error.

To test the assumptions of data missing at random (MAR) and missing completely at random (MCAR) (39), logistic regression was used to assess whether attrition at any of the three follow-up measurement periods could be predicted by bone outcome variables at the previous measurement period, independently or controlled for diet assignment, BMI, age,

gender and study site. Cox regression was also employed to determine whether attrition over the entire 12 mo course of the study could be predicted by group assignment, controlled for pre-study BMI, age, gender and study site. Cox regression indicated that the combination of diet [Exp(B)=0.57, P=0.043] and age [Exp(B)=0.95, P=0.001] predicted attrition, invalidating the MCAR assumption; however, logistic regression revealed no association between attrition and prior values of the dependent variable (all P>0.1), indicating data were MAR.

Chapter 6 References

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Table 6.1. Baseline characteristics of adults randomized to a higher protein, dairy rich diet (PRO) or a conventional higher carbohydrate diet (CARB)[1].

		PRO	CARB
Age, y	F	45 (35-51)	46 (41-52)
	M	46 (39-55)	48 (39-53)
Weight,	F	82.3 (77.6-86.5)[a]	85.8 (77.5-94.3)[a]
kg	M	94.5 (88.2-107)	98.1 (92.4-106)
BMI,	F	30.9 (28.7-34.2)	31.8 (29.6-35.0)
kg/m²	M	30.9 (29.0-34.4)	31.9 (29.9-35.0)
WB BMD,	F	1.13 (1.03-1.21)[a,b]	1.19 (1.12-1.22)a
g/cm²	M	1.26 (1.17-1.30)	1.20 (1.14-1.33)
LS BMD,	F	1.02 (0.89-1.13)	1.06 (0.99-1.14)
g/cm²	M	1.04 (0.94-1.13)	1.04 (0.98-1.11)
TH BMD,	F	0.95 (0.88-1.01)[a]	1.02 (0.94-1.06)[a]
g/cm²	M	1.07 (1.03-1.16)	1.05 (0.99-1.15)

[1] Values are median (inter quartile range). n= 64 (28 males) for PRO, 66 (31 males) for CARB. [a] Differs from males ($P<0.05$). [b] Differs from CARB ($P<0.05$).

Table 6.2. Dietary intakes in adults randomized to a higher protein, dairy rich diet (PRO) or a conventional higher carbohydrate diet (CARB) during weight loss (baseline to 4 mo) and weight maintenance (4 mo to 12 mo) periods [1].

			Baseline	4 mo	12 mo
Energy,	F	PRO	8.5 (7.7-9.4)	5.9 (5.6-6.6)[a]	6.1 (5.6-6.9)[a]
MJ/d		CARB	8.5 (7.5-9.8)	5.9 (5.3-7.1)[a]	6.3 (5.5-7.0)[a]
	M	PRO	11 (9.5-13)[b]	7.3 (6.0-8.4)[a]	8.0 (7.1-8.8)[a]
		CARB	7.9 (6.7-9.3)	6.9 (5.9-7.5)[a]	7.5 (6.5-9.5)
Protein,	F	PRO	79 (66-90)	97 (88-114)[a,b]	99 (87-116)[a,b]
g/d		CARB	76 (68-88)	61 (58-75)[a]	60 (57-69)[a]
	M	PRO	101(94-124)[b]	125 (118-149)[b]	128 (106-155)[a,b]
		CARB	82 (74-93)	74 (63-82)	74 (68-87)
Calcium,	F	PRO	0.76 (0.62-1.07)	1.05 (0.82-1.33)[a,b]	1.00 (0.82-1.21)[b]
g/d		CARB	0.89 (0.65-1.15)	0.73 (0.62-0.90)	0.69 (0.63-0.92)
	M	PRO	0.93 (0.79-1.23)	1.19 (0.91-1.53)[b]	1.26 (0.83-1.38)
		CARB	0.72 (0.61-1.16)	0.80 (0.56-1.00)	1.00 (0.67-1.15)

[1] Values are median (inter quartile range), total n=130. [a] differs from baseline ($P<0.05$). [b] differs from CARB diet within time and gender ($P<0.05$).

Supplemental Table 6.1. Pre-intervention bone mineral content (BMC) of the whole body (WB), lumbar spine (LS) and total hip (TH) [1].

		PRO diet	CARB diet
WB BMC,	F[a]	2189±58.5	2318±43.6
g	M	2851±67.3	2891±73.0
LS BMC,	F[a]	57.1±2.04	60.5±1.67
g	M	69.8±2.03	71.8±2.04
TH BMC,	F[a]	31.3±0.98	32.1±0.82
g	M	45.2±1.30	46.5±1.49

[1] Values are mean ± SEM. n=130. [a] Differs from males ($P<0.05$). Diet main effect and interaction of diet x sex were not significant ($P>0.05$).

Supplemental Table 6.2. Additional intakes in adults randomized to a higher protein, dairy rich diet (PRO) or a conventional higher carbohydrate diet (CARB) during weight loss (baseline to 4 mo) and weight maintenance (4 mo to 12 mo) periods [1].

			Baseline	4 mo	12 mo
Carb,	F	PRO	268 (226-303)	147 (129-168)[a,b]	147 (133-156)[a,b]
g/d		CARB	267 (213-289)	200 (184-249)[a]	203 (179-232)[a]
	M	PRO	319 (244-371)[b]	155 (130-203)[a,b]	179 (144-216)[a,b]
		CARB	237 (211-265)	227 (208-279)	274 (199-341)
Fat,	F	PRO	79 (63-90)	51 (46-57)[a]	59 (49-67)[a]
g/d		CARB	75 (65-87)	45 (32-57)[a]	47 (39-61)[a]
	M	PRO	100 (73-154)[b]	63 (50-83)[a,b]	76 (66-88)[a]
		CARB	69 (57-85)	38 (30-49)[a]	58 (37-71)
Protein:	F	PRO	96 (73-118)	98 (84-111)	103 (89-113)
calcium		CARB	83 (74-99)	89 (78-99)	89 (70-97)
ratio	M	PRO	109 (90-124)	104 (91-120)	116 (101-134)
		CARB	108 (80-129)	93 (76-128)	75 (69-106)

[1] Values are median (inter quartile range), total n=130. [a] differs from baseline ($P<0.05$). [b] differs from CARB diet within time and gender ($P<0.05$).

Supplemental Table 6.3. Bone mineral content (BMC) of the whole body (WB), lumbar spine (LS) and total hip (TH) after 4, 8 and 12 mo of a randomly assigned high protein, high dairy, high calcium (PRO) or conventional high carbohydrate (CARB) diet [1].

		4 mo	8 mo	12 mo
WB	PRO	2539±8.5	2522±9	2560±9.4
BMC,	CARB	2524±8.6	2500±9.4	2502±10.3
g	*P*	Diet x time: <0.01[a]		
LS	PRO	66.16±0.35	66.62±0.37	66.57±0.39
BMC,	CARB	65.58±0.34	65.23±0.38	65±0.42
g	*P*	Diet: <0.01	Time: 0.89	
TH	PRO	38.14±0.19	38.04±0.2	37.92±0.21
BMC,	CARB	37.77±0.19	37.86±0.21	37.07±0.23
g	*P*	Diet: 0.04	Time: 0.01	

[1] Estimated marginal means ± SEM, adjusted for baseline BMC, gender, study site and two- and three-way interactions of site and gender with diet and time. Only the interaction of diet x time for WB BMC wassignificant (all other $P>0.05$). Total n=130. [a] Post hoc tests revealed differences at 12 mo ($P<0.01$), but not 8 ($P=0.08$) or 4 ($P=0.19$) mo (12 mo difference robust to a Bonferroni adjustment for multiple comparisons).

Figure 6.1. Bone mineral density (BMD) during at 4, 8 and 12 mo weight of a high protein, dairy and calcium diet (PRO, ○) or a conventional higher carbohydrate diet (CARB, ●) in adults. A: whole body (WB), B: lumbar Spine (LS) and C: total hip (TH). Values adjusted for baseline BMD using a linear mixed model with random slopes, n=130. No significant two- or three-way interactions of gender or site of participation with time and diet were detected (all $P>0.05$).

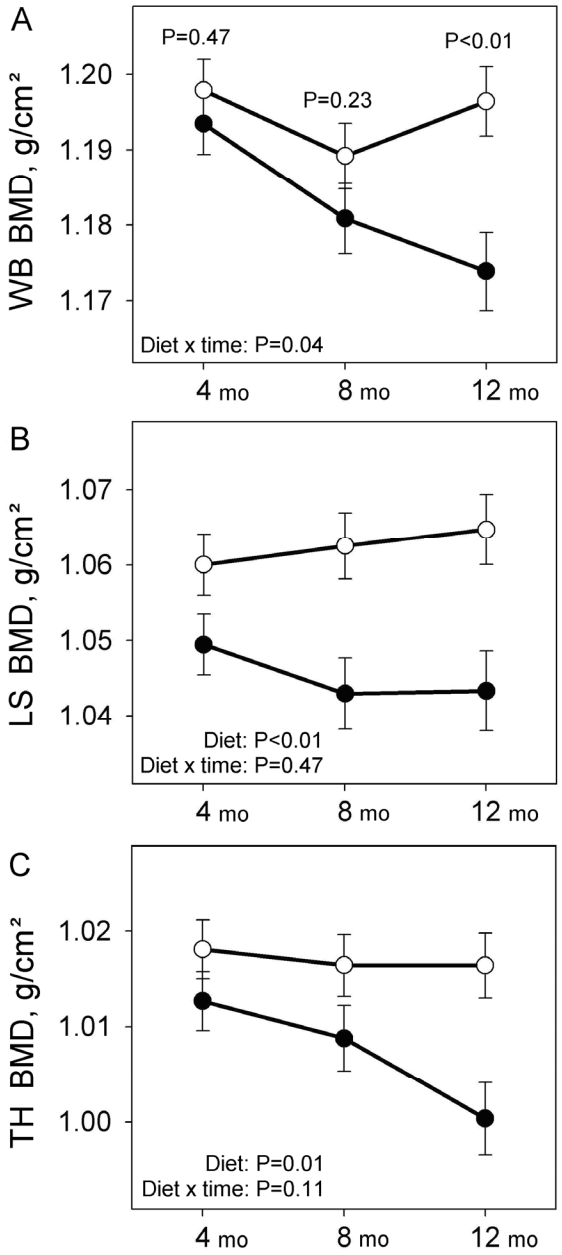
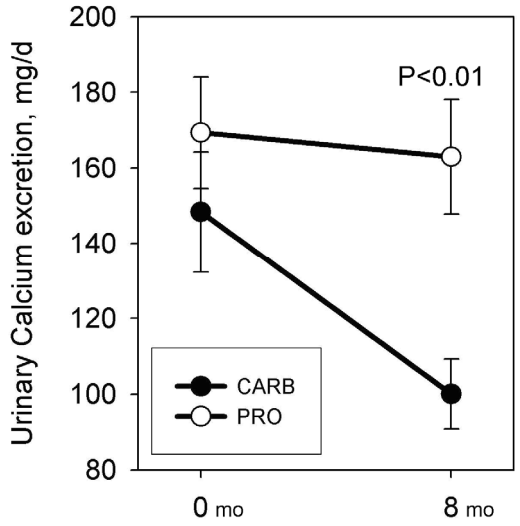


Figure 6.2. Urinary calcium excretion (mean ± SEM) at baseline and 8 mo of a high protein, high dairy, high calcium diet (PRO, ○) compared to a conventional higher carbohydrate (CARB, ●) diet in adults. Values tested using ANCOVA adjusted for baseline urinary calcium excretion, n=40.



Chapter 7

Conclusion

Summary of Chapters

The relationship between protein intake and bone health has been elusive over many decades of investigation. An emerging view of this complex association proposes that protein may have both positive and negative effects, depending on other considerations of the diet (1-4). Although this theoretical model aptly explains the available data, the model has not been formally tested using statistical models that quantify both positive and negative pathways. In the studies presented herein, the application of mediational modeling provides quantitative support for the theory, and casts light on some of the mechanisms determining whether protein's influence is positive, negative or null.

In a cross sectional investigation in postmenopausal women, protein improved BMD after adjusting for a negative effect of the sulfur containing amino acids thought to be responsible for damaging effects of protein. In rats, a similar picture emerged, with positive effects of protein mediated by IGF-1 counterbalanced by negative effects of some unmeasured pathway. A second rat study illustrates that protein sources may improve or decrease bone strength, and that these effects are mediated by changes in body composition, especially lean body mass.

During weight loss, the combination of higher protein intake and additional calcium protected adult men and women from the bone loss normally associated with energy

restriction. It is uncertain whether those effects are attributable to protein, calcium or their interaction; however, a mathematical model of bone mass change and urine calcium in this sample suggests that changes in dietary calcium were insufficient to account for improvements (Appendix A). Furthermore urine calcium, an index of the diet acid load, was negatively associated with bone density in spite of being elevated in the higher protein diet. This counterintuitive result is difficult to test in mediational modeling due to the experimental design, but is consistent again with the notion that protein concurrently stimulates negative, acid-mediated and positive changes in bone.

These data are consistent with a review of the literature that protein can improve or deteriorate bone health, with no strong effect observed on average (5). The purported benefits and harms are small, and unlikely to be of great clinical consequence, though they may be important on the scale of public health. Certainly these data refute persistent arguments against higher protein diets on the grounds of theoretical harms to bone, as a true negative effect of the protein induced acid load is likely to be offset by benefits of protein to calcium absorption and/or the IGF-1 axis.

Causal Inference

In 1965 A.B. Hill (6) proposed a system of criteria that is still the most influential system of criteria for determining causation today. These criteria are summarized in Table 7.1. Hill never proposed that all criteria must be fulfilled in order to conclude that a relationship is causal rather than spurious. On the contrary he suggests that although all criteria are important to consider, very strong evidence within any one criterion is adequate to support a causal relationship and spur appropriate public health action. By way of

example, while no long term, randomized, controlled trials are available to establish that smoking causes lung cancer, this causal relationship is hardly challenged in light of strong evidence of a strong relationship, a dose response pattern, a temporal dependence and a consistency of the association across variable populations. I propose that the balance of evidence, when weighed against Hill's criteria, support a dual pathway model of protein and bone health.

Strength

The defended research has consistently shown a statistically significant effect of protein on bone after accounting for one of the proposed mediating effects. These effects, however, have been small, and the overall average effect of higher protein intakes on bone is close to zero (5). Accordingly, the criterion of strength is consistent with a causal relationship, but insufficient by itself to rule out a spurious association.

Consistency

The consistency of support for the dual pathway model across population, animal and clinical studies is striking, and is perhaps the strongest support for a causal relationship. Across multiple studies and especially different research paradigms, we expect measurement and sampling error to "average out," allowing pure relationships to surface over repeated investigation. Extending beyond the data defended herein, a dual pathway model has also been consistent across gender and age, from youth (7) to older men and women (8), in studies which have experimentally or statistically isolated the effects of the diet acid load from those of protein or its interaction with calcium. Although additional replication is necessary to evaluate the relative importance of various proposed mediators of the protein bone

relationship, I propose the auspicious consistency of support for the dual pathway model provides strong evidence for a true causal relationship.

Specificity

In contrast, the criterion of specificity or a one-to-one relationship seems to be the most limiting, and its absence is arguably the cause of so many years of controversy surrounding the protein-bone question. While the feeding of alternate acid sources has produced predictable changes in calcium balance and bone tissue, there is no reliable one-to-one connection between a high protein diet and osteoporosis or bone fracture. In fact, by its very nature the dual pathways model is incompatible with a *specific* relationship, since many mediators are proposed to compete to produce any of beneficial, harmful or absent outcomes. Because of this complexity, it may be argued that the model explains the conflicted literature simply because it allows for essentially any result to be accepted as supporting. While not damning, the lack of specificity increases the demand on the theory to satisfy other criteria. In particular, the lack of specificity must be reconciled by legitimate biological mechanisms that would allow for the observed complexity.

Plausibility (mechanism)

Although mechanisms for the proposed positive and negative pathways are not fully understood, several specific mediators have been proposed and found to statistically explain some part of the relationship between protein and bone. It is known that protein imposes a dietary acid load (9), and that a dietary acid load from other sources causes damage to bone (10-13). In contrast, it is probable that IGF-1 (14-16), calcium absorption (17; 18) and lean body mass (19; 20) are both improved by protein intake and can improve bone health in turn.

Further work along each of these lines of inquiry is needed to clarify the optimal diet for bone health, but the theoretical plausibility and replication of each of these pathways appears sufficient to meet the burden of plausibility mandated by the non-specific relationship between protein and bone.

Coherence

Hill notes that plausibility cannot always be strictly required, as observational relationships may be apparent before science has discovered plausible biological mechanism. In the absence of plausibility, coherence demands that the proposed relationship at least not be in conflict with current knowledge. It has been argued that coherence for the negative, acid-load mediated pathway is not satisfied, because dietary manipulations of acid-base balance are unlikely to produce a noticeable impact on the pH of the bone interstitial fluid (21). Pizzorno (22) has taken care to discriminate *acidemia*, a change in the pH of the blood, from *acidosis*, a change in active acid-base regulatory systems, including buffer concentrations. Although acidemia is indeed only slightly inducible by diet (23-25), subtle changes to acid base balance may have profound effects on the regulation of bone cells (26), particularly if the bone interstitial fluid immediately adjacent to cells is near pH 7.1, as has been shown for skin (27). The regulation of bone cells in vivo in response to realistic acid loads is not determined, but at this time it appears both plausible and coherent that a diet acid load induces subtle but chronic adverse changes in bone.

Analogy

Hill discusses analogy again primarily in the context of an absent plausible mechanism. That is, where the physiological connection is unknown, can it at least be said to

be similar to some known causal relationship. In the present case, it is useful to draw analogy between the influence of dietary protein and alternate dietary sources of fixed acid. Supplementation of various non-protein acid sources, including isolated sulfur amino acids, unambiguously cause damage to bone in animal models (10-13). In contrast, fixed alkali supplements are beneficial in humans (28-31). That no consistent effect of a similar acid load from complete protein sources exists is striking, and can only be explained in one of two ways. 1) The acid load of protein is unique from other acid sources, and does not evoke the harmful response observed with other acid sources. 2) The acid load of protein exerts its expected adverse effect, but is opposed by alternate, beneficial pathways. The first option is unlikely given that sulfur amino acids do exert a negative effect independent of total protein (4; 10). The second option is supported by analogy to the reported benefits of IGF-1, calcium absorption and lean body mass.

Temporality

The animal and clinical work herein establishes the requisite temporal relationship for causal inference. Changes in the protein amount or source, the proposed cause, precede changes in bone outcomes, the proposed effect. This does not rule out the possibility of some time-varying confounder. For example in the clinical trial, it is possible that the improvement in bone outcomes is attributable to improved calcium intake accompanying increased dietary protein. Appendix A addresses this question through a mathematical model, suggesting that the difference in bone mass change between diets is too large to be explained by measured calcium intakes alone. This conclusion depends heavily on the reliability of urine and dietary calcium measures, which is tenuous, but the probable effects of bias and error in these measures would be to mask a true difference between diet groups, rather than creating a

spurious one. Furthermore, the highly controlled experimental conditions in the two animal studies aptly undermine the possibility of time varying confounders.

Dose-response

Not every causal relationship involves a dose-response relationship. Threshold effects, J-shaped curves and interactions are examples of causal relationships that may produce apparently non-linear effects, and are each ubiquitous in nutrition. In the present case, however, we have consistently observed a linear relation between bone outcomes and IGF-1, lean body mass or the sulfur amino acid load, and a corresponding effect of total protein intake that is approximately equal in magnitude but opposite in direction. This unique pattern of statistical suppression (32) seems to provide strong support for the dual-pathway model, since it is difficult to conceive a confounding variable that might explain the observed opposing relationship across multiple mediators and research paradigms.

Experiment

As alluded to under *temporality*, the experimental support for the dual pathways model provided herein lends strong support. In each case, proposed mediators were not randomly assigned but allowed to vary naturally in response to manipulations of dietary protein. Although this permits the theoretical possibility of a time-varying confounder, the tight control of experimental conditions within animal studies makes this considerably less probable. Furthermore, a test of statistical mediation must necessarily consider the effect of protein on the proposed mediator, as well as the effect of the mediator on the outcome. That a pattern of dual, mediated pathways emerged in these experimental protocols all but rules out

the possibility that some extraneous factor explains the observed positive and negative effects.

Limitations

These studies have not addressed vitamin D except in ruling it out as a confounder through experimental or statistical control. Due to these controls, it is probable that the proposed mediation of protein's influence on bone operates independently of vitamin D on average. These relationship have not, however, been tested over a considerable range of vitamin D status, therefore we cannot rule out that vitamin D may modify these associations.

Similarly, data presented herein almost invariably involve sedentary to low amounts of physical activity. It is reasonable to expect that exercise might modify these relationships, by increasing the protein requirement, by potentiating the increase in lean body mass with protein, by regulation of normal acid base physiology or by providing direct stimulation of bone growth that could potentially either enhance or obviate any influence of protein.

Also, the role of IGF binding proteins have not been tested, though they are both relevant to bone physiology (33) and regulated by protein in the diet (14; 34). It is possible that some part of the mediational role of IGF-1 is itself mediated or potentiated by nutritional regulation of its binding proteins.

Along the same line, the mediational models tested have relied on one global indicator of more complex individual pathways. The regulation of bone by dietary acid load is not completely understood in vivo, nor is the influence of IGF-1. While these results support the theoretical dual pathway model, they are not themselves mechanistic studies. These data therefore are best interpreted as identifying relevant pathways connecting protein

intake to bone and estimating reasonable effect sizes, rather than elucidating any particular pathway.

Future Directions

These data highlight the importance of sulfur amino acids, IGF-1, calcium availability and lean body mass as mediators the effect of protein on bone. The application of mediation models has allowed these relationships to be quantified. However in each investigation, only one mediator was specifically accounted for. It would be important from a public health perspective to evaluate which if any of these specific pathways predominates in a large sample of free-living humans. Measurement of calcium absorption is difficult and not feasible in large samples, however other pathways may be testable using secondary analysis of existing large data sets such as NHANES or Health ABC at low cost.

It is unclear how dietary protein influences calcium absorption. Regulation of the calcium Sensing Receptor is an emerging possibility, however the importance of this mechanism at physiological intakes has not been established. Similarly, while well defined acid-base regulatory pathways in bone are emerging in vitro, the importance of these pathways in response to realistic diet acid loads has not been tested. This would be difficult to test non-invasively, but gene expression studies in animal models may shed light. It is also important to establish whether the interstitial pH experienced by bone cells is indeed lower than the generally buffered set point of 7.4, as has been shown in skin.

The role of protein source in calcium balance and longer term bone health has not been well characterized. In epidemiology, assessment of particular key amino acids rather than blunt food groups or total protein intake would be beneficial. In clinical trials, differing

protein sources have been tested, but sample sizes have not been adequate to control type II error.

Perhaps most importantly, the specific predictions of the dual pathways model should be tested explicitly. Again, secondary use of large data sets may be most useful in comparing the relationship of protein with bone density at different levels of overall dietary acid load. Since the current evidence suggests any benefits are probably small, it would be prudent to continue clarifying these relationships through these lower cost approaches. The expense of long term randomized trials is probably not justified unless preliminary evidence indicates the benefit of some particular combination of protein source, calcium and fixed alkali is large enough to be clinically important.

Summary

I have defended the position that protein causes both positive and negative changes in bone. In terms of Hill's criteria for causal inference (Table 7.1), these effects are apparently weak though statistically significant, and are remarkably consistent across populations and research approaches. This dual pathway model by its nature does not describe a specific, one-cause to one-disease relationship, making causal inference increasingly dependent on valid biological mechanism and coherence with existing knowledge. The work of other authors providing the required plausible mechanism has been reviewed. The relationship is coherent with and analogous to other research establishing the negative influence of a diet acid load and the positive influences of IGF-1, of calcium absorption and of lean body mass. The temporal and apparently linear dose-response relationships observed in these data, as well as the support from well controlled animal models provide a sound basis for ruling out

confounding in these studies. Accordingly, on the whole, I propose that Hill's criteria are aptly met, and it is reasonable to conclude at this time that protein indeed causes positive and negative changes in bone.

On average, these effects are roughly equal in magnitude and opposite in direction, suggesting that in free living individuals any net influence of protein is likely to be modest or absent. If correct, the dual pathway model I have defended would predict that a higher protein diet, in the context of ample calcium and alkalizing fruits and vegetables, may provide modest benefits to bone.

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Table 7.1. Hill's criteria for causal inference.

Strength	**Coherence (theoretical consistency)**	**Temporality**
Consistency	**Experiment**	**Dose Response**
Specificity (1 to 1)	**Analogy**	**Biological Plausibility**

Appendix A

Dietary calcium may be more bioavailable using a weight loss diet that replaces moderate amounts of carbohydrate with protein

Weight loss causes bone mineral loss. We previously reported subjects prescribed 1.6 g/kg·d of dietary protein (PRO) consumed more dairy, excreted more calcium in urine and preserved bone mineral relative to controls consuming 0.8 g/kg·d (CARB) during weight loss. It is unclear whether attenuation of bone mineral loss was related to increases in calcium intake, availability or both.

We modeled calcium balance over 120 d for 41 adult males and females (47±7 y) randomized to PRO or CARB diets using 3 d weighed food records of calcium intake, 24 h urinary calcium (UCa) and DXA-estimated whole body bone mineral content (BMC) as a proxy for total bone calcium. We defined *availability* as the fraction of calcium intake explaining changes in bone mineral mass, after accounting for urinary calcium losses; this included unmeasured parameters absorbed calcium and endogenous intestinal calcium loss (Figure 1):

$$Availability = \left(\frac{Absorbed\,Ca}{Diet\,Ca} - \frac{Endogenous\,Fecal\,Ca}{Diet\,Ca} \right) \qquad eq.1$$

Non-bone internal calcium pools were assumed constant. Availability was solved for each subject given:

$$\Delta BMC \sim \sum_{d=1}^{120} (Ca_d) \cdot (availability) - UCa_d \qquad eq.\ 2$$

Estimates of calcium availability were higher by 22.0% (95% CI: 0.2, 43.8) in PRO vs. CARB (Figure 2) suggesting BMC change cannot be completely accounted for by

increased calcium intake with a higher protein diet; however, whether benefits to BMC are related to increased calcium absorption, reduced endogenous intestinal calcium loss or a combination is unclear.

Figure A.1. BMC, dietary Ca and urine Ca were measured. Availability was approximated by estimating change in BMC as the product of dietary calcium and availability, minus urinary calcium loss.

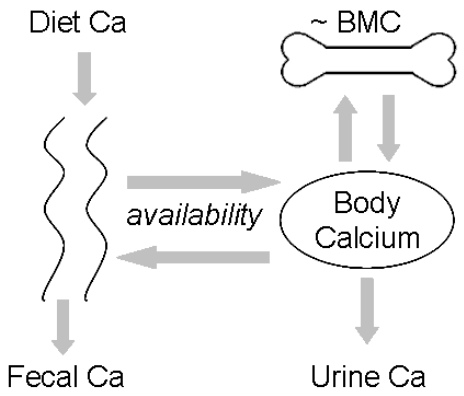
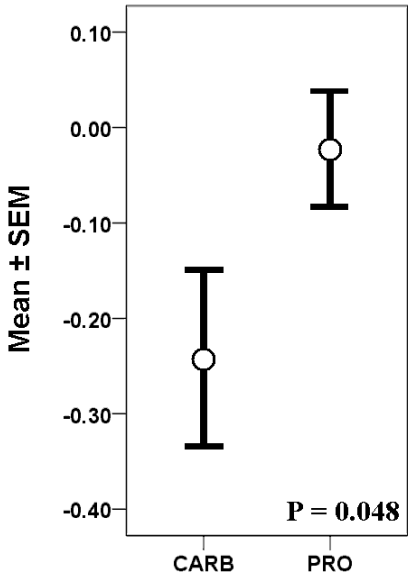


Figure A.2. Estimated availability is improved in PRO compared to CARB participants.



Appendix B

Instrumental variable analysis to adjust for non-compliance to randomized dietary interventions

Abstract

Non-compliance in randomized controlled trials (RCT) dilutes estimated treatment efficacy under intent-to-treat analysis (ITT), producing an overly conservative statistic. Another approach, instrumental variable (IV) analysis, uses measurements of compliance or dose received to estimate the treatment effect in compliant patients. The approach has gained acceptance in clinical trial methodology but is not commonly applied to dietary interventions. We use a RCT investigating the role of dietary protein on bone health, under weight loss conditions, to explore benefits and limitations of IV analysis. Patients (n=62) were randomized to weight loss diets providing 1.4 (PRO) or 0.8 (CHO) g/kg•d protein. Compliance was measured using weekly 3d weighed food records. Change in total hip bone mineral density (BMD) as a percent of baseline, measured by dual X-ray absorptiometry, was compared at 1 y. IV analysis was applied using two-stage least squares regression.

Based on ITT analysis, random assignment to PRO (without respect to compliance) caused a (standardized B±SE) 0.35 ± 0.12 (p=0.005) improvement in BMD compared to CHO. IV analysis estimated this value at 0.47 ± 0.18 (p=0.009). Accounting for non-compliance using an IV approach resulted in a 33% increase in estimated efficacy of treatment. IV analysis may provide unbiased estimates of efficacy, corrected for noncompliance, in the interpretation of RCTs. The paper presents a discussion of logic and implementation of IV analysis.

Introduction

Noncompliance is a pervasive problem in human clinical trials. In its most benign presentation, noncompliance is random, or uncorrelated with treatment assignment and outcome. Such noncompliance is said to be *ignorable*, as it does not confound the estimated treatment effect. However, in its more pernicious and perhaps more frequent presentation, noncompliance *is* correlated with other patient characteristics, with repercussions on the outcome of interest. These associations can introduce confounding into the experiment, destroying the primary advantage of the randomized, controlled trials (RCTs). Even "ignorable" noncompliance dilutes differences in outcome between treatment groups under intent-to-treat (ITT) analysis, potentially obscuring physiologic effects.

This paper reviews the problem of noncompliance, the traditional ITT estimate of treatment effect, and an alternate approach, instrumental variable (IV) analysis, to statistically adjust for bias introduced by noncompliance. It is not meant as a technical statistical description, but instead emphasizes the logic and practical considerations of dealing with noncompliance. IV analysis was developed decades ago in the field of economics, and has since become established in observational epidemiology (1). The unique design of RCTs provides special advantages to the use of IV analysis (2; 3), but the IV approach is still rarely applied to RCTs and has not yet, to the authors' knowledge, been applied to RCTs of nutritional interventions. The method requires careful measurement and evaluation of noncompliance patterns and provides further insight into the physiologic effect of a dietary intervention. An example will be drawn from real data regarding the impact of a higher protein, weight loss diet on bone density.

The rationale for randomization: Preventing confounding

Randomized, controlled trials (RCT) are accepted as the gold standard for establishing causal effects of a treatment on an outcome of interest. Specifically, randomization is intended to prevent confounding influences from introducing bias into estimates of a treatment effect. The logic of confounding is familiar, and is depicted in Figure 1. If all confounders are accurately measured and included in the model, we can obtain an accurate estimate of the causal effect of x on y:

$$y = \beta_0 + \beta_x + \beta_w + \varepsilon \qquad eq.\ 1$$

where β_0 reflects the intercept, β_x and β_w the effects of treatment x and confounders w on y, and ε the error.

In practice however we are never privy to all confounding influences. As a result, our estimate of the effect of x on y, without knowledge of w, is biased by a function of β_w (the association of w and y) and the correlation ρ between x and w (4):

$$y = \beta_0 + (\beta_x + \beta_w \rho_{xw} \frac{\sigma_w}{\sigma_x})x + \varepsilon \qquad eq.\ 2$$

Confounding, then, describes the treatment effect β_x being 'muddied' by the portion of the unmeasured variable w that is correlated with both x and y.

Randomization offers a straightforward solution to the problem of confounding. By allowing x to be determined by random chance, we have ensured that the correlation ρ_{xw} is approximately zero, and so equation 1 reduces to:

$$y = \beta_0 + \beta_x x + \varepsilon \qquad eq.\ 3$$

providing an accurate estimation of the effect of x on y.

The problem of noncompliance

Unfortunately this simple solution breaks down in face of noncompliance to randomly allocated treatment. Noncompliance creates a disjunction or imperfect correlation between the treatment *prescribed* and the treatment actually *received*. This disconnect is represented in Figure 2 by introducing a new variable, *d* for *dose received* (2). The causal effect of treatment *x* on *y* is now mediated by *d*. If noncompliance is random, that is, independent of treatment allocation, confounding variables are not introduced, and noncompliance is said to be *ignorable*. However, "ignorable" noncompliance is still problematic, as the average difference in the outcome between treatment groups is smaller than it might have been under full compliance. This is a form of misclassification bias. That is, patients in the treatment *group* have in fact received no treatment, and the outcome is biased toward the null hypothesis of no difference between groups.

Moreover, perhaps the more common scenario is for non-compliant patients to differ in some meaningful way with respect to treatment and outcome (2; 3; 5). This reintroduces confounding into the model such that the effect of dose on *y* is obscured by the pattern of noncompliance and its correlation with confounding influences (2; 3). For example, noncompliance may be higher in groups of low socioeconomic status (SES), which in turn is associated with poorer health outcomes generally. The implication is that the effect of dose on *y* will be confounded by SES in patients who do not receive the assigned treatment and happen to have poorer outcomes related to other environmental considerations.

Estimating effect size in the face of noncompliance

Common approaches to noncompliance can be grouped in familiar terms as estimation of *efficacy* or of *effectiveness* (6). As a brief review, the term effectiveness refers

to translational impact of a treatment. An estimate of effectiveness seeks to answer the question most relevant to the clinician: "What is the effect of *prescribing* the treatment to my patient?" Clearly such an estimate must account for the reality that patients do not always comply with prescription, and that the average treatment effectiveness across all patients will be diminished by the proportion of noncompliance (6). Conversely, the term efficacy denotes the physiological effect of a treatment. It answers the question of most interest to the scientist, "what is the effect of *receiving* the treatment?" or, "what is the physiologic response to the treatment?" (5; 6). In terms of the model we have defined so far, efficacy represents the causal effect of *dose* on outcome. (Note that aside from compliance, additional considerations are relevant to the distinction between effectiveness and efficacy, but these are beyond our present scope; see (7; 8) for a discussion).

The intent-to-treat (ITT) estimator is the accepted standard for estimating treatment effect (6; 9). The name indicates analysis based on the intended, or assigned dose rather than the dose actually received. In the face of noncompliance, the variable d (dose received) is ignored, and the treatment effect is estimated as the difference in y between randomly assigned treatment and control patients. In terms of our causal model, the ITT estimator is the difference in y predicted by x, without respect to d. Should the treatment arm include some patients who take less than the assigned dose, and/or some control patients receive more than the assigned dose, the ITT estimate will be diluted and conservative (2; 9).

In spite of this problem, ITT analysis is the preferred method because the original benefits of randomization remain intact. All patients are analyzed as randomized, precluding any correlation between x and w, if not between w and d. When noncompliance occurs, ITT will generally produce a conservative estimator of efficacy (3; 10). Moreover, under the

assumption that the proportion of noncompliance in the *sample* reflects the noncompliance that would be observed in a general *population* being prescribed the treatment, the ITT estimator is an unbiased measure of effectiveness (6). From this perspective, the statistic is very useful to public health officials or policymakers who wish to predict the average benefit of promoting a treatment to the population at large.

In terms of efficacy, three alternative estimators have been described (10). These are designed to emphasize the causal effect of *dose*, rather than randomly assigned treatment per se. The *as treated* (AT) estimator describes the change in *y* that is predicted by the dose actually received, irrespective of randomization. Because randomization to a treatment group is discarded in favor of the dose actually received, the estimator is in theory an estimate of efficacy. However we have also discarded the principal benefit of randomization: protection from confounding. As set out above, any factor correlated with both noncompliance and outcome will confound the estimate. In fact, the AT estimate more nearly resembles the observational cohort study design, in which exposure (dose) is longitudinally correlated to outcome. The use of this estimator may be informative, but its result cannot be properly considered a randomized, controlled trial, and is easily confounded by unmeasured variables.

A second and more common approach to efficacy is the per protocol (PP) estimator. The name refers to estimating the effect of the treatment protocol assigned, but only among compliant patients. Non-compliant patients are dropped from the data set and treated as though patients had withdrawn from the study. As a result, dose received is identical to dose prescribed in each treatment arm, and the difference in *y* between groups is, in theory, an accurate estimate of treatment efficacy. In treating noncompliant data as missing, however, we encounter the same potential sampling bias arising with any form of missing data (11; 9).

That is, any variable associated with both exclusion criteria (noncompliance) and outcomes will result in sampling bias and, again, confound the estimate. Although this bias may be easier to correct than that observed under AT analyses (11) PP estimators still lack the full benefit of randomization, since data have been excluded by non-random criteria.

Clearly noncompliance shrouds efficacy insofar as dose received is not random, but correlated with any confounder characterizing the non-compliant patient. Effectiveness may be estimated by ITT analysis where assumptions of generalizability are met. ITT is also accepted as a more conservative estimate compared to AT and PP analyses, and less prone to type I error.

Instrumental variables for estimating efficacy

For the scientist, the question of efficacy persists because it speaks more to mechanism than the alternative. Likewise, the clinician benefits from being able to tell the patient how large a difference may be expected if a treatment is taken faithfully. Answers to these questions can be approximated using an alternative method of effect size estimation. Instrumental variable (IV) analysis originated as a way to isolate the effects of two closely related variables (or more technically, to correctly estimate effects of variables whose errors are correlated with the error of the dependent variable (1). An instrumental variable, z, is defined as one which is correlated with the independent but not the dependent variable; that is, the instrument is only correlated with y indirectly by way of its association with the independent variable. This variable can be used as an instrument to isolate or purify the estimate of effect size.

As a practical nutritional example of the procedure, we have applied IV analysis to a clinical trial of the effect of a higher protein diet (PRO) compared to a conventional higher

carbohydrate diet (CARB) on bone density in 62 adults. The results of this trial were originally published in the Journal of Nutrition in June 2008 (12), using only ITT analysis. In order to illustrate IV analysis here, we simplify the analysis by ignoring other important statistical considerations, such as missing data and multiple follow-up assessments.

A randomized clinical trial offers an ideal setting for implementation of IV analysis. We are interested in efficacy, or the effect of protein "dose" on bone mineral density (BMD). Confounding variables collectively represented as *w* are correlated with *d* and *y*, due to unique characteristics of noncompliant patients. The assigned treatment *x,* diet group, becomes our instrument, and since it was assigned by randomization, we are confident that it is not correlated with *y* or *w* except through its influence on protein dose. The assumptions of the IV approach are met, and we can now use *x* to purify the estimate of the causal effect of dose on *y*.

Subjects and Methods

Adults, n=130 (59 males), aged 46±9y were randomized to PRO and CARB treatments using a parallel-arm design. Participants were excluded based on medical history of smoking, cancer, heart disease, frank diabetes, renal disease or use of bone active medications. Patients were block-randomized following matching by age and BMI into isocaloric PRO and CARB weight loss diets, designed to achieve roughly 10% weight loss over 4 mo, then maintain weight for an additional 8 mo. The CARB diet was modeled on the Food Guide Pyramid, prescribing ~15% of energy as protein and 30% as fat. The PRO diet substituted some lean meats and dairy for starchy staples, prescribing roughly 30% energy as protein and 30% as fat. Patients were instructed in keeping 3-d weighed food records and met weekly with a registered dietitian for diet education and monitoring of diet records. Bone

mineral density (BMD) was measured using dual x-ray absorptiometry (DXA; Hologic QDR 4500A or 4500W). Institutional Review Boards of participating centers reviewed and approved all study protocols, which were in accordance with their ethical standards for human experimentation. Additional details of the intervention may be found in Thorpe, *et al.* (12).

As in many long term weight loss studies (13-15), we experienced substantial attrition over the 1 y time course, such that 62 subjects (37 in the PRO arm) remained. For simplicity of this example we employ listwise deletion of cases with missing data and restrict our analysis to BMD of the total hip at 1 y. Weight loss was similar between groups among males and females ($p>0.2$). To account for baseline variation in BMD, we analyze BMD change as a percent of baseline values. This approach has the advantage of adjusting for many baseline covariates impacting bone health which are not changed in response to the intervention (16). As a result of this adjustment, change scores did not differ according to gender or the interaction of gender with treatment group. By using change scores, this approach provides a more parsimonious set of variables for the implementation of IV analysis. As the measure of dose, we used the continuous variable percent energy consumed as protein (pro%), estimated using 3-d weighed food records, to account for any influence of energy intake, which was also manipulated by the diet. The distributions of pro% in each group are illustrated in Figure 3.

Statistics: ITT and IV estimates

The ITT effect was estimated by ordinary least squares regression using a treatment dummy code (CHO=0, PRO=1). This estimate ignores dose, and is therefore free from

confounding influences correlated with dose, but underestimates the physiologic effect of the diet, due to overlap in actual protein intakes between groups (Figure 3).

Several approaches to the implementation of IV analysis exist and are reviewed in Dunn *et al.* (9).Perhaps the most conceptually straightforward approach is to use a pair of ordinary least squares regression models, using the residuals from the first to adjust the estimates of the second (3). Because this approach effectively illustrates the logic of IV analysis, it will be emphasized here. First we use regression to predict protein intake based on randomly assigned diet dummy codes. We save the residuals from this regression, which reflect noncompliance, or that part of variance in protein intake that is *not* accounted for by treatment assignment. This is the share of protein intake that may be correlated with confounding variables. Next, we perform a second regression, predicting BMD using protein dose and covarying for the residuals saved from the first regression. By statistically adjusting the effect of protein dose for this extra, potentially confounded variation, we have purified the effect estimate, or removed the contaminating influence of that part of dose that may be confounded. The result is a better approximation of the true causal effect of dose on y than is available using ITT, AT or PP analytic approaches.

Following this piece-wise implementation of IV analysis, the IV estimate was obtained using the two-stage least squares (2SLS) procedure in SPSS (version 16.0, Chicago, IL). The 2SLS procedure is specifically designed for IV analysis. The procedure simultaneously performs the two part regression sequence described above, yielding an identical effect size but slightly adjusted standard errors to account for correlations in the data. Comparable procedures are available in most contemporary statistical packages. The present example uses continuous dose and outcome variables; however the same process may

be applied to categorical dose or outcome using the appropriate logistic regression models (3; 9).

Results

The Table summarizes the effect sizes estimated by the two approaches. The ITT statistic is interpreted as the average effect of *assignment* to the PRO diet on BMD. The IV statistic is interpreted as the average effect of the PRO diet *among compliant patients* (9). These standardized estimates translate into a predicted 1.8% benefit to BMD under ITT, or 2.4% under IV analysis.

As expected, IV analysis produced a larger effect size than the generally conservative ITT statistic. Provided the assumptions of IV analysis are met, this statistic more closely estimates physiologic efficacy (effect of dose) of the dietary treatments on bone health. Conversely the ITT statistic, if the pattern of noncompliance in the sample is representative of the general population, more nearly reflects effectiveness (effect of prescription) on bone health. Figure 4 illustrates the results of the IV analysis. Values are standardized regression estimates. Although effects of w are unknown, they are uncorrelated with d after adjusting for residuals r.

Note in the figure that the ITT statistic, 0.35, is the product of the IV estimate (effect of d on y) and the effect of treatment assignment x on *dose*. Conceptually this is because noncompliance dilutes the effect of treatment assignment by the proportion of compliance in the sample. Accordingly, where compliance is perfect, the effect of x on d is 1 and the ITT and IV estimates are identical.

Discussion

In this data set, the use of IV analysis produced a 33% increase compared to the conventional ITT estimate of the causal effect of the higher protein diet on bone density during weight loss. The ITT estimate is known to be conservative in the face of noncompliance (5), and this offers particular advantages. First, type I error is usually less likely under ITT, though at the cost of increased type II error risk. Second, ITT preserves the full benefits of randomization, ensuring that treatment x is not correlated with confounders w. Additionally, ITT can be used to answer the question, how much benefit was derived in the sample on average, without respect to compliance? If the pattern of noncompliance in the sample is generalizable to the population of interest, this statistic speaks to the effectiveness of the intervention. Despite these favorable properties, the presence of noncompliance makes ITT a biased estimate of efficacy, since outcomes in patients receiving no treatment are averaged with the rest of the experimental group. This limitation tempers its usefulness where the primary research question deals with the physiological effect of the treatment.

We have so far taken for granted the validity of measurements of noncompliance in this illustrative analysis. In practice this is not a perfect assumption. Intuitively, patients who do not comply with diet prescriptions may not comply with food records or recalls. It is not even unheard of for counterfeit packet or pill counts to be engineered by patients who wish to maintain a facade of compliance, for reasons of social desirability or fear of losing benefits of participation (9). Biochemical indicators or tracers of intake offer one possible solution, however even these are not completely unambiguous. For example, a simulation study observed that a staggering variety of consumption patterns might explain given blood levels using a dual-tracer monitoring approach (17). Specifically, tracer levels might just as easily

be produced by excellent compliance in the three days prior to testing as by consistent compliance throughout the study period. Frequent or random biochemical tests may resolve these difficulties, but add expense and complexity to a trial. The gold standard of compliance monitoring is direct observation of each dose by research staff, however this also adds expense, complexity and subject burden to a trial, and is often not practical in long term studies in free-living subjects.

Though difficult, compliance measurement is critical to the interpretation of efficacy in nutritional RCTs, regardless of the mode of analysis. Recall that even the ITT statistic depends on the generalizability of compliance patterns to perform well as an estimate of effectiveness. We therefore recommend that RCTs employ at least two alternate methods of monitoring compliance, in order to triangulate the true dose. The combination of food records or packet counts with some biochemical marker, for example, would allow greater confidence in the validity and reliability of compliance measures.

We have emphasized that the ITT statistic estimates effectiveness when the pattern of noncompliance in the sample is reflective of the general population. Again, in practice, this assumption is sometimes untenable. In fact the subpopulation of individuals willing to volunteer for clinical trials differs in important ways from the population at large (18, 19). They tend to be more health conscious and more likely to comply with treatment prescription. In addition, exclusion of patients with multiple health conditions tends to create a sample that is healthier on average than the population at large. These differences tend to make estimates of treatment effect more conservative, as control participants will have better outcomes on average than a truly random, untreated sample (19). On the whole these problems are inevitable, as research ethics mandate volunteer participation by way of

informed consent. In theory, the ITT statistic as an estimate of effectiveness would be improved if no greater efforts were made to maintain compliance in the study than would be made in the clinic. Naturally both investigators and funding agencies are eager to maintain compliance as much as possible. These caveats should be kept in mind when interpreting the ITT statistic.

In light of these considerations, we propose the following steps for dealing with noncompliance in clinical trials: 1) Noncompliance should be prevented to the greatest extent possible, and then measured. The limitations of methods of monitoring compliance should be explicitly recognized and taken into account when drawing inference. 2) Having accepted some indicator(s) of compliance as roughly accurate, the pattern of noncompliance should be explored and described, as it is an important element of interpretation of the data. 3) Once the pattern of noncompliance is understood, it should be incorporated into the statistical model. In general, the scientific community regards the ITT estimate as the gold standard which should serve as the primary hypothesis test for most clinical trials. Reporting the IV statistic in addition to the ITT can add valuable information regarding the physiologic efficacy of a treatment. 4) Any estimate of treatment effect must be interpreted with appropriate attention to its assumptions and any deviation from them. Departures from assumptions, or disagreement between significant tests of two estimators, leave us in murky statistical territory. This uncomfortable but familiar scenario should be navigated by careful assessment of the pattern of noncompliance, which of course requires adequate measurement.

Appendix B References

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Table B.1. Intent-to-treat (ITT) and Instrumental Variable (IV) statistics for the influence of protein intake on bone health, with and without adjustment for noncompliance[1].

Statistic	Standardized estimate	(95% Confidence Interval)
ITT	0.35	(0.11-0.60)
IV	0.47	(0.14-0.82)

[1] Statistics derived from dummy-coded ordinary least squares (for ITT) and 2 stage least squares (for IV) regression models of the influence of a higher protein relative to a conventional higher carbohydrate weight loss diet on bone mineral density of the total hip in 62 middle aged adults.

Figure B.1. Confounders *w* cause part or all of the relationship between *x* and *y*.

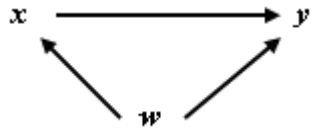


Figure B.2. The effect of *x* on *y* is mediated by dose *d*, which may be confounded.

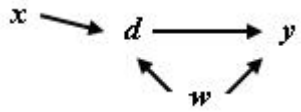


Figure B.3. Distributions of protein intake as a percent of total energy.

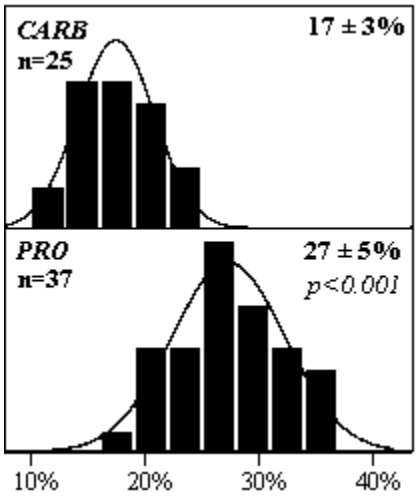
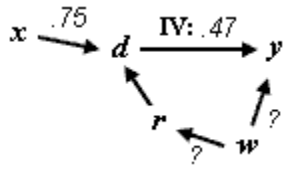


Figure B.4. Schematic of the IV analysis[1].



[1] The ITT statistic, 0.35, can be derived as the product of the IV statistic and the effect of treatment assignment on dose.

Author's Biography

Matt Thorpe was raised in Orangeville, UT, where a traffic jam is three cars behind a tractor and the "Welcome" and "Sorry you're leaving" signs are on the same signpost. Here Matt enjoyed the benefit of several excellent teachers and, more importantly, a family who loved learning and literature. Through high school, Matt was active in drama, speech and debate and student leadership of the Utah chapter of the Technology Student Association, and spent one summer as a page for the U.S. House of Representatives. Though not scientific per se, these experiences turned out to be invaluable training in scientific communication.

Following high school, Matt spent two years in volunteer service as a missionary for the Church of Jesus Christ of Latter-day Saints (Mormons) in Puerto Rico, where he learned to love another culture and to relate to people of diverse backgrounds.

In 2002 Matt began college at Brigham Young University on a full-ride National Merit Scholarship, where he completed dual degrees in Nutritional Science and Family and Human Development. Matt was fortunate to fall into early research training under the mentorship of Randy Day, who put a great deal of faith and investment in undergraduate researchers. Matt completed a senior thesis on the influence of parental involvement on adolescent physical activity and eating behaviors using the National Longitudinal Survey of Adolescent Health. Matt graduated BYU in 2006, *magna cum laude*.

Matt began in the University of Illinois MD/PhD program in Nutritional Sciences in 2006, under the outstanding co-mentorship of Ellen Evans and John Erdman. Here Matt discovered a strong interest in statistics and the philosophy of causal reasoning in science.

Matt is a husband to the beautiful and talented April, and father to an imaginative and curious preschooler, Taylor.