Special Article

Dietary protein and bone health: harmonizing conflicting theories

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A precise understanding of the role of dietary protein in bone health has been evasive despite decades of research. It is known that a dietary acid load is harmful to bone, and sulfur-containing amino acids are metabolized to provide such an acid load. It is also known that protein elevates urine calcium loss. However, recent clinical studies and a meta-analysis have indicated either no effect or a modest benefit associated with higher protein intakes. These contradictory considerations may be explained by the existence of a two-faced relationship between protein and bone, with simultaneous positive and negative pathways. In opposition to the negative effects of dietary acid load, protein may exert positive effects related to improving calcium absorption, increasing insulin-like growth factor 1, or improving lean body mass, which, in turn, improves bone strength. Putative mechanisms behind these pathways are reviewed here, and some limitations in the historical literature as well as suggested measures to counter these in the future are identified. When positive and negative pathways are considered in tandem, protein may offer modest benefits to bone in the presence of adequate dietary calcium and acid-neutralizing fruits and vegetables.

INTRODUCTION

A debate regarding the relationship between dietary protein and bone health has unrolled over more than 50 years. Animal, epidemiological, and clinical works 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 contrast, equally diverse research paradigms support that dietary protein increases calcium absorption or bioavailability,[8–13] casting the net effect of high-protein diets on the calcium economy into doubt. Furthermore, trials have persuasively demonstrated that increased protein intake 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] found no overall effect in observational studies, excepting a modest benefit to bone mineral density of the lumbar spine. However, 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 any negative influence of protein-related dietary acid load is opposed by increased calcium absorption or the 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

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Significant portions of this manuscript have been included in the doctoral dissertation of MP Thorpe. Dissemination of that dissertation outside of the University of Illinois has been embargoed pending scholarly publication.

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Key words: acid base balance, bone, dietary protein, osteoporosis, protein

doi:10.1111/j.1753-4887.2011.00379.x

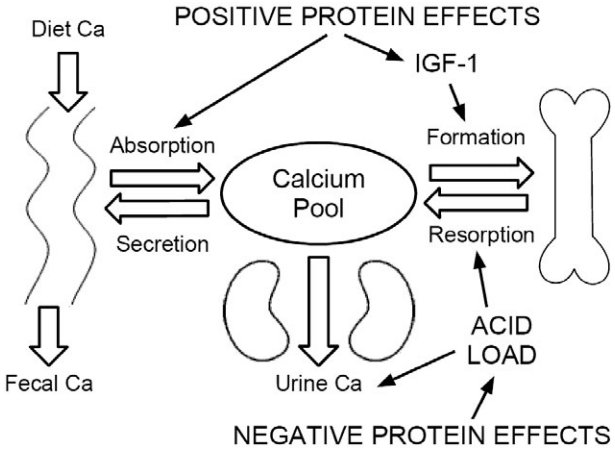

Figure 1 **Pathways for theoretical positive and negative influences of dietary protein on bone health.**

model is attractive in its capacity to harmonize apparently conflicting data, providing a sound theoretical basis to reconcile divergent views (Figure 1).

In this review, the history and current state of the literature on dietary protein in urinary calcium loss, intestinal availability, dietary acid load, and bone turnover are summarized. An attempt is also made to illustrate that none of these factors is sufficient to explain the complex relationship of dietary protein and bone health. Throughout this review, the influence of protein intake at levels above the current RDA of 0.8 g protein/kg body weight is discussed. 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 the scope of this review.

If the competing pathways model is correct, it might predict that additional manipulations of the diet, such as providing ample calcium or acid-neutralizing foods like fruits and vegetables, may "tip the scales"[23] towards 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 input and output.[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 the following equation, which explains 49% of the variation in urinary calcium across 26 studies:

$$\text{Urine Ca, mmoL/d} = (3.208 * 10^{-2}) * \text{protein, g/d} + 1.501 \quad (1)$$

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; this would represent 1.8% loss per year from roughly 1 kg of skeletal calcium in a typical adult.[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 conducted by Lemann et al. in the 1960s[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 the release of buffer from bone tissue, including bicarbonate and phosphate, would accompany the release of calcium observed in the urine, and that an 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 the combustion of food, which corresponds 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, [32–36] including over a time span of up to 36 months.[37]

Dietary protein increases 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.[38] (Eq. 3) developed prediction equations for NAE based on analysis of their own data in addition to a meta-analysis of available, sufficiently detailed reports; the authors concluded that across 20 diets and 141 subjects, NAE increases 1 mEq for each additional 0.91 g protein consumed on average, when holding potassium constant ($r^2 = 0.67$):

$$\text{NAE} = 0.91(\text{Pro, g/d}) - 0.57(\text{K, mEq/d}) + 21 \quad (2)$$

The range of NAE occurring in the 20 diets studied by Frassetto et al.[38] was approximately 15 mEq/d to 115 mEq/d. Accordingly, an increase of mixed protein intake from 60 to 100 g/d, as described before, would be expected to increase the NAE by roughly 36.4 mEq/d, or over one-third of the complete range of NAE encountered across the low to high extremes of realistic diets.

Protein acid load explains protein-induced urine calcium loss

A 1980 study in rats compared the effect of various protein sources on urine calcium and found that the SAA or acidogenic fraction accounted for the effect.[46] To verify, the authors fed sulfate to the rats 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 et al.[51] illustrated that across 25 clinical trials meeting selection criteria, 86% of the average change in urine calcium could be explained by the experimentally manipulated change in NAE. Across the 25 studies reviewed, regression of urine calcium change onto change in the NAE (without discrimination of protocols for acidification of urine) yielded the following:

$$\text{Urine Ca change, mmoL/d} = 0.289 + 0.027(\text{NAE change, mEq/d}) \quad (3)$$

A secondary analysis of the data summarized by Fenton et al.[51] was performed (excluding two extreme values) to test whether the slope of urine calcium change regressed onto NAE differed between protein and non-protein sources of acid. No difference in the slope of protein versus other acid sources was found ($P = 0.37$), suggesting that the rise in urine calcium observed with increasing dietary protein can be completely explained by the dietary acid load of the protein (Figure 2).

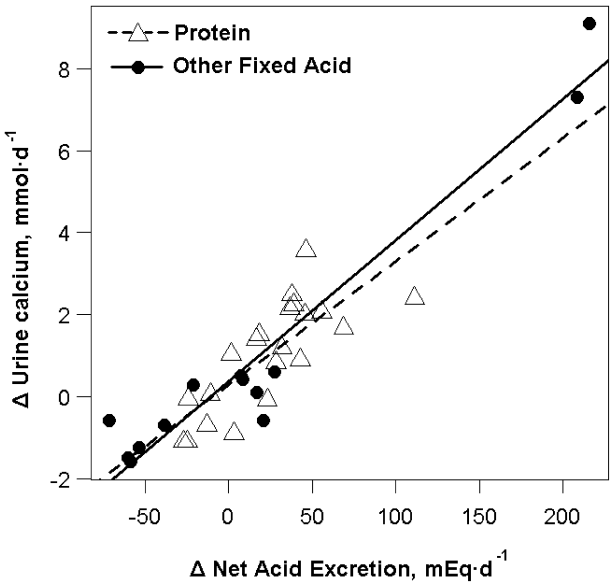

Figure 2 **Elevations of urine calcium with protein intake can be attributed to the dietary acid load of protein. Data from Fenton et al.[51]**

Reinforcing this idea, the authors compared the value of urine calcium predicted from protein intake (using the meta-analysis of Kerstetter et al.,[7] 50 mg/d with a 40 g/d increase in protein, Eq. 1) and that predicted from the combination of protein-induced increase in NAE and NAE-induced urine calcium (using Eq. 2 from Frassetto et al.[38] and Eq. 3 from Fenton et al.,[51] respectively). Again, for a realistic 40-g increase in protein intake, the combination of Eqs. 2 and 3 would predict an increase in NAE of 36.4 mEq and a corresponding increase in urine Ca of 55 mg, values that are well within the confidence limits of the 50 mg predicted by Kerstetter et al.[7] 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 demonstrated 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 transporter 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 might also 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 the 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, which would normally lead to a decrease in urinary calcium excretion, i.e., urine calcium that is increased when statistically holding PTH constant). This suggests protein causes an obligate calcium loss that is not simply a by-product 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 recovered through other pathways.

PROTEIN AND CALCIUM ABSORPTION

Clinical effect of protein on calcium absorption

Over time, several randomized studies have noted improvements in calcium absorption with higher protein intakes[9,57,62,65,66]; however several other studies have seen no such effect.[67–70] This discrepancy has been attributed to inadequacies in older methods of tracing calcium[8,71] compared to modern dual stable isotope methods, though even this approach has not resulted in an observed effect in every study.[70] A well-controlled animal study by Bell et al. in 1975[10] showed that excretion of radiolabeled ^{45}Ca in 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[12] 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-Stomberg et al.[13] 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- versus low-protein animals, which more than compensated for protein-induced urine losses.

Protein effects on calcium absorption in relation to parathyroid hormone and vitamin D

Kerstetter et al.[42,72,73] have demonstrated in humans that protein intakes below 0.9 g/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 1.0 g/kg and 2.1 g/kg, despite improvements in calcium absorption. Similarly, other randomized trials have not observed differences in calcitriol[48] or PTH[53,62,69] with higher compared to normal protein intakes. Conigrave[74] 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 et al.,[7] 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[75] 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.[76] did not directly measure absorption, but observed no changes in bone turnover in spite of increased urinary calcium in patients randomized to a fivefold 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, it is estimated using food composition tables that the aromatic, aliphatic, and polar amino acids shown to most potently regulate the CaR[77] 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 the 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.

Potential masking of protein effects on absorption by alternate homeostatic mechanisms

It is also possible that protein effects on absorption, including the 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% versus 26.0% at lower protein), such that the net calcium balance was only slightly negative. In contrast, high protein levels did not alter calcium absorption in subjects consuming 1,510 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 the calcium absorption fraction in response to total calcium intake, as has been described previously.[78] Specifically, if dietary calcium is sufficiently high to meet requirements, 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,[42,72,73] 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 the protein source.

PROTEIN AND BONE

Regulation of bone cells 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,[30,79,80] which reasonably accounts for the acid gap described by Lemann et al.[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,[81] 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[82] but osteoblasts are strongly inhibited,[83] whereas at pH 7.4, osteoclasts are turned off and osteoblasts demonstrate high expression and activity of alkaline phosphatase.[83] These changes appear to occur through well-developed cell-signaling cascades that are not fully understood but include prostaglandin E2 and stimulation of receptor activator for nuclear factor kappa B ligand.[84–86] These effects are potentiated by tumor necrosis factor α,[87] and nuclear factor of activated T cells 1.[88] 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.[89]

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[4,82] 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 (by Bonjour[22] as well as others) 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[81,90–92] and humans,[93–95] a change greater than 0.014 on the pH scale was not observed in our review of the literature.[94]

In spite of the small effect of diet acid load on blood pH, this effect may still be clinically meaningful. Arnett[82] notes that changes in pH on the order of 0.05 can double resorption pit formation. Additional studies by Sebastian and Maurer[32,96] illustrate that supplementation of alkali to neutralize acid equivalents results in suppression of bone turnover. 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,[82] 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.[97]

In rats, NH_4^+ loading causes an increase in serum ionized calcium, even in parathyroidectomized animals.[98] 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[99] also observed increasing ionized calcium in serum in response to acute acid loading. This effect has been reported in one trial of high protein intake[22]; however, most studies have not seen a change in serum calcium with high protein intakes.[53,66,100]

Effect of diet acid load on bone health

In animals, the finding that feeding acid ash damages bone over time has been reasonably consistent,[91,101,102] though not in all studies.[103] Barzel[104] 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 et al.[105] 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 the administration of alkaline supplement.[106] 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 1 year of therapy with potassium citrate. A more recent trial[108] indicates supplementation with base may independently promote calcium absorption.

Epidemiology supports the notion that a high acid load in the diet 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 greater alkaline contribution) for potassium, magnesium, and calcium.[109] The estimated NEAP and PRAL are adversely correlated with bone density[110,111] and broadband ultrasound attenuation of the heel,[112,113] as well as bone turnover markers,[110,111] though not in all studies.[114]

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.[19,115] 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,[116] epidemiological,[117] and clinical[118] investigations; however, positive associations have been much more frequently reported in cross-sectional studies.[119–125] Hannan et al.[126] 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 notice-

able effect of protein intake on change in bone density over time,[124,127,128] which suggests 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. From a study with a large sample ($n = 8,178$), Weikert et al.[129] reported that while vegetable protein positively predicted broadband ultrasound attenuation of the heel, animal protein was inversely associated. Similarly, Beasley et al.[127] reported that low intake of vegetable protein in young women corresponded to lower bone density of the spine, while no association was observed for animal or total protein. In contrast, Promislow et al.[130] reported 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[20] 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 100 g 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 that 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 as well as 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 an 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[131] and postmenopausal women.[132] The possibility of a beneficial interaction of protein with calcium has been emphasized by Dawson-Hughes et al.[133] In a nested cohort investigation of 342 men and women randomized to calcium and vitamin D supplementation, the researchers 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.[134] reported a positive correlation between protein intake and bone accrual over approximately11.4 years in growing adolescents, and an augmentation of that benefit when calcium intake exceeded 1 g/d. A recent study[135] observed an increase in the risk of fracture at high versus low quartiles of animal protein intake, but this association evaporated when calcium intake was high.

Protein intake and bone turnover

The effects of higher protein intakes on bone turnover markers are more equivocal. Allen et al.[69] 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 et al.[136] also found no effect on bone metabolism markers in response to 12% versus 20% energy from protein, with meat accounting for the difference. Dawson-Hughes et al.[76] also failed to show any effect of aromatic amino acids compared to branched-chain amino acids on turnover markers.

Hunt et al.[62] reports a beneficial uncoupling of turnover given 20% versus 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 et al.[137] found that relative to 0.7 g/kg protein, 2.1 g/kg increased N-telopeptide (another marker of bone degradation) and decreased bone-specific alkaline phosphatase. Reddy et al.[138] 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,[139] 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 (as reviewed by Wengreen et al.[142]); however, this is not always the case. Turnover is related to fracture risk along a J-shaped curve,[139] such that both low and high levels may be detrimental. For example, within the present review, protein or energy deficiency was found to cause a depression of both formation and resorption markers, which may remain well coupled, as in two studies by Mardon et al.[140,141] Despite

decreased turnover, the long-term impact of such deficiency is known to be harmful.[22,142–144] Conversely, increases in non-extreme exercise can elevate formation and resorption in tandem, yet produce improvements in bone health over time, as in the studies of Karlsson et al.[145] and Wallace et al.[146] Accordingly, the long-term impact on bone health of a well-coupled increase in turnover depends on the underlying cause of the turnover changes. The long-term consequence of protein-induced turnover changes are not yet understood and probably differ when comparing high-to-adequate versus adequate-to-deficient intakes. Given the unambiguous link between protein and acid load[38] and between acid load and bone resorption,[104] the absence of a consistent negative effect of protein reemphasizes that protein may influence bone turnover in multiple and opposing ways.

Protein intake and fracture

Fracture rates have been reported to increase[147–150] and decrease[142,151,152] in relation 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 a 12-year period) observed no association between protein intake and hip fracture, but even this tremendous sample size yielded only 234 incident hip fractures.[150] The same study did observe a linear increase in wrist fracture with protein intake, with a 22% increase in risk at higher intake levels (>95 g/d) compared to lower intake levels (<68 g/d). This association was attributable to animal, and not vegetable, protein, which is consistent with the acid-ash hypothesis. A similar increase in fracture incidence with high animal-protein intake was observed by Dargent-Molina et al.,[135] but fracture risk was not elevated when calcium intake was high. Darling et al.[17] summarized the available data for hip fracture, finding no association of protein intake in a meta-analysis weighted by sample size. This was true for total, animal, and vegetable protein, but only three studies were included in each analysis.

Heaney and Rafferty[153] have recently discussed the standard of "preponderance of evidence" in drawing conclusions from conflicting reports. 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 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 the available literature on these outcomes, causing significant findings in both directions to be over-reported with respect to null results. Alternatively, as introduced previously, various researchers[18,19,21,154] have supported a competing pathway model, in which dietary protein may cause an increase or a 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 a 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

IGF-1 has been shown to hold predictive value in osteoporosis in older individuals[155] and in bone accrual in young males.[156] 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.[157] Dietary protein is known to modify both IGF-1 and some of its binding proteins[158]; it has therefore been an attractive candidate as a mediator of protein's influence on bone.[22,61,154,159]

Schurch et al.[160] 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 recently reported[161] 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. However, these patients were initially somewhat protein deficient, and it is not uniformly supported that additional protein, above the threshold of adequate intake, increases IGF-1.[162,163]

Three randomized trials implicate protein intakes above the RDA as relevant to IGF-1 regulation. Ballard et al.[164] 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 intake levels by Hunt et al.[62] discovered improved IGF-1 with protein, independently of calcium intake. Two studies by Dawson-Hughes et al.[14,76] shed additional light. The first shows an increase in IGF-1 and a 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 was observed with branched-chain amino acid supplementation.

IGF-1 may also link protein nutrition to muscle mass.[158] It has been proposed elsewhere[165] that documented increases in lean body mass in response to the dietary protein level[166,167] 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.[168]

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.[63,169–175] The primary difficulty in interpreting studies 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 the enrichment of fruits and vegetables in the diet. Zerwekh et al.[116] illustrated in rats the ability of supplemental base, potassium citrate, to ameliorate the osteomalacia induced by a high-casein diet, while potassium chloride 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,[32,35,36,96,176–179] 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.[35,36,176,177] This effect is also achieved with alkaline, compared to relatively more acidogenic mineral water.[178] 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.[179]

Taken together, observational studies 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, it is proposed 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.[180] In epidemiological studies, opposing effects may be investigated using mediational or path analysis.[181] 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.[181] In experiments, appropriate controls should be incorporated to isolate potentially conflicting effects.

Estimation of the diet acid load in bone studies

Fenton et al.[182] 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 the result refutes the acid-ash hypothesis, since phosphate is included in those dietary components thought 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.[183,184] In 1981, Linkswiler et al.[185] reviewed consistent evidence that phosphorus intake depressed urine calcium loss. Heaney et al.[186–188] have since shown repeatedly 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] a negative influence was observed of diet acid load from SAA on bone density of the spine after adjusting for total dietary protein. No similar association was observed 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.[169,170,189,190] Similarly, estimated NEAP[38] accurately predicts NAE but does not account for possible positive roles of protein independent 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 three, rather than two levels of intake where possible: one level that is marginally deficient, one level that is sufficiently high to preclude frank deficiency, and one that is higher to explore the impact of protein above adequate levels. For example, the intakes 0.7 g/kg, 1.0 g/kg, and 2.1 g/kg were used by Kerstetter et al.[57,73,137] and resulted in more decisive interpretation. Where three parallel arms are not feasible, care should be taken to unambiguously compare high with adequate, or adequate with deficient protein intakes, as the comparison between high and deficient intakes is not useful in evaluating the possibility of harm to bone of higher protein intakes.

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), since reporting only percent 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., by Eastell et al.[191]) and are helpful in evaluating the likely net effect of changes in turnover to bone health. The authors recommend reporting uncoupling as the ratio of percent change from baseline for formation and resorption markers, as follows:

$$Uncoupling\ Ratio = \frac{\left(\begin{matrix} end\ formation - \\ baseline\ formation \end{matrix}\right)}{baseline\ formation} \Big/ \frac{\left(\begin{matrix} end\ resorption - \\ baseline\ resorption \end{matrix}\right)}{baseline\ resorption} \quad (4)$$

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.

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,[192–194] 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). While 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.[195] Applying such a model for the possible interaction of protein and calcium could be done as follows, with BMD representing bone mineral density:

$$\begin{aligned} BMD = {} & B_0 + B_1(protein) + B_2(calcium) + \\ & B_3(protein * calcium) \end{aligned} \quad (5)$$

This model avoids the disadvantages outlined above but still provides all the information afforded by a ratio and is more easily interpreted. For a non-technical discussion of dealing with interaction in regression, see the publication of Aiken and West.[195]

CONCLUSION

The present review finds ample evidence that dietary protein may have both positive and negative effects on bone. In many scenarios, these opposing effects may cancel one another out, thus explaining the lack of a definitive result in the meta-analysis performed by Darling et al.[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 the positive and negative effects. This may permit the benefits of dietary protein to bone to be enjoyed without contradictory adverse effects.

Acknowledgment

Declaration of interest. The authors have no relevant interest to declare.

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