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Dietary Protein and Bone Health

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1. Introduction

Dietary proteins represent 10 to 20% of energy consumption. The recommended daily minimum intake of protein and amino acids in adults is 0.8 g per kg of body weight. However, no upper limit has been identified. In industrialized countries, the main sources of protein are milk, eggs and meat. The nutritional value of protein is influenced by several factors, especially the amino acid (AA) composition, protein digestibility, protein digestion kinetics and the ability to transfer AA for protein synthesis. Diets based on either animal or vegetable products supply proteins of differing quality in differing quantities. Plant proteins are often deficient or low in some specific indispensable AAs. Soy protein is reported as a "complete" protein but its overall indispensable AA content is relatively low (~85% lower than milk) (Wilson & Wilson, 2006).

Epidemiological data support a positive association between protein intake and bone health. Protein is the precursor of AAs used for bone matrix protein synthesis. Moreover, studies evaluating the relationship between dietary protein and bone turnover support the hypothesis that high protein intake may decrease bone resorption. IGF-1 is a key mediator of bone growth (Geusens & Boonen, 2002) and dietary protein is an important regulator of circulating IGF-1 levels (Bonjour *et al.*, 2001). However, protein is also known to be calciuric, though the origin of the increased calcium excretion is debated. According to the acid ash hypothesis, the protein-induced acid load would have a deleterious effect on bone (Barzel & Massey, 1998). Finally, some proteins, due to their amino acid content, have specific effects on bone metabolism by acting directly on bone cells or through indirect pathways.

Even if the effect of nutrition on bone is not as dramatic as that of pharmaceuticals, it can be of great help when considering bone loss. Indeed nutrition is a lower-cost, longer-term, higher-compliance and wider-spread approach than pharmaceutical treatment. The nutritional intervention can be done alone preventively or in combination with a therapy in more severe cases. Some nutritional components such as calcium and vitamin D are recognized to have a positive effect on bone. However, the effect of protein on bone health is much more debated.

This chapter reviews the literature on the subject, giving an overview of the data available and comparing the proposed mechanisms of action. Attention is drawn to protein quality and to the specific effect of some soy, collagen and milk peptides on bone metabolism.

2. Action of protein on bone

The mechanism underlying the debate on the effect of protein on bone is the hormonal anabolic effect. It is based on the increase of bone anabolic hormone IGF-1 with the consumption of protein.

2.1 Bone protein metabolism and turnover

Type I collagen is the major structural protein distributed throughout the whole body accounting for 25% of total body protein and for 80% of total conjunctive tissue in humans. The different factors involved in bone strength include not only mineral density, crystal characteristics, micro architecture, geometry and morphology (size and shape), but also protein matrix and collagen fiber quality. Cortical bone strength, whose main role is to protect bone integrity, is influenced not only by porosity, presence of micro damage and mineral composition, but also by the orientation of collagen fibers, extent and nature of collagen cross-linking and number and composition of cement lines (Burr, 2002; Currey, 2001; Seeman & Delmas, 2006; Turner, 2006). Collagen is an important component of bone, being the main extra cellular matrix protein for calcification and playing a role in osteoblast differentiation (Takeuchi *et al.*, 1996, 1997).

Markers of bone resorption, which measure the release of peptide resulting of the degradation of mature modified type 1 collagen into the serum and urine, are commonly used to evaluate the relationship between dietary protein and bone turnover. However, to better understand the effect of feeding on bone collagen turnover, a better knowledge of the physiology of bone collagen synthesis is needed (Babraj Smith 2005).

2.2 Hormonal anabolic effect

Protein has an anabolic effect on bone metabolism by upregulating Insulin-like Growth Factor 1 (IGF-1). IGF-1 is a peptide hormone mainly produced by the liver under the action of the Growth Hormone. A small percentage of IGF-1 is also produced locally by different cell types throughout the body, including osteoblasts (Ohlsson *et al.*, 2009). There is good evidence of the anabolic effect of IGF-1 on bone and muscle, especially when considering age-related disorders (Perrini *et al.*, 2010). Studies measuring IGF-1 levels under different protein intakes consistently report that a high protein intake increases IGF-1. This fact has been shown over a six-month period in young exercising subjects (Ballard *et al.*, 2005) and evidence also exists in older populations of both sexes (Arjmandi *et al.*, 2005; Dawson-Hughes *et al.*, 2004; Hunt *et al.*, 2009).

There is some evidence that this hormonal response is based on the quality of the protein ingested. Dawson-Hughes and colleagues showed that a fivefold increase of aromatic AA intake (phenylalanine and histidine) for 24 days induced an increase of IGF-1 level. On the other hand, a fivefold increase of branched chain AA (leucine and isoleuine) induced no effect on IGF-1, indicating that the protein intake effect on IGF-1 is probably AA-dependent (Dawson-Hughes *et al.*, 2007). Moreover, two studies comparing milk and soy protein reported that soy induces a greater increase of IGF-1 (Arjmandi *et al.*, 2003; Khalil *et al.*, 2002). However, it is important to note that the soy protein contained isoflavone in both studies, thus the effect on IGF-1 could be attributed to this molecule rather than to the AA content.

The effect of protein intake on IGF-1 level could involve Calcium-Sensing Receptor (CaSR) which was also reported to be implicated in the calciuric effect of protein (Conigrave *et al.*, 2008). *In vitro* investigations showed that CaSR is expressed in hepatocytes (Canaff *et al.*, 2001). The activation of CaSR in the liver by AA could explain the increased IGF-1 level due to protein consumption. Moreover, osteoblasts also express CaSR and produce IGF-1 under its regulation. CaSR also upregulates numerous bone forming mechanisms and inhibits bone resorption (Marie, 2010). Aromatic AA supplementation induces an increase in IGF-1 while branched chain AAs have no effect (Dawson-Hughes *et al.*, 2007). This is consistent with the findings of Conigrave *et al.*, that aromatic AAs are the most potent activators of CaSR, while branched chain AAs are the less potent ones (Conigrave *et al.*, 2000).

There is no doubt that protein induces an increase of IGF-1 level. By doing so and according to what is known of IGF-1, protein is likely to have an anabolic effect on bone. The proposed mechanism would be that a high protein intake (especially one rich in aromatic AAs) would activate CaSR in hepatocytes and osteoblasts and stimulate IGF-1 production, which would in turn exert an anabolic effect on bone.

2.3 Bone turnover markers

Bone turnover markers are measured in blood or urine; they give information on bone formation or on bone resorption. Both types of markers are needed in a study in order to assess bone turnover.

The recent meta-analysis of Darling *et al.* concludes that there is no clear evidence of an effect of protein intake on bone markers (Darling *et al.*, 2009). Some studies reported an increase in bone resorption while bone formation remained stable (Kerstetter *et al.*, 1999; Roughead *et al.*, 2003; Zwart *et al.*, 2005). However, in one of them, the authors report an increase of hydroxyproline (a bone resorption marker) that could be related to the protein source: a collagen-rich meat (Roughead *et al.*, 2003). On the other hand, three studies considering both formation and resorption markers concluded that there was a positive effect of protein on bone. One of them observed a decrease of desoxypyridinoline (a bone resorption marker) with stable formation, indicating a positive decoupling (Hunt *et al.*, 2009). In the second study, both formation and resorption markers increased in a protein-supplemented group compared to control during 6 months of exercise. According to the authors, bone formation will increase faster than bone resorption over time (Ballard *et al.*, 2005). In the final study, a one year soy protein supplementation only increased bone formation markers while resorption markers remained at the same level (Arjmandi *et al.*, 2005). Finally, two intervention studies reported a decrease of resorption with protein supplementation but provided no data on bone formation (Dawson-Hughes *et al.*, 2004; Ince *et al.*, 2004).

3. Protein and calcium metabolism

3.1 Urine calcium

Urinary calcium excretion is the major pathway for body calcium loss, along with that of feces and sweat. A high urinary calcium level has been linked to increased bone loss (Giannini *et al.*, 2003).

Numerous studies have reported a positive linear relationship between dietary protein and urinary calcium (Kerstetter & Allen, 1990; Kerstetter *et al.*, 2003; Whiting *et al.*, 1997). The rise in urinary calcium with protein intake has also been observed in more recent studies (Ceglia *et al.*, 2009; Hunt *et al.*, 2009; Jajoo *et al.*, 2006). In one study, no effect of protein on calciuria was observed (Roughead *et al.*, 2003). The mechanism underlying the effect of protein on calcium excretion is debated. First, a high protein diet induces an increase of glomerular filtration rate which will exacerbate any increased calcium excretion (Kerstetter *et al.*, 1998). According to the acid ash hypothesis, the calciuric effect of a high protein intake would be caused by the protein-induced acid load. A meta-analysis by Fenton *et al.* of over 25 studies concluded that changes in acid excretion modulate calcium excretion (Fenton *et al.*, 2008). Other studies showed that urinary calcium could be reduced by decreasing the acid load (L. Frassetto *et al.*, 2005; Lemann *et al.*, 1991). The systemic acidosis generated by the protein load inhibits TRPV5 calcium channel expression in the kidney. Because TRPV5 participates in renal calcium reabsorption, its inhibition increases urinary calcium (Nijenhuis *et al.*, 2006).

Another mechanism for the protein-induced calcium excretion was proposed by Conigrave and colleagues, involving the extracellular CaSR (Conigrave *et al.*, 2008). CaSR found on cells of the parathyroid glands and in the renal tubules are sensitive not only to calcium, but also to some AAs (Conigrave *et al.*, 2000). Through this double sensitivity, a link is formed between protein and calcium metabolism. According to these findings, AAs would activate the CaSRs, reducing parathormone (PTH) production and decreasing renal calcium reabsorption, leading eventually to an increased calcium excretion (Conigrave *et al.*, 2008). Indeed, PTH level has been shown to be influenced by protein intake (Kerstetter *et al.*, 1997, 1998, 2006).

Urinary calcium loss due to a high protein diet was attributed to enhanced bone resorption (Kerstetter & Allen, 1990); however, this view has been discussed as the link between protein and bone resorption is unclear (Bonjour, 2005; M. Thorpe & Evans, 2011). Urinary calcium excretion is mainly related to bone as the main calcium storage compartment. However, an increase of the excreted calcium could also be related to an increase in calcium absorption through the diet. Hence the effect of protein on intestinal calcium absorption should also be considered.

3.2 Calcium absorption

While many studies claim that high-protein diets cause calciuria, there is no clear relationship between protein intake and calcium absorption. Some studies report a positive effect of protein on calcium absorption (Cao *et al.*, 2011; Hunt *et al.*, 2009; Kerstetter *et al.*, 1998, 2005). One study of 13 women observed an increase in calcium absorption that explained 93% of the calcium excretion (Kerstetter *et al.*, 2005). Another study reported an increased calciuria and a non-significant increase in calcium absorption with protein intake. But the net difference between calcium absorption and excretion with high or low protein intake remained stable (Cao *et al.*, 2011). However, other studies observed a decrease of calcium absorption with protein (Dawson-Hughes & Harris, 2002; Heaney, 2000), or no effect at all (Roughead *et al.*, 2003). It should be pointed out that all studies showing a positive effect were short term studies (2 to 7 weeks) whereas the two studies with a negative effect were long-term studies (years). It is thus possible that protein increases calcium absorption at first and that this effect is reversed in the long run.

The effect of protein on calcium absorption could also be conditioned by the calcium intake itself. Two studies tested the effect of protein intake on intestinal calcium absorption at different levels of calcium intake. One observed a positive effect of protein for 675 mg Ca/d and no effect at 1510 mg Ca/d (Hunt *et al.*, 2009) whereas an other study observed a negative effect at 871 mg/d and no effect at 1346 mg/d.

Moreover, Kerstetter et al. repeatedly showed that, in the context of low protein intake, there is both an increase of PTH and a decrease in calcium absorption (Kerstetter *et al.*, 1997, 1998, 2006). Those two findings seem incompatible because PTH is known to increase calcium absorption (Heaney, 2007). However, Conigrave *et al.*, as a part of his theory on amino acid-sensing CaSR, proposes another mechanism not involving PTH. CaSR is also present in the gastro-intestinal tract, where it promotes gastric acid secretion directly and indirectly through gastrin release (Conigrave & Brown, 2006). High-protein diets induce the acidification of the intestinal content which helps calcium salts to dissolve into Ca^{2+}, thereby facilitating its absorption even if PTH level is low (Conigrave *et al.*, 2008).

3.3 The acid-ash theory

According to the acid-ash theory, some foods such as protein would induce a metabolic acidosis whereas other foods such as vegetables and fruits would counteract this process. The acidosis resulting from a high protein diet would be deleterious to bone and induce bone loss.

Dietary proteins, and more specifically their sulphur AA content, are part of the acid-forming nutrients. Consequently, high protein consumption would lead to metabolic acidosis (Frassetto *et al.*, 1998; Kerstetter *et al.*, 2006). pH is closely regulated in the body and any increase of the acid load is buffered through different pathways. The main one is through lung excretion of CO_2, followed by kidney acid excretion. Bone has also been proposed to take part by releasing Ca^{2+} (Lemann *et al.*, 2003). This last pathway is debated because the lungs' and the kidneys' buffering capacity are considered to be sufficient to compensate for the protein-induced acidosis (Bonjour, 2005). The idea that an acidic diet is deleterious for bone is supported by observational studies linking acid production to a reduced BMD (New *et al.*, 2004; Rahbar *et al.*, 2009; Wynn *et al.*, 2008). Intervention studies on this topic usually correct the acid diet of the subjects by supplementing them with alkalinizing molecules. The results consistently show an improvement of bone health with the supplementation. This improvement occurs at the level of urinary calcium excretion (Frassetto *et al.*, 2005; Lemann *et al.*, 1991), bone turnover markers (Dawson-Hughes *et al.*, 2009; He *et al.*, 2010; Maurer *et al.*, 2003) or Bone Mineral Density (BMD) (Domrongkitchaiporn *et al.*, 2002). Despite all this evidence of the beneficial effect of alkalinizing the diet, it should be noted that according to a recent meta-analysis, no causal link could be established between dietary acid load and bone disease (Fenton *et al.*, 2011). It is also important to consider that the findings supporting the acid-ash hypothesis are based on an alkalinisation of the diet and are not directly related to protein intake.

A decrease of pH is probably one of the main activators of bone-resorbing osteoclasts and an inhibitor of bone matrix deposition by osteoblasts, which explains the acid-induced bone reabsorption (Arnett, 2008). Extrapolation of these results to the clinical level is risky as it compares the *in vitro* pH variation to a high protein-induced acidosis. Indeed, the pH

variation induced by protein intake is likely to be too small to induce any effect in the bone micro-environment (Bonjour, 2005).

Although they lead to different conclusions, the acid-ash theory and the hormonal anabolic theory are not mutually exclusive. A dual-pathway model for the effect of protein on bone has been proposed (M. Thorpe & Evans, 2011). Although it has been recently criticized in a meta-analysis (Fenton *et al.*, 2011), it seems that there is a beneficial effect of diet alkalinisation. This can be achieved by lowering acidic or by increasing alkaline food consumption. When considering protein, the second method is more beneficial. Indeed, even if protein is an acid nutrient, it is also an activator of bone anabolic hormones and its consumption should not be lowered. This is especially true in populations already consuming a low protein diet such as the elderly. The acid load resulting from the protein consumption should be compensated by an increased consumption of alkalinizing fruits and vegetables.

Some diets designed to promote weight loss rely on high protein consumption for a quick effect on weight. It is hard to estimate what is the effect of such diets on bone. Indeed, hyperproteic diets imply also potentially confounding effect such as spontaneous caloric restriction and a possible lack of micronutrients. Moreover, the wide variety of hyperproteic diets and the potential lack of compliance of the subjects further increase the difficulty of designing an adequate study on the subject.

4. Protein and bone parameters

4.1 Bone Mineral Density

BMD is not the only determinant of skeletal fragility; the spatial distribution of the bone mass (as cortical and trabecular bone) and the intrinsic material properties of bone are also major components (Bouxsein & Seeman, 2009). Measuring BMD or Bone Mineral Content (BMC) is the easiest way of directly assessing bone strength in humans.

Most observation studies report a positive association between protein and bone density (Hannan *et al.*, 2000; Promislow *et al.*, 2002) or between protein and BMD change (Dawson-Hughes & Harris, 2002; Vatanparast *et al.*, 2007). Specific studies showing a positive correlation between protein and bone density cover a broad population: premenopausal women (Teegarden *et al.*, 1998), postmenopausal women (Devine *et al.*, 2005; Ilich *et al.*, 2003; Rapuri *et al.*, 2003; M. Thorpe *et al.*, 2008b), men (Whiting *et al.*, 2002) and children (Alexy *et al.*, 2005; Chevalley *et al.*, 2008).

On the other hand, two studies on premenopausal women concluded that protein intake had no relation with BMD (Beasley *et al.*, 2010; Mazess & Barden, 1991). One study reported a negative association with BMC (Metz *et al.*, 1993). Finally, a case-control study compared 134 osteoporotic women with 137 controls and identified total protein intake as a risk factor for osteoporosis. A meta-analysis by Darling and colleagues conclude that there is no evidence of a negative effect of protein intake on bone when looking at observation studies; in fact there is probably a small positive effect of protein on bone (Darling *et al.*, 2009).

There are very few interventional studies comparing high vs. low protein intake. One of them focused on the effect of an AA and carbohydrate supplement during bed rest on 13

men. The study lasted 28 days and the authors reported a decrease in BMC in the supplemented subjects whereas BMC in control subjects remained stable (Zwart *et al.*, 2005). It should be noted that energy intake was different between the two groups due to an energy-free placebo. Another study supplemented hospitalized elderly men and women for 38 days with 20.4g of protein. Along with other positive effects (lower complication rate, shorter hospital stay), the authors reported a decrease in BMD loss with the protein supplement (Tkatch *et al.*, 1992). Finally, a year-long study supplementing 62 postmenopausal women with 25g of soy protein observed no effect on total hip BMD or BMC (Arjmandi *et al.*, 2005).

4.2 Fracture risk

Fracture risk is the ultimate clinical outcome when considering bone. A longitudinal study of 32 050 postmenopausal women observed a reduced relative risk of hip fracture when protein intake was increased (Munger *et al.*, 1999). Another one observed that a reduction in wrist fracture is positively associated with the consumption frequency of high protein food in 1865 perimenopausal women (D. L. Thorpe *et al.*, 2008a). Finally, in a case-control study on 1167 cases of hip fractures and 1334 controls of both sexes aged 50-89, the odds ratio for hip fracture decreased with the increase of protein intake (Wengreen *et al.*, 2004).

According to these three studies, protein would have a beneficial effect on bone, but some other studies found conflicting results (Dargent-Molina *et al.*, 2008; Feskanich *et al.*, 1996; Meyer *et al.*, 1997). However, it should be noted that the negative relationship between protein and fracture risk was significant only in the lowest quartiles of calcium intake (<400 mg/d) and that no association was observed for higher calcium intake (Dargent-Molina *et al.*, 2008; Meyer *et al.*, 1997). Pooling four studies in a meta-analysis, Darling found no significant effect of protein on the risk ratio of fracture (Darling *et al.*, 2009). To our knowledge, it seems that as long as calcium intake is sufficient there is a protective effect of protein on bone that lowers the fracture risk.

The question of the effect of protein on fracture healing has also been addressed in some randomised controlled trials. All four trials were conducted by the same group and reported that a protein supplement improved the patients' condition after a low-trauma femoral neck fracture (Delmi *et al.*, 1990; Tkatch *et al.*, 1992) or hip fracture (Chevalley *et al.*, 2008; Schurch *et al.*, 1998). All the clinical trials had very similar design: patients were about 80 years old and the protein supplement given was 20g. According to the two studies on femoral neck fracture, patients taking the protein supplementation had better clinical outcomes and reduced rate of complication and mortality during the hospital stay and 6 months after (Delmi *et al.*, 1990; Tkatch *et al.*, 1992). After hip fracture, the protein supplement increased IGF-1, attenuating bone loss at 6 months and shortening hospital stay (Schurch *et al.*, 1998). The IGF-1 increase is independent of the type of protein given (casein, whey protein or whey protein and amino acids) (Chevalley *et al.*, 2010). The beneficial effect of protein is probably linked to both the increased IGF-1 level and the correction of the patient nutritional state.

5. Effect of specific protein source on bone

Each protein bares some information in the form of its chain of amino acids. This information can relate to specific effects of some proteins. When protein goes through the

intestinal barrier, 50% are completely degraded as simple amino acids, 40% are partly degraded as peptides and 10% remain intact (Mallegol *et al.*, 2005). This observation means that half of the digested protein reaching blood is still bearing some information in the form of the AA chain. Much research has tried to investigate what might be the best source of protein for bone health.

5.1 Animal vs. vegetable protein

Some observational studies considered the nature of protein source when measuring the effect on bone. One of them studied the relationships between the animal/vegetable protein ratio and bone parameters on 1035 postmenopausal women, showing that the ratio is positively associated with bone loss and hip fracture risk (Sellmeyer *et al.*, 2001). However, the fact that a ratio was used in this study instead of the absolute values has been criticized (Bonjour, 2005; M. Thorpe & Evans, 2011). Other observational studies provide conflicting results. BMD was positively associated with animal protein intake and negatively to vegetable intake in one study (Promislow *et al.*, 2002) but the opposite association was found when considering bone ultrasound attenuation (Weikert *et al.*, 2005). Low levels of both protein types have been associated with deleterious effects on bone: low animal protein is related to bone loss (Hannan *et al.*, 2000) and low vegetable protein is related to low BMD (Beasley *et al.*, 2010). These results suggest that a minimum intake of both proteins is required regardless of the source. Finally, when considering fracture risk, a positive relationship was found with both animal (Dargent-Molina *et al.*, 2008; Feskanich *et al.*, 1996; Meyer *et al.*, 1997) and vegetable protein (Munger *et al.*, 1999; D. L. Thorpe *et al.*, 2008a). It should be noted that the studies finding a positive relationship between animal protein and fracture risk also found a positive relationship with total protein. On the other hand, those finding a positive relationship between vegetable protein and fracture risk reported a negative one with total protein. Hence the results for animal and vegetable protein are not obtained in the same context.

The intervention studies comparing animal and vegetable protein always focus on specific types of protein, usually soy and casein. Hence it is the specific effect of those proteins that is evaluated and not the one of the animal and vegetable food groups. The only whole-diet intervention study was based on meat or protein-rich vegetables such as nuts and grains. Only urinary parameters were measured and urinary calcium was similar for the two diets (Massey & Kynast-Gales, 2001). More studies of this type are needed to address this issue.

Mechanistically, the reason for differentiating animal from vegetable protein is their differing sulphur AA content and the consequent modulation of potential acid load (Sellmeyer *et al.*, 2001). The influence of sulphate content on protein efficiency has been emphasized in a cross-sectional study on 161 postmenopausal women. The results show a positive association of total protein intake with lumbar spine and total hip BMD; however, in lumbar spine this benefit is suppressed by the sulphur-containing AAs (M. Thorpe *et al.*, 2008b). The authors conclude that an excessive consumption of sulphur AAs is likely to be deleterious for bone. However, as underlined by Massey, the assumption that animal protein contains more sulphur AA than vegetable protein is not always true. As an example, potential acidity from sulphur AA in milk or beef is lower than that of whole wheat or white rice (Massey, 2003). Hence, even if sulphur AAs were proven to have a negative effect on bone, the extrapolation to animal and vegetable food groups is likely to give incorrect

results because of the broad variety of foods in these groups. In future research, it would be more accurate to consider an estimation of the sulphate content of each protein-containing food rather than focusing only on a crude animal-vegetable distinction.

5.2 Milk proteins

Milk contains many bioactive factors, including growth hormones, enzymes, antimicrobials, anti-inflammatory agents, transporters and peptide or nonpeptide hormones. Milk, because it contains bioactive molecules, extends beyond applications in infant nutrition and was considered as a possible source of factors with anabolic effects on bone.

5.2.1 Milk Basic Proteins (MBP)

Clinical trials showed that MBP supplementation increased BMD and decreased bone resorption biomarkers in healthy women (Aoe *et al.*, 2001; Uenishi *et al.*, 2007; Yamamura *et al.*, 2002), menopausal women (Aoe *et al.*, 2005), and healthy older women (Aoyagi *et al.*, 2010). In particular, MBP suppresses osteoclast-mediated bone resorption and leads to reduced osteoclast number in animal studies (Morita *et al.*, 2008). Morita *et al*. reported that the protein fraction responsible for the observed activities of MBP is the bovine angiogenin which acts as a bone resorption-inhibitory protein (Morita *et al.*, 2008).

However, not all studies have shown a beneficial effect of MBP. In one study, 84 healthy young women were divided into three groups receiving placebo, whole milk, or milk containing 40 mg MBP for 8 months. Compared with the baseline values, total BMD significantly increased in all groups. There was a significant decrease of bone resorption marker N-teleopeptides of type-I collagen (NTx) while bone formation remained stable in both milk groups. (Zou *et al.*, 2009).

HPLC analysis of the major proteins in the MBP fraction identified the presence of the glycoprotein lactoferrin in most of these fractions (Naot *et al.*, 2005).

5.2.2 Lactoferrin

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein of the transferrin family. This molecule has been demonstrated to inhibit *in vitro* osteoclast-mediated bone resorption (Lorget *et al.*, 2002). LF was also demonstrated to have *in vitro* anabolic, differentiating and anti-apoptotic effects on osteoblasts, and to inhibit osteoclastogenesis. Moreover *in vivo* local injection of LF above the hemicalvaria increases bone formation and bone area in adult mice (Cornish *et al.*, 2004). LF has a role in host non-specific defense (Gahr *et al.*, 1991; Legrand *et al.*, 2004). In addition to its direct antimicrobial effects, LF is believed to modulate the inflammatory process mainly by preventing the release of inflammatory cytokines which induce recruitment and activation of immune cells at inflammatory sites (Legrand *et al.*, 2005).

Investigations of our group and others established that LF at physiological concentrations can stimulate proliferation of primary osteoblasts and osteoblastic-cell lines and increase osteoblast differentiation (Blais *et al.*, 2009; Cornish, 2004; Takayama & Mizumachi, 2008). Studies using 3-week culture of primary rat osteoblasts show that LF dose-dependently increases the number of nodules and the area of mineralized bone formed (Cornish, 2004). Our *in vitro* experiments demonstrated that LF could directly act on bone cells. Bovine LF

(bLF) at low physiological concentrations (5 µg/ml) stimulates growth and activity of osteoblastic MC3T3-E1 cells and primary culture of murine osteoblast bone cells (Blais *et al.*, 2009). Low density lipoprotein receptor-related protein 1 and 2, which are present on osteoblastic cells, have been shown to be partially responsible for LF's mitogenic effect in osteoblasts (Grey *et al.*, 2004). Moreover, Grey *et al.*, showed that LF is able to protect osteoblastic cells from apoptosis induced by serum withdrawal (Grey *et al.*, 2006).

LF action on osteoclasts is strikingly different since it produces an important arrest of osteoclastogenesis (Cornish, 2004; Lorget *et al.*, 2002). However, LF does not modulate mature ostoeclast activity. bLF at a concentration ranging from 10 to 1000 µg/ml was found to inhibit pre-osteoclastic established RAW cell growth. These results were confirmed in mixed primary culture of murine bone cells (Blais *et al.*, 2009). In contrast, there was no effect of LF on bone resorption when tested on isolated mature osteoclasts, or in organ cultures that can detect mature osteoclast activity (Grey *et al.*, 2006).

In vivo bone effects of LF were first studied using local injection of LF above the hemicalvaria which increased bone formation and bone area in adult mice (Cornish, 2004). Few recent studies using ovariectomized (OVX) rodents as a model for post menopausal bone loss measured the effect of dietary supplementation on bone (Blais *et al.*, 2009; Guo *et al.*, 2009; Malet *et al.*, 2011). LF administered orally to OVX rats for three months protected them against the OVX-induced reduction of bone volume and BMD and increased the parameters of mechanical strength, increased bone formation and reduced bone resorption (Guo *et al.*, 2009). Our studies using OVX mice demonstrated that the dietary bLF transfer into peripheral blood. LF supplementation increases BMD and bone strength compared to the OVX control group. This study supports a direct effect of LF on bone cells (Blais *et al.*, 2009).

Recent animal studies demonstrated that estrogen deficiency causes bone loss by mechanisms associated with inflammatory and oxidative processes (Grassi *et al.*, 2007; Lean *et al.*, 2003; Muthusami *et al.*, 2005). TNFα is one of the cytokines responsible for the augmented osteoclastogenesis (Suda *et al.*, 1999). Indeed, ovariectomy causes an increase in TNF production from T-cells which in turn increases macrophage colony-stimulating factor and RANKL levels, leading to osteoclastogenesis (Cornish *et al.*, 2004; Suda *et al.*, 1999). Moreover the presence of increased levels of TNFα was reported in the bone marrow of OVX animals and in blood cells of postmenopausal women (Oh *et al.*, 2007; Shanker *et al.*, 1994). Postmenopausal osteoporosis should also be regarded as the result of an inflammatory process (Weitzmann & Pacifici, 2007). It has been shown that bLF plays a role in host non-specific defense and modulates the inflammatory process mainly by preventing the release of inflammatory cytokines that induce recruitment and activation of immune cells at inflammatory sites (Debbabi *et al.*, 1998; Legrand *et al.*, 2006). Indeed, bLF enriched diet ingestion can reduce release of pro-inflammatory cytokines and increase anti-inflammatory cytokine production. Our studies showed that bLF ingestion decreases bone loss and bone resorption markers. A decreased TNFα mRNA expression associated with a TNFα production inhibition on peripheral T-lymphocytes was observed with a bLF supplementation in OVX mice. Furthermore, bLF can prevent lymphocyte activation and cytokine release in the bone micro-environment. Production and release of TNFα were strongly down-regulated by LF. These immune modulations were spatially and temporally correlated with reduced bone loss. We suggested that bLF modulates the inflammatory process via specific TNFα

inhibition in order to improve bone loss (Malet *et al.*, 2011). Recently a clinical trial of 38 healthy postmenopausal women randomized into placebo or ribonuclease-enriched-LF (R-ELF) groups evaluated bone health status over a period of 12 months. The authors demonstrated that R-ELF supplementation reduced bone resorption and increased osteoblastic bone formation; however BMD was not evaluated (Bharadwaj *et al.*, 2009).

In conclusion, LF has a positive effect on bone health and might be useful in pathological states of reduced bone density. The molecular mechanisms are not fully understood but our studies suggest that dietary bLF supplementation can have a beneficial effect on postmenopausal bone loss not only via a direct effect but also by modulating immune function. The development of pharmaceutical or nutriceutical compounds that are based on LF will require a better understanding of LF's mechanism of action on bone.

5.3 Collagen

Collagen has a unique triple helix configuration with a repeating sequence $(Gly\text{-}X\text{-}Y)_n$, with X and Y being mostly proline and hydroxyproline (Hyp) (Bos *et al.*, 1999; Ramshaw *et al.*, 1998). Some studies suggest that a hydrolyzed collagen-enriched diet improves bone collagen metabolism and BMD. Oral administration of Hydrolyzed Collagen (HC) increased BMC and BMD in rats and mice fed a calcium or protein deficient diet (Koyama *et al.*, 2001; Wu *et al.*, 2004).

5.3.1 Ingestion of collagen

Oral administration of HC was demonstrated to increase the quantity of type I collagen and proteoglycans in the bone matrix of ovariectomized rats (Nomura *et al.*, 2005). Moreover, in patients with osteoporosis, oral intake of HC with calcitonin had a stronger inhibitory effect on bone resorption than calcitonin alone (Adam *et al.*, 1996). Oesser *et al*. demonstrated the intestinal absorption and cartilage accumulation of collagen-derived peptides (Oesser *et al.*, 1999). It has been generally assumed that collagen–rich diets interact with the bone matrix. Indeed, collagen-derived di- and tripeptides rich in hydroxyproline such as Hyp, Pro-Hyp, Pro-Hyp-Gly or Gly-Pro-Val have been detected in human blood following the ingestion of HC (Iwai *et al.*, 2005). The PEPT1 proton-dependent transporter assures the transport of Pro-Hyp across the intestinal barrier (Aito-Inoue *et al.*, 2007).

A study of Ohara *et al*. compared quantity and structures of food-derived gelatin hydrolysates in human blood from fish scale, fish skin and pork skin type I collagen in a single blind crossover study (Ohara *et al.*, 2007). Amounts of free Hyp and Hyp-containing peptide were measured over a 24-h period. Hyp-containing peptides comprised approximately 30% of all detected Hyp. However, efficiency of HC ingestion depends not only on collagen origin but also on the molecular size of the HC. Collagen needs to be hydrolysed to be able to interact with bone metabolism.

5.3.2 *In vivo* studies

Our *in vivo* studies indicate that ingestion of HC diet induced the growth of the external diameter of the bone cortical zone in OVX mice (Guillerminet *et al.*, 2010, 2011). The increased cortical area was correlated with a significant increase in the femur external

diameter, without modification of the size of the medullar area. Therefore, the increased size of the cortical area was induced by a periosteal apposition of bone on the mouse femur. Due to this increase in bone size, the ultimate strength of femurs of OVX-mice ingesting HC was significantly greater than the control OVX mice. The increase of the external diameter was also related to a higher level of bone ALP during the first month of HC ingestion. However, the effect was transient; after three months no significant ALP increase was reported. Moreover, HC ingestion was able to increase the bone non-mineral content. There was no significant modification of Young's modulus but bone stiffness increased. Assuming that the stiffness of bone is correlated to the amount of type I collagen present (Burr, 2002; Mann *et al.*, 2001), and since some previous studies showed an increase of type I collagen and proteoglycan excretion for mice fed hydrolyzed collagen, we can propose that HC ingestion increases type I collagen formation in mouse bone.

5.3.3 *In vitro* studies

The *in vitro* results obtained with primary tissue culture of murine bone cells confirmed that HC was able to stimulate cell growth and ALP activity. In our studies, all the tested collagens were able to increase osteoblast activity but the 2kDA porcine HC was the most efficient *in vitro* (Guillerminet *et al.*, 2010). Similar observations were also reported with osteoblasts grown on collagen type I films compared to a plastic support with an improvement in various bone markers including increased ALP activity and an accelerated and uniform mineralization of the bone matrix (Lynch *et al.*, 1995). Moreover, our work using the *in vitro* BD BioCoat™ Osteologic™ bone cell culture system showed that PCH-N hydrolyzed collagen did not modify osteoclast growth but reduced osteoclast differentiation (Guillerminet *et al.*, 2010). This effect, combined with increased osteoblast activity is likely to modulate bone turnover leading to the growth of the external diameter of cortical bone.

5.3.4 Mechanism of action

Several potential mechanisms can be proposed to explain the influence of HC-derived peptides on bone metabolism. Some results have suggested that ingestion of type I hydrolyzed collagen leads to the production and absorption of collagen-derived peptides similar to peptides released from type I collagen *in situ* during bone resorption. Those peptides also act on bone cell metabolism (Adam *et al.*, 1996). Osteoblast activity involves three steps including proliferation, matrix protein synthesis (type I collagen and proteoglycans) and mineralization of the bone matrix (Owen *et al.*, 1991; Quarles *et al.*, 1992; Stein & Lian, 1993). Several hormones and cytokines can modulate osteoblast and osteoclast differentiation and activity. The cytokine TGF-β which is stored in a latent form in the bone matrix, and secreted during the bone resorption phase, is believed to exert such an effect (Oreffo *et al.*, 1989). TGF-β stimulates type I collagen and proteoglycan production while inhibiting that of hydroxyapatite. Interestingly, the type I collagen-derived peptide DGEA (asparagine, glycine, glutamine and alanine), was shown to interact with α2β1 integrin located on the osteoblast cell membrane. This interaction leads to inhibition of TGF-β and consequently bone matrix protein synthesis (Oesser *et al.*, 1999; Takeuchi *et al.*, 1996, 1997; Xiao *et al.*, 1998). Moreover, Hyp is an aromatic AA, and an increase of its concentration can, as suggested previously, increase IGF-1 levels which consequently attenuates bone loss.

Taken together, the results indicate that hydrolyzed collagen modulates bone formation and mineralization of the bone matrix by stimulating osteoblast growth and differentiation while reducing osteoclast differentiation. These effects lead to growth of the external diameter of the cortical zone.

5.4 Isoflavone-containing soy protein

Soy contains isoflavones able to bind to estrogen receptors (Folman & Pope, 1969). They have received considerable interest as a possible alternative to conventional Hormone Replacement Therapy (HRT). However, the efficiency of phytoestrogens such as soy isoflavone on bone is still to be proven.

Epidemiological studies suggest that populations with high soy intake (such as Asian populations) have a lower incidence of osteoporotic fractures (Adlercreutz & Mazur, 1997; Schwartz *et al.*, 1999). Asian women typically consume about 20g of soy daily which provides 40 mg of isoflavones (Chen *et al.*, 1999; Ho *et al.*, 2003). However, lower rates of fracture in these populations may not be fully attributed to soy consumption as ethnic related variation in fracture rates can also be explained by differences in bone structure (Bouxsein, 2011).

Many animal studies show that soy protein and/or its isoflavones have positive effects on bone mineral density (BMD) (Arjmandi *et al.*, 1998a, 1998b). However, clinical trial results ranged from no significant changes (Alekel *et al.*, 2000; Dalais *et al.*, 1998; Gallagher *et al.*, 2004; Kreijkamp-Kaspers *et al.*, 2004; Potter *et al.*, 1998) to a slight increase (Chiechi *et al.*, 2002; Lydeking-Olsen *et al.*, 2004; Potter *et al.*, 1998) in BMD. The bone protective effects of soy and/or its isoflavones are at best inconclusive.

5.4.1 Types of isoflavones

The major isoflavones in soy foods include genistein and diadzein. Genistein 2 has one-third of the potency of estradiol 1 when it interacts with estrogen receptor-b (ER-b), and one-thousandth of the potency of estradiol 1 when it interacts with ER-a. Hence Genistein 2 can induce a small estradiol-like response in bone tissues (Adlercreutz & Mazur, 1997; Zhou *et al.*, 2003). Another isoflavone, called equol, is not present in soybean but is a metabolic product of the biotransformation of diadzein by gut bacteria (Setchell *et al.*, 2002). 80% of the Asian population are equol producers (Fujimoto *et al.*, 2008; Morton *et al.*, 2002). In contrast, as few as 25% of individuals in North America and Europe are able to make S-equol (Lampe *et al.*, 1998).

5.4.2 Isoflavone and bone fracture

A number of reviews describe the effects of dietary soy and isoflavones on bone (Jackson *et al.*, 2011; Messina, 2010; Reinwald & Weaver, 2010). Among the studies exploring the effect of isoflavone-containing food on BMD in postmenopausal women, few report a relationship between soy consumption and the risk of bone fracture. A clinical trial conducted by Marini *et al*. found that in postmenopausal osteopenic Italians receiving 54mg/day genistein for two years, spinal BMD increased by 5.8% (n=150), whereas it decreased in the placebo group by 6.3% (n=154). Similar effects were reported for the hip (Marini *et al.*, 2007). However,

recently published long-term trials do not confirm these results; only the trial conducted by Alekel *et al*. reports a modest effect at the femoral neck with 120mg/d isoflavone but no effect with 80mg/d (Alekel *et al*., 2009).

In contrast clinical trials investigating associations between soy-food intake and BMD in Japanese or Chinese healthy postmenopausal women report that higher isoflavone consumption is associated with lower risk of bone fracture (Ho *et al*., 2003; Ikeda *et al*., 2006; Kaneki *et al*., 2001). Analysis of fracture incidence in the Shanghai cohort (Zhang *et al*., 2005) and of hip fracture in the Singapore cohort (Koh *et al*., 2009) shows in both studies one-third reductions in fracture risk when comparing high- with low-soy consumers.

5.4.3 Factors modulating the effect of soy on bone health

The effectiveness of dietary adaptation of western populations which rarely consumed soy must be considered. East Asian participants in epidemiological studies did not require an adaptation period or an interruption of life-long dietary habits like a western population would. Hence the observation cannot be extrapolated from one population to another.

It may be less difficult to determine bone effects following a life-long intake of traditional foods compared with intermittent intakes of soy. Traditional soy foods are a complex blend of isoflavones, protein, lipids, vitamins, minerals and other bioactive compounds that may act individually and/or synergistically to exert healthy effects. Supplements included in western diets provide quantities of individual soy components. Types of whole soy food consumed (fermented vs. nonfermented) and/or ethnicity (equol producers) may also affect outcome interpretation of soy bone effects.

Long-term observational studies in Asian populations support a benefit of traditional soy food consumption on bone health in this population. The health effects of soy-bean phytoestrogens in non-Asian postmenopausal women are promising. No conclusive evidence supports that the isoflavones from the sources studied do have beneficial effects on bone health. More researches are needed to clarify the role of dietary phytoestrogen in osteoporosis prevention.

6. Conclusion

Protein acts on bone metabolism at different levels and through different mechanisms. There is little evidence that a high-protein diet will increase bone loss. Protein is well-known to be calciuric, yet there are conflicting data on whether the excreted calcium comes from an increase of calcium absorption or from bone resorption. The direct effects of protein on bone turnover markers and BMD seem to be positive when considering observational studies, but interventional studies do not provide significant outcomes to conclude. Finally, when considering fracture rate, there seems to be a small positive effect of protein on bone as long as calcium levels remain adequate.

Two mechanisms are proposed to explain the action of protein on bone: the acid-ash theory and the hormonal anabolic effect through IGF-1 and CaSR. The hormonal anabolic mechanism supports the fact that protein is beneficial to bone by increasing IGF-1. On the other hand, the acid-ash theory considers that the acid load due to protein consumption is harmful to bone. If both mechanisms occur at the same time, it is possible to benefit from the

protein-induced IGF-1 without the negative effect of the acid load by compensating the diet with adequate alkalinizing foods.

Dietary protein quality adds complexity to the protein debate. It has been hypothesized that animal protein would be more deleterious to bone than vegetal protein. However, studies show no real difference between those two protein sources. Similarly, long-term observational studies support a benefit of traditional soy food consumption on bone health, but no conclusive evidence supports the hypothesis that this is due to the isoflavones. On the other hand, some peptides obtained from protein digestion have been shown to be helpful to prevent bone loss. Recent results indicate that HC could be of potential interest for nutritional intervention in the prevention of bone loss. Moreover, LF has been reported to have a positive effect on bone health and might be useful in pathological states of reduced bone density. The molecular mechanisms are not fully understood but our studies suggest that dietary bLF supplementation can have a beneficial effect on postmenopausal bone loss not only by acting on bone cells but also by modulating immune function.

7. References

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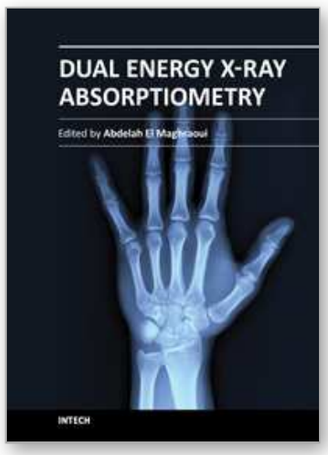

Dual Energy X-Ray Absorptiometry

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The World Health Organization (WHO) has established dual-energy x-ray absorptiometry (DXA) as the best densitometric technique for assessing bone mineral density (BMD) in postmenopausal women and has based the definitions of osteopenia and osteoporosis on its results. DXA enables accurate diagnosis of osteoporosis, estimation of fracture risk and monitoring of patients undergoing treatment. Additional features of DXA include measurement of BMD at multiple skeletal sites, vertebral fracture assessment and body composition assessment, including fat mass and lean soft tissue mass of the whole body and the segments. This book contains reviews and original studies about DXA and its different uses in clinical practice (diagnosis of osteoporosis, monitoring of BMD measurement) and in medical research in several situations (e.g. assessment of morphological asymmetry in athletes, estimation of resting energy expenditure, assessment of vertebral strength and vertebral fracture risk, or study of dry bones such as the ulna).

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