

Biomarkers of bone health appropriate for evaluating functional foods designed to reduce risk of osteoporosis

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Osteoporosis is a growing global problem. The health care costs and decreased productivity and quality of life are staggering. Much research is invested in life-style approaches to build peak bone mass during growth to prevent osteoporosis as well as to treat the disease in later life. Functional foods have enjoyed a niche in bone health. Foods fortified with Ca are most popular. Other bone nutrients such as vitamin D, Mg and vitamin K are sometimes added. Future products are likely to include enhancers of Ca absorption such as inulin or whey proteins. Dietary factors that reduce urinary Ca loss (plant proteins) or suppress bone resorption (possibly phyto-oestrogens) are also gaining attention. Methodologies for evaluating the effectiveness of functional foods on bone health include measures of bone quality such as bone densitometry or measures of Ca metabolism, particularly absorption. Biochemical markers for bone turnover are less satisfactory for diet-related effects. Use of a rare isotope, ⁴¹Ca, and accelerator mass spectrometry offers a new approach for assessing the ability of functional foods to suppress bone resorption.

Osteoporosis: Bone health: Food fortification: Biochemical biomarkers: Calcium

Introduction

Biomarkers to evaluate the effectiveness of functional foods for bone health are quite advanced compared with many other diseases. Yet, simple, inexpensive and rapid methods for evaluating large numbers of people are still lacking. The appropriate choice of an outcome measure depends on the mechanism of action of the functional food and the targeted population. Functional foods designed to prevent osteoporosis may work by providing a key nutrient important to bone development and maintenance, by enhancing Ca absorption or retention, by building peak bone mass or by suppressing bone turnover and, therefore, bone loss. Because functional foods for bone health are largely targeted towards increasing Ca intake or utilization of Ca, the focus of this review will be on strategies to improve Ca nutrition and methods to evaluate their effectiveness.

Importance of adequate dietary calcium

There is a renewed interest in the importance of adequate Ca intake, because of a greater appreciation of its role in health and disease prevention. The role of Ca in bone health is best understood as the primary cation in bone (Bryant *et al.* 1999). Requirements of Ca are based on

intakes adequate for bone accretion and maintenance. There are many other health reasons to achieve adequate Ca intake. The role of Ca and dairy product consumption in vascular tone and blood pressure control was reviewed recently (McCarron & Reusser, 1999). Observational studies suggest adequate Ca decreases risk of kidney stones, probably by decreasing urinary oxalate level (Heller, 1999). Increasing Ca and dairy food intakes appear to reduce risk of colon cancer, probably through lowering faecal bile acid and fatty acid concentrations, which lowers cytotoxicity (Holt, 1999). The reversal of dietary fat-induced hyperplasia and hyperproliferation in mammary and colonic tissues in mice when Ca and vitamin D were supplemented led to the recent suggestion that Ca is also important in preventing breast cancer (Lipkin & Newmark, 1999). A multi-centre trial showed that Ca supplementation significantly ($P < 0.05$) decreased premenstrual symptoms relative to those observed in a placebo-controlled group (Thys-Jacobs *et al.* 1998). More recently, adequate Ca intake has been associated with maintenance of body fat and body weight (Lin *et al.* 2000; Zemel *et al.* 2000).

Ca intakes for most groups over the age of 11 years fall short of the recommended intakes in North America (Food and Nutrition Board, 1997) and many other parts of the world. Ca absorption and retention are inefficient. Ca

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absorption in adults averages only 30% (Heaney *et al.* 1988) and losses through endogenous secretions approximate 120 mg/d regardless of intake (Wastney *et al.* 1996). Early man is thought to have consumed liberal amounts of Ca compared with the intake of modern man (Eaton & Konner, 1985). Because osteoporosis and the other diseases described above typically occur post reproduction, there is little evolutionary pressure to adapt to the current low intake of Ca. Increasing Ca intake is a prudent solution to the Ca deficit, but improving absorption and retention could also improve Ca nutriture.

Absorption: mechanisms and methodology

Ca bioavailability is frequently equated to Ca absorption. Absorption is the first barrier to achieving Ca homeostasis. Dietary factors that affect excretion or bone resorption, i.e. net retention, will be discussed in later sections. Ca is

absorbed both by active, transcellular and by passive, paracellular processes (Fig. 1). Active absorption dominates at low Ca intake, but owing to its saturable nature and subsequent down-regulation at adequate intake, this pathway becomes less important with increasing Ca intake. Ca absorption efficiency is inversely related to Ca load over a wide range of intake, although the absolute quantity of Ca absorbed increases with increased load (Heaney *et al.* 1990b).

Methods to assess Ca bioavailability include Ca balance, determination of bone (or whole-body) Ca retention, and the use of intestinal loops, Caco-2 cells and isotopic tracers. These span studies in man, animal models and *in vitro* techniques. Ca balance studies are expensive and can lead to erroneous results unless sufficient attention is paid to ensuring the completeness of food intake and urine and faecal collections. An additional challenge is the demarcation of faeces to time intervals that can be

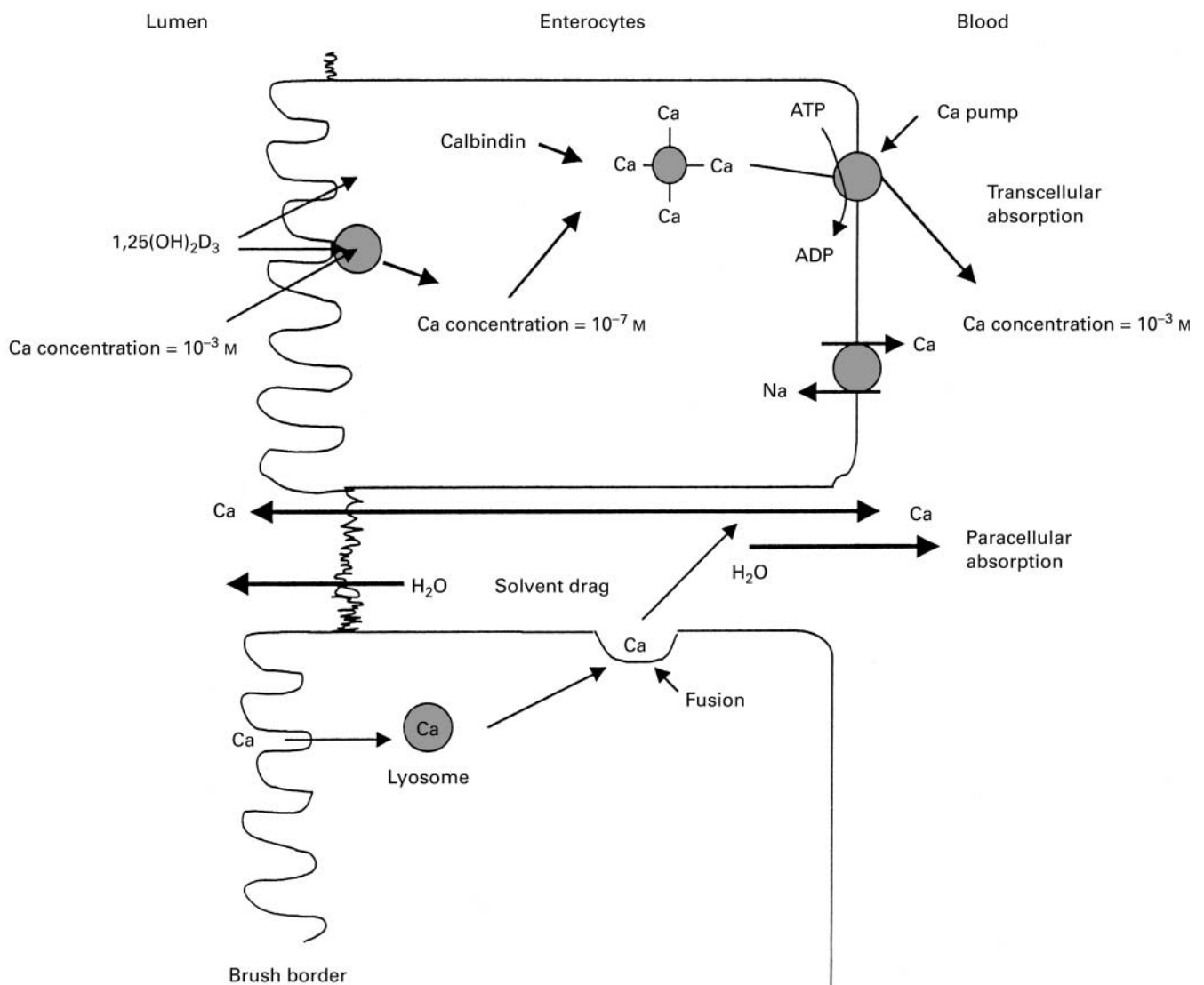


Fig. 1. Calcium absorption from intestinal lumen through intestinal epithelium to blood. Calcium can be absorbed (1) transcellularly, by a process mediated by $1,25(\text{OH})_2$ vitamin D-induced carrier, calbindin D_9 , or by endocytosis of acidic lysosomes; or (2) paracellularly, a passive process.

related to specific Ca intake; validity is improved markedly in this regard when faecal results are corrected for recovery of a quantitative faecal marker (Hargreaves & Rose, 1965). Appropriate uses of balance studies include comparisons of the bioavailability of different sources of Ca and to help quantify complex nutrient interactions which affect bioavailability (Mertz, 1987). Ca balance studies have contributed to the body of literature that suggests that both oxalate (Kelsay & Prather, 1983; Liebman & Doane, 1989) and phytate (Morris & Ellis, 1985; Liebman & Landis, 1989) are important inhibitors of Ca absorption. Balance studies can determine net Ca absorption, but not true absorption.

In animal models, the bone (femur) uptake method compares femur accumulation of an oral dose of an isotope tracer with a dose injected intraperitoneally. This method is based on the assumption that the intraperitoneal injection behaves as an oral dose with 100% absorption. With use of the whole-body counting technique, absorption of Ca from an oral dose of an isotopic tracer is based on extrapolating the linear portion of a retention curve to time zero (Koo *et al.* 1993). Koo *et al.* (1993) demonstrated that assessments of radiotracer activity of Ca in femur or whole body are equally accurate for comparing bioavailability of Ca among sources. Not having to collect excreta or multiple blood samples is a clear advantage of these methods.

Ca absorption can also be assessed with use of the *in situ* loop and everted sac methods. Studies based on these methods clearly established the existence of two Ca absorptive processes, a vitamin D-dependent, saturable, transcellular pathway that predominates in the duodenum and a non-saturable, paracellular pathway that occurs throughout the entire length of the small intestine (Pansu *et al.* 1983; Bronner *et al.* 1986). However, they are insensitive to the important factor of exposure time of the chyme to the mucosa.

Pinto *et al.* (1983) demonstrated that cultured Caco-2 cells, a human colon adenocarcinoma cell line, exhibit structural and functional differentiation patterns characteristic of mature enterocytes. Yee (1997) reported a strong correlation between *in vitro* permeability across Caco-2 cells and *in vivo* (small intestinal) absorption for a variety of compounds. It has also been confirmed recently that vitamin D-mediated Ca transport in these cells is a specific, transcellular process that requires transcriptional events normally mediated through the vitamin D receptor (Fleet & Wood, 1999). However, in culture, these cells form a membrane with tight junctions that interfere with measurement of transport.

Some have tried to use rise in urinary Ca to determine relative bioavailability, but this is a weak approach especially for Ca loads less than 500 mg. For example, variability in rise in urinary Ca was 77–99% compared with 38–60% for serum Ca following ingestion of three Ca salts (Heaney *et al.* 2001). Increments in serum Ca are also less sensitive than following the fate of isotopic tracers of Ca. Isotopic tracers have been used to label dietary sources of Ca for the measurement of Ca bioavailability in both animal models and man. Ca has two useful radioisotopes (^{45}Ca and ^{47}Ca), several useful stable isotopes (^{42}Ca , ^{43}Ca , ^{44}Ca , ^{46}Ca and ^{48}Ca), as well as one

long-lived radioisotope (^{41}Ca). Tracers can be followed by appearance in plasma, excretion or by whole-body retention in the case of ^{47}Ca . Use of tracers for determining Ca absorption gives true absorption, in contrast to net absorption determined by balance. Intrinsic labelling of a broad array of hydroponically grown plants, milk made into a variety of dairy products and salts has enabled determination of Ca absorption in man from most Ca-rich foodstuffs in the Western diet (Weaver *et al.* 1999). Isotopic tracers can also be used to determine transfer rates, sites of absorption and pathway of absorption. When absorption is plotted *v.* various Ca loads, a curvilinear relationship suggests active transport and a linear relationship suggests paracellular absorption (see Fig. 2).

Modifying calcium absorption

In a recent review, Bronner & Pansu (1999) stated: 'It is obvious that calcium must be ionized and in solution to be absorbed'. Typically, Ca must be dissociated from its ligands in a foodstuff prior to absorption. However, there is recent evidence that Ca from a small-molecular-weight compound did not require dissociation prior to absorption (Hanes *et al.* 1999*a,b*). This was apparent because Ca and oxalic acid have very different serum appearance profiles, yet doubly labelled ^{45}Ca - ^{14}C oxalate showed parallel serum profiles of the two labels until the unabsorbed salt reached the colon, where bacterial hydrolysis presumably occurs. It is tempting to conclude that absorption of the salt occurred paracellularly, but calcium oxalate could conceivably transverse the epithelial membrane because it is apolar. The implications of this finding might be greatest for individuals with defective active Ca absorption capacity.

Solubilization is a reasonable assumption prior to Ca absorption, at least at the absorptive surfaces. Yet, *in vitro* solubility of Ca salts at neutral pH over a wide range had little impact on Ca absorption (Heaney *et al.* 1990*a*). For example, Ca absorption from CaCO_3 with a solubility of 0.14 mmol/l is as good as from tricalcium phosphate, which is nearly an order of magnitude more soluble at 0.97 mmol/l. Only salts that are at the extreme

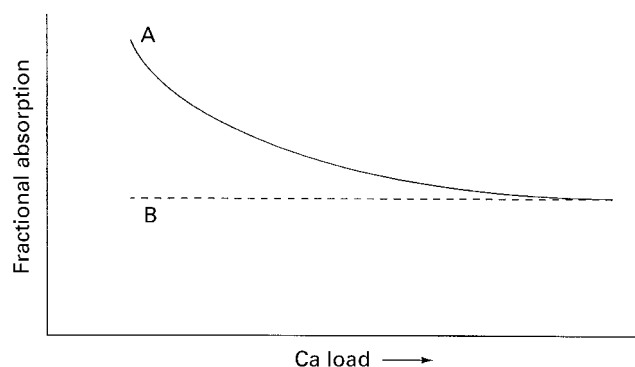


Fig. 2. Illustration of (A) saturable and (B) non-saturable components of calcium kinetics curves. At large calcium loads, active transport approaches a small value. The linear portion of the curve reflects constant and paracellular absorption.

ends of solubility have appreciably different Ca absorption efficiencies.

Other factors that influence Ca bioavailability include the presence of inhibitors and enhancers in the diet. Functional foods for bone health may decrease the content of inhibitors and increase the content of enhancers. The most potent inhibitor of Ca absorption is oxalate (Heaney & Weaver, 1989). Fibre has been labelled as an inhibitor of Ca absorption since the early balance studies of McCance & Widdowson (1942). However, research with purified fibres has suggested that fibre does not influence Ca absorption appreciably (Heaney & Weaver, 1995). This is supported by the finding that Ca absorption efficiency from fibre-rich brassica vegetables is higher than from other foodstuffs (Weaver *et al.* 1999). The presence of an enhancer of Ca absorption in brassica vegetables is suspected, but not yet identified. In contrast to the lack of fibre effect, phytates associated with cereal and legume fibres can decrease Ca absorption (Heaney *et al.* 1991; Weaver *et al.* 1991). The capacity of phytate-rich extruded wheat bran cereal to bind Ca is linear over a wide range of Ca intake (Weaver *et al.* 1996).

Few Ca absorption enhancers have been identified. Most research has concentrated on searching for highly absorbable Ca salts. Only a few have been identified, including calcium citrate malate with a solubility of 80 mmol/l (Miller *et al.* 1988), calcium gluconate glycerophosphate (Schanler & Abrams, 1995) and calcium ascorbate (Tsugawa *et al.* 1999). Additionally, a few Ca absorption enhancers have been identified. Consumption of 15 g oligofructose/d increased stable isotopic tracer Ca absorption from 47.8% during a placebo period to 60.1% (van den Heuvel *et al.* 1999). Feeding of 40 g inulin/d increased apparent Ca absorption in adults

participating in a balance study from 21.3 to 33.7% (Coudray *et al.* 1997). Certain amino acids, notably lysine, and casein phosphopeptides, digestive products from milk proteins, have Ca absorption-enhancing effects under some conditions such as in women with low absorption efficiency (Heaney *et al.* 1994). Yet another type of Ca absorption enhancer is the hydrolysis product of phytic acid, 1,2,3,6-inositol tetrakisphosphate, which improved ^{45}Ca absorption efficiency from 26.2 to 30.7% in rats using calcium ascorbate as the Ca source (Shen *et al.* 1998).

Enhancement of Ca absorption has typically been attributed to the formation of soluble complexes with Ca which prevent precipitation by P in the gut. Other mechanisms of enhancing Ca absorption deserve to be explored. Increasing paracellular absorption is promising because it is not limited by becoming saturated, it is vitamin D-independent, and it occurs throughout the length of the intestine in contrast to active absorption which is dominant in the duodenum. If the intercellular junction spaces illustrated in Fig. 1 can be widened, more Ca could be absorbed. Or, if solvent drag could be increased, even though water would flow bidirectionally, given the large volume of blood that would serve to dilute ions extruded from the basolateral membrane, ions would have a net movement from lumen to blood. Ideal compounds would be those that could be incorporated into Ca-containing food to enhance absorption of Ca but would have only a transient effect, so that transfer of undesirable organisms and ions would be minimized. Pappenheimer & Reiss (1987) provided evidence that glucose and amino acids in the lumen of the small intestine increase solvent drag through paracellular channels by fuelling the contraction of epithelial cytoskeletal elements, thereby opening tight junctions to allow mass transport of nutrients.

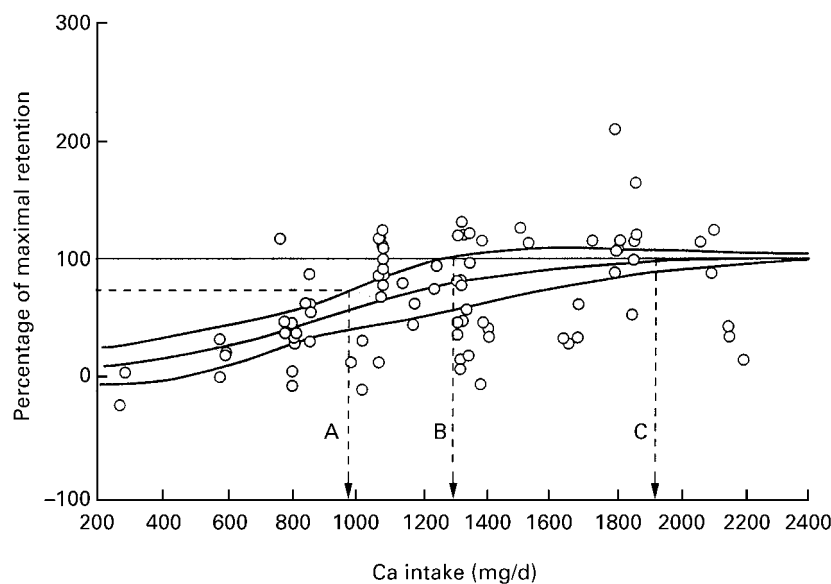


Fig. 3. Relationship between calcium retention and calcium intake in adolescent Caucasian girls (mean and 95% confidence intervals). Calcium intakes required to achieve: (A) 70% of maximal retention at the lower edge of the confidence interval; (B) 100% of maximal retention at the upper edge of the confidence interval; and (C) 100% of maximal retention at the mean.

Modifying calcium retention

Dietary factors that alter Ca absorption or Ca excretion can modify Ca retention. Nutrients with the greatest impact on Ca loss include salt and protein, both of which increase urinary Ca output. Ironically, Ca intake influences urinary Ca output only modestly; only 6% of the variance in urinary Ca in adolescents was explained by Ca intake (Jackman *et al.* 1997). Data on the effect of dietary variables on Ca retention are collected largely in adults. We know little of these relationships across the life span or of the impact of race or other genetic determinants.

Perhaps a more useful way of determining the impact of dietary variables on Ca retention is to determine how Ca requirements might be affected by various dietary patterns and for different populations. The relationship between Ca retention and Ca intake has been examined for various populations (Matkovic & Heaney, 1992). For all populations, there is a linear relationship up to a certain intake after which there is a levelling off of the curve so that further increases in Ca intake produce little further gain in retention. The intake at which this plateau occurs is a reference value for setting requirements to maximize development of peak bone mass during growth and minimize bone loss during ageing. There is no consensus on whether requirements should be set at 100% of maximum retention or at some lower target. Nor is it clear how to determine intake for a given percentage retention taking into account variability. Figure 3 shows the mean and 95% confidence interval of the relationship between Ca retention and Ca intake in adolescent girls using the data of Jackman *et al.* (1997). Should the Ca requirement be set at extrapolated value A (70% of maximal retention taken at the lower limit of the 95% confidence interval), value B (100% maximal retention taken at the lower limit of the 95% confidence interval), value C (the mean

100% maximal retention), or some other value? The first value is arbitrary. The second value is dependent on sample size and inter-subject variability, which influences the confidence interval. The third value may be impractically high.

Regardless of how the relationship of Ca retention to Ca intake is used to set requirements, knowing the relationship is the first step. Understanding how the relationship can be influenced by dietary life-style and genetic factors is the next area of needed research. Theoretically, dietary patterns which include high bioavailable Ca, low salt and low protein can shift the curve to the left (Fig. 4), whereas diets characterized by low Ca bioavailability, high salt and high protein can shift the curve to the right (Fig. 5). Similarly, the curve might be shifted according to race, ethnic group, physical activity level, smoking and other factors.

Modifying bone turnover

Functional foods that promote health by modifying bone turnover may work by enhancing bone formation or suppressing bone resorption. Increasing Ca intake during adolescence results in increased Ca absorption, which suppresses bone resorption by the equivalent amount (Wastney *et al.* 2000). Phyto-oestrogens may provide some protection against bone resorption similar to oestrogen, although this is not yet well documented (Weaver *et al.* 2001). Suppression of bone resorption can lead to increased Ca retention.

Use of Ca isotopic tracers and kinetic analysis allows quantification of bone formation and bone resorption in units of Ca such as mg of Ca per day. Use of just one dose of the rare isotope, ^{41}Ca , and accelerator mass spectrometry opens the possibility of determining types of diet changes that might suppress bone resorption in

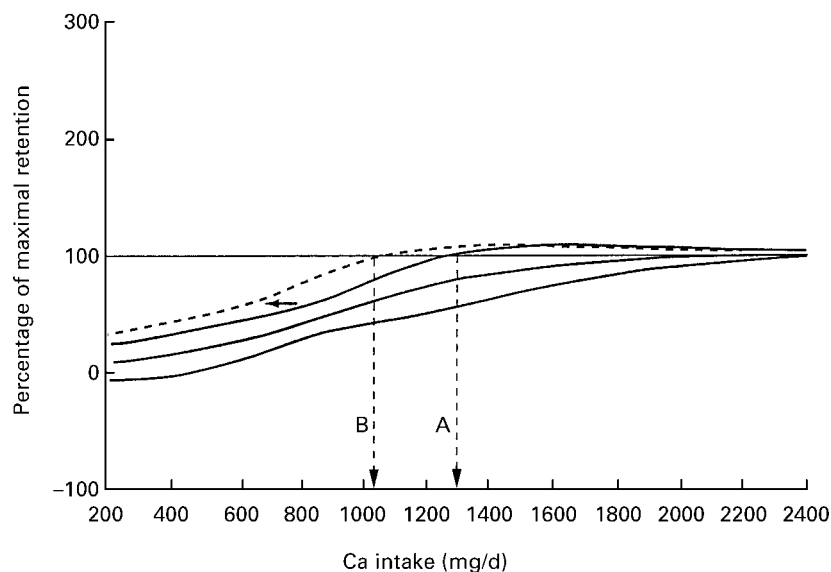


Fig. 4. The curve of calcium retention *v.* calcium intake shifts to the left with high bioavailability, low-salt and low-protein diets. A is the original intake to achieve maximal retention at the upper edge of the confidence interval and B is the new intake when the curve is shifted to the left.

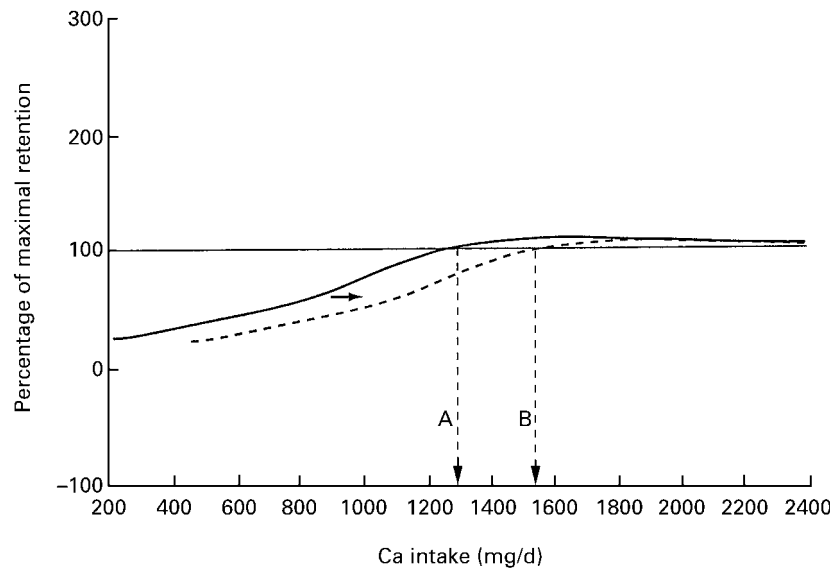


Fig. 5. The curve of calcium retention *v.* calcium intake shifts to the right with low bioavailability, high-salt and high-protein diets. A is the original intake to achieve maximal retention and B is the new intake when the curve is shifted to the right.

individuals followed longitudinally. Approximately two months after dosing, the appearance of ^{41}Ca in the urine directly reflects bone resorption. The sensitivity of accelerator mass spectrometry allows the tracer to be followed for years, thereby allowing assessments of changes in diet or other life-style factors.

Biochemical markers of bone turnover have also been used to determine qualitative changes in bone turnover (Weaver, 1998). Some common biochemical markers of bone formation include serum osteocalcin and bone alkaline phosphatase. Biochemical markers of bone resorption are typically urinary crosslinks of collagen. These assays are not in units of bone or Ca and results are highly variable. Larger sample sizes are required to find significant treatment effects. Frequently, the subtle effects of diet cannot be detected.

Bone mineral density

Bone mineral density is a strong biomarker for fracture. To evaluate the effectiveness of functional foods using this method, the intervention period needs to be much longer than for evaluating parameters of Ca metabolism. Ideally, interventions would have the duration of three to four sigmas. Each sigma, the period for a complete cycle of bone resorption and formation, is about four months in man. Trials of short duration fail to show the long-term impact on bone health. One study showed the benefit of one year of Ca-fortified foods on bone measures in growing children (Bonjour *et al.* 1997).

Conclusions

Increasing the Ca intake of the general population is the most effective strategy for using functional foods for bone health. Despite education efforts and the increased

availability of an array of Ca-fortified foods and supplements, intake of Ca remains inadequate. The role of Ca bioavailability and the ability to maximize absorption and retention are less important under conditions of adequate intake. A variety of methods are available to evaluate the effectiveness of functional foods for bone health. Their effect on Ca absorption can be determined by *in vitro* techniques, use of animal models or in man. Use of Ca isotopic tracers offers a specific way to determine the point of Ca metabolism affected. Understanding mechanisms of Ca absorption and how to increase absorption and retention efficiency are also important.

In the next decade, we will understand better the dietary and life-style factors that influence Ca absorption by both transcellular and paracellular routes. We will understand better how dietary factors influence the relationship between Ca retention and Ca intake. We will understand better the genetic factors that influence Ca absorption and retention. That Ca retention has a large genetic component was demonstrated by the greater response to Ca of three generations of women who were from osteoporotic families compared with women from healthy families (O'Brien *et al.* 1998). A beginning in identifying a specific gene that may influence Ca absorption is the significant association of vitamin D receptor gene *Fok1* polymorphism with Ca absorption and bone mineral density in children aged 7.5–12 years (Ames *et al.* 1999). Perhaps one day we will be able to tailor the food supply for identifiably vulnerable segments of the population.

Acknowledgements

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