Doctoral Thesis

Effects of *Lactobacillus helveticus* fermented milk and milk-derived bioactive peptides (CPP, IPP and VPP) on calcium and bone metabolism

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LIST OF ORIGINAL PUBLICATIONS

I Narva M, Collin M, Lamberg-Allardt C, Kärkkäinen M, Poussa T, Vapaatalo H, Korpela R. Effects of long-term intervention with *Lactobacillus helveticus* fermented milk on bone mineral density and bone mineral content in growing rats. Ann Nutr Metab 2004;48:228-234.

II Narva M, Collin M, Jauhiainen T, Vapaatalo H, Korpela R. Effects of *Lactobacillus helveticus* fermented milk and its bioactive peptides on bone parameters in spontaneously hypertensive rats. Milchwissenschaft, in press, 2004.

III Narva M, Rissanen J, Halleen J, Vapaatalo H, Väänänen K, Korpela R. Effects of bioactive peptide, valyl-prolyl-proline (VPP), and *Lactobacillus helveticus* fermented milk containing VPP on bone loss in ovariectomised rats. Life Sci, submitted.

IV Narva M, Halleen J, Väänänen K, Korpela R. Effects of *Lactobacillus helveticus* fermented milk on bone cells *in vitro*. Life Sci 2004;75:1727-1734.

V Narva M, Nevala R, Poussa T, Korpela R. The effect of *Lactobacillus helveticus* fermented milk on acute changes in calcium metabolism in postmenopausal women. Eur J Nutr 2004;43:61-68.

VI Narva M, Kärkkäinen M, Poussa T, Lamberg-Allardt C, Korpela R. Caseinphosphopeptides in milk and fermented milk do not affect calcium metabolism acutely in postmenopausal women. J Am Coll Nutr 2003;22:88-93.

MAIN ABBREVIATIONS

AAS	atom absorption spectrometry
ACE	angiotensin-converting enzyme
Ang	angiotensin
ANOVA	analysis of variance
AUC	area under curve
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CPP	caseinphosphopeptides
DXA	dual energy X-ray absorptiometry
HRT	hormone replacement therapy
ICTP	type I collagen amino terminal telopeptide
IGF	insulin-like growth factor
IPP	isoleucyl-prolyl-proline
OVX	ovariectomised
PGE_2	prostaglandin E_2
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
SEM	standard error of mean
SHR	spontaneously hypertensive rats
VPP	valyl-prolyl-proline

ABSTRACT

Fermentation of milk with lactic acid bacteria causes degradation of milk proteins into small peptides, some of which are biologically active and are therefore called bioactive peptides. These bioactive peptides can also be formed in the gastrointestinal tract during digestion. In the present study, the effects of the *L. helveticus* fermented milk and its two bioactive peptides, isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP), were studied on bone metabolism in spontaneously hypertensive (SHR) and ovariectomised (OVX) rat models, and on osteoblasts and osteoclasts *in vitro*. In addition, the effects of the *L. helveticus* and caseinphosphopeptides (CPP) on calcium metabolism in postmenopausal women were studied.

The *L. helveticus* supported bone mineral density in growing SHR and prevented trabecular bone loss by 16% in OVX after the 12-14-week interventions. The *L. helveticus* fermented milk whey increased bone formation of osteoblasts compared to normal sour milk whey, suggesting that there are active components formed during *L. helveticus* fermentation. The pure IPP and VPP peptides in water increased bone formation *in vitro* 4-6-fold, but did not have any effect on bone metabolism *in vivo*. In postmenopausal women, the *L. helveticus* fermented milk increased serum calcium concentration and decreased PTH concentration compared to normal sour milk, suggesting enhanced calcium absorption after the ingestion of *L. helveticus* fermented milk. Caseinphosphopeptides given in milk did not show any difference on the acute changes in calcium metabolism compared to normal milk in postmenopausal women.

According to these results, *L. helveticus* fermented milk supports bone mineral density. This effect may be mediated through the bioactive peptides formed during fermentation or through increased bioavailability of protein. The bioactive peptides increase bone formation *in vitro*, but not *in vivo*. It is possible that the IPP and VPP peptides are not bioavailable when given in water. Another explanation is that *L. helveticus* fermented milk affects bone through enhanced calcium absorption after ingestion of *L. helveticus* fermented milk, which causes changes in calcium-

dependent hormones such as PTH and 1,25 dihydroxyvitamin D. The increased calcium absorption may be caused by caseinphosphopeptides that could be formed in the gastrointestinal tract.

In conclusion, *L. helveticus* fermented milk supports bone mineral density in animal models and increases calcium absorption in postmenopausal women. These results encourage further clinical investigations of the use of *L. helveticus* fermented milk in osteoporosis prevention.

1 INTRODUCTION

Osteoporosis is defined as a disorder resulting from compromised bone strength predisposing to increased fracture risk. The incidence of osteoporosis is increasing as the number of old people in the western countries increases.

The ways of preventing osteoporosis are through maximising peak bone mass and attenuating bone loss during the aging process. A 5-10% increase in bone mass is related to a 25-50% decrease in fracture risk (Heaney et al. 2000). Adequate calcium intake has been shown to support bone growth and prevent bone loss during the aging process. Milk and milk products provide 75% of calcium intake in western countries (Osler et al. 1997). However, the use of milk products has been declining over the past decade (Black et al. 2002), thus increasing the risk groups of low-calcium intake. In epidemiological studies milk consumption has been related to higher bone mineral density. In addition to calcium, milk also consists of many other nutrients, such as vitamin D, phosphorus and magnesium, that are important for bone health.

Caseinphosphopeptides (CPP) formed from milk protein are known to increase calcium absorption *in vitro* by preventing the precipitation of calcium into an insoluble form (Sato et al. 1986). However, in human studies caseinphosphopeptides have shown inconsistent results (Heaney et al. 1994, Hansen et al. 1997a, Hansen et al. 1997b). Other bioactive peptides formed from milk protein during food processing or in the gastrointestinal tract have not been studied on calcium or bone metabolism. During *Lactobacillus helveticus* fermentation, isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP) peptides are formed, which have been shown to possess angiotensin coverting enzyme (ACE)-inhibitory activity (Nakamura et al. 1995b, Sipola et al. 2001). ACE inhibition is an effective means of preventing hypertension, and according to *in vitro* studies (Hagiwara et al. 1998, Hatton et al. 1997), ACE-inhibition could have an impact on bone as well.

The purpose of the present thesis was to study the effects of *L. helveticus* fermented milk and milk-protein-derived peptides, caseinphosphopeptides, isoleucyl-prolyl-proline and valyl-prolyl-proline on calcium and bone metabolism in rat, in *in vitro* and in human studies.

2 REVIEW OF THE LITERATURE

2.1 PREVENTION OF OSTEOPOROSIS

During growth, bone formation exceeds bone resorption, resulting in bone mass accumulation. Peak bone mass is defined as the full genetic potential of the bone mass (for review see Heaney et al. 2000). Peak bone mass is reached during the late twenties, with different bone sites reaching peak bone mass at different times (Bonjour et al. 1991, Matkovic et al. 1994, Teegarden et al. 1995, Weaver et al. 1999). A high peak bone mass does not inhibit postmenopausal and age-related bone loss but it could maintain bone mass above the fracture risk level at which bone loss occurs (Weaver et al. 1999). Genetic variation determines 60-70% of bone mineral density (for review see Prentice 2001). After achieving peak bone mass, the rate of bone formation and resorption remains constant, until later in life, particularly after the menopause in women, the rate of bone resorption overtakes bone formation, and bone loss occurs. Estrogen deficiency causes alterations in calcium absorption and bone metabolism (Reid 1999). This may partly explain bone loss in both sexes, although the mechanism of bone loss is not fully understood.

Osteoporosis is a condition in which decreased bone mass and bone strength lead to increased fracture risk. Because bone strength cannot be measured *in vivo*, bone mineral density is used to diagnose osteoporosis. Bone mineral density has been shown to correlate well with fracture risk (Cummings et al. 1993, Kröger et al. 1995). Osteoporosis is defined as a bone mineral density value of 2.5 SD or more below the value of young adults (Kanis 2002). The risk factors for osteoporosis are: female gender, age, estrogen deficiency, low body weight and smoking (National Institute of Health, 2000). The prevention of osteoporosis should include both maximising peak bone mass and the prevention of age-related bone loss.

Calcium in osteoporosis prevention

The main environmental factors affecting peak bone mass accumulation are nutrition and physical activity. Of the nutrients, calcium intake is most often compromised. The recommended calcium intake is 1000 mg/d, varying between countries and age groups. Low calcium intake is detrimental for bone; however, intake greater than 1300 mg/d does not increase calcium retention (Weaver 2000). It has been suggested that calcium intake accounts for 5-10% of the peak bone mass (Weaver 2000), which causes a 25-50% difference in hip fracture rate (Heaney et al. 2000). Childhood calcium intake correlates with higher bone mineral content in both childhood and adulthood (Molgaard et al. 2001, Wang et al. 2003). A supplementation of calcium increases bone mineral density in childhood (Johnston et al. 1992, Lee et al. 1996, Lloyd et al. 1996) but most often the positive effect is lost when the supplementation ceases (Lee et al. 1996). In a study by Bonjour et al. (2001), it was found that with milk-extracted calcium, the higher bone mineral density remained for 3.5 years after the intervention period (Bonjour et al. 2001).

Calcium intake is associated with higher bone mineral density during the perimenopause and with reduced bone loss during the menopause (Suzuki et al. 1996, Macdonald et al. 2004). In cohort studies the correlation between calcium intake and fracture risk has not been conclusive (Suzuki et al. 1996, Cumming and Nevitt 1997, Michaelsson et al. 2003). It is possible that those with higher risk of fracture increased their calcium intake, thus making it difficult to draw conclusive interpretations of the results. In calcium deficiency, calcium supplementation has been shown to reduce fractures (Recker et al. 1996). One meta-analysis concludes that calcium intake has a small but significant effect on bone mineral density (Shea et al. 2002). This meta-analysis examined the effect of calcium supplementation with minimal vitamin D. Since vitamin D deficiency is common in the elderly (Chapuy et al. 1997), the effect of calcium supplementation could have been greater if the vitamin D intake had been adequate. Simultaneous vitamin D and calcium supplementation preserves bone mineral density and protects from fractures in the elderly (for review see Rodriguez-Martinez and Garcia-Cohen 2002).

Pharmacological therapy in bone loss

The most effective methods of protecting against bone loss are the drug therapies (for review see Cranney et al. 2002a). Details of different drug therapies on bone loss are shown in Table 1. In postmenopausal women hormone replacement therapy (HRT) is effective in reducing bone loss at all the sites of the skeleton. In a meta-analysis, HRT showed a 4-6% difference in the bone mineral density compared to the control group (for review see Wells et al. 2002). Adequate intake of calcium has been shown to improve the efficacy of HRT (Sirola et al. 2003). Bisphosphonates affect bone by reducing the formation of the osteoclastic enzymes that resorb bone. The use of bisphosphonates has been followed for 5-10 years. A 5-year use of risedronate showed a 5-8% increase in bone mineral density (Sorensen et al. 2003). After a 10-year use of alendronate the bone mineral density 5-14% increased compared to the placebo (Bone et al. 2004). Selective estrogen receptor modulators (SERMs) act as estrogen agonists or antagonists, depending on the target tissue. Raloxifene is an estrogen agonist in bone, and increases bone mineral density in the spine and the femoral neck by 2-2.5% (for review see Cranney et al. 2002b). Calcitonin increases bone mineral density by 3-4% through blocking the activity of osteoclasts (for review see Cranney et al. 2002c). Most pharmacological agents in the treatment of osteoporosis act against osteoclast activity and bone resorption. A new therapy with a parathyroid hormone (PTH) increases bone formation. One 3-year treatment with PTH showed an increase in bone mineral density of 2.7% in the hip and 13% in vertebral bone (Lindsay et al. 1997). Although drug treatments are efficient in osteoporosis treatment, they are always accompanied by a risk of side effects, such as thromboembolic disease, gastrointestinal adverse effects or even cancer, and the duration of therapy is often limited (for review see Delmas 2002)

In conclusion, both maximising peak bone mass and preventing age-related bone loss are important ways of preventing osteoporosis. Calcium plays an important role in peak bone mass accumulation. In the elderly both calcium and vitamin D intake should be secured. During the most dramatic phase of increased bone loss, drug therapies are often essential for preventing osteoporosis.

Table 1: The effect of drug therapies on bone metabolism and on vertebral and non-vertebral bone mineral density, assessed in postmenopausal women.

Therapy	**Drug**	**Mechanism**	**Treatment period**	**Vertebral BMD**	**Non-vertebral BMD**	**Reference**
HRT	estrogen	Decreases osteoclastic activity, increases calcium absorption	2 years	+ 6.8%	+ 4.1%	Wells et al. 2002
Bisphosphonates	alendronate	Decreases osteoclastic activity	10 years	+ 13.7%*	+ 5.4%*	Bone et al. 2004
	risedronate		5 years	+ 7.9%	+ 4.5%	Sorensen et al. 2003
SERM	raloxifene	Decreases osteoclastic activity	1-3 years	+ 2.5%	+ 2.1%	Cranney et al. 2002b
Calcitonin	calcitonin	Decreases osteoclastic activity	1-3 years	+ 3.7%	+ 3.8%	Cranney et al. 2002c
PTH	teriparatidi	Increases bone formation	3 years	+ 13.0%	+ 2.7%	Lindsay et al. 1997

HRT = hormone replacement therapy, SERM = selective estrogen receptor modulators, PTH = parathyroid hormone, BMD = bone mineral density, * = compared to baseline.

2.2 CALCIUM METABOLISM

Calcium is important in the regulatory systems of the body: in cell metabolism, muscle contraction, enzyme production and activation, and also in blood coagulation and bone mineralisation. The body contains approximately 1200 g of calcium, of which 99% is stored in bone. Less than one per cent of the calcium in bone is in constant interaction with extracellular calcium. Serum calcium is either bound to serum proteins (45%) or other ions (10%) or is in an ionised form (45%). Ionised calcium is the active form of calcium and its concentration in serum is tightly regulated (for reviews see Bronner et al. 1993, Guyton and Hall 1996).

Calcium absorption at different calcium intakes varies between 20-40%, increasing with lower intakes (for reviews see Heaney 1989a, Weaver 1992). Calcium is mainly absorbed in the duodenum and the jejunum by a saturable active mechanism, which requires 1,25 dihydroxyvitamin D. In the ileum, calcium is passively absorbed depending on the concentration of calcium in the intestinal fluid. During growth, pregnancy and lactation, calcium absorption increases. With aging, intestinal resistance to 1,25 dihydroxyvitamin D occurs (Pattanaungkul et al. 2000), leading to impaired calcium absorption (Wishart et al. 2000). This may be partly due to reduced levels of estrogen, in particular in women after the menopause (Heaney and Recker 1986). The unabsorbed calcium is excreted in the feces together with the endogenous calcium excreted into the lumen (for review see Gueguen and Pointillart 2000). All the excess absorbed calcium that is not stored in the bone is excreted by glomerular filtration in the kidneys. From the glomerular filtration more than 95% of calcium is reabsorbed.

Calcium bioavailability is an expression of calcium absorption together with the utilisation of calcium in bone (for review see Bronner 1993). As almost all calcium absorbed in the body is used in bone metabolism, bone calcium content can be considered as a measurement of calcium bioavailability, which can be most reliably measured from bone after long-term feedings (Poneros-Schneler and Erdman 1989, Peterson et al. 1992, 1995, Tsuchita et al. 1993, Gueguen and Pointillart 2000).

Regulation of calcium metabolism

Calcium metabolism is regulated mainly by the parathyroid hormone (PTH), by 1,25 dihydroxyvitamin D and by calcitonin (Figure 1). PTH is excreted from the parathyroid glands when ionised calcium concentration falls below a certain limit. PTH sustains the concentration of calcium in serum by increasing calcium excretion from bone, decreasing calcium excretion in the kidneys and increasing calcium absorption indirectly by activating 1,25 dihydroxyvitamin D, which in its turn stimulates the formation of a calcium-binding protein necessary for the absorption of calcium. Calcium absorption has been shown to vary according to serum vitamin D levels (Heaney et al. 2003). Calcitonin is an endogenous hormone with opposite effects from those of PTH. It regulates serum calcium levels by increasing urinary calcium excretion and reducing calcium absorption and calcium release from bone.

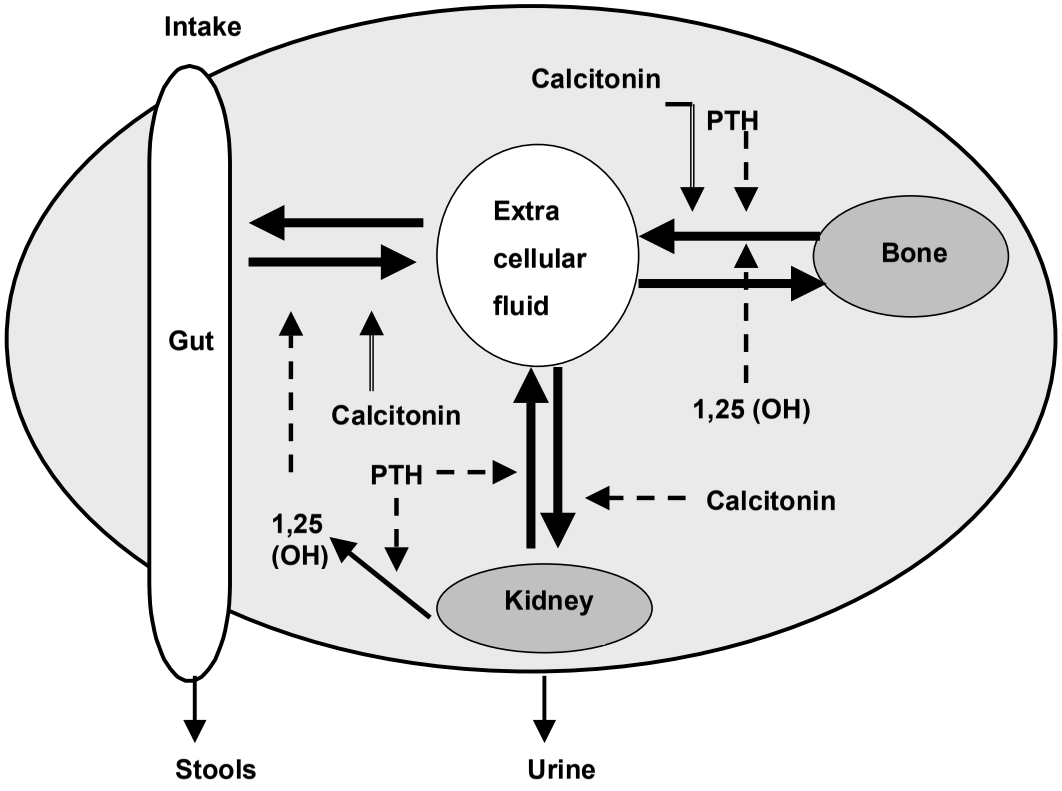

Figure 1: The regulation of calcium metabolism by PTH and 1,25 (OH) vitamin D. PTH=parathyroid hormone, 1,25 (OH)=1,25 dihydroxyvitamin D. Solid line = decreasing effect, dotted line = increasing effect.

Nutritional factors affecting calcium metabolism

Calcium absorption is affected by dietary factors; fibre, phytates and oxalates have been shown to inhibit calcium absorption by forming insoluble calcium salts (for review see Gueguen and Pointillart 2000). Calcium ingested with a meal increases calcium absorption by 10-30% (Heaney et al. 1989b), which may be because of slower gastric emptying. Fermentation of milk has also been postulated to increase calcium absorption by slowing down the gastrointestinal emptying rate or by increasing soluble calcium as a result of increased acidity (Renner et al. 1983, Mahé et al. 1994, Chonan et al. 1998). In previous studies fermentation has been shown to increase serum ionised calcium and decrease intact PTH levels acutely (Kärkkäinen et al. 1997, Talbot et al. 1999), but in studies using isotope methods fermentation did not increase calcium absorption (Smith et al. 1985, Recker et al. 1988, Nickel et al. 1996).

Sodium, caffeine, alcohol and excess intake of protein all increase the urinary loss of calcium. Proteins tend to increase calcium excretion mainly due to their sulphur-containing amino acids, which cause acidic conditions. Potassium, on the other hand, produces alkaline conditions, and thus decreases the excretion of urinary calcium (Lemann et al. 1989). Phosphorus enhances the reabsorption of calcium in the glomerus and increases faecal calcium excretion (Whiting et al. 2002), thus the net effect of phosphorus on calcium balance is likely to be small.

The effects of nutrients on calcium bioavailability are dependent on the factors affecting absorption, but only a limited number of studies have examined the actual effect on bioavailability measured as the mineral content of the femur (for review see Gueguen and Pointillart 2000, Camara-Martos and Amaro-Lopez 2002). The effect of fermentation on calcium bioavailability has been shown to be augmented by cheese compared to normal milk (Ghanem and Hussein 1999). However, in the study of Delisle et al., yoghurt did not increase calcium bioavailability compared to milk, although calcium absorption increased (Delisle et al. 1995). Therefore the effect of fermentation on calcium bioavailability remains uncertain.

2.3 METHODS OF ASSESSING CALCIUM ABSORPTION

Several methods are available for studying calcium absorption in humans (for review see Charles 1992). The balance study determines the net absorption of calcium after a calcium load by analysing the calcium excreted in the urine and the feces. The method does not make a distinction between endogenously-excreted calcium and non-absorbed calcium, thus it is not an accurate method. The isotope balance study measures the absorption of labelled calcium, which can be correlated with labelled calcium given intravenously. This method measures absolute calcium absorption, and is thus the most accurate method available. However, due to the radioactive nature of the calcium it is less often used.

Biological markers in the blood and urine can be used to obtain an indirect measurement of calcium absorption (Reid et al. 1986, Guillemant et al. 2000a). An oral calcium load suppresses PTH concentrations, which can be measured acutely (Kärkkäinen et al. 2001). Within two to three hours after the ingestion of calcium, 95% of calcium is absorbed (Kärkkäinen et al. 2001). Passive absorption peaks at six hours after the ingestion of calcium. A strong correlation exists between the acute intake of calcium and urinary calcium excretion, indicating intestinal calcium absorption. This method has been commonly used to measure the effect of calcium supplementation on calcium metabolism in short-term studies (Reid et al. 1986, Horowitz et al. 1994, Guillemant et al. 2000b, Kärkkäinen et al. 2001).

2.4 BONE METABOLISM

Bone is composed of an organic matrix (30%) and minerals (70%). Most of the matrix is type 1 collagen. In addition, other proteins, such as osteocalcin, osteonectin and osteopontin, are also present. The main minerals in bone are calcium and phosphate, present in a form of hydroxyapatite. Carbonate, citrate, magnesium, sodium, fluoride and strontium are also found in the hydroxyapatite crystals.

There are two types of bone: cortical and trabecular bone (Figure 2) (for review see Baron 1999). Cortical bone is dense and organised in the form of osteons, bone structural units which are connected to one another by blood vessels. Trabecular bone, which is more metabolically active, is made up of thin trabeculae, which are attached to each other, forming a stable network.

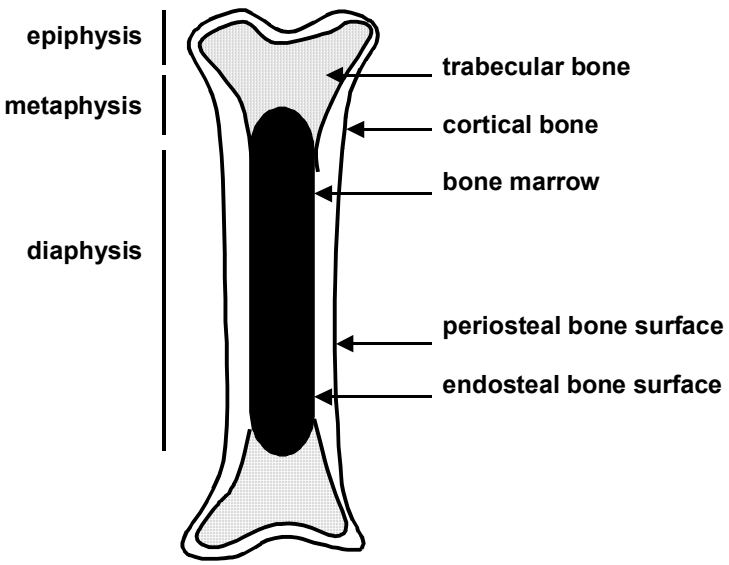

Figure 2: The composition and different structural segments of bone.

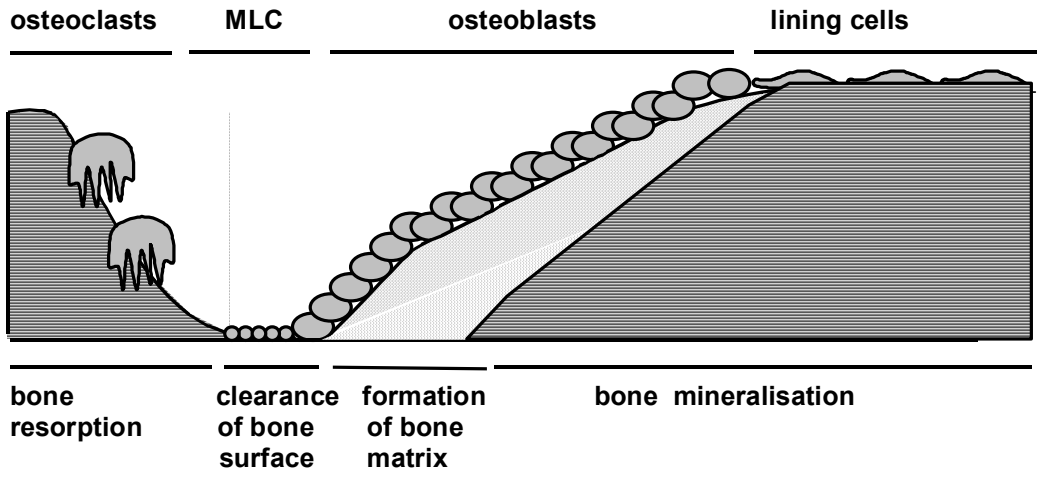

Figure 3: The process of bone remodelling. MLC = macrophage-like cells. (Modified from Mundy 1999)

The cells that affect bone are osteoblasts, osteoclasts, osteocytes and lining cells, each with its own structure and function (for review see Baron 1999). Osteoclasts are bone-resorbing cells that differentiate from precursors influenced by hormones and several local factors, such as cytokines. The bone-forming cells, the osteoblasts, on the other hand, differentiate in the presence of different hormones and growth factors. Osteocytes are osteoblasts that are embedded within the new bone. The function of osteocytes is not
clear, although they are the most common cells in bone. It has been suggested that they respond to mechanical stress by increasing bone formation. Lining cells are osteoblasts that are not in the process of forming bone.

Bone remodelling

Bone remodelling is a process that takes place when old bone is replaced by new, allowing the bone to repair microfractures, renew itself, and adapt to the physical needs of mechanical support and calcium balance (Figure 3) (for review see Lian et al.

1999). Remodelling replaces 2% of cortical bone and 10% of trabecular bone in one year. It takes place throughout the skeleton in a remodelling unit, where bone is remodelled independently of the other bone remodelling units. Remodelling begins with bone resorption, when osteoclasts resorb bone by excreting hydrogen ions which dissolve bone minerals and form an optimal environment for the enzymes. The enzymes, mainly cathepsins and collagenases, digest the organic matrix. The bone resorption process activates the bone formation process of the osteoblasts. The mechanism, through which the coupling occurs, is still unknown. The resorbed bone surface is covered with macrophage-like cells that adapt the surface so that it is able to bind new bone. Osteoblasts synthesise new bone by releasing matrix proteins that are mineralised. After new bone is formed, the osteoblasts are calcified to form osteocytes. The resorptive phase takes approximately 10 days. Bone formation, on the other hand, lasts 3 months.

Regulation of bone remodelling

Remodelling is under the control of both systemic and local hormones (Figure 4). The main hormones regulating bone remodelling are PTH and 1,25 hydroxyvitamin D, but other hormones, such as calcitonin, glucocorticosteroids, insulin, growth factors, thyroid hormones and androgen and estrogen, also affect bone remodelling (for review see Mundy 1999, Russell 2001). PTH increases calcium release from bone, but it also stimulates the differentiation of osteclasts and activates the preformed osteoclasts. 1,25 dihydroxyvitamin D activates mature osteoclasts and thus stimulates bone resorption. It is also involved in osteoblastic differentiation and activity. Estrogens increase the activation of 1,25 hydroxyvitamin D and the production of calcitonin. They also affect osteoclasts directly by decreasing interleukin 1-induced osteoclast formation and increasing osteoclast apoptosis (Qu et al. 1998, 1999). Calcitonin inhibits osteoclast formation and bone resorption.

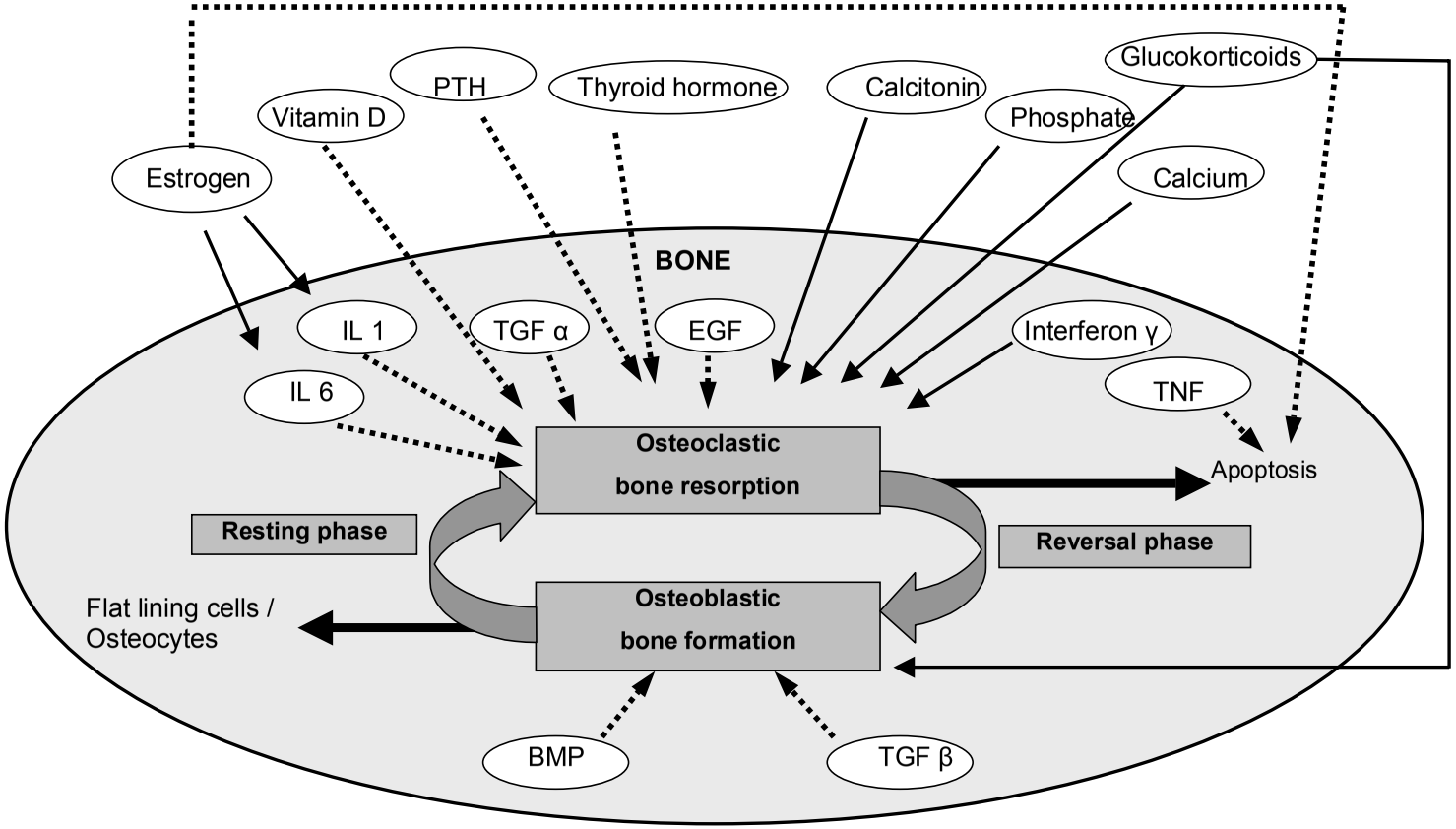

Figure 4: The regulation of bone remodelling. Solid line = decreasing effect, dotted line = increasing effect, PTH = parathyroid hormone, IL = interleukin, TGF = transforming growth factor, EGF = epidermal growth factor, TNF = tumor negrosis factor, BMP = bone morphogenic protein.

Nutritional factors affecting bone metabolism

Nutrition plays an essential role in bone health. Milk products, fruit and vegetables have been shown to be important for bone growth and the prevention of bone loss (Macdonald et al. 2004, Whiting et al. 2004). Since nutrition is a combination of several nutrients, the assessment of the effects on bone is complex. Nutrients important for bone growth are proteins, vitamins D, C and K, and minerals such as calcium and phosphate, and also magnesium, potassium, iron, copper and zinc (for review see Heaney et al. 2000).

The role of protein is discussed in Chapter 2.4. In foods, vitamin D is present in two different forms depending on the source of the vitamin. Cholecalciferol (D_3) is derived from animal sources and ergocalciferol (D_2) from plant sources. Vitamin D deficiency is common, especially during the winter season (Lamberg-Allardt et al. 2001, Chapuy et al. 1997). In a meta-analysis, vitamin D treatment has been shown to prevent vertebral fractures (Papadimitropoulos et al. 2002). Vitamin C is needed for collagen formation (Rowe et al. 1999). Vitamin K is essential for the carboxylation of bone matrix proteins (for review see Zittermann 2001). Low vitamin K intake is not correlated to bone mineral density but to an increased risk of fracture (Booth et al. 2000). Phosphate is needed for the mineralisation of bone. Low intakes of phosphate may compromise bone mineralisation; however, high phosphate intakes may be harmful to bone (for review see Eastell and Lampert 2002). Magnesium is present in the hydroxyapatite crystals in bone. Magnesium and potassium have been shown to prevent bone loss during the perimenopause and the menopause (New et al. 2000, Macdonald et al. 2004). Zinc stimulates the formation of insulin-like growth factor I. Zinc deficiency has been correlated to increased risk of fracture (Elmstahl et al. 1998).

Excess intake of certain nutrients may compromise bone health. Serum retinol levels correlate positively with fracture risk in men (Michaelsson et al. 2003a), although the effect of a dietary intake of vitamin A on fracture risk has shown conflicting results (positive: Melhus et al. 1998, Feskanich et al. 2002, negative: Lim et al. 2004). Both an excess intake and a deficiency of vitamin A are suggested as being detrimental to bone health (Promislow et al. 2002). A low sodium diet decreases bone turnover markers compared to a high sodium diet in adults (Evans et al. 1997, Lin et al. 2003)

but not in young adults (Ginty et al. 1998). It has been concluded that dietary sodium does not have adverse effects on bone in healthy individuals (for review see Cohen and Roe 2000).

2.5 METHODS OF ASSESSING BONE

Bone mineral density is one of the parameters used in *in vivo* assessments of bone. It has been shown to correlate with the strength of bone (Ammann and Rizzoli 2003). The most commonly used technique for measuring bone mineral density is dual energy X-ray absorptiometry (DXA) (Mazess et al. 1990). The measurement of bone mineral density by DXA determines the areal density of bone (g/cm^2) without taking into account the size of the bone. A quantitative computed tomography (QCT) is used to measure the volumetric bone mineral density (g/cm^3). Quantitative ultrasound has also been used to assess bone quality. Advanced imaging modalities such as microcomputed tomography (microCT), can be applied to examine the three-dimensional architecture of bone.

Bone turnover can also be assessed *in vivo* by using biomarkers of bone metabolism in serum or urine (for review see Khosla and Kleerekoper 1999). These are mainly used to monitor the effect of osteoporosis treatment, but also to study short-term effects on bone metabolism. Bone resorption is reflected in collagen degradation products both in urine and in serum. Pyridinoline and deoxypyridinoline as well as amino- and carboxyterminal telopeptides (NTX, CTX) can be measured from urine. The carboxyterminal telopeptide can also be measured from serum (ICTP). The tartrate resistant acidic phosphatase 5b, secreted by the osteoclasts, can be measured from serum. Bone formation is expressed as either amino- or carboxyterminal propeptide type 1 collagen (PINP, PICP). The non-collagenous proteins, such as alkaline phosphatase and osteocalcin, are released from osteoblasts during bone formation and in the mineralisation process.

From excised bone, its strength, histomorphometry and ash weight can be measured. Bone strength reflects the ability of bone to resist mechanical stress. It is determined by bone mass, geography and the intrinsic material properties. Changes in the

microarchitecture of bone, bone mineral composition, organic matrix and turnover rates also affect bone strength (Ammann and Rizzoli 2003). Properties such as maximal energy, stiffness, and absorbed energy can be determined by mechanical loading until failure occurs. The polar moment of inertia, derived from pQCT measurements, reflects the rigidity of the bone, affected by its cross-sectional geometry. Bone structure and cellular changes in bone remodelling, such as the bone formation rate and the mineral apposition rate, can be measured using static and dynamic histomorphometry. Ash weight reflects the mineralisation of bone. Different ratios of bone minerals can be measured by analysing the calcium, phosphorus and magnesium contents of the ashes.

2.6 MILK PROTEIN AND BONE

Correlations with milk consumption and bone

Milk is a good source of several nutrients, such as protein, calcium, phosphate, magnesium, potassium and zinc, that are considered important for bone health. In epidemiological studies milk consumption in childhood and during adolescence has been related to higher bone mineral density in adulthood (Sandler et al. 1985, Hirota et al. 1992, Stracke et al. 1993, Murphy et al. 1994, Soroko et al. 1994, Teegarden et al. 1995, New et al. 1997, Kalkwarf et al. 2003) (Table 2). This effect has been particularly established in white women (Opotowsky and Bilezikian 2003). In cross-sectional studies milk intake has been shown to correlate to bone mineral density in children (Black et al. 2002), in adult men (Egami et al. 2003) and in women (Lacey et al. 1991, Tylavsky et al. 1992, Davis et al. 1996, Hawker et al. 2002) (Table 2).

Intervention studies show that milk and milk products increase bone mineral density in adolescents, both in girls (Chan et al. 1995, Cadogan et al. 1997, Merrilees et al. 2000) and in boys (Renner et al. 1998) (Table 3). In pre- and postmenopausal women, 2-4-year interventions with milk and milk products prevented the bone loss that occurred in the control group, who had no dietary intervention, although there was no increase in bone mineral density (Baran et al. 1990, Chee et al. 2003). The control groups of these intervention studies did not have adequate calcium intake, thus the

results of the interventions may be the result of a higher calcium intake by the groups receiving milk products. In calcium-controlled studies, calcium supplementation and ingestion of dairy products increased bone mineral density in adolescents (Matkovic et al. 2004) and reduced bone loss in postmenopausal women (Prince et al. 1995). Dairy products have also been shown to increase not only bone mineral density but also bone growth (Matkovic et al. 2004).

The effect of milk and milk products on fracture risk has been evaluated in children and in women. It has recently been found that children with low milk intake are at higher risk of prepubertal bone fractures (Goulding et al. 2004). Higher milk consumption by women during the past 12-14 years has been shown to prevent fractures in a Japanese population (Fujiwara et al. 2003). In North America retrospectively obtained information about childhood milk intake was associated with lower fracture risk in females over the age of 50 (Kalkwarf et al. 2003), but milk consumed during adulthood did not relate to fracture risk (Feskanich et al. 1997, 2003).

These results show that the consumption of milk and milk products correlates with higher bone mineral density during growth and prevents age-related bone loss. This may be the result of a higher calcium intake, in view of the fact that in western countries milk contributes 80% of dietary calcium (Fleming and Heimbach 1994). However, there are other components in milk that may contribute to the effect on bone, and these will be the subject of the present studies.

Table 2: Epidemiological studies on the effect of milk and milk products on bone mineral density

N (sex)	Age	Method	Bone measure-ment	Dietary assessment	Milk consumption correlated to BMD	References
255 (F)	49-66	Retrospective/ Cross-sectional	CT	FFQ, 3-d food record	Childhood and adolescence	Sandler et al. 1985
178 (F)	35-40, 55-60	Cross-sectional	SPA	3-d food record	Postmenopause	Lacey et al. 1991
705 (F)	18-22	Cross-sectional	SPA	FFQ	High school and college	Tylavsky et al. 1992
161(F)	19-25	Retrospective/ Cross-sectional	SPA	FFQ, 3-d food record	Childhood	Hirota et al. 1992
98 (F), 33 (M)	mean 57	Retrospective	SPA	FFQ	Childhood and adolescence but not adulthood	Stracke et al. 1993
284 (F)	44-74	Retrospective	DXA	24-hour diet recall	To the age of 25	Murphy et al. 1994
624 (F)	60-79	Retrospective	DXA	FFQ	Youth and adulthood	Soroko et al. 1994
421 (F)	25-34	Retrospective	DXA	FFQ	Youth	Davis et al. 1996
994 (F)	45-49	Retrospective/ Cross-sectional	DXA	FFQ	Early adulthood	New et al. 1997
224 (F)	18-31	Retrospective/ Cross-sectional	DXA	FFQ, 24-hour diet recall	Adolescence	Teegarden et al. 1999
30 (F), 20 (M)	3-10	Cross-sectional	DXA	FFQ	Negative correlation with milk avoiders	Black et al. 2002
963 (F)	19-35	Cross-sectional	SXA	FFQ	Negative association with lack of milk consumption	Hawker et al. 2002
3251 (F)	>20	Retrospective/ Cross-sectional	DXA	FFQ	Negative correlation with childhood	Kalkwarf et al. 2003
143 (M)	18-22	Retrospective/ Cross-sectional	CXD	FFQ	Correlation in junior high school	Egami et al. 2003

BMD = bone mineral density, DXA = dual energy X-ray absorptiometry, SXA = single X-ray absorptiometry, CXD = computed X-ray densitometry, FFQ = food frequency questionnaire, SPA = single photon absorptiometry, CT = computed tomography, F = female, M = male.

Table 3: Intervention studies on the effects of milk and milk products on bone mineral density

N (sex)	Age (years)	Intervention	Bone measurement	Main result	References
37 (F)	30-42	3 years, 0.5 g calcium (dairy products)	DXA	Prevented bone loss, >2% in vertebra	Baran et al. 1990
48 (F)	11	1 year, appr. 800 mg calcium (dairy products)	DXA	Increased BMD, 6.6% in total body	Chan et al. 1995
168 (F)	57-68	2 years, 1 g calcium (milk powder)	DXA	Prevented bone loss, + 0.5% in femoral neck	Prince et al. 1995
82 (F)	12	1.5 years, 300ml/d milk	DXA	Increased BMD, 1.1% in total body	Cadogan et al. 1997
91 (F)	15-18	2 years, appr. 500 mg calcium (dairy products), 1 year follow-up	DXA	Increased BMD, 4.8% in femoral neck	Merrilees et al. 2000
200 (F)	55-65	2 years, 1.2 g calcium (milk powder)	DXA	Prevented bone loss, +1.7% in femoral neck	Chee et al. 2003

DXA=dual energy X-ray absorptiometry, BMD=bone mineral density, F=female.

Protein intake and bone

Protein intake is important for bone health. Both inadequate and excess intakes have been postulated as having detrimental effects on bone (for reviews see Dawson-Hughes 2003, Ginty 2003). The effect of protein on bone is dependent on the intake of calcium (Meyer et al. 1997). Increase in protein intake causes increased calcium excretion through acid production, but this effect is not significant when calcium intake is adequate (Heaney 1998, Dawson-Hughes and Harris 2002, Dawson-Hughes et al. 2004). In addition to the effect on calcium, protein affects bone through insulin-like growth factor 1 (IGF-1) (for review see Bonjour et al. 1997), which regulates bone growth and bone mineral density (Yakar et al. 2002). Protein supplementation increases serum IGF levels (Schürch et al. 1998) and a restriction of protein decreases IGF levels (Bourrin et al. 2000). The intake of milk has been associated with higher IGF-1 levels, possibly due to the effects of protein (Cadogan et al. 1997, Heaney et al. 1999).

Protein intake has been associated with higher bone mineral density in adults (Promislow et al. 2002), in postmenopausal women (Kerstetter et al. 2000) and in the elderly (Hannan et al. 2000, for review see Bell and Whiting 2002). The effect of protein on fracture risk is controversial (negative: Feskanich et al. 1996, Sellmeyer et al. 2001, positive: Munger et al. 1999).

To conclude, protein intake is important for bone health. However, without adequate calcium intake, a high protein intake can be harmful to bone.

Whey protein and bone

3% of milk is protein, of which 80% is casein and 20%, whey protein. Whey proteins, such as alpha lactalbumin and beta lactoglobulin, bind calcium, but this has no effect on calcium balance and retention *in vivo* (Takada et al. 1997a, for review see Gueguen and Pointillart 2000). In fact, whey protein has been shown to increase the bone-breaking energy of rats compared to casein, by increasing the total amino acid content of the femur (Takada et al. 1997a). In growing rats, a whey protein supplementation did not make any difference to femoral density, bone mineral content

or the biomarkers of bone metabolism after a 7-week intervention (Kelly et al. 2003). However, in the same study the whey protein-supplemented casein diet increased bone formation after 14 days in a model of ectopic bone formation (Kelly et al. 2003), implying that the intervention was too short to demonstrate the effect on bone. These results suggest that whey protein does not increase mineral bioavailability but supports bone by increasing bone protein, especially bone collagen content.

Whey protein reduces the formation of osteoclasts and their activity (Takada et al. 1997b). Osteoclast formation measured by multi-nuclei cell formation decreased dose-dependently with the highest concentration of 1 mg/ml whey. Whey protein has also been shown to activate osteoblasts (Takada et al. 1996).

The effect of whey protein on bone could be derived from the basic part of the protein (milk basic protein, MBP). Isolated MBP decreases bone deterioration in ovariectomised rats (Kato et al. 2000). In human studies short-term ingestion of MBP increases radial and calcaneus BMD and decreases bone resorption markers (Aoe et al. 2001, Toba et al. 2001, Yamamura et al. 2002). The active component of MBP has been found to be kininogen, belonging to high-mobility-group-like proteins (Yamamura et al. 1999, 2000). One of the mechanisms by which this active compound affects bone is through cystatin C, which inhibits osteoclast activity by decreasing the secretion of collagen-digesting cathepsin (Matsuoka et al. 2002).

To summarise the effect of milk on bone, milk consumption has a positive correlation with bone mineral density. In addition to its calcium content, milk contains proteins, which are beneficial to bone health in the presence of adequate calcium.

2.7 MILK-DERIVED BIOACTIVE PEPTIDES ON CALCIUM AND BONE METABOLISM

In the gastrointestinal tract, food protein is digested into smaller peptides, some of which have physiological effects (for review see Korhonen and Pihlanto 2003). These bioactive peptides can be formed by enzymatic hydrolysis with digestive enzymes but also by fermentation with starter cultures (for review see Korhonen and Pihlanto

2003). In fermentation, the formation of peptides depends on the bacteria used (Matar et al. 1996). The active peptides are usually small, consisting of 3-20 amino acid residues. Bioactive peptides have been found in many different foods, such as milk, eggs, beans, fish and corn (for review see Kitts and Weiler 2003), but milk protein is the most important source of bioactive peptides (for review see Korhonen and Pihlanto 2003).

The bioavailability of peptides most often requires that they should not be digested in the gastrointestinal tract. The absorption of small peptides is well known (for review see Fricker and Drewe 1996). Peptides can be absorbed through the gastrointestinal wall by different mechanisms, such as by passive diffusion through the enterocytes, paracellularly, through cytosis or through a carrier (for review see Fricker and Drewe 1996). Some peptides, such as caseinphosphopeptides, express their activity in the gastrointestinal tract without being absorbed.

Bioactive peptides have been shown to have various physiological effects both *in vitro* and *in vivo* (for reviews see Pihlanto-Leppälä 1999, Korhonen and Pihlanto 2003, Meisel and FitzGerald 2003). Opioid peptides from casein or whey proteins possess an affinity to opiate receptors as well as opiate-like effects. They act either as agonists or antagonists in the opioid receptors. Opioid peptides influence the nervous system, gastrointestinal transit time, nutrient intake and the secretion of insulin and glucagons. Antithrombotic peptides derived from casein have been shown to suppress platelet aggregation. Immunomodulating peptides stimulate the proliferation of human lymphocytes and the phagocytic activities of macrophages. Antimicrobial effects have been shown with small whey proteins binding iron, an essential nutrient of micro-organisms (for review see Clare et al. 2003). Other casein-derived antimicrobial peptides have shown anticariogenic effects (for review see Aimutis 2004). The antioxidative and hypocholesterolemic effects of bioactive peptides have only recently been discovered (for review see Korhonen and Pihlanto 2003). One bioactive peptide may have several physiological effects, e.g. casein-derived β-casomorphin 7 has both angiotensin converting enzyme (ACE)-inhibitory effects and opioid-like effects (for review see Meisel and Bockelmann 1999). The only milk-derived bioactive peptides that have been studied on calcium metabolism are caseinphosphopeptides (CPP) (for review see Scholz-Ahrens and Schrezenmeier 2000).

Caseinphosphopeptides (CPP)

CPP are a large group of peptides that have a phosphoseryl residue in common. Phosphopeptides are formed either from casein by proteolytic enzymes during fermentation or in the gastrointestinal tract. CPP increase calcium absorption by forming a hydrophobic complex with calcium, thus preventing the formation of insoluble calcium phosphates (Meisel and Bockelmann 1999). *In vitro* studies have shown the effects of CPP on calcium absorption by inhibiting the precipitation of calcium in the intestine (Sato et al. 1986).

In animal studies, the effect of CPP has produced inconsistent results. In most of the studies the CPP have increased calcium absorption (Mykkänen and Wasserman 1980, Lee et al. 1980, 1983, Kitts et al. 1992, Hirayama et al. 1992, Tsuchita et al. 1993, 2001, Bennett et al. 2000), but some studies failed to show any effect on calcium absorption (Brommage et al. 1991, Kopra et al. 1992). Only one study examined the effect of CPP on bone. In this study CPP was found to prevent bone loss in ovariectomised rats (Tsuchita et al. 1996).

As early as 1950 Mellander reported the first finding of the effects of CPP in humans in his study on rachitic children (Mellander 1950). Increased calcium absorption was independent of the effect of vitamin D, suggesting that CPP increase calcium absorption in the distal small intestine. In later human studies, the effect of CPP has been shown to be influenced by the calcium status of the subjects and by other nutrients affecting calcium absorption (Heaney et al. 1994, Hansen et al. 1997a, 1997b). A CPP preparation increased calcium absorption in women whose calcium absorption rate was low (Heaney et al. 1994). The effect of the addition of 1-2 g of CPP to different food products has produced conflicting results on calcium absorption. Calcium was shown to be more efficiently absorbed from a rice meal compared to whole-grain whey (Hansen et al. 1997a); however, in a later study CPP did not show any greater effect on calcium absorption from low or high phytate foods (1g) (Hansen et al. 1997b).

In conclusion, CPP increase calcium absorption in animal studies but the effect in humans is not conclusive.

ACE-inhibitory bioactive peptides

ACE is responsible for the generation of angiotensin II (Ang II), a vasopressive, and for the inactivation of bradykinin, a vasodepressor. Many milk-derived bioactive peptides have shown ACE-inhibitory activity (for review see FitzGerald and Meisel 2000), though most of these peptides are too large to be absorbed through the gastrointestinal tract.

Fermentation of milk with *Lactobacillus helveticus* (*L. helveticus*) releases, in particular, proline-containing peptides from casein, mainly isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP), through the action of an X-prolyl dipeptidyl aminopeptidase enzyme (Matar et al. 1996, Nakamura et al. 1995a). IPP and VPP peptides have been shown to be absorbed through the gastrointestinal tract, as tested *in vitro* (Masuda et al. 1996) and *in vivo* (Satake et al. 2002), possibly as a result of the proline-proline bonds that are resistant to mammalian proteolytic enzymes (Vanhoof et al. 1995). IPP and VPP peptides possess ACE-inhibitory activity (Nakamura et al. 1995b, Sipola et al. 2001). A long-term feeding with *L. helveticus* fermented milk has been shown to lower blood pressure, indicating an *in vivo* effect of the IPP and VPP peptides, in rats (Sipola et al. 2002) and in humans (Hata et al. 1996, Seppo et al. 2002, 2003). The peptides given in the fermented milk attenuated blood pressure more than the peptides given purely in water, thus there may be other mechanisms in *L. helveticus* fermented milk, such as mineral availability, that affect blood pressure.

Polymorphism in ACE causes lower ACE activity, and this has been shown to correlate with higher bone mineral density (Perez-Castrillon et al. 2003). Ang II has been reported to affect bone by decreasing osteoblast differentiation and increasing osteclastic bone resorption (Hagiwara et al. 1998, Hatton et al. 1997). The mechanisms through which ACE-inhibitory peptides may affect bone are presented in Figure 5. The inhibition of ACE increases the formation of the vascular endothelial growth factor (Higgins et al. 2003), which is important for bone formation (Pufe et al. 2003). On the other hand, Ang II has been reported to increase prostaglandin synthesis (Segawa et al. 2003).

In addition to converting Ang I to Ang II, ACE inactivates bradykinin. ACE inhibitors have even been shown to multiply the activity of bradykinin (Tom et al. 2002). The IPP sequence has also been found in bradykinin potentiator peptides (Chi et al. 1985). Bradykinin receptors are present in osteoblastic cells (Brechter and Lerner 2002). Although bradykinin itself has no effect on osteoblast proliferation (Frost et al. 1999), it stimulates prostaglandin synthesis (Goldstein and Wall 1984) and releases nitric oxide (Figini et al. 1996), which has an anabolic effect on bone formation (for review see van't Hof and Ralston 2001). Prostaglandin E_2 (PGE_2) has been shown to affect bone by stimulating both osteoblastic differentiation and bone formation (Suda et al. 1996, Keila et al. 2001), but also by increasing osteoclast differentiation (Collins and Chambers 1992) and bone resorption *in vitro* (Lader and Flanagan 1998, Suzawa et al. 2000). *In vivo,* PGE_2 stimulates bone formation (Suponitzky and Weinreb 1998, Jee et al. 1990, Keila et al. 2001).

In conclusion, the IPP and VPP peptides produced during *L. helveticus* fermentation have ACE-inhibitory activity *in vivo* and *in vitro.* ACE inhibition can affect bone through angiotensin II inhibition or bradykinin activation.

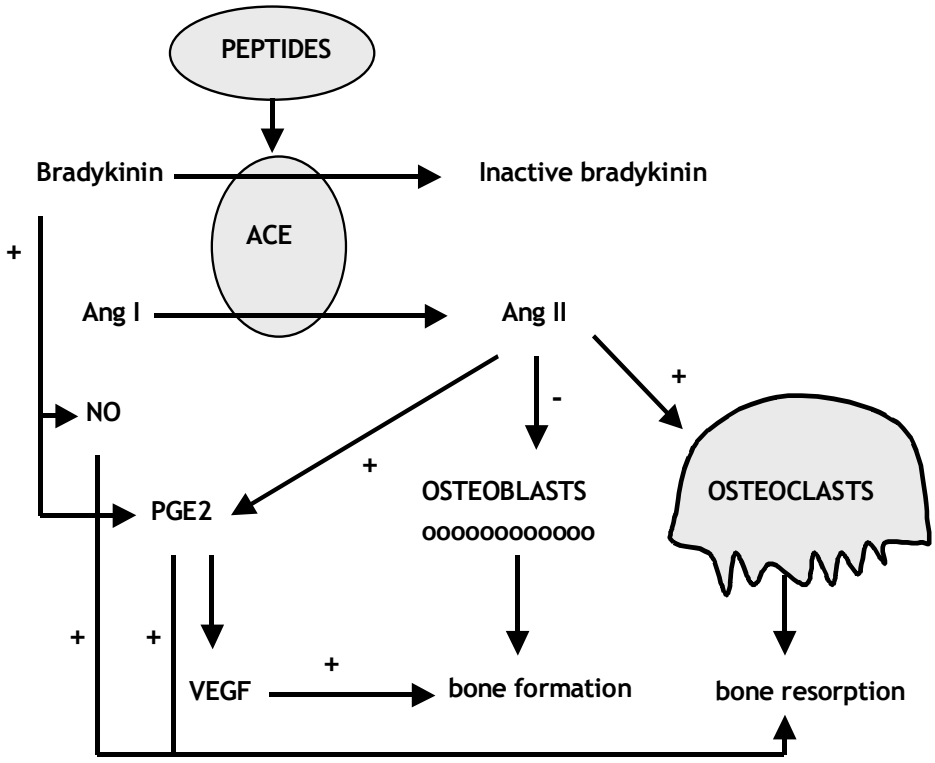

Figure 5: The hypothesis of the effects of bradykinin and ACE inhibition on bone formation and bone resorption. ACE = angiotensin converting enzyme, Ang I = angiotensin I, Ang II = angiotensin II, NO = nitric oxide, PGE2 = prostaglandin E 2, VEGF = vascular endothelial growth factor.

3 AIMS OF THE STUDY

According to the current literature, milk consumption correlates to higher bone mineral density. In addition to calcium, milk contains many nutrients essential for bone health. The effect of caseinphosphopeptides on calcium metabolism has been studied in humans but the results are not conclusive. The effects of bioactive peptides on bone have not been previously studied. The bioactive peptides formed during *L. helveticus* fermentation have been postulated as having ACE-inhibitory activity and as increasing mineral absorption. In the present thesis the aim was to explore the effect of *L. helveticus* fermented milk and its bioactive peptides on calcium and bone metabolism. The detailed aims were:

1. To investigate the effect of long-term feeding with *L. helveticus* fermented milk and its components on bone parameters in spontaneously hypertensive rats with increased bone turnover (Studies I and II)

2. To determine the effect of *L. helveticus* fermented milk and VPP peptide on bone loss in ovariectomised rats (Study III)

3. To specify the effect of *L. helveticus* fermented milk and its components on osteoclast differentiation and bone formation *in vitro* (Study IV)

4. To evaluate the acute effect of *L. helveticus* fermented milk and caseinphosphopeptides on the markers of calcium metabolism in postmenopausal women (Studies V and VI)

4 MATERIALS AND METHODS

4.1 ASSESSMENTS OF BONE PARAMETERS IN VIVO (STUDIES I, II, III)

Spontaneously hypertensive rats (SHR; Harlan, Indianapolis, IN, USA) (Studies I, II) and ovariectomised normotensive rats (Harlan Sprague Dawley, Indianapolis, IN, USA) (Study III) were used to investigate the effect of *L. helveticus* fermented milk and its bioactive peptides on bone parameters following the 12-14-week interventions. The body weight-matched rats were divided into different treatment groups (n=10) and housed five to a cage (Studies I, II) or in individual cages (Study III) in standard experimental laboratory. The consumption of the study products and food was monitored throughout the study. The amount of calcium and peptides in the study products was adjusted at the beginning of the interventions according to the amount of *L. helveticus* fermented milk consumed by the rats. The protocols were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland (Studies I and II) and the Department for Social Welfare and Health Services of the Provincial State Office of Western Finland (Study III).

Bone measurements

In the SHR, the bone mineral density and the bone mineral content were measured from the right femur by dual-energy X-ray absorptiometry (PIXImus, GE Medical Systems LUNAR, Helsinki, Finland) immediately after the intervention. In OVX rats the bone mineral density, tissue and bone area measurements and the polar moment of inertia were analysed from the proximal end of the right tibia and the tibial diaphysis, using peripheral quantitative computed tomography (pQCT) *in vivo* before the operation, four and twelve weeks after the operation. To determine the ash weight, the bones were ashed on a metal plate in an electric furnace.

The mechanical properties of the bone were tested from the femoral head-neck complex by a cantilever bending test (Studies II, III). The maximal load, energy absorbed and stiffness were measured by loading the femoral head with a force parallel to the shaft until failure occurred (Peng et al. 1994).

Histomorphometrical analyses

For static histomorphometry, the specimens were fixed in increasing concentrations of ethanol and embedded in methylmetacrylate. Longitudinal undecalcified sections of the tibia were prepared and stained by the Masson-Goldner-trichrome method. The trabecular bone volume, trabecular thickness, the number of osteoclasts per tissue area and osteoclast surfaces per bone surfaces were determined from the right proximal tibia. The bone marrow area and the bone area of the cortical bone were evaluated from the tibial diaphysis.

The dynamic histomorphometric parameters of bone formation rate and mineral apposition rate were measured from the tibia of labelled animals at 12 weeks, following the suggestions of the American Society for Bone and Mineral Research (Parfitt 1987). The animals were labelled with tetracycline (20 µg/kg in 0.9% NaCl) 9 days before termination, and with calcein (20 µg/kg in 0.9% NaCl) 5 days before termination.

OsteoMeasure software (Osteometrics Inc., Atlanta, GA, USA) was used for the histomorphometric analyses. The analyses were made by a single investigator to eliminate the possibility of interindividual variation in the evaluation of the results.

4.2 ASSESSMENTS OF BONE FORMATION AND OSTEOCLAST DIFFERENTIATION IN VITRO (STUDY VI)

Osteoblast activation and osteoclast differentiation were tested with bone marrow-derived osteoblast and osteoclast precursor cells from the tibia and femora of 8-12-week-old female NMRI mice (University of Turku, Turku, Finland). Osteoblast activation was tested by culturing the precursor cells for 7 days, following which subcultures were prepared. After the subcultures had been cultured for 14 days, the deposited calcium was removed with 0.6 M HCl from the bone nodules that had formed, and analysed. To determine the osteoclast differentiation, the osteoclast precursor cells were cultured in the presence of PTH for 7 days. Osteoclast formation

was determined by measuring tartrate resistant acidic phosphatase (TRAP) activity from the culture medium at the end of the test (Alatalo et al. 2000).

4.3 ASSESSMENT OF CALCIUM METABOLISM IN VIVO (STUDIES IV AND V)

Healthy postmenopausal women who were not taking hormone replacement therapy were recruited to the studies, which examined the effect of the *L. helveticus* fermented milk and CPP on calcium absorption acutely (Table 4). The exclusion criteria included medication affecting calcium and bone metabolism (Studies V, VI), and the use of ACE inhibitors (Study VI). The use of vitamin and mineral supplements was not allowed during the studies.

The studies were conducted as double-blind randomised cross-over studies. One intervention consisted of two separate days with a one-week washout period in between (Figure 6). On the day prior to the study day the subjects consumed a restricted amount of milk products (600 mg calcium) in order to control the amount of calcium that might affect the results of the study day (Run-in). The product was given in randomised order after baseline blood and urinary samples had been collected. During the follow-up period of 6-8 hours, standardised meals were served. The effect of the products was evaluated by the area under curve (AUC), maximal change and change in two hours, of serum ionised calcium, parathyroid hormone (PTH), calcium and phosphate and urinary calcium. A three-day food record was analysed by the Nutrica program (Social Insurance Institution of Finland, 2000), to estimate the habitual calcium intake of the subjects (Study V).

The protocols were approved by the Ethical Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (Study V) and by the Ethical Committee of the University of Helsinki (Study VI).

Table 4: The characteristics of the postmenopausal women in studies V and VI.

	N	Age, years	BMI	Calcium intake, mg/d	S-D-25, nmol/l
Study V	20	65 (50-78)	26 (22-31)	1180 ± 400	18-77
Study VI	9	57 (49-66)	25 (22-29)	na	na

BMI = body mass index, S-D-25 = Serum 25-hydroxyvitamin D, na = not assessed.

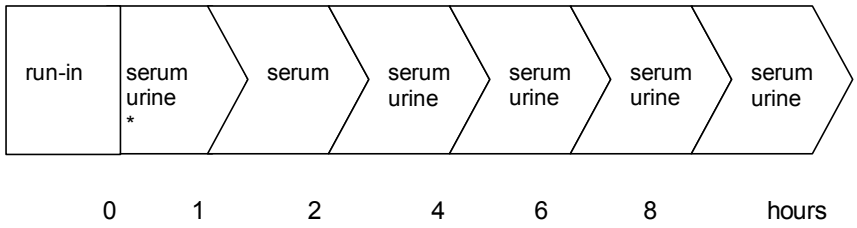

Figure 6: The study day design. After the 24-hour run-in period, the product was given after the baseline serum and urine collection. * = study sample.

4.4 PRODUCTS

The main products used in the different studies are shown in Table 5. The *L. helveticus* milk was produced by fermenting normal milk with *L. helveticus* 16 H strain and supplementing the milk with the solid content of the same milk. As a reference product, normal milk and normal sour milk were used (Valio Ltd., Helsinki, Finland). In the fermentation of the sour milk, a *Lactococcus sp* mixed culture was used. Caseinphosphopeptide powder, 1000 mg (Phospho Peptide CE 90 CPP, DMV International, Veghel, Netherlands) was used as caseinphosphopeptide enrichment. The IPP and VPP peptides (Bachem AG, Bubendorf, Switzerland) used in these studies were diluted in tap water. Plain tap water was used as a control.

Table 5: The main study products.

	L. helveticus	**Sour milk**	**Milk**	**CPP**	**IPP / VPP**	**Water**
Study I	**X**	**X**	**X**			**X**
Study II	**X**	**X**			**X**	**X**
Study III	**X**				**X (VPP)**	**X**
Study IV	**X**	**X**			**X**	
Study IV	**X**	**X**				
Study VI			**X**	**X**		

CPP = caseinphosphopeptides, IPP = isoleucyl-prolyl-proline, VPP = valyl-prolyl-proline

In the first SHR study (I) the *L. helveticus* fermented milk was given in the form of whey after the casein was separated by centrifuge. In the cell study the *L. helveticus* and sour milk were filtrated through a 1000 D membrane and diluted in water. The concentrations of the IPP and VPP peptides and of calcium chloride were the same as in the solutions of the *L. helveticus* whey.

4.5 BIOCHEMICAL ANALYSES

The analyses of the products were carried out at the laboratories of Valio Ltd. (R & D, Helsinki, Finland). The mineral composition of all the study products was determined by atom absorption spectrometry (AAS). The IPP and VPP contents of the *L. helveticus* fermented milk and the peptide water were determined by a modified method of Masuda et al. (Masuda et al. 1996), collecting the peptide fraction by gel filtration chromatography (Superdex Peptide HR 10/30 Amersham Pharmacia Biotech, Bucks, UK) and analysing it by reversed phase HPLC (Waters Alliance HPLC system, Milford, MA, USA) monitored at 214 nm, and quantifying it by using synthesised peptides as standards.

The mineral contents of the femur were analysed by ICP-MS (inductively coupled plasma-mass spectrometry) (Elan 6100, Perkin Elmer, Boston, MA, USA) (Studies I, II).

The serum ionised calcium concentration was measured with an ion selective analyser (ISE cCa 2+/pH Analyzer 634, Halstead, UK), the serum intact PTH concentration by an immunoradiometric assay using Allegro kits (Nichols Institute, San Juan Capistrano, CA, USA), and the serum phosphate, serum total calcium, urinary calcium and creatinine by routine laboratory methods (Studies V, VI). The serum ICTP was analysed using a radioimmunoassay (Risteli et al. 1993) (Study V).

4.6 STATISTICAL ANALYSES

Analysis of variance (ANOVA) was used to compare the results between the study groups in the animal and cell studies (Studies I-III). One-way ANOVA (Studies I-III) was used to compare the *L. helveticus* group, separately, to the other study groups.

The change in the area under curve (AUC) and maximal change were calculated for response variables in the cross-over studies (Studies V, VI). A paired t-test (Study VI) and ANOVA for cross-over (Study V) were used to compare the interventions.

The results are expressed as means ± SEM. A value of $p<0.05$ was considered statistically significant.

5 RESULTS

5.1 *L. HELVETICUS* FERMENTED MILK AND BONE PARAMETERS

Effects on bone composition and mechanical properties (Studies I, II, III)

In the first SHR study (I) the *L. helveticus* fermented milk group showed higher bone mineral density in relation to body weight than the water group (0.08 vs 0.07 mg/cm^2/100g, p=0.006) and the sour milk group (0.08 vs 0.07, p=0.01). In the second SHR study (II) the *L. helveticus* fermented milk had higher bone mineral density compared to the sour milk group (0.23 vs 0.20 g/cm^2, p=0.014) but not to the water group (0.23 vs 0.23 mg/cm^2, p=0.7). In the histomorphometry the *L. helveticus* showed a 17% higher trabecular thickness (p=0.03) and a 34% higher trabecular bone volume (p=0.07) compared to the water group. In the mechanical testing with the cantilever bending test there were no differences between the groups.

The ovariectomy caused a 57% loss in the trabecular bone mineral density (p<0.001) as well as loss in the cortical bone mineral density (Study III). The treatment with *L. helveticus* fermented milk resulted in a 16% higher trabecular bone mineral density (p<0.001) than that of the OVX control group (Table 7). The reduction in cortical bone mineral density (1255 vs 1267 mg/cm^3, p=0.04) and cortical bone area (5.4 vs 5.8 mm^2, p=0.01) was also attenuated with the *L. helveticus* fermented milk. In the tibial diaphysis the cortical tissue area and bone volume increased in both the pQCT and the histomorphometrical measurements. The polar moment of inertia was higher in the *L. helveticus* group, but there was no effect in the mechanical testing of the femoral neck. The *L. helveticus* fermented milk reduced bone turnover by decreasing the bone formation rate in the proximal tibia and endocortical surface of the tibial diaphysis, but there was no effect on the osteoclasts.

In conclusion, *L. helveticus* fermented milk shows a clear trend of supporting bone in growing rats (Table 6). In OVX rats, *L. helveticus* fermented milk prevents bone loss in the trabecular and cortical bone by decreasing the bone turnover rate (Table 7).

Table 6: Summary of the main results of SHR rat studies after a long-term feeding with *L. helveticus* fermented milk and IPP and VPP peptides. (Studies I, II)

	L. helveticus	**Peptides**
BMD	↑	↔
BMC	↔	↔
Strength	↔	↔
Histomorphometry	↑	↔

BMD = bone mineral density, BMC = bone mineral content. ↑ = increased trend
↔ = no effect

Body weight gain and the intake of energy and nutrients (Studies I, II, III)

The daily intake of energy and nutrients in the rat studies is shown in Table 8. In SHR the body weight gain was higher in the water group than in the *L. helveticus* group, although there was no difference in the energy intake between these two groups. The *L. helveticus* group received the lowest amount of protein and the highest amount of calcium (Study I) and phosphorus.

The ovariectomised groups gained more weight than the sham group, as was expected. The weight gain was higher in the *L. helveticus* group than in the OVX control group, reflecting the higher energy intake. Fluid consumption was significantly higher in the *L. helveticus* group, which also caused variance in the intake of nutrients, mainly of protein and calcium.

Table 7: Summary of the effects on OVX of *L. helveticus* fermented milk and VPP peptide (30 mg/l, 60 mg/l) on bone measurements after a long-term feeding ($p<0.05$).

	OVX vs Sham	***L. helveticus* vs OVX**	**VPP 30 / 60 vs OVX**
Proximal tibia			
trabecular BMD	↓	↑	↔/↔
cortical BMD	↓	↑	↔/↔
cortical bone area	↑	↑	↔/↔
tissue area	↔	↑	↔/↔
Tibial diaphysis			
cortical BMD	↔	↔	↔/↔
cortical bone area	↔	↑	↔/↔
tissue area	↔	↑	↔/↔
Ash weight	↔	↑	↔/↔
Strength	↓	↔	↔/↔
Static histomorphometry			
bone volume	↓	↑	↔/↔
trabecular thickness	↔	↔	↔/↔
diaphysis bone area	↓	↔	↔/↔
diaphysis tissue area	↓	↑	↔/↔
osteoclast number	↑	↔	↔/↔
Dynamic histomorphometry			
proximal BFR	↑	↓	↔/↔
proximal MAR	↑	↔	↔/↔
endocortical BFR	↑	↔	↔/↔
endocortical MAR	↑	↓	↔/↑
periosteal BFR	↑	↔	↔/↔
periosteal MAR	↔	↔	↔/↔

OVX = ovariectomised, BMD = bone mineral density, BMC = bone mineral content, BFR = bone formation rate, MAR = mineral apposition rate, ↑ = increased effect, ↓ = decreased effect, ↔ = no effect.

Table 8: Mean intake of rats of feed, drinking fluids, energy, protein, calcium, phosphorus and peptides of the different groups during the whole study period in the SHR and OVX studies. The results are shown as mean ± SEM. Results of studies I and II show the mean intake from the rats, five to a cage. In study III the results are from rats in individual cages.

	Study I			Study II				Study III		
Variable	***L. helv.***	**Sour milk**	**Water**	***L. helv.***	**Sour milk**	**Peptides**	**Water**	***L. helv.***	**VPP 60**	**OVX**
Feed intake, g/d	15 ± 1	18 ± 0.0	21 ± 0.1	11 ± 0.2	15 ± 0.4	18 ± 0.6	19 ± 0.4	8 ± 0.4	18 ± 0.5	18 ± 0.2
Fluid intake, ml/d	25 ± 0.7	32 ± 0.4	31 ± 0.4	41 ± 1.0	29 ± 1.7	29 ± 0.7	26 ± 0.5	60 ± 2.5	24 ± 0.6	26 ± 0.9
Energy, kJ/d	232 ± 2.6	284 ± 0.9	260 ± 0.6	257 ± 3	247 ± 7	252 ± 8	262 ± 5	297 ± 4	266 ± 7	271 ± 2
Protein, g/d	3.0 ± 0.0	4.1 ± 0.0	3.8 ± 0.0	3.1 ± 0.0	3.8 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	2.9 ± 0.0	2.7 ± 0.1	2.7 ± 0.0
Calcium, mg/d	260 ± 4.4	218 ± 0.7	203 ± 0.5	201 ± 3	217 ± 10	186 ± 6	193 ± 4	185 ± 3	129 ± 4	132 ± 1
Phosphorus, mg/d	na	na	na	406 ± 8	367 ± 17	120 ± 4	124 ± 2	88 ± 1	90 ± 3	92 ± 1
IPP or VPP, mg/d	0.9 ± 0.0	-	-	0.7 ± 0.0	-	1.0 ± 0.0	-	1.0 ± 0.0	1.4 ± 0.0	-

L. helv. = *L. helveticus* fermented milk, VPP 60 = 60 mg/l VPP, na = not assessed, - = no detectable amounts.

Effects on bone cells (Study IV)

The bone formation rate of the osteoblasts measured with the level of calcium accumulated in the formed bone matrix varied between baseline and the different treatment groups of *L. helveticus* (Table 9). *L. helveticus* whey increased bone formation consistently by 20-40% with different solutions. Sour milk whey and calcium chloride had no effect on bone formation when the treatment groups were compared with baseline.

Osteoclast differentiation did not differ between baseline and the different treatment groups with *L. helveticus* whey and sour milk whey. When the different solutions of *L. helveticus* were compared with baseline, osteoclast differentiation decreased more than 25% with all the solutions (p=0.01-0.07). The sour milk whey had similar results, although not dose-dependently.

Based on these results, it appears that *L. helveticus* fermented milk whey promotes bone formation *in vitro* and that this is due to components other than those found in sour milk whey.

5.2 IPP AND VPP PEPTIDES AND BONE PARAMETERS

Effects on bone composition and mechanical properties (Studies II, III)

In SHR (Study II) the IPP and VPP peptides given in water (70% of the intake in the *L. helveticus* group) had no effect on bone mineral density or mechanical properties (Table 6). In the histomorphometry the rats receiving IPP and VPP peptides showed a 10% higher trabecular thickness than the rats receiving water. There were no changes in any of the other histomorphometrical measurements.

In the OVX rats the two doses of VPP in water (80% and 140% of the intake in the *L. helveticus* group) did not protect against bone loss measured as bone mineral density and mechanical properties. Nor were there any differences in the histomorphometry. (Study III) (Table 7).

According to these studies, pure IPP and VPP peptides given in water have no clear effects on bone *in vivo*.

Effects on bone cells (Study IV)

The IPP and VPP peptides increased bone formation *in vitro* (Table 9) (Study IV). The maximal increase with IPP was seen with the 10^{-6} M concentration ($p<0.001$). However, the highest concentration decreased bone formation ($p=0.001$). The VPP peptide increased bone formation with all the concentrations. The highest, a 6-fold increase, was observed with the 10^{-8} M concentration ($p<0.001$). The IPP and VPP peptides had no effect on osteoclast differentiation.

To summarise, IPP and VPP peptides increase bone formation in vitro, without any effect on osteoclast differentiation.

Table 9: Summary of the main effects of the different solutions of *L. helveticus* fermented milk whey and sour milk whey and the different concentrations of IPP and VPP peptides on bone formation and on the inhibition of osteoclast differentiation ($p<0.05$).

	Bone formation			Osteoclast differentiation	
Dilution	***L. helveticus***	**IPP**	**VPP**	***L. helveticus***	**Sour milk**
10^{-10}	-	↑	↑	↔	↔
10^{-8}	-	↑	↑	↔	↓
10^{-6}	↔	↑	↑	↓	↔
10^{-5}	↑	↓	↑	-	-
10^{-4}	↑	-	-	↓	↓
10^{-3}	↑	-	-	-	-

↑ = increased effect, ↓ = decreased effect, ↔ = no effect, - = not assessed.

5.3 *L. HELVETICUS* FERMENTED MILK AND CPP ON CALCIUM METABOLISM (STUDIES V AND VI)

Two hours after the *L. helveticus* fermented milk intake, serum PTH decreased (-14.3 vs. –8.1 ng/l, p=0.02) and calcium increased (0.04 vs. –0.01 mmol/l, p=0.03) compared to the normal sour milk (Study V). During the whole study period PTH decreased by 13% (p=0.01) after the *L. helveticus* fermented milk compared to the sour milk. A decreasing trend in the maximal change in serum PTH (-28.8 vs -15.4, p=0.07) and an increasing trend in serum calcium (0.09 vs 0.05, p=0.05) was seen after the ingestion of the *L. helveticus* fermented milk. The changes in serum ionised calcium, phosphate, ICTP and urinary calcium were similar in the two interventions.

Table 10: Summary of the main effects of *L. helveticus* fermented milk and CPP-enriched milk on markers of calcium metabolism in postmenopausal women ($p<0.05$).

	L. helveticus **vs sour milk**	**CPP milk vs milk**
Serum ionised calcium	↔	↔
Serum PTH	↓	↔
Serum total calcium	↑	↔
Urinary calcium	↔	↔

CPP = caseinphosphopeptide, PTH = parathyroid hormone, ↑ = increased effect, ↓ = decreased effect, ↔ = no effect.

To examine the effect of CPP enrichment on acute changes in calcium metabolism, the CPP-enriched milk was compared to normal milk (Study VI). Both milks caused an equal reduction in PTH and an equal increase in ionised calcium. The maximal changes in ionised calcium, PTH, total calcium, phosphate and urinary calcium were similar between the CPP milk and the normal milk groups.

To summarise, the *L. helveticus* fermented milk showed positive effects on the acute response of calcium metabolism in the postmenopausal women. The response to CPP-enriched milk was similar to that of normal milk (Table 10).

6 DISCUSSION

During milk fermentation, bioactive peptides with physiological effects are formed. These peptides have been shown to enhance such processes as mineral absorption. Nevertheless, there are no prior studies on the effects of bioactive peptides and bone. The aim of the present study was to clarify the effect of *L. helveticus* fermented milk, isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP) peptides, and casein-phosphopeptides on the bone metabolism of rats *in vivo*, on osteoblast and osteoclast precursor cells *in vitro*, and on acute changes in calcium metabolism in man.

6.1 *L. HELVETICUS* FERMENTED MILK AND BONE METABOLISM

In the present study, the effect of *L. helveticus* fermented milk was assessed on bone by using bone cells, and SHR and OVX rat models, as well as acute biochemical markers of bone resorption, in postmenopausal women (Table 11).

With rat models it is possible to study the effects of feeding in a controlled setting. SHR are a model commonly used in hypertension studies. Adult SHR have been shown to have abnormal calcium metabolism (Wright et al. 2000), increased serum parathyroid hormone levels and a higher number of osteoclasts in the bone (Merke et al. 1989). These abnormalities may be due to decreased serum 1,25 vitamin D (Lucas et al. 1986). As a result the SHR develop osteoporosis with advancing age (Izawa et al. 1985, Lucas et al. 1986, Merke et al. 1989, Ma et al. 1997). In the present study with SHR, bone mineral density was measured only at the end of the study. The method used is therefore a cross-sectional observation of a 12-14-week feeding effect, and thus could not detect changes as easily as an intervention study in which bone mineral density was measured both at baseline and at the end of the study.

Table 11: Summary of the main results of *L. helveticus* fermented milk and its components on bone in bone cells, animal models and humans.

	L. helveticus	**IPP and VPP**	**Sour milk**
Bone cells *in vitro*			
osteoclast	↔	↔	↔
differentiation	↑	↑	↔
bone formation			
Animal models			
SHR BMD	↑	↔	↔
histomorphometry	↑	↔	↔
OVX BMD	↑	↔	na
histomorphometry	↓	↔	na
Postmenopausal women			
ICTP	↓	na	↓

SHR = spontaneously hypertensive rats, OVX = ovariectomised rats, BMD = bone mineral density, ICTP = bone resorption marker, na = not assessed, IPP = isoleucyl-prolyl-prolyl, VPP = valyl-prolyl-prolyl, ↑ = increased trend, ↓ = decreased trend, ↔ = no effect.

After ovariectomy, estrogen deficiency induces a dramatic loss of trabecular bone in the proximal tibia as a result of increased bone resorption. Cortical bone is not as sensitive to estrogen deficiency, although the osteoblasts are reported to increase on the endocortical surface. The changes in the bone are similar to those in postmenopausal women, therefore the OVX rat is considered a suitable model for testing the potential of new drugs to prevent or reverse bone loss in postmenopausal osteoporosis (for review see Kalu 1991).

In the present study, the bone mineral density in SHR was higher after *L. helveticus* fermented milk compared to normal sour milk. However, there was no significant difference between the groups receiving *L. helveticus* or water. The results of the present study were obtained with DXA, which does not take into account the size of the bone, thus the higher weight gain of the water group may conceal the real effect of the *L. helveticus* fermented milk. In histomorphometry, the *L. helveticus* fermented milk increased trabecular thickness compared to the water group, indicating that the *L. helveticus* fermented milk had beneficial effects on bone despite the difference in body weight.

In OVX rats the treatment with *L.* helveticus fermented milk maintained a 16% higher trabecular bone mineral density in the proximal tibia than in the OVX control group. The ash weight of the femur was significantly higher in the *L. helveticus* group, suggesting a larger bone size, which may have caused the rise in the polar moment of inertia in both the proximal tibia and the tibial diaphysis. Nevertheless, in the mechanical testing there was no difference in the strength measured by maximal load and energy from the femoral neck. However, both the cortical tissue area and bone volume had increased in the tibial diaphysis, thus the measurement of mechanical strength from the diaphysis with a three-point bending test might have given different results.

In the OVX study the calcium content of the feed was twice as high as what is considered the optimal amount for bone quality (for review see Kalu 1991), thus the feed was enough to ensure normal bone growth for the groups. The calcium intake was significantly higher in the *L. helveticus* group than in the OVX control group. A 10-15 times higher calcium intake has been shown to prevent OVX-induced bone loss compared to a lower calcium intake (Geng et al. 2000, Shirai et al. 2002). However, studies using a 2-5 times higher calcium intake do not show any effect on bone mineral density in OVX rats (Kalu and Orhii 1999, Gala et al. 2001). In the present study, the calcium intake was 1.4 times higher in the *L. helveticus* group than in the OVX control group, suggesting that the differences in bone mineral density are not dependent on the calcium intake.

In human studies the effect of an intervention can be examined with biomarkers of bone metabolism acutely (Reid et al. 1986, Horowitz et al. 1994, Rubinacci et al. 1996, Guillemant et al. 2000a, 2000b, Scorpacasa et al. 2000). In the present study, both the *L. helveticus* fermented milk and the sour milk caused a decreasing trend in the bone resorption marker, ICTP, but there was no significant difference between the two. This result accords with previous studies, which showed no effect on ICTP after a 1000 mg calcium intake (Sairanen et al. 1994, Kärkkäinen et al. 2001). The negative results are disputed by other studies using different markers of bone resorption, in which a 1000 mg calcium load has been shown to decrease bone resorption measured by urinary hydroxyproline and deoxypyridinoline (Reid et al. 1986, Horowitz et al. 1994). Despite the difficulties of collecting urine, carboxytelopeptide (CTX) has been shown to be more responsive to treatment than ICTP measured from serum (Guillemant et al. 2000a, 2000b, for review see Leary 2001). Thus the ICTP marker may not have been the best biomarker available. According to the results of these studies, the potential beneficial effect of *L. helveticus* fermented milk on bone metabolism in humans cannot be confirmed.

The findings of the rat studies were supported by the results of the bone cell culture studies, in which the *L. helveticus* whey increased bone formation. In previous studies, milk-derived whey protein has been shown to stimulate osteoblastic cell proliferation and to suppress osteoclastic cell formation (Takada et al. 1996, Takada et al. 1997b). In our study, both the *L. helveticus* fermented milk and the sour milk showed some trend towards decreasing osteoclast differentiation, which accords with a study with whey proteins (Takada et al. 1997b). The effect of whey protein on osteoblast stimulation has been postulated as being mediated through the insulin-like growth factor I (IGF-I), since the effect on osteoblast cell proliferation was deactivated in the presence of both IGF-I and IGF-II antibodies (Takada et al. 1996). In ovariectomised rats a low calcium diet supplemented with whey protein has been shown to increase the breaking strength of the femoral diaphysis after a 4-week intervention compared to a control diet of casein (Takada et al. 1997a). In the present study, the use of sour milk as a control rules out the possibility that the mechanism has an effect through the components of whey protein. However, there may be differences in the protein bioavailability of different milk products. The biological value of the milk protein varies depending on the processing, the heat treatment, the starters used and the

fermentation conditions (for review see Alm 1980). An amino acid supplementation has been shown to increase bone strength and decrease bone loss in ovariectomised rats compared to rats receiving casein (Ammann et al. 2002). In *L. helveticus* fermentation the protein is digested mainly into small peptides which are likely to be absorbed, while in the fermentation of normal sour milk small peptides are not formed to such an extent. Milk protein consists of different growth factors (Cox and Bürk 1991), although these are not likely to be available in the gastrointestinal tract. Nevertheless, protein intake has been shown to increase IGF-1 levels in serum (Schürch et al. 1998) and, accordingly, protein restriction depresses IGF-1 levels (Bourrin et al. 2000). Thus the effect of *L. helveticus* fermented milk through IGF-1 formation is possible. Other mechanisms through which *L. helveticus* fermented milk may affect bone are the IPP and VPP peptides, as discussed below.

In conclusion, *L. helveticus* fermented milk has beneficial effects on bone in rats, a result which is supported by *in vitro* studies. But there are so far no conclusive results of the effects of *L. helveticus* fermented milk on bone in humans.

6.2 IPP AND VPP PEPTIDES AND BONE METABOLISM

The IPP and VPP peptides are absorbed in the gastrointestinal tract (Nakamura et al. 1995, Satake et al. 2002), enabling an effect on bone. However, in our study the IPP and VPP peptides did not show significant effects on bone mineral density or histomorphometry when given in water. These results accord with previous *in vivo* studies with ACE-inhibitory drugs (Stimpel et al. 1995, Ma et al. 1997, Broulik et al. 2001). Although the present studies were considerably longer than the earlier ones, the ACE-inhibitory effect of the IPP and VPP peptides has been reported as being clearly weaker compared to that of the pharmacological preparations (Sipola et al. 2001). However, in previous studies, the IPP and VPP peptides have been shown to attenuate the development of blood pressure in rats (Sipola et al. 2001), showing an *in vivo* effect of the peptides**.** The difference in these results may be due to the difference in the site of action for blood pressure and bone.

The IPP and VPP peptides increased the bone formation of bone cells when this was measured as the accumulation of calcium on bone matrix. The effect of IPP and VPP was significantly higher than that of *L. helveticus* fermented whey, if one takes into account the amount of peptides. There may therefore be components in the *L. helveticus* whey formulation that inhibit the effect of IPP and VPP. In previous studies an ACE-inhibitory drug, captopril, showed no effect on osteoblast differentiation when it was tested in an osteoblastic cell line MC3T3-E1 (Nishiya and Sugimoto, 2001). It is possible that ACE inhibition does not affect the differentiation of osteoblasts, but their activity. On the other hand, an ACE inhibitor, enalapril, has been shown to stimulate the release of PGE_2 from cultured rabbit renomedullary interstitial cells (Zusman 1981). PGE_2 has been shown to increase osteoblast activity *in vitro* (Keila et al. 2001). The *in vivo* effect of enalapril on PGE_2 formation measured from urine is not conclusive (Vlasses et al. 1983, Mittman et al. 1985, Broulik et al. 2001). The effect of IPP and VPP peptides on prostaglandin formation remains hypothetical.

Another possible reason for the effect of IPP and VPP peptides on bone formation is the content of proline. Proline is essential for the formation of the bone matrix. In a recent study, the effect of synthesised prolyl-proline (PP), prolyl-valyl-proline (PVP) and leucyl-prolyl-proline (LPP) peptides was examined on bone formation (Narva et al. unpublished results, 2004). In this study all the peptides were found to activate bone formation. These results raise the question as to whether the peptides are digested during incubation, and whether the effect of the peptides is dependent on proline alone.

To summarise, the IPP and VPP peptides were shown to increase bone formation of bone cells *in vitro* but no effect was seen in *in vivo* experiments.

6.3 *L. HELVETICUS* FERMENTED MILK, CPP AND CALCIUM METABOLISM

The acute changes in calcium metabolism were measured by serum calcium, ionised calcium, parathyroid hormone and urine calcium for six to eight hours after the administration of a study milk in postmenopausal women. Calcium absorption decreases and bone turnover increases in postmenopausal women, thus these

women are a sensible subject group in which to study calcium absorption and bone metabolism. Active calcium absorption is known to occur within 2.5 hours and passive absorption six hours after small calcium loads (Reid et al. 1986). The acute method has been commonly used for measuring the effect of calcium supplementation on calcium metabolism acutely (Reid et al. 1986, Kärkkäinen et al. 1997, Guillemant et al. 2000a, 2000b). The response of calcium metabolism is affected by habitual calcium intake, other foods ingested, vitamin D status, and the phase of the menopause (Heaney and Recker 1986). To eliminate these effects in the present studies, intra-individual comparison was used with women with at least one postmenopausal year. The effect of other nutrients was excluded by keeping the study meals identical in the interventions. The studies were carried out within a period of one month, which eliminated the seasonal variation of vitamin D levels.

In the present study, *L. helveticus* fermented milk reduced serum PTH and increased serum calcium concentrations acutely compared to normal fermented milk. In previous studies *L. helveticus* fermented milk has been shown to attenuate the development of hypertension in rats (Sipola et al. 2001, Sipola et al. 2002) and in man (Hata et al. 1996, Seppo et al. 2002, 2003). The effect on blood pressure has been postulated to be partly dependent on the ACE-inhibiting peptides, IPP and VPP. However, *L. helveticus* fermented milk prevented the development of hypertension more than IPP and VPP peptides in water (Sipola et al. 2001), suggesting that the peptides are less biologically available from water or that there are other mechanisms, such as mineral bioavailability, through which *L. helveticus* fermented milk affects blood pressure. In the present study, the effect of the *L. helveticus* fermented milk on the markers of calcium absorption in postmenopausal women was confirmed. Although, in the study with SHR, the *L. helveticus* fermented milk did not increase the femoral calcium content more than the group receiving normal sour milk, indicating no effect on calcium bioavailability. The effect may be explained through CPP formation in the intestine. This, however, remains a hypothesis since the formation of CPP after *L. helveticus* ingestion has not been studied.

On the other hand, the markers of calcium metabolism did not differ between the CPP-enriched milk and normal milk. Both groups received 500 mg of calcium, which lowered the level of serum PTH and increased ionised calcium, showing the effect on

calcium metabolism. It can therefore be concluded that CPP did not affect calcium metabolism acutely compared to normal milk. In previous human studies the effect of CPP as part of a rice- or grain-based meal has shown controversial effects on calcium absorption (Heaney et al. 1994, Hansen et al. 1997a, Hansen et al. 1997b). The present study is the first to investigate the effect of CPP given in a milk formula. In a recent study Meisel et al. (2003) examined the amount of CPP in the intestine after the ingestion of milk or CPP in water. The study showed that CPP formed in the intestine from milk is more effective than preformed CPP given in water. Ileostomy patients given 1520 mg of preformed CPP or 250 g of milk were shown to have higher amounts of CPP in the ileum after ingestion of milk compared to the preformed CPP. According to calculations, 250 g of milk accounts for 120 mg of available CPP (Meisel et al. 2003). In the present study, the subjects were given 280 g of milk equivalent of approximately 130 mg of CPP or CPP milk enriched with 1000 mg of preformed CPP. According to the present study CPP enrichment of milk does not increase calcium absorption acutely, possibly because of the inactivation of CPP by the gastrointestinal enzymes.

An early study with CPP has suggested that CPP increase passive calcium absorption (Mellander, 1950). Vitamin D is essential for the active absorption of calcium, and thus the effect of CPP on calcium absorption could be seen more clearly in vitamin D-deficient subjects. In our studies, the effect of CPP was tested in June, when the vitamin D status in Finland is known to be higher than in winter, which might have diluted the effect of CPP. The *L. helveticus* study was conducted in April, when the levels of vitamin D were within the normal range (18-77 nmol/l).

Fermentation itself may increase calcium absorption by slowing down the gastrointestinal emptying rate due to the difference in the consistence of the milk (Mahe et al. 1994). In the present studies the response to CPP-enriched fermented milk did not differ from the response to CPP-enriched normal milk. Furthermore, the *L. helveticus* fermented milk showed a greater effect on calcium metabolism than normally fermented sour milk, eliminating the possibility that the effect of *L. helveticus* depended only on the fermentation. In previous studies calcium absorption has been reported to increase acutely after the ingestion of fermented dairy products, yoghurt and cheese, compared to normal milk, when similar calcium loads were used

(Kärkkäinen et al. 1997, Talbot et al. 1999). Conversely, in a stable isotope study there has been no difference in the absorption of calcium from milk, yoghurt or cheese (Recker et al. 1988). The effect of fermentation on calcium bioavailability has been assessed in rat bone mineral content with inconclusive results (Delisle et al. 1995, Ghanem and Hussein 1999).

In conclusion, *L. helveticus* fermented milk increases calcium absorption acutely, possibly due to CPP formation. However, preformed CPP do not increase calcium absorption acutely. After a long-term intervention, calcium bioavailability is not enhanced after *L. helveticus* fermented milk compared to normal sour milk.

7 CONCLUSIONS

In the present study, the effects of the *L. helveticus* fermented milk and the IPP, VPP and CPP peptides were studied on calcium and bone metabolism. The following conclusions can be drawn from the present study:

1. *L. helveticus* fermented milk supports bone mineral density in growing rats and reduces bone loss in ovariectomised rats. The effect of *L. helveticus* fermented milk may be mediated through the IPP and VPP peptides formed during fermentation or the increased bioavailability of milk proteins.

2. The IPP and VPP peptides increase the bone formation of osteoblastic precursor cells. However, the IPP and VPP peptides given in water have no effect on bone metabolism *in vivo* in animal models.

3. *L. helveticus* fermented milk increases calcium absorption in postmenopausal women acutely. However, it does not increase calcium bioavailability, measured as the calcium content of the bones in rats.

4. Preformed CPP added to a milk solution do not increase calcium absorption acutely more than normal milk. It is likely that the gastrointestinal enzymes deactivate the preformed CPP, while the milk-derived CPP are formed during gastrointestinal digestion.

To summarise, *L. helveticus* fermented milk increases calcium absorption and supports bone mineral density. This study suggests that *L. helveticus* fermented milk may have an additional value as part of a healthy diet in achieving high peak bone mass and in preventing bone loss during the aging process.

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