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Investigating the role of natural phyto-oestrogens on bone health in postmenopausal women

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Research on the bone effects of natural phyto-oestrogens after menopause is at a relatively early stage. Published studies are few, difficult to compare and often inconclusive, due in part to design weaknesses. Currently, many questions remain to be answered including to what extent a safe daily intake may prevent postmenopausal bone loss. These questions can only be addressed by conducting well-planned, randomised clinical trials that take into consideration present knowledge in the oestrogen, phyto-oestrogen and bone fields. This review is intended to provide hints for critical decision-making about the selection of subjects, type of intervention, suitable outcome measures and variables that need to be controlled.

Isoflavones: Menopause: Clinical trials

Introduction

Osteoporosis, one of the most prevalent diseases in the Western Hemisphere (Cooper & Melton, 1996), is also a leading cause of disability among the elderly in developed countries (Kanis, 1993; Chrischilles *et al.* 1994) and its related care and rehabilitation expenses pose a major socio-economic burden. While there is no cure, the progression of osteoporosis can be delayed and its onset prevented (Dawson-Hughes *et al.* 1997; McClung *et al.* 1998). Although usually diagnosed between the ages of 50 and 70 years, osteoporosis is increasingly regarded as a lifetime disease that affects predominantly women, for whom the incidence of osteoporotic fractures is twice that for men (Valtueña *et al.* 2001). Consequently, the urgent need for suitable preventive strategies has intensified bone mineral research, especially in the population subgroups that are in the process of achieving peak bone mass (children) or at the highest risk for bone loss (peri- and postmenopausal women).

In postmenopausal women, oestrogen deficiency is associated with enhanced bone turnover and accelerated bone loss, leading to increased susceptibility to bone fractures (Riggs & Melton, 1986; Nguyen *et al.* 1995). Although several intervention strategies have been tested to attenuate menopausal bone loss, including selective oestrogen receptor modulators, bisphosphonates and nutritional supplements, hormone replacement therapy (HRT) appears the single most effective treatment (Lindsay *et al.* 1976; Riggs & Melton, 1986; Komulainen *et al.* 1999). However, even though HRT is still a first-choice option in the prevention of postmenopausal osteoporosis, alternatives are continually being sought because low acceptance, relevant side-effects and contra-indications are limiting its use to no more than 10–30% of postmenopausal women in Europe (Colditz *et al.* 1995; Beresford *et al.* 1997), of whom only a small proportion comply with the treatment for more than a year.

Recently, attention has focused on the so-called phyto-oestrogens, non-steroidal compounds occurring naturally in foods of plant origin (especially soya foods) that are

Abbreviations: ALP, alkaline phosphatase; BBRI, breaking bending resistance index; BMC, bone mineral content; BMD, bone mineral density; CTX, C-terminal cross-linked peptide of type I collagen; DXA, dual-energy X-ray absorptiometry; ER, oestrogen receptor; HRT, hormone replacement therapy; NTX, N-terminal cross-linked peptide of type I collagen; PINP, procollagen type I N-propeptide; RFLP, restriction fragment length polymorphism; TRAP, tartrate-resistant acid phosphatase; VENUS, Vegetal Estrogens in Nutrition and the Skeleton.

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able to compete with the principal oestrogens of most mammals (17β-oestradiol and oestrone) for binding to oestrogen receptors (ER). Because of their low, but significant, oestrogenic activity, natural phyto-oestrogens have been proposed as a safe alternative to HRT in attenuating menopause-related symptoms including bone loss, although data supporting an eventual bone-sparing effect of phyto-oestrogens is limited essentially to *in vitro* experiments, animal studies and epidemiological associations. Only a few human studies have been conducted that generally were of short duration, small in size and, since they used variable doses of soya foods, are difficult to compare. In addition, this preliminary evidence took no account of the potential bioavailability of phyto-oestrogens in foods, nor were studies conducted to define the optimal dose for bone-preserving effects.

The present review aims to provide hints in planning future research on the effect of natural phyto-oestrogens on bone metabolism in Caucasian, menopausal women, with typically low intakes of soya and soya products. The rationale for decision-making regarding subject selection, study design, type of intervention, suitable endpoints and possible confounding factors is presented.

Current evidence on the bone-sparing effects of natural phyto-oestrogens after menopause

Phyto-oestrogens have shown a relative molar binding affinity for the α subtype of ER, ERα, between 100 and 1000 times lower than that of 17β-oestradiol (Martin *et al.* 1978). However, they generally have a higher specificity for the recently cloned β subtype, ERβ (Kuiper *et al.* 1997), which is preferentially expressed in tissues such as bone, brain, vascular endothelia and bladder. Genistein and coumestrol are able to stimulate the transcriptional activity of both ER subtypes at concentrations of 1–10 nM and 10–100 nM (Kuiper *et al.* 1998), respectively, to generate a response similar in magnitude to that of physiological levels of oestradiol. In oestrogen-deficient rat models, low doses of soya isoflavones, one class of phyto-oestrogens, prevent ovariectomy-induced bone loss as effectively as 5 μg/d of conjugated equine oestrogens (Anderson & Ambrose, 1995; Arjmandi *et al.* 1996, 1998*a*,*b*; Draper *et al.* 1997; Fanti *et al.* 1998; Ishimi *et al.* 1999; Coxam, 2003), but data in primates are less consistent in this direction (Jayo *et al.* 1997).

Human studies documenting the effects of phyto-oestrogen intake on bone are strikingly few, mostly limited to epidemiological associations (Ross *et al.* 1991; Melton, 1995; Horiuchi *et al.* 2000; Somekawa *et al.* 2001) and focused primarily on postmenopausal women (Dalais *et al.* 1998; Kardinaal *et al.* 1998; Potter *et al.* 1998). A number of short-term interventions suggest beneficial effects of phyto-oestrogen-enriched diets on bone turnover after menopause. A 12-week study on forty-three postmenopausal women who consumed soya milk or soya nuts to deliver 60–70 mg of total isoflavones/d showed a 14 % decrease in urinary excretion of the bone resorption marker N-terminal cross-linked peptide of type I collagen (NTX); serum osteocalcin, a marker of osteoblastic activity, increased significantly, whereas another marker of bone formation, bone-specific alkaline phosphatase (ALP), was unaffected (Scheiber *et al.* 2001). These findings are consistent with reduced bone resorption and increased bone formation, and resemble the effects of the synthetic isoflavone ipriflavone, rather than the oestrogens in HRT, which have little effect on osteoblastic activity (Arjmandi *et al.* 2000). No effects on bone ALP or NTX were observed in a 24-week, randomised, whey-controlled intervention trial with either an isoflavone-rich (80·4 mg aglycones/d) or an isoflavone-poor (4·4 mg aglycones/d) soya protein diet (Alekel *et al.* 2000). Similar results were obtained from a three-arm, randomised crossover trial with soya protein isolates providing 7, 65 and 132 mg isoflavones/d in seventeen postmenopausal women. Osteocalcin tended to decrease in the high isoflavones diet, bone ALP decreased significantly in the 65 and 132 mg isoflavones/d diets and no diet effects were observed in the urinary excretion of deoxypyridinoline or C-terminal cross-linked peptide of type I collagen (CTX), both markers of bone resorption (Wangen *et al.* 2001).

Some conflicting data have also been obtained for intervention studies with bone mineral density (BMD) or bone mineral content (BMC) as primary endpoint. A longitudinal, non-randomised, non-controlled study conducted in thirty-seven postmenopausal women taking isoflavone supplements twice daily for 6 months (150 mg/d) reported no significant changes in calcaneous BMD (Hsu *et al.* 2001). Dalais *et al.* (1998) reported a significant increase of 5·2 % in total body BMC after a 12-week diet with soya grits containing 52 mg isoflavones/d. However, these results should be interpreted with caution because total body BMC also increased by 3·8 % in the control (wheat) group, an effect that is unlikely in untreated postmenopausal women (Greendale *et al.* 2000).

Postmenopausal women consuming isolated soya protein over a 6-month period were found to have significant increases in BMC compared with women on a diet with casein (Potter *et al.* 1998). Daily intake of 40 g isolated soya protein with 2·25 g isoflavones/g protein was associated with significant increases in BMC and lumbar spine BMD, but isolated soya protein with 1·29 mg isoflavones/g protein was not protective (Potter *et al.* 1998). It should be remembered that, although frequently cited as a reference in the bone field, the study of Potter *et al.* was originally designed to investigate the effects of isoflavone intake on blood lipids. Similarly, soya isoflavones, and not soya protein *per se*, were identified as responsible for the bone-sparing effect in the only study available on perimenopausal women: a 24-week, double-blind, randomised controlled trial where sixty-nine subjects were assigned to consume 40 g of whey protein/d (controls) or 40 g of soya protein isolate/d with either high (80·4 mg aglycones/d) or low (4·4 mg aglycones/d) isoflavone content (Alekel *et al.* 2000). BMD and BMC of the lumbar spine did not change in the soya groups, whereas they decreased in controls by 1·28 % and 1·73 %, respectively. After adjustment for covariates by regression analysis, only the soya protein with high isoflavone content showed a positive effect on change in BMD (5·6 %) and BMC (10·1 %). This observation is consistent with previous studies in oestrogen-deficient rats (Arjmandi *et al.* 1998*a*,*b*).

Planning future research on the effects of dietary phyto-oestrogens on bone health in postmenopausal women

The ultimate research question about the role of phyto-oestrogens in osteoporosis prevention is whether these compounds may decrease the incidence of osteoporotic bone fractures, either by preventing postmenopausal bone loss or by other mechanisms. A satisfactory answer, which is anticipated to be complex, requires multiple studies designed to address not only the effects of phyto-oestrogens on a certain outcome, but also the circumstances in which that positive or negative result has been achieved, as well as its biological relevance.

The generic term 'postmenopausal women' is used to describe a subgroup of the population that is tremendously heterogeneous with respect to variables that may well modulate, or even determine, the susceptibility of bone to the action of natural phyto-oestrogens, such as genetic background, endocrine status and life-style. Similarly, the phyto-oestrogens family includes a large group of compounds with diverse chemical structures that are contained in a variety of food matrices and are likely to differ in bioavailability, metabolism, oestrogenic action, effects on bone and minimum dose at which those effects might be observed (Messina, 1999; Setchell & Cassidy, 1999). On the other hand, several markers are available to measure the bone response to natural phyto-oestrogens in terms of the magnitude, type, nature, size and preferential location of the effect (Faulkner, 2001; Faulkner & Pocock, 2001) that, in turn, may depend on the population studied and the phyto-oestrogen source used. Therefore, the research question above may be broken down as follows. Have phyto-oestrogens a bone-sparing effect in postmenopausal women? Which compounds, dose and mode of delivery achieve that effect? How big is the effect? Is it transient or sustained? When should the intervention start — after menopause occurs? Is it the result of reduced bone breakdown, increased formation, or both? Which sites of the skeleton receive the largest benefit? Are the changes in bone metabolism and density relevant in terms of fracture risk reduction?

Selection of subjects

Postmenopausal status is associated with low circulating levels of endogenous oestrogens. At the beginning of menopause, oestrogen levels are unstable and drop definitively by the time of the last menses. Postmenopausal is defined as starting 12 months after the last menses. Bone loss increases discretely with the irregular bleedings phase, further during the menopausal transition, and overwhelmingly in the first year after menopause, mainly due to an increase in bone resorption. However, as the skeleton adapts to the low oestrogen concentration, the rates of bone loss decline as well. By the sixth year after last menses, the bone loss rate stabilises at about 1 % per annum until the process is completed, generally before the age of 70 years (Nilas, 1993; Mazzuoli *et al.* 2000). Overall, 30–50 % of the peak bone mass will be lost due to menopause (Hui *et al.* 1982), about half of which occurs within the first 5 years (Mazzuoli *et al.* 2000).

It is well established that the responsiveness of bone to oestrogen deficiency changes markedly over the natural history of menopause, as does its sensitivity to HRT and, probably, to natural phyto-oestrogens. The benefit to be obtained from treatment, as the relative amount of bone mass that can be 'spared' with the intervention, also depends on the time after menopause, together with the biological significance of a given effect. For example, the greatest benefit from HRT is achieved within the first 12 months after menopause probably because the body is not yet well adapted to low oestrogen levels and the bone loss rate, in the absence of treatment, would be higher at that time than later on. In other words, evaluation of the efficacy of natural phyto-oestrogens in preventing postmenopausal bone loss requires complete characterisation of the population being studied with regard to their hormonal status and expected rate of bone loss, since these characteristics will strongly affect both the results and their interpretation (Hadjidakis *et al.* 1999). For research purposes, the following cut points may be used to make study populations homogeneous:

1. Perimenopausal women. This category will include any women 45 years of age or older with irregular menses and presence of spontaneous vaginal bleeding within the last 6 months. Rates of bone loss are increased with respect to premenopausal women, but not as high as in early menopause.
2. Early postmenopausal women. Women within 6 and 60 months since last menses. Phase of rapid bone loss. Menopausal status should be confirmed by biochemical assessment of the hormonal profile.
3. Late postmenopausal women. Any women more than 60 months postmenopausal, 65 years of age or younger. Studies in the elderly assessing age-related bone loss have traditionally used this age as cut-off point.

Study design

Only randomised, controlled clinical trials are appropriate to establish a cause–effect relationship between an intervention and an outcome. In clinical studies on bone metabolism including postmenopausal women, block randomisation is essential to ensure comparability between control and intervention groups regarding age, race, hormonal status and BMI, all known to have an important effect on bone mass (National Institutes of Health Consensus Statement, 2000).

When the effects of a nutrient in food are to be tested, the control group should ideally be given the same food without the nutrient of interest, in a double-blind fashion, to control for the influence that the research conditions themselves, independent of the intervention, may have on subjects' behaviour and therefore on the outcome. Clinical studies on the effects of natural soya isoflavones on bone use wheat as the control equivalent of soya flour (Dalais *et al.* 1998) and isoflavone-free whey or casein as the soya protein control (Potter *et al.* 1998; Alekel *et al.* 2000). In both cases, foods introduced in the study groups can easily be matched regarding calories and macronutrients, which is important in the eventuality of weight gain or loss due to the novel diet. It should be

remembered that weight changes not only may have an effect on bone mass by themselves, but also make difficult the interpretation of BMD fluctuations measured by dual-energy X-ray absorptiometry (DXA; Gotfredsen *et al.* 1997; Van Loan *et al.* 1998). However, protein itself modulates bone metabolism in many ways, one of which is the acid load that sulphur amino acids generate (Massey, 1998). Animal protein, such as casein, promotes Ca excretion to a greater extent than vegetal protein, and therefore is likely to have a negative impact on bone metabolism, while soya protein is basic and will not have this effect (Heaney, 1998; Valtueña *et al.* 2001). A possible solution to this problem lies in the recently marketed soya isoflavone concentrates, which contain up to 40 % isoflavones by weight and can be incorporated into a wide range of foods without changing substantially their composition in macronutrients.

Type of intervention

With regard to the intervention arm, comprehensive analyses of the isoflavone level in numerous soya foods (the richest identified source of the isoflavone phyto-oestrogens) have been reported in the literature and generally indicate that most contain 0·1–3·0 mg of total isoflavones/g (Reinli & Block, 1996; Kiely *et al.* 2003). Soya germ products derived from the hypocotyledon provide one of the most concentrated sources (>20 mg/g) of isoflavones. In addressing the matrix effect, studies are currently underway to determine the effect of varying the dietary intake of isoflavones in a single food on the resulting plasma and urinary levels and on the pharmacokinetics. Further studies will address the effects of chemical composition, and liquid *v.* solid matrix from a known dose of isoflavones, on these parameters. These data will help determine what level of intake is required to deliver physiologically relevant levels of these compounds to target tissues.

Numerous isoflavone supplements (from soya and red clover) are commercially available. However, recent data suggest that, in some products, the actual contents differ significantly from the levels documented on the packaging (Setchell *et al.* 2001). In addition, the supplements have not been fully evaluated in relation to their biological or clinical efficacy and recent evidence suggests they have a different pharmacokinetic profile to soya isoflavones in foods (Setchell *et al.* 2001). Furthermore, there are obvious concerns regarding the potentially adverse effects that could result from megadosing with these bioactive compounds, a practice all too common in the supplements area.

Numerous short-term studies have been conducted in the phyto-oestrogen field to address the potential health benefits of phyto-oestrogen-rich diets. The interest in the field has been caused predominantly by the early data suggesting that feeding diets rich in phyto-oestrogens resulted in significant endocrine effects in healthy premenopausal women (Cassidy *et al.* 1994). A recent consensus statement collated by key international experts in the phyto-oestrogen field suggested specific levels of food fortification for specific health effects, based on the current scientific information. For example, for the relief of menopausal symptoms, 60 mg aglycones/d were proposed, while, for improvement in bone density, consumption of 60–100 mg aglycones/d over a minimum period of 6 months was suggested for any potential gains.

In determining the choice of food, matrix or supplement in designing a clinical study, many lessons can be learned from the cardiovascular research that has been carried out in this area (Potter *et al.* 1998; Scheiber *et al.* 2001). Although it is well established that dietary soya modifies risk factors for cardiovascular disease, the component of soya responsible for the heart benefits is not clearly established. Strong evidence exists to suggest that the intact soya isoflavones (when interacting with the protein) are important for cardiovascular protection, as studies conducted with alcohol-washed soya (alcohol washing removes the isoflavones and also other molecules like the saponins) do not show CHD benefits. However, although there is a general consensus that soya protein in combination with the isoflavones has potential health benefits on the cardiovascular system, little experimental evidence exists to suggest that the purified isoflavone supplements modify cardiovascular risk factors (Vitolins *et al.* 2001).

Outcomes

The ultimate objective of intervention strategies in osteoporosis research is to reduce the risk of bone fractures. However, large sample sizes and long follow-ups are needed to prove such a benefit, especially in individuals younger than 65 years. Repeated measurements of BMD by DXA have been the primary means of assessing future fracture risk, and are now accepted as an intermediate endpoint in the evaluation of nutritional and pharmacological regimens (Cummings *et al.* 1993, 1995; WHO, 1994; National Institutes of Health Consensus Statement, 2000). Changes in BMD can often be measured meaningfully by DXA as early as 6 to 12 months from baseline at low radiation exposure in almost all population groups (Roubenoff *et al.* 1993). Recent data from large prospective studies have, however, indicated that biochemical markers represent independent fracture risk predictors (Garnero *et al.* 1996*a*, 1999; van Daele *et al.* 1996), and that combination of the two techniques enhances the risk prediction (Garnero *et al.* 1996*a*). It is clear that increased bone turnover in itself constitutes a high risk for fracture and that this trend increases progressively with age (Garnero *et al.* 1996*b*). As with most assessment techniques, however, the performance of biochemical markers as fracture risk predictors in the individual is rather weak (Bauer *et al.* 1999).

To evaluate the efficacy of natural phyto-oestrogens in preventing osteoporosis-related disability, it is essential to select the outcome variables providing the most relevant information at the lowest cost for both the patient and researcher. Since markers of efficacy are many but the resources available usually scarce, the selection process may be difficult. Here we present a brief overview.

Short-term endpoints: bone turnover markers. Monitoring bone metabolism involves measurements of bone formation and bone resorption markers, which reflect osteoblastic and osteoclastic activities, respectively. Although bone is a relatively active tissue with a turnover

rate through remodelling of about 10% per annum in adults and much higher rates with the additional modelling processes in children, the changes in balance that lead to bone loss or accretion are long-term. Thus, when measuring markers of bone turnover to predict future changes in the rates of bone loss, all possible sources of variation need to be minimised. Considerable advances in the development of new markers have been made in recent years. Generally, however, each marker has different advantages and disadvantages that have to be taken into account in selecting the most appropriate ones for monitoring dietary supplementation with phyto-oestrogens. A summary of the primary bone markers and their characteristics is given in Table 1 (for recent recommendations on bone marker nomenclature, see Delmas, 2001).

For bone formation, attention has focused on specific proteins or enzyme activities produced by osteoblasts, such as osteocalcin (Delmas, 1990) or bone-specific ALP (Gomez *et al.* 1995), or on production of the N-terminal (Melkko *et al.* 1996) or C-terminal (Melkko *et al.* 1990) precursors of collagen type I, since this protein represents over 90% of bone matrix proteins. The procollagen type I C-propeptide has proved to be rather insensitive to changes in bone remodelling, whereas more recent data using procollagen type I N-propeptide (PINP) have been more encouraging. These differences are probably associated with the way in which the two analytes are cleared from the circulation. Interpretation of osteocalcin results is often difficult, particularly for dietary studies, because of the influence of vitamins D and K on osteocalcin expression. Also, as osteocalcin is relatively unstable in serum and is cleaved within a C-terminal site, it is important to use assays that recognise the cleaved products (termed N-terminal mid fragments or OC [1–43]; Garnero *et al.* 1994). Bone ALP has traditionally been a difficult assay to perform technically, but the advent of relatively specific monoclonal antibodies (Garnero & Delmas, 1993) has led to new immunoassays (Gomez *et al.* 1995) that appear to be sensitive to changes in bone metabolism.

Because of its preponderance in bone matrix, collagen type I is the primary source of analytes for monitoring bone resorption, including not only pyridinium collagen cross-links (Robins *et al.* 1994; Gomez *et al.* 1996) but also peptides associated with cross-linking at the N-terminal (NTX; Hanson *et al.* 1992) or C-terminal (CTX; Bonde *et al.* 1994) end. The recent discovery of age-related changes in the cross-linking regions involving isoaspartyl transformations (Fledelius *et al.* 1997; Brady *et al.* 1999) has provided an opportunity to gain additional information on bone collagen metabolism with specific assays that reflect the age of the molecules being degraded. Recent data on the equilibration rates of isoaspartyl formation (Cloos & Fledelius, 2000) suggest, however, that the application of these types of assay may be limited to situations involving high turnover rates and they should not be considered for monitoring subtle changes in normal bone remodelling in adults. By contrast, it has been shown that the ratio of β- to α-aspartyl is distinctly different in children compared with adults (Brady *et al.* 1999) and these types of assay may have significant applications in monitoring growth.

Most pyridinium cross-link and telopeptide assays have been performed in urine and the results expressed relative to creatinine. Serum assays for NTX (Clemens *et al.* 1997) and CTX (Rosenquist *et al.* 1998) are now available and methods for the measurement of both free and total pyridinium cross-links in serum have been described (James & Perrett, 1998). However, the latter are technically demanding and unsuitable for large-scale clinical trials. It was hoped that, because of their lower intrinsic variability compared with urinary assays, serum measurements could provide more reliable assessments of bone metabolism. Initial data for serum NTX, however, indicated that the lower variability of the serum assay is matched by smaller differences between groups and in response to a change (Eastell *et al.* 2000). Serum assays of bone cross-linking components may not, therefore, provide any real advantages over urinary measurements.

Newer markers such as bone sialoprotein are also being developed (Seibel *et al.* 1996; Karmatschek *et al.* 1997) and, although interpretation of the data is still not fully understood, the serum assay appears to represent a marker better suited to monitoring pathological bone resorption. Bone sialoprotein and other acidic proteins may be bound in serum by the complement component, factor H (Fedarko *et al.* 2000), and a modified assay for total bone sialoprotein has been devised (Fedarko *et al.* 2001). Tartrate-resistant acid phosphatase (TRAP) represents another serum resorption marker that has been improved markedly recently through optimisation of assays using antibodies specific for the osteoclast-derived, TRAP 5b isotype (Halleen *et al.* 2000).

With current knowledge, the most appropriate markers to study the potential effects of phyto-oestrogens on bone metabolism are bone ALP, PINP and total urinary pyridinium cross-links. The latter are chosen because many of the peptide-fragment assays are crucially dependent on potential changes in degradative metabolism of the collagen released from bone. Interpretation of the results obtained with the cross-links assay has fewest uncertainties, and data can be related to absolute rates of bone resorption.

Medium-term endpoints: markers of bone strength. The use of areal BMD alone to predict bone strength has been questioned lately for several reasons. First, bone strength is proportional to the square of volumetric bone density (not measured directly by conventional DXA) which could be very different in bones with identical areal BMD but different depth. Second, small errors in the calculation of BMD imply a larger error in the prediction of bone strength, given the exponential relationship that exists between both variables (Ebbsesen *et al.* 1999). Third, bone strength is determined not only by the composition (degree of mineralisation), but also by the shape, size and organisation (architecture) of the bone structure. Actually, bone mass alone can account for about 65 to 80% of the observed variation in strength of human bones from cadavers, whereas the incorporation of bony architecture parameters may increase the predictability up to 94% (Siffert *et al.* 1996; Jiang *et al.* 1998).

In vitro studies reveal that geometrical indices of mechanical strength that can be calculated from areal

Table 1. Biochemical markers of bone metabolism

Marker	Abbreviation	Assay type	Fluid	Comments	Reference
Bone formation					
Osteocalcin	OC	EIA, RIA	serum or plasma	synthesis dependent on vitamins D and K; unstable in serum; exhibits immunological heterogeneity	Garnero *et al.* (1994)
Procollagen type I C-propeptide	PICP	RIA	serum or plasma	rather insensitive to changes in bone remodelling	Melkko *et al.* (1990)
Procollagen type I N-propeptide	PINP	RIA	serum or plasma	as well as an intact, 100 kDa component, there is a 30 kDa fragment in blood, not measured in all assays	Melkko *et al.* (1996); Brandt *et al.* (1999)
Bone-specific alkaline phosphatase	Bone ALP	RIA, immunocapture	serum or plasma	specific antibodies allow measurement of either enzyme protein or activity; the latter provides comparability with total ALP assays	Garnero & Delmas (1993); Gomez *et al.* (1995)
Bone resorption					
Deoxypyridinoline	DPD	EIA, HPLC	urine	total or free may be assayed	Robins *et al.* (1994)
Pyridinoline	PYD	EIA, HPLC	urine or serum	total or free may be assayed; immunoassay measures both PYD and DPD	Gomez *et al.* (1996)
N-terminal cross-linked peptide of type I collagen	NTX	EIA	urine or serum	antigen isolated from human urine	Hanson *et al.* (1992); Clemens *et al.* (1997)
C-terminal cross-linked peptide of type I collagen	CTX (α and β forms)	EIA, RIA	urine or serum	synthetic peptide antigens; assays measure the natural (α) or isomerised (β) forms of aspartyl residues	Bonde *et al.* (1994); Fledelius *et al.* (1997); Rosenquist *et al.* (1998)
Tartrate-resistant acid phosphatase	TRAP	EIA	serum	antibodies specific for osteoclast-specific form of the enzyme, isotype 5b	Halleen *et al.* (2000)
Bone sialoprotein	BSP	RIA	serum	assay data correlate with other resorption markers	Seibel *et al.* (1996)

EIA, enzyme-linked immunoassay; RIA, radioimmunoassay.

measurements provided by DXA, such as the breaking bending resistance index (BBRI), discriminate better between postmenopausal patients with hip and vertebral fractures than BMD (Augat *et al.* 1996). Interestingly, BMC correlates better with BBRI than BMD in elderly populations (Bouxein *et al.* 1994). Additional exploration of the shape, size and organisation of bone structures, which may have an independent effect on fracture risk and are susceptible to modification by the ageing process and (probably) by nutritional interventions, is now possible *in vivo* by using three-dimensional imaging techniques (Lang *et al.* 1998). The latest advances in quantitative computed tomography allow precise assessment of volumetric trabecular and cortical density in peripheral bones (peripheral quantitative computed tomography), whereas ultra-high resolution computed tomography scanners can image the trabecular structure of vertebrae and some peripheral locations (Lang *et al.* 1997; Augat *et al.* 1998). Similarly, high-resolution magnetic resonance can be used to assess trabecular texture peripherally, which, when combined with BMD estimates, improves discrimination between subjects with osteoporotic fractures and normal controls (Majumdar *et al.* 1999). The sensitivity and specificity of these techniques, compared with DXA-derived areal BMD, as predictors of fracture risk and their utility as markers of bone status in longitudinal studies are, however, a matter of controversy and intense research (Ross *et al.* 1989; Lang *et al.* 1997; Formica *et al.* 1998).

BMD and BMC, measured by DXA, are the variables that have been used most widely to estimate changes in bone mineral as a surrogate of bone strength over time. New DXA instruments are relatively easy to use and allow detection of small changes in BMD, but the collection of meaningful longitudinal BMD data and their correct interpretation require a profound knowledge of the DXA technology and bone dynamics. It is currently accepted that changes in BMD occurring over a period shorter than 6–12 months would not reflect the effects of an intervention on steady-state bone balance, but rather a change in the bone remodelling space (Heaney, 1996). Therefore, follow-up BMD measurements should be conducted at least 6–12 months after the phyto-oestrogen-rich diet or phyto-oestrogen supplementation has been started. In addition, it is important to remember that estimation of the significance of bone loss or gain over time relies on precision errors greater than the CV for repeated measurements provided by DXA manufacturers. These need to be calculated by each laboratory, including also the potential changes in soft tissue overlying bone (subject variability), subject's positioning, differences in scan area and interpretation (operator variability) and stability of the instrument over time (technical error; Fuleihan *et al.* 1995; Nielsen *et al.* 1998).

Direct comparability of results obtained with different DXA instruments, calibration phantoms and software programs is not yet possible, although this is a much desired goal for large, multi-centre intervention trials. Cross-calibration procedures between different DXA machines and software versions using the same phantom are giving relatively promising results (Pearson *et al.* 1995; Cawte *et al.* 1999).

Factors that may modulate the individual responsiveness of bone to phyto-oestrogen intake

Impact of individual genetic variation

Although postmenopausal HRT is effective in preventing bone loss, individual variation exists in the response to HRT and several studies have demonstrated the existence of non-responders (Hassager *et al.* 1994; Han *et al.* 1997; Salmen *et al.* 2000). Although non-responders have not been reported with regard to the effect of phyto-oestrogen therapy on bone, there is variation in individuals' skeletal response to dietary phyto-oestrogen supplementation (Dalais *et al.* 1998; Potter *et al.* 1998) or to synthetic phyto-oestrogen therapy (e.g. ipriflavone; Gambacciani *et al.* 1997; Gennari *et al.* 1997, 1998*a*). This variation could be explained by a genetically determined response to HRT and phyto-oestrogen therapy. One possible explanation that has been widely investigated is that variants in the ER gene could be related to oestrogenic (and possibly phyto-estrogenic) resistance.

Most studies have focused on the ERα gene, which has two restriction fragment length polymorphisms (RFLP) located in the first intron. Following an initial report demonstrating a relationship between these RFLP and BMD in healthy, postmenopausal Japanese women (Kobayashi *et al.* 1996), there has been much conflicting evidence on the influence of ERα genotype on BMD and/or bone loss and/or bone turnover. For example, positive associations were reported between ERα genotype and BMD in pre-/perimenopausal (Mahonen *et al.* 1997; Mizunuma *et al.* 1997; Ongphiphadhanakul *et al.* 1998; Willing *et al.* 1998) and postmenopausal (Sano *et al.* 1995; Deng *et al.* 1998, 1999; Ongphiphadhanakul *et al.* 2000) women, whereas other studies have found no such associations (Han *et al.* 1997, 1999; Gennari *et al.* 1998*b*; Vandevyver *et al.* 1999; Bagger *et al.* 2000). Significant gene–gene interactions between polymorphisms at the ER genotypes and other locations may also be a significant factor contributing to variable responses. The vitamin D receptor has been studied extensively in this respect (Deng *et al.* 1998; Gennari *et al.* 1998*b*; Willing *et al.* 1998) and associations with P450 aromatase, known to be regulated by vitamin D (Nawata *et al.* 1995), may combine to influence the balance between androgens and oestrogens in peripheral tissues (Willing *et al.* 1998). These studies suggest that genetic variation at the ERα locus, singly and in relation to the vitamin D receptor gene, influences attainment and maintenance of peak bone mass in younger women and changes in bone mass in elderly women, which in turn may render some individuals more susceptible to osteoporosis than others.

To date, no studies have investigated the influence of ERα genotype on the responsiveness of bone to phyto-oestrogen therapy. For the ERβ gene, known to reside on human chromosome 14 (Enmark *et al.* 1997), a dinucleotide (cytosine–adenine) repeat polymorphism located in the flanking region has been linked to differences in BMD in healthy postmenopausal women (Ogawa *et al.* 2000). In addition, several other mutations and polymorphisms in the ERβ gene have been reported (Rosenkranz *et al.*

1998). However, association studies attempting to link these polymorphisms to BMD have yet to be carried out.

From current evidence we may conclude that, as phyto-oestrogens bind to both ERα and ERβ, polymorphisms in both receptor subtypes may influence the response of bone to phyto-oestrogen therapy. However, future research is needed to investigate the potential impact of genetic variation at the ER gene loci on the responsiveness of bone to phyto-oestrogen therapy.

Overall diet

When investigating the relationship between phyto-oestrogens and bone health in a (double-blind) placebo-controlled study, the effects of the diet need to be considered in several respects, as summarised in Table 2:

1. as carrier of the phyto-oestrogens (test product) and the control product;
2. intake of phyto-oestrogens in the background diet; and
3. intake of other nutrients as potential confounders of the relationship between phyto-oestrogens and bone.

Test and control product. An adequate description of the food products used to provide isoflavones is essential with respect to the chemical composition (aglycones *v.* glycones), the food matrix or purified supplement, and the actual content of isoflavones (preferably analysed values). In a double-blind study, compliance should be monitored by counting the portions of test and control products that have not been consumed.

Phyto-oestrogen intake in the background diet. Available data suggest that habitual Western diets contain very low levels (about 1 mg/d) of phyto-oestrogens (both isoflavones and lignans; Valsta *et al.* 2003; van Erp-Baart *et al.* 2003). These levels are unlikely to interfere with the higher doses of an active intervention (above 60 mg/d; North American Menopause Society, 2000). Moreover, randomisation of study subjects will result in random distribution of background dietary intake over the active and placebo treatment groups. Special cases are subjects with deviating dietary habits and/or high baseline intake of phyto-oestrogens, either from natural food or as supplements. These should be either excluded from the study or asked to restrict their consumption of specific phyto-oestrogen-rich foods, although populations with background phyto-oestrogen-rich diets may be not the most suitable for investigating the effect of increasing phyto-oestrogen intake.

To control for background consumption of phyto-oestrogens, information about the isoflavone or lignan content of generic foods is available in several databases (Kiely *et al.* 2003). High intakes of phyto-oestrogens can, therefore, easily be screened and monitored by using a food frequency-type questionnaire for specific phyto-oestrogen-rich foods, whereas high phyto-oestrogen consumption can be easily avoided by restricting consumption of relatively few well-defined products (foods and supplements).

Confounding influence of other dietary compounds. A large number of macro- and micronutrients are known to affect bone metabolism. As for other conditions being modulated by several nutritional factors (e.g. hypertension; Appel *et al.* 1997), intervention studies on diet and bone focusing on a single nutrient are challenging. On one hand, single nutrients may have an effect on bone too small to be detected clinically, but be relevant collectively to bone health in the context of an entire diet. Moreover, the individual effects of nutrients that tend to cluster into food groups (e.g. phyto-oestrogens, minerals K and Mg, fibre and vitamin C in fruits and vegetables) are difficult to isolate. On the other hand, the precision of current tools for dietary assessment in outpatients is generally low and food composition tables available for analysis of intake contain imprecise and/or insufficient information relative to some nutrients of interest (e.g. phyto-oestrogens, vitamin K).

Of several methods available to compare dietary intake between the intervention groups, validated food frequency questionnaires, dietary records and diet histories are all appropriate, but have differing advantages and disadvantages. This topic has recently been extensively reviewed (Biro *et al.* 2002).

In multi-centre studies where data are collected in different countries, the comparability of dietary intake data is somewhat complicated (Biro *et al.* 2002). The most relevant variable is the validity of intake assessment methods within a country, which should allow comparison of intake between the intervention and control groups in

Table 2. Factors to be considered in human dietary studies on phyto-oestrogens

Factor	Important considerations	Reference
Test and placebo product characteristics	indistinguishable in appearance and taste	
Background phyto-oestrogen intake	unlikely to be significant in Western diets	Valsta *et al.* (2003); van Erp-Baart *et al.* (2003)
Compliance and monitoring	serum/urine monitoring may be misleading due to interindividual variability monitoring not appropriate for a blinded study	Rowland *et al.* (2003)
Confounding influence of other dietary components	effects on bone of many macro- and micronutrients (e.g. protein, Ca, vitamins K, C and A, Zn, Cu, etc.) many components cluster in food groups which makes it difficult to characterise their effects individually	Heaney (1996)
Assessments of dietary intakes	stability and reliability needed for long-term studies, with extra precautions for multi-centre studies in different countries	Biro *et al.* (2002)

that country. These intake assessment methods should focus on the dietary habits and common foods of that country, and make use of local food composition databases for intake calculations. It may be more difficult quantitatively to compare intakes of specific nutrients or other diet compounds among countries because, when dietary habits differ, intake questionnaires must differ as well. This is especially the case for food frequency questionnaires that have not been properly validated for cross-comparisons between the populations considered. Diet record methods are more suitable for international comparisons of intake but some variation may be introduced by a persistent lack of harmonisation of food composition tables.

Physical activity

Weight-bearing physical activity is known to modulate the architecture, geometry, mass and turnover of bone by stimulating matrix deposition and mineralisation (Lanyon, 1996). Exercise training of moderate intensity induces changes in serum levels of bone collagen markers (Thorsen *et al.* 1996) and consistently prevents bone loss by 1 % per annum in postmenopausal women, as assessed by DXA-measured BMD (Wolff *et al.* 1999). The bone-sparing effect of exercise is evidenced mostly at the weight-bearing sites of the skeleton and especially at the lumbar spine, where oestrogen-related bone loss is more pronounced (Berard *et al.* 1997). At present, the precise inter-relationship between oestrogens (and probably phyto-oestrogens) and mechanical stress in menopausal women is unclear. As a precaution, however, physical activity patterns should be recorded accurately and, if possible, controlled for, in clinical trials on phyto-oestrogens and bone loss during menopause.

Conclusion

Natural phyto-oestrogens (especially the isoflavones class) show some promise in the prevention of menopausal osteoporosis, but convincing evidence for their bone-sparing efficacy is still lacking in man. Further intervention studies should clarify the minimal effective dose and the magnitude of the effect in well-characterised subjects with respect to their usual dietary intake, physical activity and menopausal status. Background isoflavone intake in a typical Western diet is too low to be a concern in intervention trials. High phyto-oestrogen intakes can easily be screened and monitored by using a food frequency-type questionnaire for specific phyto-oestrogen-rich foods, whereas high phyto-oestrogen consumption can easily be avoided by restricting consumption of relatively few well-defined products (foods and supplements). Regarding the outcomes, the most appropriate markers to study the potential effects of phyto-oestrogens on bone metabolism are bone ALP, PINP and total urinary pyridinium cross-links. BMD by DXA is the method of choice to monitor bone changes, especially when combined with new techniques to assess the micro-architecture of bone. Finally, polymorphisms in ERα and ERβ, usual diet and the pattern of physical activity may be partially responsible for variations between individuals in the response to nutritional phyto-oestrogens.

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References

Alekel D, St. Germain A, Peterson C, Hanson K, Stewart J & Toda T (2000) Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *American Journal of Clinical Nutrition* **72**, 844–852.

Anderson J & Ambrose W (1995) Orally dosed genistein from soy and prevention of cancellous bone loss in two ovariectomized rat models. *Journal of Nutrition* **125**, Suppl., 799S.

Appel LJ, Moore TJ, Obarzanek E, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH & Karanja N (1997) A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *New England Journal of Medicine* **336**, 1117–1124.

Arjmandi B, Birnbaum R, Goyal N, Getlinger M, Juma S, Alekel L, Hasler C, Drum M, Hollis B & Kukreja S (1998*a*) Bone-sparing effect of soy protein in ovarian hormone-deficient rats is related to its isoflavone content. *American Journal of Clinical Nutrition* **68**, Suppl., 1364S–1368S.

Arjmandi B, Birnbaum R, Juma S, Barengolts E & Kukreja S (2000) The synthetic phyto-oestrogen, ipriflavone, and oestrogen prevent bone loss by different mechanisms. *Calcified Tissue International* **66**, 61–65.

Arjmandi B, Getlinger M, Goyal N, Alekel L, Hasler C, Joma S, Drum M, Hollis B & Kukreja S (1998*b*) Role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone deficiency in rats. *American Journal of Clinical Nutrition* **68**, Suppl., 1358S–1363S.

Arjmandi B, Slekel L, Hollis B, Amin D, Stacewicz-Sapuntzakis M, Guo P & Kukreja SC (1996) Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *Journal of Nutrition* **126**, 161–167.

Augat P, Gordon C, Lang T, Iida H & Genant H (1998) Accuracy of cortical and trabecular bone measurements with peripheral quantitative computed tomography (pQCT). *Physics in Medicine and Biology* **43**, 2873–2883.

Augat P, Reeb H & Claes L (1996) Prediction of fracture load at different skeletal sites by geometric properties of the cortical shell. *Journal of Bone and Mineral Research* **11**, 1356–1363.

Bagger Y, Jorgensen H, Heegaard A, Bayer L, Hansen L & Hassager C (2000) No major effect of oestrogen receptor gene polymorphisms on bone mineral density or bone loss in postmenopausal Danish women. *Bone* **26**, 111–116.

Bauer D, Sklarin P, Stone K, Black D, Nevitt M, Ensrud K, Arnaud C, Genant H, Garnero P, Delmas P, Lawaetz H & Cummings SR (1999) Biochemical markers of bone turnover and prediction of hip bone loss in older women: the study of osteoporotic fractures. *Journal of Bone and Mineral Research* **14**, 1404–1410.

Berard A, Bravo G & Gauthier P (1997) Meta-analysis of the effectiveness of physical activity for the prevention of bone

loss in postmenopausal women. *Osteoporosis International* **7**, 331–337.
Beresford S, Weiss N, Voight L & McKnight B (1997) Risk of endometrial cancer in relation to use of oestrogen combined with cyclic progestagen therapy in postmenopausal women. *Lancet* **349**, 458–461.
Biro G, Hulshof K, Oversen L & Amorim Cruz J (2002) Selection of methodology to assess food intake. *European Journal of Clinical Nutrition*, in press.
Bonde M, Qvist P, Fledelius C, Riis BJ & Christiansen C (1994) Immunoassay for quantifying type I degradation products in urine evaluated. *Clinical Chemistry* **40**, 2022–2025.
Bouxein M, Myburg K, van der Meulenm M, Linderberg E & Marcus R (1994) Age-related differences in cross sectional geometry of the forearm bones in healthy women. *Calcified Tissue International* **54**, 113–118.
Brady J, Ju J & Robins S (1999) Isoaspartyl bond formation within N-terminal sequences of collagen type I: implications for their use as markers of collagen degradation. *Clinical Science* **96**, 209–215.
Brandt J, Krogh T, Jensen C, Frederiksen J & Teisner B (1999) Thermal instability of the trimeric structure of the N-terminal propeptide of human procollagen type I in relation to assay technology. *Clinical Chemistry* **45**, 47–53.
Cassidy A, Bingham S & Setchell K (1994) Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *American Journal of Clinical Nutrition* **60**, 333–340.
Cawte SA, Pearson D, Green DJ, Maslanka WB, Miller CG & Rogers AT (1999) Cross-calibration, precision and patient dose measurements in preparation for clinical trials using dual energy X-ray absorptiometry of the lumbar spine. *British Journal of Radiology* **72**, 354–362.
Chrischilles E, Shireman T & Wallace R (1994) Cost and health effects of osteoporotic fractures. *Bone* **15**, 337–386.
Clemens J, Herrick M, Singer F & Eyre D (1997) Evidence that serum NTX (collagen-type I N-telopeptides) can act as an immunochemical marker of bone resorption. *Clinical Chemistry* **43**, 2058–2063.
Cloos P & Fledelius C (2000) Collagen fragments in urine derived from bone resorption are highly racemized and isomerized: a biological clock of protein aging with clinical potential. *Biochemical Journal* **345**, 473–480.
Colditz G, Hankinson S, Hunter D, Willett W, Manson J, Stampfer M, Hennekens C, Rosner B & Speizer F (1995) The use of oestrogens and progestins and the risk of breast cancer in postmenopausal women. *New England Journal of Medicine* **332**, 1589–1593.
Cooper C & Melton LI (1996) Magnitude of the impact of osteoporosis and fractures. In *Osteoporosis*, pp. 419–434 [R Marcus, D Feldman and J Kelsey, editors]. San Diego, CA: Academic Press.
Coxam V (2003) Prevention of osteopaenia by phyto-oestrogens: animal studies. *British Journal of Nutrition* **89**, Suppl. 1, S75–S85.
Cummings S, Black D, Nevitt M, Browner W, Cauley J, Ensrud K, Genant H, Palermo L, Scott J & Vogt T (1993) Bone density at various sites for prediction of hip fractures. *Lancet* **341**, 72–75.
Cummings S, Nevitt M, Browner W, Stone K, Fox K, Ensrud K, Cauley J, Black D & Vogt T (1995) Risk factors for hip fracture in white women. *New England Journal of Medicine* **332**, 767–773.
Dalais F, Rice G, Wahlqvist M, Grehan M, Murkies A, Medley G, Ayton R & Strauss B (1998) Effects of dietary phyto-oestrogens in postmenopausal women. *Climateric* **1**, 124–129.
Dawson-Hughes B, Harris S, Krall E & Dallal G (1997) Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. *New England Journal of Medicine* **337**, 670–676.
Delmas P (1990) Biochemical markers of bone turnover for the clinical assessment of metabolic bone disease. *Endocrinology and Metabolism Clinics of North America* **19**, 1–18.
Delmas PD (2001) Bone marker nomenclature. *Bone* **28**, 575–576.
Deng H, Li J, Li JL, Johnson M, Gong G & Recker R (1999) Association of VDR and oestrogen receptor genotypes with bone mass in postmenopausal Caucasian women: different conclusions with different analyses and the implications. *Osteoporosis International* **9**, 499–507.
Deng HW, Li J, Li JL, Johnson M, Gong G, Davis K & Recker RR (1998) Change of bone mass in postmenopausal Caucasian women with and without hormone replacement therapy is associated with vitamin D receptor and oestrogen receptor genotypes. *Human Genetics* **103**, 576–585.
Draper C, Edel M, Dick I, Randall A, Martin G & Prince R (1997) Phyto-oestrogens reduce bone loss and bone resorption in ovariectomized rats. *Journal of Nutrition* **127**, 1795–1799.
Eastell R, Mallinak N, Weiss S, Ettinger M, Pettinger M, Cain D, Fressland K & Chesnut CR (2000) Biological variability of serum and urinary N-telopeptides of type I collagen in postmenopausal women. *Journal of Bone and Mineral Research* **15**, 594–598.
Ebbsesen E, Thomsen J, Beck-Nielsen H, Nepper-Rasmussen H & Mosekilde L (1999) Age- and gender-related differences in vertebral bone mass, density, and strength. *Journal of Bone Mineral Research* **14**, 1394–1403.
Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M & Gustafsson J-A (1997) Human oestrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82**, 4258–4265.
Fanti P, Monier-Faugere M, Geng Z, Schmith J, Morris P, Cohen D & Malluch H (1998) The phyto-oestrogen genistein reduces bone loss in short-term ovariectomized rats. *Osteoporosis International* **8**, 274–281.
Faulkner KG (2001) Update on bone density measurement. *Rheumatic Diseases Clinics of North America* **27**, 81–99.
Faulkner KG & Pocock N (2001) Future methods in the assessment of bone mass and structure. *Best Practice Research in Clinical Rheumatology* **15**, 359–383.
Fedarko NS, Fohr B, Robey PG, Young MF & Fisher LW (2000) Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. *Journal of Biological Chemistry* **275**, 16666–16672.
Fedarko NS, Jain A, Karadag A, Van Eman MR & Fisher LW (2001) Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. *Clinical Cancer Research* **7**, 4060–4066.
Fledelius C, Johnsen A, Cloos P, Bonde M & Qvist P (1997) Characterization of urinary degradation products derived from type I collagen. Identification of a beta-isomerized Asp–Gly sequence within the C-terminal telopeptide (a1) region. *Journal of Biological Chemistry* **272**, 9755–9763.
Formica CA, Nieves JW, Cosman F, Garrett P & Lindsay R (1998) Comparative assessment of bone mineral measurements using dual X-ray absorptiometry and peripheral quantitative computed tomography. *Osteoporosis International* **8**, 460–467.
Fuleihan G, Testa M, Angell J, Porrino N & Leboff M (1995) Reproducibility of DXA absorptiometry: a model for bone loss estimates. *Journal of Bone and Mineral Research* **10**, 1004–1014.
Gambacciani M, Ciaponi M, Cappagli B, Piaggesi L & Genazzani A (1997) Effects of combined low dose of the isoflavone derivative ipriflavone and oestrogen replacement on bone

mineral density and metabolism in postmenopausal women. *Maturitas* **28**, 75–81.
Garnero P & Delmas P (1993) Assessment of the serum levels of bone alkaline phosphatase with a new immunoradiometric assay in patients with metabolic bone disease. *Journal of Clinical Endocrinology and Metabolism* **77**, 1046–1053.
Garnero P, Grimaux M, Seguin P & Delmas P (1994) Characterisation of immunoreactive forms of human osteocalcin generated in vivo and in vitro. *Journal of Bone and Mineral Research* **9**, 255–264.
Garnero P, Hausherr E, Chapuy M, Marcelli C, Grandjean H, Muller C, Cormier C, Breart G, Meunier P & Delmas P (1996*a*) Markers of bone resorption predict hip fracture in elderly women: the EPIDOS prospective study. *Journal of Bone and Mineral Research* **11**, 1531–1538.
Garnero P, Sornay-Rendu E, Chapuy M & Delmas P (1996*b*) Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *Journal of Bone and Mineral Research* **11**, 337–349.
Garnero P, SornayRendu E, Duboeuf F & Delmas P (1999) Markers of bone turnover predict postmenopausal forearm bone loss over 4 years: the OFELY study. *Journal of Bone and Mineral Research* **14**, 1614–1621.
Gennari C, Adami S, Agnusdei D, Bufalino L, Cervetti R, Crepaldi G, Di Marco C, Di Munno O, Fantasia L, Isaia G, Mazzuoli G, Ortolani S, Passeri M, Serni U & Vecchiet L (1997) Effect of chronic treatment with ipriflavone in postmenopausal women with low bone mass. *Calcified Tissue International* **61**, S19–S22.
Gennari C, Agnusdei D, Crepaldi G, Isaia G, Mazzuoli G, Ortolani S, Bufalino L & Passeri M (1998*a*) Effect of ipriflavone — a synthetic derivative of natural isoflavones — on bone mass loss in the early years after menopause. *Menopause* **5**, 9–15.
Gennari L, Becherini L, Masi L, Mansani R, Gonnelli S, Cepollaro C, Martini S, Montagnani A, Lentini G, Becorpi A & Brandi M (1998*b*) Vitamin D and oestrogen receptor allelic variants in Italian postmenopausal women: evidence of multiple gene contribution to bone mineral density. *Journal of Clinical Endocrinology and Metabolism* **83**, 939–944.
Gomez B, Ardakani S, Evans B, Merrell L, Jenkins D & Kung V (1996) Monoclonal antibody assay for free urinary pyridinium cross-links. *Clinical Chemistry* **42**, 1168–1175.
Gomez B, Ardakani S, Ju J, Jenkins D, Cerelli M, Daniloff G & Kung V (1995) Monoclonal antibody assay for measuring bone-specific alkaline phosphatase activity in serum. *Clinical Chemistry* **41**, 1560–1566.
Gotfredsen A, Baeksgaard L & Hilsted J (1997) Body composition analysis by DEXA by using dynamically changing samarium filtration. *Journal of Applied Physiology* **82**, 1200–1209.
Greendale GA, Wells B, Marcus R & Barrett Connor E (2000) How many women lose bone mineral density while taking hormone replacement therapy? Results from the Postmenopausal Oestrogen/Progestin Interventions Trial. *Archives of Internal Medicine* **160**, 3065–3071.
Hadjidakis D, Kokkinakis E, Sfakianakis M & Raptis S (1999) The type and time of menopause as decisive factors for bone mass changes. *European Journal of Clinical Investigation* **29**, 877–885.
Halleen JM, Alatalo SL, Suominen H, Cheng S, Janckila AJ & Vaananen HK (2000) Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. *Journal of Bone and Mineral Research* **15**, 1337–1345.
Han K, Choi J, Moon I, Yoon H, Han I, Min H, Kim Y & Choi Y (1999) Non-association of oestrogen receptor genotypes with bone mineral density and bone turnover in Korean pre-, peri-, and postmenopausal women. *Osteoporosis International* **9**, 290–295.
Han K, Moon I, Kang Y, Chung H, Min H & Han I (1997) Non-association of oestrogen receptor genotypes with bone mineral density and oestrogen responsiveness to hormone replacement therapy in Korean postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **82**, 991–995.
Hanson D, Weis M, Bollen A, Maslan S, Singer F & Eyre D (1992) A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *Journal of Bone and Mineral Research* **7**, 1251–1258.
Hassager C, Jensen S & Christiansen C (1994) Non-responders to hormone replacement therapy for the prevention of postmenopausal bone loss: do they exist? *Osteoporosis International* **4**, 36–41.
Heaney R (1996) Nutrition and risk for osteoporosis. In *Osteoporosis*, pp. 483–509 [R Marcus, D Feldman and J Kelsey, editors]. San Diego, CA: Academic Press.
Heaney R (1998) Excess dietary protein may not adversely affect bone. *Journal of Nutrition* **128**, 1054–1057.
Horiuchi T, Onouchi T, Takahashi M, Ito H & Orimo H (2000) Effect of soy protein on bone metabolism in postmenopausal Japanese women. *Osteoporosis International* **11**, 721–724.
Hsu CS, Shen WW, Hsueh YM & Yeh SL (2001) Soy isoflavone supplementation in postmenopausal women. Effects on plasma lipids, antioxidant enzyme activities and bone density. *Journal of Reproductive Medicine* **46**, 221–226.
Hui S, Wiske H, Norton JA & Johnston CC Jr (1982) A prospective study of change in bone mass with age in postmenopausal women. *Journal of Chronic Diseases* **35**, 715–725.
Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T & Ikegami S (1999) Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by oestrogen deficiency. *Endocrinology* **140**, 1893–1900.
James I & Perrett D (1998) Automated on-line solid-phase extraction and high-performance liquid chromatographic analysis of total and free pyridinium crosslinks in serum. *Journal of Chromatography* **79A**, 159–166.
Jayo M, Anthony M, Register T, Rankin S, Vest T & Clakson T (1997) Dietary soy isoflavones and bone loss in ovariectomized monkeys. *FASEB Journal* **11**, 228, Abstr.
Jiang Y, Zhao J, Augat P, Ouyang X, Lu Y, Majumdar S & Genant H (1998) Trabecular bone mineral and calculated structure of human bone specimens scanned by peripheral quantitative computed tomography: relation to biomechanical properties. *Journal of Bone and Mineral Research* **13**, 1783–1790.
Kanis J (1993) The incidence of hip fracture in Europe. *Osteoporosis International* **1**, Suppl., 10S–15S.
Kardinaal A, Morton M, Bruggermann-Rotgans I & Van Beresteijn E (1998) Phyto-oestrogen excretion and rate of bone loss in postmenopausal women. *European Journal of Clinical Nutrition* **52**, 850–855.
Karmatschek M, Maier I, Seibel M, Woitge H, Ziegler R & Armbruster F (1997) Improved purification of human bone sialoprotein and development of a homologous radioimmunoassay. *Clinical Chemistry* **43**, 2076–2082.
Kiely M, Faughnan M, Wähälä K, Brants H & Mulligan A (2003) Phyto-oestrogen levels in foods: the design and construction of the VENUS database. *British Journal of Nutrition* **89**, Suppl. 1, S19–S23.
Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M & Orimo H (1996) Association of bone mineral density with polymorphism of the oestrogen receptor gene. *Journal of Bone and Mineral Research* **11**, 306–311.

Komulainen M, Kröger H, Tuppurainen M, Heikkinen A-M, Alhava E, Honkanen R, Jurvelin J & Saarikoski S (1999) Prevention of femoral and lumbar bone loss with hormone replacement therapy and vitamin D3 in early postmenopausal women: a population-based 5-year randomized trial. *Journal of Clinical Endocrinology and Metabolism* **84**, 546–552.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of oestrogen receptors alpha and beta. *Endocrinology* **138**, 863–870.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B & Gustafsson JA (1998) Interaction of oestrogenic chemicals and phyto-oestrogens with oestrogen receptor beta. *Endocrinology* **139**, 4252–4263.

Lang T, Augat P, Majumdar S, Ouyang X & Genant H (1998) Noninvasive assessment of bone density and structure using computed tomography and magnetic resonance. *Bone* **22**, Suppl. 5, 149S–153S.

Lang TF, Keyak JH, Heitz MW, Augat P, Lu Y, Mathur A & Genant HK (1997) Volumetric quantitative computed tomography of the proximal femur: precision and relation to bone strength. *Bone* **21**, 101–108.

Lanyon LE (1996) Using functional loading to influence bone mass and architecture: objectives, mechanisms, and relationship with oestrogen of the mechanically adaptive process in bone. *Bone* **18**, 37S–43S.

Lindsay R, Hart D, Aitken J, MacDonald E, Anderson J & Clarke A (1976) Long-term prevention of postmenopausal osteoporosis by oestrogen: evidence for an increased bone mass after delayed onset of oestrogen treatment. *Lancet* **1**, 1038–1041.

McClung M, Clemmesen B, Daifotis A, Gilchrist N, Eisman J, Weinstein R, Fuleihan G, Reda C, Yates A & Ravn P (1998) Alendronate prevents postmenopausal bone loss in women without osteoporosis. *Annals of Internal Medicine* **128**, 253–261.

Mahonen A, Turunen A, Kroger H & Maenpaa P (1997) Oestrogen receptor polymorphism is associated with bone mineral density in perimenopausal Finnish women. *Journal of Bone and Mineral Research* **12**, T614.

Majumdar S, Link T, Augat P, Lin J, Newitt D, Lane N & Genant H (1999) Trabecular bone architecture in the distal radius using magnetic resonance imaging in subjects with fractures of the proximal femur. Magnetic Resonance Science Center and Osteoporosis and Arthritis Research Group. *Osteoporosis International* **10**, 231–239.

Martin P, Horwitz K, Ryan D & McGuire W (1978) Phyto-oestrogen interaction with oestrogen receptors in human breast cancer cells. *Endocrinology* **103**, 1860–1867.

Massey L (1998) Does dietary protein adversely affect bone? *Journal of Nutrition* **128**, 1048–1050.

Mazzuoli G, Acca M, Pisani D, Diacinti D, Scarda A, Scarnecchia L, D'Erasmo E, Minisola S, Bianchi G & Manfredi G (2000) Annual skeletal balance and metabolic bone marker change in healthy early postmenopausal women: results of a prospective study. *Bone* **26**, 381–386.

Melkko J, Kauppila S, Niemi S, Risteli L, Haukipuro K, Jukkola A & Risteli J (1996) Immunoassay for intact amino-terminal propeptide of human type I procollagen. *Clinical Chemistry* **42**, 947–954.

Melkko J, Niemi S, Risteli L & Risteli J (1990) Radioimmunoassay of the carboxy terminal propeptide of human type I procollagen. *Clinical Chemistry* **36**, 1328–1332.

Melton LD (1995) Epidemiology of fractures. In *Osteoporosis: Etiology, Diagnosis, and Management*, pp. 225–247 [B Riggs and LD Melton, editors]. Philadelphia, PA: Lippincott-Raven.

Messina M (1999) Legumes and soybeans: overview of their nutritional profiles and health effects. *American Journal of Clinical Nutrition* **70**, Suppl., 439S–450S.

Mizunuma H, Hosoi T, Okano H, Soda M, Tokizawa T, Kagami I, Miyamoto S, Ibuki Y, Inoue S, Shiraki M & Ouchi Y (1997) Oestrogen receptor gene polymorphism and bone mineral density at the lumbar spine of pre- and postmenopausal women. *Bone* **21**, 379–383.

National Institutes of Health Consensus Statement (2000) *Osteoporosis Prevention, Diagnosis, and Therapy*, vol. 17. Bethesda, MD: National Institutes of Health.

Nawata H, Tanaka S, Tanaka S, Takayanagi R, Sakai Y, Yanase T, Ikuyama S & Haji M (1995) Aromatase in bone cell: association with osteoporosis in postmenopausal women. *Journal of Steroid Biochemistry and Molecular Biology* **53**, 65–74.

Nguyen T, Jones G, Sambrook P, White C, Kelly P & Eisman J (1995) Effects of oestrogen exposure and reproductive factors on bone mineral density and osteoporotic fractures. *Journal of Clinical Endocrinology and Metabolism* **80**, 2709–2714.

Nielsen P, Barenholdt O, Diessel E, Armbrust S & Felsenberg D (1998) Linearity and accuracy errors in bone densitometry. *British Journal of Radiology* **71**, 1062–1068.

Nilas L (1993) Calcium intake and osteoporosis. In *Osteoporosis: Nutritional Aspects*, 1st ed. pp. 1–27 [A Simopoulos and C Galli, editors]. *World Review of Nutrition and Dietetics* vol. 73. Basel: Karger.

North American Menopause Society (2000) The role of isoflavones in menopausal health: consensus opinion of the North American Menopause Society. *Menopause* **7A**, 215–229.

Ogawa S, Hosoi T, Shiraki M, Orimo H, Emi M, Muramatsu M, Ouchi Y & Inoue S (2000) Association of oestrogen receptor beta gene polymorphism with bone mineral density. *Biochemical and Biophysical Research Communications* **269**, 537–541.

Ongphiphadhanakul B, Chanprasertyothin S, Payatikul P, Tung S, Piaseu N, Chailurkit L, Chansirikarn S, Puavilai G & Rajatanavin R (2000) Oestrogen-receptor-alpha gene polymorphism affects response in bone mineral density to oestrogen in post-menopausal women. *Clinical Endocrinology* **52**, 581–585.

Ongphiphadhanakul B, Rajatanavin R, Chanprasertyothin S, Piaseu N, Chailurkit L, Sirisriro R & Komindr S (1998) Oestrogen receptor gene polymorphism is associated with bone mineral density in premenopausal women but not in postmenopausal women. *Journal of Endocrinological Investigation* **21**, 487–493.

Pearson J, Dequeker J, Reeve J, Felsenberg D, Henley M, Bright J, Lunt M, Adams J, Diaz Curiel M & Galan F (1995) Dual X-ray absorptiometry of the proximal femur: normal European values standardized with the European Spine Phantom. *Journal of Bone and Mineral Research* **10**, 315–324.

Potter S, Baum J, Teng H, Stillman R, Shay N & Erdman J Jr (1998) Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *American Journal of Clinical Nutrition* **68**, Suppl., 1375S–1379S.

Reinli K & Block G (1996) Phyto-oestrogen content of foods — a compendium of literature values. *Nutrition and Cancer* **26**, 123–148.

Riggs B & Melton LJ III (1986) Involutional osteoporosis. *New England Journal of Medicine* **14**, 1676–1686.

Robins S, Woitge H, Hesley R, Ju J, Seyedin S & Seibel M (1994) Direct, enzyme-linked immunoassay for urinary deoxypyridinoline as a specific marker for measuring bone resorption. *Journal of Bone and Mineral Research* **9**, 1643–1649.

Rosenkranz K, Hinney A, Ziegler A, Hermann H, Fichter M, Mayer H, Siegfried W, Young J, Remschmidt H & Hebebrand J (1998) Systematic mutation screening of the oestrogen receptor beta gene in probands of different weight

extremes: identification of several genetic variants. *Journal of Clinical Endocrinology and Metabolism* **83**, 4524–4527.
Rosenquist C, Fledelius C, Christgau S, Pedersen B, Bonde M, Qvist P & Christiansen C (1998) Serum CrossLaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. *Clinical Chemistry* **44**, 2281–2289.
Ross P, Heilbrun L, Wasnich R, Davis J & Vogel J (1989) Perspectives: methodological issues in evaluating risk factors for osteoporotic fractures. *Journal of Bone and Mineral Research* **4**, 649–656.
Ross PD, Norimatsu H, Davis JW, Yano K, Wasnich RD, Fujiwara S, Hosoda Y & Melton LJ (1991) A comparison of hip fracture incidence among native Japanese, Japanese Americans, and American Caucasians. *American Journal of Epidemiology* **133**, 801–809.
Roubenoff R, Kehayias J, Dawson-Hughes B & Heymsfield S (1993) Use of dual-energy x-ray absorptiometry in body composition studies: not yet a 'gold standard'. *American Journal of Clinical Nutrition* **58**, 589–591.
Rowland I, Faughnan M, Hoey L, Wähälä K, Williamson G & Cassidy A (2003) Bioavailability of phyto-oestrogens. *British Journal of Nutrition* **89**, Suppl. 1, S45–S58.
Salmen T, Heikkinen A, Mahonen A, Kroger H, Komulainen M, Saarikoski S, Honkanen R & Maenpaa P (2000) Early postmenopausal bone loss is associated with Pvu II oestrogen receptor gene polymorphism in Finnish women: effect of hormone replacement therapy. *Journal of Bone and Mineral Research* **15**, 315–321.
Sano M, Inoue S, Hosoi T, Ouchi Y, Emi M, Shiraki M & Orimo H (1995) Association of oestrogen receptor dinucleotide repeat polymorphism with osteoporosis. *Biochemical and Biophysical Research Communications* **215**, 378–383.
Scheiber MD, Liu JH, Subbiah MT, Rebar RW & Setchell KD (2001) NAMS Fellowship Findings: Dietary inclusion of whole soy foods results in significant reductions in clinical risk factors for osteoporosis and cardiovascular disease in normal postmenopausal women. *Menopause* **8**, 384–392.
Seibel M, Woitge H, Pecherstorfer M, Karmatschek M, Horn E, Ludwig H, Armbruster F & Ziegler R (1996) Serum immunoreactive bone sialoprotein as a new marker of bone turnover in metabolic and malignant bone disease. *Journal of Clinical Endocrinology and Metabolism* **81**, 3289–3294.
Setchell K & Cassidy A (1999) Dietary isoflavones: biological effects and relevance to human health. *Journal of Nutrition* **129**, 758S–767S.
Setchell KD, Brown NM, Desai P, Zimmer Nechemias L, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A & Heubi JE (2001) Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *Journal of Nutrition* **131**, 1362S–1375S.
Siffert R, Luo G, Cowin S & Kauffman J (1996) Dynamic relationships of trabecular bone density, architecture, and strength in a computational model of osteopenia. *Bone* **18**, 197–206.
Somekawa Y, Chiguchi M, Ishibashi T & Aso T (2001) Soy intake related to menopausal symptoms, serum lipids, and bone mineral density in postmenopausal Japanese women. *Obstetrics and Gynecology* **97**, 109–115.
Thorsen K, Kristoffersson A & Lorentzon R (1996) The effects of brisk walking on markers of bone and calcium metabolism in postmenopausal women. *Calcified Tissue International* **58**, 221–225.
Valsta L, Kilkkinen A, Mazur W, Nurmi T, Lampi A-M, Ovaskainen M-L, Korhonen T, Adlercreutz H & Pietinen P (2003) Phyto-oestrogen database of foods and average intake in Finland. *British Journal of Nutrition* **89**, Suppl. 1, S31–S38.
Valtueña S, Sette S & Branca F (2001) Influence of Mediterranean diet and Mediterranean lifestyle on calcium and bone metabolism. *International Journal for Vitamin and Nutrition Research* **71**, 189–202.
van Daele P, Seibel M, Burger H, Hofman A, Grobbee D, van Leeuwen J, Birkenhaeger JC & Pols HAP (1996) Case–control analysis of bone resorption markers, disability and hip fracture risk: the Rotterdam study. *British Medical Journal* **312**, 482–483.
Vandevyver C, Vanhoof J, Declerck K, Stinissen P, Vandervorst C, Michiels L, Cassiman J, Boonen S, Raus J & Geusens P (1999) Lack of association between oestrogen receptor genotypes and bone mineral density, fracture history, or muscle strength in elderly women. *Journal of Bone and Mineral Research* **14**, 1576–1582.
van Erp-Baart AMJ, Brants HAM, Kiely M, Mulligan A, Turrini A, Sermoneta C, Kilkkinen A & Valsta LM (2003) Isoflavone intake in four different European countries: the VENUS approach. *British Journal of Nutrition* **89**, Suppl. 1, S25–S30.
Van Loan M, Johnson H & Barbiery T (1998) Effects of weight loss on bone mineral content and bone mineral density in obese women. *American Journal of Clinical Nutrition* **67**, 734–738.
Vitolins M, Anthony M & Burke G (2001) Soy protein isoflavones, lipids and arterial disease. *Current Opinion in Lipidology* **12**, 433–437.
Wangen KE, Duncan AM, Xu X & Kurzer MS (2001) Soy isoflavones improve plasma lipids in normocholesterolemic and mildly hypercholesterolemic postmenopausal women. *American Journal of Clinical Nutrition* **73**, 225–231.
Willing M, Sowers M, Aron D, Clark M, Burns T, Bunten C, Crutchfield M, Dagostino D & Jannausch M (1998) Bone mineral density and its change in white women: oestrogen and vitamin D receptor genotypes and their interaction. *Journal of Bone and Mineral Research* **13**, 695–705.
Wolff I, van Croonenborg JJ, Kemper HC, Kostense PJ & Twisk JW (1999) The effect of exercise training programs on bone mass: a meta-analysis of published controlled trials in pre- and postmenopausal women. *Osteoporosis International* **9**, 1–12.
World Health Organization (1994) *Assessment of Fracture Risk and Its Application to Screening for Postmenopausal Osteoporosis*. Geneva: World Health Organization.