OSTEOCALCIN

Novel insights into the use of osteocalcin as a determinant of bone metabolism

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ABSTRACT

Bone is continuously being remodeled by the coupled processes of bone resorption and formation in order to maintain skeletal integrity. Disturbances in the dynamic balance of remodeling may result in bone diseases, such as osteoporosis, which is characterized by loss of bone and increased susceptibility to fracture.

Bone metabolism can be monitored by measuring biochemical markers of bone turnover in blood or urine. Markers provide dynamic and rapid measures of skeletal status and may have applications in e.g. monitoring of osteoporosis therapy or estimation of fracture risk. Measurement of circulating levels of osteocalcin, a bone-specific protein produced during bone formation, is used to assess bone formation rate in clinical investigations. However, the use of serum osteocalcin has been limited by discrepancies in the ability of assays to detect multiple forms of osteocalcin in the circulation. Osteocalcin is also found in urine but its clinical significance has remained unclear due to the lack of suitable methods for its detection.

In this study, the molecular forms of urine osteocalcin were clarified, immunoassays for their detection were developed and urine osteocalcin was evaluated as a novel marker of bone metabolism. Osteocalcin concentration in urine was closely associated with bone turnover rate and it rapidly decreased in response to osteoporosis therapy. Furthermore, urine osteocalcin was found to be a promising new marker for the prediction of forthcoming fractures, especially vertebral fractures. The clinical performance of urine osteocalcin assays appeared to be distinct from serum osteocalcin assays. To better understand the origin of osteocalcin in serum, different bone cell cultures were evaluated. Results obtained with osteoclasts, the cells responsible for bone degradation, demonstrated that, in addition to production by bone-forming osteoblasts during synthesis of new bone, osteocalcin is also released during bone resorption. Therefore, the potential contribution of osteoclastic resorption to circulating osteocalcin has to be considered, and serum osteocalcin should preferentially be considered a marker of bone turnover instead of a pure marker of bone formation.

The results provide novel insights into the origin and characteristics of osteocalcin and its degradation products and to the use of osteocalcin as a marker of bone metabolism. The measurement of osteocalcin in urine clearly offers an additional method for monitoring bone turnover and may have potential applications in diagnostics related to bone diseases, particularly in the monitoring of osteoporosis therapy and improving the prediction of fracture risk.

Keywords: bone, osteoporosis, osteocalcin, bone turnover, fractures

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YHTEENVETO

Luu on elävää kudosta, jota muokataan jatkuvasti hajottamalla vanhaa luukudosta ja korvaamalla se uudisluulla. Luukadossa eli osteoporoosissa luun hajotuksen ja muodostuksen välinen tasapaino on järkkynyt ja luuta hajotetaan enemmän kuin uutta luuta muodostuu. Tämä johtaa vähitellen luumassan pienenemiseen ja luun hienorakenteen vaurioitumiseen sekä lisääntyneeseen alttiuteen luun murtumille.

Luun hajotus- ja muodostusnopeutta voidaan arvioida mittaamalla luun aineenvaihdunnasta vapautuvia tekijöitä veri- tai virtsanäytteestä. Näiden ns. merkkiaineiden pitoisuus elimistössä kuvaa luukudoksen aineenvaihdunnan vilkkautta ja niiden käyttömahdollisuuksia esimerkiksi luukadon lääkehoidon vaikutusten seurannassa pidetään lupaavina. Eräs luun uusiutumisnopeutta kuvaavista merkkiaineista on osteokalsiini, jota muodostuu uuden luun syntypaikoilla. Verinäytteestä mitattua osteokalsiinia on käytetty luun muodostumisnopeuden kuvaajana, mutta eri määritysmenetelmien vaihteleva kyky mitata osteokalsiinin lukuisia pilkkoutumistuotteita on vaikeuttanut tulosten tulkintaa. Verenkierrosta osteokalsiini erittyy virtsaan, mutta virtsanäytteen osteokalsiinin tutkimista on rajoittanut sen määrittämiseen soveltuvien menetelmien puuttuminen.

Tässä tutkimuksessa selvitettiin virtsaan erittyvän osteokalsiinin rakennetta, kehitettiin mittausmenetelmät erilaisten osteokalsiinimuotojen määrittämiseen ja tutkittiin virtsan osteokalsiinimittausten käyttökelpoisuutta luun aineenvaihduntaa kuvaavana merkkiaineena. Virtsanäytteestä mitattu osteokalsiini soveltui hyvin kuvaamaan luun uusiutumisnopeutta. Virtsaan erittyvän osteokalsiinin pitoisuus pieneni nopeasti luustoon vaikuttavan lääkehoidon seurauksena ja lisäksi virtsan lisääntyneeseen osteokalsiinipitoisuuteen liittyi selvästi suurentunut murtumariski. Kohonneella osteokalsiinitasolla oli erityisesti yhteys selkänikamien luhistumamurtumien riskiin.

Potilasnäytteiden lisäksi työssä tutkittiin eri luusolujen merkitystä elimistön osteokalsiinitasoihin. Luun muodostumisessa erittyvän proteiinin lisäksi osteokalsiinia havaittiin vapautuvan myös luun hajotuksen yhteydessä. Tämän perusteella osteokalsiinituloksia olisikin tulkittava luun kokonaisaineenvaihdunnan kuvaajina eikä ainoastaan luun muodostumisnopeuden merkkiaineena.

Tutkimuksen tulokset antavat uutta tietoa elimistön osteokalsiinin rakenteesta, alkuperästä ja kliinisestä käytöstä luun aineenvaihdunnan merkkiaineena. Virtsan osteokalsiinipitoisuuden mittaaminen soveltunee uudeksi vaihtoehdoksi luun uusiutumisnopeuden määrittämiseen ja yksinkertaista virtsanäytteestä tehtävää mittausta voidaan käyttää luusairauksien laboratoriodiagnostiikassa esimerkiksi lääkehoidon vaikutuksen nopeaan havainnointiin ja murtumariskin alustavaan arviointiin.

Avainsanat: luu, osteoporoosi, luukato, osteokalsiini, luun merkkiaineet, murtumat

CONTENTS

ABBREVIATIONS

aBMD	areal bone mineral density
ALP	alkaline phosphatase
α-MEM	α-modified minimum essential medium
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenetic protein
BoneALP	bone-specific alkaline phosphatase
BSA	bovine serum albumin
CI	confidence interval
CTX	C-terminal cross-linked telopeptides of type I collagen
CV	coefficient of variation
DPD	deoxypyridinoline
DXA	dual energy X-ray absorptiometry
ELISA	enzyme-linked immunoassay
FBS	fetal bovine serum
FGF	fibroblast growth factor
Gla	γ-carboxyglutamic acid
GR	glucocorticoid receptor
HEPES	1M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hOC	human osteocalcin
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
I-OC	assay for intact osteocalcin in cell culture supernatants
IRMA	immunoradiometric assay
LSC	least significant change
MAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
M-CSF	macrophage-colony stimulating factor
MGP	matrix Gla protein
M-OC	assay for the majority of two-site detectable osteocalcin in cell culture supernatants
OC	osteocalcin, bone Gla protein
OPG	osteoprotegerin
OR	odds ratio
PBS	phosphate-buffered saline
PICP	C-terminal propeptides of type I collagen
PINP	N-terminal propeptides of type I collagen
PTH	parathyroid hormone
PYD	pyridinoline
(p)QCT	(peripheral) quantitative computed tomography
RANK	receptor activator of nuclear factor κ B
RANKL	ligand for receptor activator of nuclear factor κ B
RIA	radioimmunoassay
Runx2	osteoblast-specific transcription factor
S-	serum
SD	standard deviation
S-GlaOC	assay for total γ-carboxylated osteocalcin in serum, S-cOC
S-IntactOC	assay for intact osteocalcin in serum, S-OC[1-49]

S-OC	serum osteocalcin
S-TotalOC	assay for total osteocalcin in serum
TFA	trifluoroacetic acid
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
T-OC	assay for total two-site detectable osteocalcin in cell culture supernatants
TRACP5b	tartrate-resistant acid phosphatase isoenzyme 5b
U-	urine
U-LongOC	assay for the longest osteocalcin fragments in urine
U-MidOC	assay for the predominant two-site detectable osteocalcin fragments in urine
U-OC	urinary osteocalcin
U-TotalOC	assay for total osteocalcin in urine
V-ATPase	vacuolar-type proton adenosine triphosphatase
VDR	vitamin D receptor
VDRE	vitamin D responsive element
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I – IV). In addition, unpublished results are included.

I Ivaska KK, Hellman J, Likojärvi J, Pettersson K, Käkönen S-M, Gerdhem P, Åkesson K, Obrant KJ, Väänänen HK (2003) Identification of novel proteolytic forms of osteocalcin in human urine. **Biochem Biophys Res Commun**. 306:973-980.

II Ivaska KK, Käkönen S-M, Gerdhem P, Obrant KJ, Pettersson K, Väänänen HK (2005) Urinary osteocalcin as a marker of bone metabolism. **Clin Chem.** in press.

III Gerdhem P, Ivaska KK, Alatalo SL, Halleen JM, Hellman J, Isaksson A, Pettersson K, Väänänen HK, Åkesson K, Obrant KJ (2004) Biochemical markers of bone metabolism and prediction of fracture in elderly women. **J Bone Miner Res**. 19:386-393.

IV Ivaska KK, Hentunen TA, Vääräniemi J, Ylipahkala H, Pettersson K, Väänänen HK (2004) Release of intact and fragmented osteocalcin molecules from bone matrix during bone resorption in vitro. **J Biol Chem**. 279:18361-18369.

1 INTRODUCTION

Bone tissue is continuously being remodeled by the coupled processes of bone resorption and formation. Remodeling allows bone to adapt to changes in the distribution of mechanical forces in response to mechanical and physiological stresses and to repair the microdamages which accumulate in bone matrix. A dynamic balance between bone formation and bone resorption is essential for the maintenance of skeletal integrity. Changes in bone remodeling and an imbalance between bone degradation and formation are responsible for most metabolic disorders of the skeleton, including osteoporosis. Osteoporosis is a disease characterized by low bone mass and deterioration of bone microarchitecture leading to increased bone fragility and consequently to increased susceptibility to fracture. It is undoubtedly a major public health problem. High fracture rates result in the loss of quality of life of patients, but also places an economic burden on society.

The key diagnostic tool in osteoporosis is the quantification of bone mineral density (BMD) by dual-energy x-ray absorptiometry. Another approach to evaluate skeletal status involves the measurement of biochemical markers in blood or urine that are produced or released during bone turnover. The analytes used as bone markers are generally separated into those associated with bone formation and those associated with resorption. Biochemical markers are not substitutes for the measurement of BMD and at present, the diagnosis of osteoporosis cannot be based solely on marker levels. However, markers reflect the total body skeletal metabolism whereas the regional BMD measurement provides information only from a fraction of the skeleton and therefore, bone markers may provide more dynamic and rapid measures of skeletal status. Markers may be useful tools in monitoring osteoporosis therapy and in assessing the rate at which bone loss occurs. Furthermore, there is increasing evidence that at least some markers could be applied for the determination of fracture risk.

Osteocalcin is a bone-specific protein produced primarily by osteoblasts during bone formation. Although the majority of osteocalcin secreted by osteoblasts is deposited in the extracellular matrix of bone, a small amount of it enters the blood where it can be detected. Circulating osteocalcin has been used in clinical investigations as a marker of bone metabolism while the protein expression has served as an index of osteoblastic phenotype and bone formation *in vitro*. However, the introduction of serum osteocalcin assays to routine clinical practice has been limited by the existence of multiple forms of osteocalcin in the circulation and by discrepancies in the ability of available assays to detect them. Further limitations include instability and a lack of consensus regarding the most clinically informative fragment(s) of osteocalcin.

In addition to circulation, osteocalcin can be found in the urine. It is excreted to urine as fragments as a result of glomerular filtration and degradation. The clinical significance of urine osteocalcin has remained unclear, presumably due to the lack of commercially available assays to detect these small fragments.

A better insight into the structure and origin of different fragments of osteocalcin would clarify the use of osteocalcin as a diagnostic tool. Therefore, the initial aims of the present study were to clarify the molecular forms of osteocalcin in urine and to evaluate urine osteocalcin as an additional marker of bone metabolism.

2 REVIEW OF THE LITERATURE

2.1 Composition of bone

2.1.1 Macroscopic structure

Bone tissue, together with the cartilage, comprises the skeletal system in vertebrates. It provides mechanical support, protects bone marrow and vital organs, and provides sites of muscle attachment for locomotion. It also carries a metabolic function by serving as a reserve of ions, especially calcium and phosphate, thereby playing an essential role in homeostasis. The large surface of the skeleton is also able to adsorb toxins and heavy metals in order to minimize their effects on other tissues.

Two types of bone tissue can be distinguished: cortical bone and trabecular bone. Cortical or compact bone comprises about 80% of the skeleton. It is predominantly located at the external part of bones and in the diaphysis of long bones where it encloses the medullary cavity for the hematopoietic bone marrow. In cortical bone, dense layers of calcified tissue arrange into concentric lamellae and form osteons with a Haversian canal in the middle. Trabecular or cancellous or spongy bone is predominantly located in the inner parts of small bones and at the epiphysis of long bones. It is also prominent in the vertebral column. It is formed of a network of thin, calcified trabeculae with more loosely organized and less dense matrix. Cortical bone provides mainly mechanical and structural support and protection whereas the trabecular bone fulfills a metabolic function. Despite the structural, distributional and functional differences, both cortical and trabecular bone are composed of the same matrix components and cells.

Bones can be divided anatomically into flat bones such as the skull bones and long bones such as the femur and tibia. These two bone types are produced during growth and development by two distinct processes. Intramembraneous ossification predominates in skull bones and occurs in condensations within the embryonic connective tissue by proliferation and differentiation of mesenchymal progenitor cells directly into bone-forming cells. The resulting woven bone is later remodeled and replaced by mature lamellar bone. Endochondral ossification occurs in the growth plate of limb bones and is dependent on a cartilage template. The cartilage cells become hypertrophic and die and the cartilage is calcified. Calcified cartilage is partially resorbed and a layer of bone is formed on the cartilaginous remnants. This primary spongiosa is later subjected to remodeling in which the woven bone and cartilage remnants are replaced with lamellar bone. (for review, see Marks and Odgren, 2002, Baron, 2003).

2.1.2 Extracellular matrix

Connective tissues are characterized by the relatively large amount of extracellular matrix compared to the number of cells. The extracellular matrix of bone tissue has the unique ability to become calcified and it is composed of inorganic and organic matrix components. The inorganic matrix consists of mineral, predominantly calcium and phosphate, and provides mechanical rigidity and load bearing strength organic matrix of collagen fibers and non-collagenous proteins. The organic matrix provides elasticity and flexibility as well as determines structural organization while the inorganic matrix. (Marks and Odgren, 2002).

Collagen network. Over 90% of the total protein in organic bone matrix is composed of collagens. The network of collagen fibers serves as scaffold and provides strength as well as binds and orients other proteins that nucleate mineral deposition thereby playing a dominant role in maintaining the integrity and providing the structural framework for the skeleton.

Collagens comprise a large family of multimeric proteins with at least 20 different collagens (Myllyharju and Kivirikko, 2001). The fiber network in bone consists predominantly of type I collagen, although trace amounts of other collagens may be present at least in some developmental stages. Collagens are triple-helical molecules and the type I collagen triplex contains two α1(I) chains and one α2(I) chain. Collagen chains are characterized by Gly-X-Y repeats and due to this sequence, each chain is coiled in a left-handed helix. In type I collagen, X in Gly-X-Y repeat is usually proline and Y is often hydroxyproline or hydroxylysine. Hydroxylation and glycosylation are common post-translational modifications on collagens and the addition of hydroxyl groups to prolyl and lysyl increases the thermal stability of the molecule. After biosynthesis and post-translational modifications, collagen chains form a nucleus at the C-terminus and assemble into a right-handed triple helix in a zipper-like fashion. When the chains are assembled, they exit the cells and the globular propeptide regions at both ends are cleaved resulting in decrease in the solubility of collagen. Collagen is further modified in the extracellular space via the formation of intra- and intermolecular covalent cross-links. (for review, see Rossert and de Crombrugghe, 2002).

Non-collagenous proteins. The non-collagenus proteins in bone matrix can be classified into at least four groups which are proteoglycans, glycosylated proteins containing an RGD-sequence, non-RGD containing glycoproteins and γ-carboxylated proteins. They may contribute to the strength, stability and structural integrity or be involved in bone turnover. Furthermore, some serum-derived proteins are also adsorbed to bone matrix. For example, albumin and HS-glycoprotein, both synthesized in hepatocytes, are concentrated several-fold in bone compared to circulation (Robey, 2002).

Proteoglycans are large molecules that contain glycosaminoglycans attached to core proteins. Two small chondroitin sulphate proteoglycans, decorin and biglycan, are heavily enriched in bone matrix. They bind to type I collagen and several growth factors, such as transforming growth factor beta (TGFβ), and are assumed to be needed for the integrity of the matrix and/or be important in modulating the activity of growth factors (Robey, 2002). Biglycan deficient mice develop a decrease in trabecular bone mass indicating that proteoglycans may be positive regulators of bone formation (Xu *et al.*, 1998).

Glycosylated matrix proteins synthesized by bone cells may mediate cell attachment. These include e.g. fibronectin, vitronectin, and thrombospondin, as well as some members of the family of small intergrin-binding ligands with N-linked glycosylation (SIBLING) such as osteopontin, bone sialoprotein, and dentin matrix protein 1 (Robey, 2002). Many of these are phosphorylated and all contain an RGD-sequence (Arg-Gly-Asp), the cell-attachment consensus sequence that binds to the integrin class of cell-surface molecules (Ruoslahti, 1996). The RGD-sequence in matrix proteins is assumed to interact with the vitronectin receptor $\alpha_v\beta_3$ on cell surface and mediate the attachment of various cell types on bone (Flores *et al.*, 1996). Osteopontin is an acidic glycoprotein that is localized in a variety of tissues including bone where it is predominantly expressed in osteogenic cells at late stages of maturation. It presumably acts within the extracellular matrix to promote osteoclast attachment to mineralized surfaces (Butler, 1989). In addition to mediating attachment, osteopontin has also been implicated as a nucleator for mineralization (McKee and Nanci, 1996). The skeleton of osteopontin-deficient mice appears normal, but in older animals the trabecular bone volume is increased and the mice are resistant to ovariectomy-induced bone resorption (Yoshitake *et al.*, 1999). Bone sialoprotein is a highly glycosylated phophoprotein that, similar to osteopontin, contains integrin-binding sites and may play a role in cell-matrix adhesion processes. Bone

sialoprotein is a product of osteoblasts and odontoblasts and almost exclusively found in mineralized connective tissues. The expression of bone sialoprotein coincides with the appearance of mineral deposition and thus, bone sialoprotein may also play a role in matrix mineralization in addition to mediating attachment (Ganss *et al.*, 1999). It is not clear why there are so many RGD-containing proteins in bone. However, their expression pattern as well as the expression pattern of different integrins varies during development and cell-matrix interactions may change as a function of maturational stage (Robey and Boskey, 2003).

Non-RGD containing glycoproteins found in bone matrix include proteins such as osteonectin and alkaline phosphatases. Osteonectin is a phosphorylated glycoprotein highly enriched in bone. It binds collagen, hydroxyapatite and growth factors and has been suggested to provide a link between the collagen network and the mineral phase (Termine *et al.*, 1981). Osteonectin-deficient mice display decreased bone remodeling and a defect in bone formation causing osteopenia (Delany *et al.*, 2000). Alkaline phosphatase is an enzyme usually located on the surface of osteoblasts but the enzyme can be cleaved from the surface and also found within the mineralized matrix in the membrane of matrix vesicles (Väänänen *et al.*, 1987). It probably plays a role in the first steps of matrix formation and especially mineralization but the role of it is not yet conclusively elucidated (Robey and Boskey, 2003).

Vitamin K-dependent proteins or γ-carboxylated proteins are characterized by the presence of γ-carboxyglutamic acid (Gla) residues. The formation of Gla occurs via a post-translational modification of specific glutamate residues in selected proteins and provides these proteins with the ability to bind calcium. Osteocalcin, or bone Gla protein, is one of the most abundant non-collagenous proteins in bone and among the ten most abundant proteins in human body (Gallop *et al.*, 1980). Because of specific interaction with bone mineral, it has been thought to participate in mineralization (Romberg *et al.*, 1986). It may also play some role in regulating bone mineral turnover since osteocalcin-deficient mice develop progressive increase in bone mass (Ducy *et al.*, 1996) and impaired mineral maturation (Boskey *et al.*, 1998). Osteocalcin is highly enriched in bone matrix while the other member of the family, matrix Gla protein (MGP), is found in bone, cartilage and arteries and also in several soft tissues (Price, 1989). MGP is a powerful inhibitor of mineralization in arteries and cartilage (Luo *et al.*, 1997). Because it is abundantly found in bone it appears not to be sufficient to inhibit mineralization of bone but rather, it may serve to control the degree of mineral deposition in normally calcified tissue (Gundberg, 2003).

Inorganic bone matrix. The mineral accounts for up to 70% of adult bone by weight. It predominantly contains calcium and phosphorus in the form of hydroxyapatite crystals which provide mechanical rigidity and load bearing strength to the organic bone matrix. The mineral crystals in bone are extremely small and soluble compared to geological hydroxyapatite facilitating bone to act as a reservoir for calcium and phosphate and other ions (Eppell *et al.*, 2001). Bone mineral also contains impurities such as carbonate which tend to make the crystals smaller and more soluble. Carbonate becomes incorporated into the crystal whenever apatite is formed in an environment that contains CO_2, such as body fluids. Dietary cations such as magnesium and strontium can also be incorporated into the bone mineral and substitute calcium in the crystal lattice yielding crystals which are smaller and less perfect. Cadmium also has similar effects on bone mineral. With time, the crystals enlarge and become more perfect and contain fewer impurities and are more resistant to resorption than smaller and younger crystals (Robey and Boskey, 2003).

Growth factors and cytokines. Bone matrix also serves as a reservoir for several growth factors and cytokines needed in the bone environment. This allows growth factors to participate in the remodeling process and they may mediate e.g. the coupled actions of osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) play an important role in the regulation of bone induction, maintenance and repair, and can induce the formation of new bone when implanted into non-skeletal sites *in vivo* (Reddi, 1998). Also TGFβ and fibroblast growth factors (FGFs) are abundantly present in bone matrix (Fromigue *et al.*, 2004). Bone matrix proteins such as proteoglycans, bind to growth factors and cytokines and may affect their bioavailability (Young, 2003).

2.1.3 Bone cells

Osteoclasts. The osteoclast is a multinucleated cell specialized in bone resorption. Osteoclasts have several unique features such as the capacity to polarize on bone and the development of a ruffled border, a deep folding of plasma membrane in the area facing the bone matrix. The osteoclast originates from the progenitor cells of the monocyte/macrophage lineage. The proliferation of osteoclast precursors is followed by commitment to the osteoclast phenotype, fusion of mononuclear progenitors and eventually the degradation of bone matrix by mature cells (Teitelbaum, 2000). The differentiation of osteoclasts is dependent of the presence of marrow stromal cells or osteoblasts (Udagawa *et al.*, 1990) which express the ligand for receptor activator of nuclear factor kappa B (RANKL) on their surface (Lacey *et al.*, 1998, Yasuda *et al.*, 1998). RNAKL stimulates the RANK receptor on the plasma membrane of osteoclast progenitors and is the key interaction between osteoblasts and osteoclasts needed for the osteoclastogenesis (Lacey *et al.*, 1998, Yasuda *et al.*, 1998, Hsu *et al.*, 1999). Osteoprotegerin (OPG), a soluble factor produced by osteoblasts/stromal cells, is structurally similar to RANK and functions as a decoy receptor for RANKL thus inhibiting osteoclastogenesis (Simonet *et al.*, 1997, Yasuda *et al.*, 1998). The balance in the RANK/RANKL/OPG system and especially, the balance between the stimulator of osteoclastogenesis (RANKL) and of the inhibitor (OPG) determine the degree of activation of RANK receptor and subsequently the pool size of active osteoclasts and the amount of resorption (Hofbauer *et al.*, 1999). Another factor essential for osteoclastogenesis is macrophage colony-stimulating factor (M-CSF) which binds to its receptor on osteoclast precursors and provides signals for survival and proliferation. Both RANKL and M-CSF are essential for osteoclastogenesis as mice lacking either RANKL (Kong *et al.*, 1999) or RANK (Dougall *et al.*, 1999) or M-CSF (Yoshida *et al.*, 1990) fail to produce osteoclasts. In addition, several other factors affect macrophage and the osteoclast differentiation pathway. Transcription factor PU-1 is essential for the early differentiation of macrophage precursors and for their determination to the myeloid lineage. M-CSF functions later in the differentiation pathway than PU-1 and is needed for the proliferation and survival of immature macrophages to macrophages. The commitment to osteoclast lineage is mediated via RANKL and its downstream effectors whereas molecules such as $\alpha_v\beta_3$ integrin and c-Src appear to be involved in the polarization of cells. Finally, the expression of genes encoding proteins essential for bone resorption, such as cathepsin K, vacuolar H^+-adenosine triphosphatase and carbonic anhydrase II delineate the final stages of the differentiation pathway (Teitelbaum, 2000). Mature osteoclasts perform their bone resorptive function via the ruffled border membrane which seals an extracellular compartment between the osteoclast and bone surface. Osteoclast acidifies the extracellular compartment by secreting protons across the ruffled border membrane. The low pH results in the dissolution of hydroxyapatite crystals and the residual organic matrix is digested by proteolytic enzymes secreted by the osteoclast. The remnants of the digestion are

internalized, transported across the cell, and finally released to the surroundings (for review, see Väänänen and Zhao, 2002).

Osteoblasts. The osteoblast originates from a mesenchymal stem cell. The commitment of mesenchymal cells to tissue-specific cell types is coordinated by transcriptional regulators which serve as master switches. The differentiation into osteoblastic cells requires transcription factors Runx2 (also known as Cbfa1) and Osterix (Lian *et al.*, 2003). Runx2 expression precedes osteoblast differentiation and is restricted to mesenchymal cells destined to become chondroblasts or osteoblasts (Ducy *et al.*, 1997). Osterix acts downstream of Runx2 and is required in later stages of osteoblastic differentiation (Nakashima *et al.*, 2002). The maturation of osteoblasts is also influenced by local growth factors such as BMPs, TGFβ, and FGFs as well as homeobox binding factors (Ducy *et al.*, 2000). During bone formation, osteoblasts secrete type I collagen and other bone matrix proteins in a directed manner toward the bone-forming front and produce an unmineralized osteoid (Lian *et al.*, 2003). Bone formation occurs sequentially in periods of proliferation, development and maturation of the extracellular matrix, and finally, mineralization (Owen *et al.*, 1990a). Osteoblasts are usually found as clusters along the bone surface, with ~100-400 cells per bone-forming site (Baron, 2003). On quiescent bone surfaces undergoing neither formation nor resorption, single layers of flattened osteoblastic cells also known as bone lining cells are observed. Currently, very little is known about the functions of these cells but they can differentiate into osteogenic cells representing a possible source of osteogenic precursors and may be involved in the propagation of the activation signal that initiates bone resorption and bone remodeling (Miller *et al.*, 1989).

Osteocytes. The most abundant cell type in bone is the osteocyte. It is embedded deep within the mineralized bone matrix in contrast to osteoclasts and osteoblasts which are located at bone surfaces. The osteocyte is considered the terminal differentiation stage of the osteoblast. It probably originates from osteoblastic cell which becomes trapped in the synthesized bone matrix. It lies embedded in mineralized bone within individual lacunae and is connected to other osteocytes and bone surface osteoblasts with long cell processes which pass through the matrix via small canals. The canals form a network of thin canaliculi permeating the entire bone matrix and gap junctions between cells allow direct cell-cell communication. (for review, see Nijweide *et al.*, 2002). Osteocytes are long-living cells and can reside in healthy bone even decades if the particular bone site where they reside in has a slow turnover rate (Lian *et al.*, 2003). In aging bone, empty osteocytic lacunae are observed as a result of osteocyte apoptosis (Noble *et al.*, 1997). The vertebral skeleton needs to adapt to the prevailing mechanical needs of the organism and the osteocyte network is believed to be the mechanosensory system of skeleton (Klein-Nulend *et al.*, 1995b). Experimental studies indicate that osteocytes respond to mechanical deformation and loading of bone and transduce the stress to biological osteogenic activity (Skerry *et al.*, 1989, el Haj *et al.*, 1990). Potential stimuli include the flow of extracellular fluid throughout the canalicular network, hydrostatic pressure or direct cell strain in response to mechanical forces. They may induce several responses in osteocytes including e.g. the induction of prostaglandin synthesis and the production of nitrous oxide (Klein-Nulend *et al.*, 1995a, Ajubi *et al.*, 1996, Fox *et al.*, 1996) but most of the steps in the signalling cascades that are involved still have to be elucidated.

2.2 Bone remodeling

The skeleton is a metabolically active organ which undergoes continuous remodeling throughout life by the coupled processes of bone resorption and formation. Up to 10% of the adult human skeleton is remodeled annually. The remodeling allows vertebrates to renew bone

and is necessary to maintain the structural integrity of the skeleton and to fulfil its metabolic function. The remodeling allows the bone to adapt to changes in the distribution of mechanical forces and to repair microdamage. Furthermore, remodeling is needed for the mobilization of calcium and other components from the bone matrix as well as incorporation of them to bone when needed.

Most of the metabolism occurs at the bone surfaces. Due to the higher surface area of trabecular bone compared to cortical bone, most of the turnover takes place at the endosteal surface of trabecular bone where it is in contact with bone marrow. To some extent remodeling also takes place in the Haversian channels in cortical bone but in general, the remodeling occurs in cortical bone at a lower rate (~2% annually) than in trabecular bone (~10%). Remodeling takes place in distinct areas. It is dependent of the interactions of cells of osteoblastic and osteoclastic lineages and requires a coordinated action of osteoclast to degrade and remove bone and osteoblasts to replace it by new bone matrix. The coupling of bone formation to previous bone resorption occurs faithfully and in the normal adult skeleton, bone formation only occurs at sites where bone has previously been resorbed (Eriksen, 1986). The complete remodeling cycle at each microscopic site takes about 3-4 months (Mundy *et al.*, 2003) (Fig.1).

Bone resorption. The initial stage involves the recruitment of osteoclastic cells to the site of incoming resorption. The signals that guide osteoclasts to certain resorption sites are still unclear but probably osteocytes and bone lining cells mark these sites (Väänänen and Zhao, 2002). E.g. the contraction of lining cells may allow the access of osteoclasts to the resorption site. Proteolysis of the matrix surface to uncover a mineralized surface may also be needed in preparing the bone for subsequent resorption by the incoming osteoclasts and this may be performed by the bone lining cells (Jones and Boyde, 1976). Bone resorption by recruited osteoclasts is a multistep process. The initial event is the attachment of the osteoclast to the target matrix and generation of an isolated microenvironment between the cell and the bone surface (Silver *et al.*, 1988). This is defined by a sealing zone, which mediates the tight attachment of the osteoclast to bone matrix and separates the resorption area from its surroundings (Väänänen and Horton, 1995). Osteoclasts secrete acid and proteases to the bone surface which act sequentially to degrade the bone matrix. Demineralization is the first phase and involves the acidification of the isolated extracellular microenvironment. This is mediated by a vacuolar H^+-adenosine triphopshatase (V-ATPase) located at the ruffled border membrane facing bone surface (Väänänen *et al.*, 1990). The ruffled border also contains chloride channels which allow a flow of chloride anions into the resorption lacuna in order to maintain electro-neutrality. This results in overall secretion of HCl into resorption lacuna causing a decrease in the pH. Cytosolic pH is maintained by the action of carbonic anhydrase which generates protons and bicarbonate from CO_2 and H_2O while a chloride-bicarbonate exchanger exports bicarbonate and imports chloride. (Väänänen *et al.*, 2000). The acidic environment in the lacuna mobilizes bone mineral and putatively some proteins may be released when mineral component of the matrix is removed. Dissolution of the bone mineral is followed by the degradation of organic component of bone by proteases, primarily by cathepsin K, a lysosomal proteinase (Inui *et al.*, 1997). The products of bone degradation are endocytosed and transported through the cell via transcytosis and finally released at the basolateral side via a functional secretory domain (Salo *et al.*, 1997). In addition to cathepsin K, other osteoclastic enzymes such as matrix metalloproteinases and other cathepsins may contribute to the process (Väänänen and Zhao, 2002). Furthermore, tartrate-resistant acid phosphatase (TRACP) may facilitate the fragmentation of endocytosed bone material in the transcytotic vesicles by producing reactive oxygen species which are able to destroy collagen and other proteins (Halleen *et al.*, 1999b).

The resorptive step of the remodeling cycle has been estimated to last up to 10 days (Mundy *et al.*, 2003).

Reversal phase. During the intermediate phase between matrix resorption and bone formation, the resorbed surface is being prepared for the bone formation. The exact molecular events taking place before bone formation are currently not completely understood but this process has been suggested to be mediated by a mononuclear cell population (Rifkin and Heijl, 1979). Recently, osteoblast-like cells have also been reported to be capable of functioning as matrix degrading cells and finalizing the resorption phase before synthesizing new mineralized matrix (Mulari *et al.*, 2004). During the reversal phase, a layer rich in osteopontin (known as cement line) is formed in the resorbed area. This structure links the old and new bone and marks the boundaries of a new osteon and has been suggested to function in interaction between the old and the new bone matrix (McKee and Nanci, 1996).

Bone formation. Osteoclastic resorption is followed by a more prolonged phase of bone formation mediated by the osteoblasts. In the adult skeleton, bone formation occurs normally only at sites where bone resorption has occurred and these processes are thus coupled both in time and in space (Eriksen, 1986). Osteoblast precursors are attracted to the resorbed sites by chemotactic molecules, probably released from the resorbed bone matrix (Mundy *et al.*, 1982). These may include fragments of structural proteins or growth factors enriched in the bone matrix. Osteoblast precursors proliferate at the remodeling site by the influence of local growth factors and the mature osteoblasts lay down component of organic bone matrix to the resorbed bone area in a sequential manner. The collagen network is synthesized first followed by incorporation of non-collagenous proteins. The production of type I collagen and formation of the collagenous matrix is followed by an increased expression of alkaline phosphatase. This gradually decreases when matrix mineralization is progressed. Osteocalcin appears at the latest stages of differentiation pathway, approximately at the onset of mineralization (Owen *et al.*, 1990a). The newly synthesized organic matrix which has not yet been mineralized is known as osteoid and it is observed due to a time lag between matrix formation and its subsequent calcification which is approximately 10 days in humans (Baron, 2003).

Mineralization. Extracellular mineralization is a physiologic process in bone, teeth, and hypertrophic cartilage, whereas in other locations it must be inhibited. Bone mineralization occurs in two stages. Primary mineralization is a rapid increase in mineral to about 70% of its final density due to nucleation and crystal multiplication. This is followed by a secondary mineralization leading to gradual completion of mineralization due to crystal growth. This part of the remodeling cycle takes approximately 3 months (Mundy *et al.*, 2003). In general, crystals are formed when the ions come together with proper orientation and sufficient energy to generate the first stable crystal (critical nucleus). The formation of the initial miniature crystal is the most energy-demanding step and the nucleation is followed by the addition of ions to the nucleus resulting in crystal growth (Robey and Boskey, 2003). In woven bone and cartilage, the primary mineralization is initiated in matrix vesicles that bud from osteoblast plasma membrane (Morris *et al.*, 1983, Väänänen *et al.*, 1983). In lamellar bone, the macromolecular organization of type I collagen is a factor facilitating bone mineralization and calcification is initiated in spatially distinct nucleation sites. Calcium phosphate is initially deposited in a highly organized fashion at distinct sites in the matrix and it has been postulated that the sites for nucleation reside in the gaps, or “hole zones” between collagen molecules (Miller, 1984). Later, the rest of the space within collagen fibrils are filled (Glimcher, 1987). Body fluids are undersaturated for apatite, thus apatite will not precipitate spontaneously but the bone must contain components

that facilitate the mineral deposition (Robey and Boskey, 2003). The functions of non-collagenous proteins have often been attributed in bone mineralization with some proteins serving as crystal nucleators and other controlling the size and speed of crystal growth (Roach, 1994). During bone maturation the mineral crystals become larger and more perfect and the amount of impurities decreases. The increase in crystal dimension is due to both the crystal growth by addition of ions and the aggregation of crystals (Robey and Boskey, 2003).

Skeletal remodeling can be triggered by changes in mechanical forces or microdamage (Turner, 1998) and by hormonal responses to changes in calcium and phosphorus supplies (Mundy and Guise, 1999). These include the systemic calcium-regulating hormones, parathyroid hormone (PTH) and $1,25(OH)_2D_3$ (vitamin D), as well as calcitonin and a number of local hormones and cytokines (Mundy *et al.*, 2003). Sexual steroid hormones such as estrogens are involved by negatively affecting osteoclast differentiation and thereby bone resorption although the mechanisms of action are incompletely understood (Riggs *et al.*, 1998). Additional systemic control is provided by leptin hormone synthesized by adipocytes which functions indirectly via binding to its receptor in the hypothalamus and subsequently signalling to bone (Ducy *et al.*, 2000).

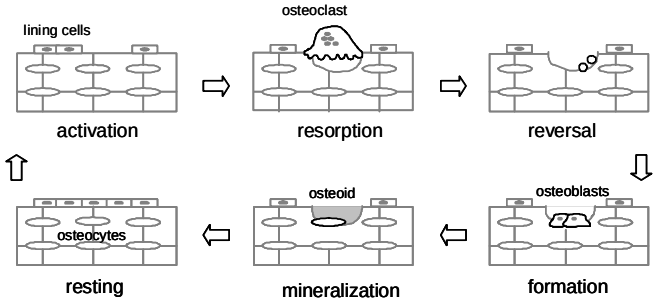

Figure 1. *Bone remodeling cycle.* Bone remodeling consists of a series of sequential steps that are highly regulated. Bone resorption by osteoclasts is followed by the recruitment of osteoblasts and formation of new bone matrix. The newly synthesized matrix is then slowly mineralized. See text for details.

2.3 Osteoporosis

Osteoporosis is a common metabolic disorder of the skeleton which is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to an increase in bone fragility and susceptibility to fracture (Anonymous, 1993). It is more common in females than in males due to the greater bone loss and longer life span of women compared to men. Osteoporosis is undoubtedly a major public health problem. The high fracture rates result in the loss of quality of life of elderly women and the medical costs of osteoporosis and fractures generate a burden on the society. The costs are expected to increase dramatically in the future as a result of the increased life expectancy and the rise in the proportion of elderly population (Cooper, 2003). Therefore, it will be imperative to develop inexpensive and widely applicable methods for diagnosis, prevention, and treatment to limit the increase in osteoporotic fractures.

2.3.1 Pathogenesis of bone fragility

The universal loss of bone that occurs with age is due to the negative balance of osteoblastic relative to osteoclastic activity. The pathogenesis of bone loss and osteoporosis is complex and involves genetic predisposition and alterations in systemic and local hormones together with

environmental influences. The predominant cause of osteoporosis in women is the decrease in estrogen that accompanies menopause and cessation of ovarian function. Bone loss in postmenopausal women occurs in two phases (Riggs *et al.*, 1998). At menopause, women undergo an accelerated transient phase which lasts for ~5-10 years and accounts for significant losses of trabecular bone, about 3% per year in the spine. The slow phase of bone loss which also begins at menopause becomes dominant after the first phase. It involves a more generalized bone loss of both trabecular and cortical bone, about ~0.5% per year at many sites of the skeleton. It continues throughout the remaining lifespan and affects also men. Traditionally, osteoporosis in women has been divided into two distinct syndromes, type I, or postmenopausal osteoporosis and type II, or senile osteoporosis (Riggs and Melton, 1983). The processes leading to type I and II syndromes correspond well to the two phases of bone loss described above.

The pathogenesis of both types of osteoporosis appears to be manifestations of the underlying estrogen deficiency (Riggs *et al.*, 1998). The decreased level of circulating estradiol at the time of menopause is the major cause of the initial rapid bone loss around menopause. The mechanisms of action of estrogen on bone are incompletely understood but apparently bone loss in postmenopausal women results from both an increase in the bone remodeling rate as well as from an imbalance between bone resorption and bone formation (uncoupling) (Riggs *et al.*, 1998). Bone remodeling rates are approximately doubled at menopause and remain elevated in osteoporosis (Recker *et al.*, 2004). Estrogen may play a direct role in the activity of differentiated bone cells. It may also affect indirectly through an action on extraskeletal calcium homeostasis and systemic hormones or modulate the activity of local factors, including growth factors and cytokines and thereby affecting osteoblasts and/or osteoclasts (Eastell, 2003b). Estrogen withdrawal is associated with an increase in the activation frequency of bone remodeling, increased resorption depths, and an increase in osteoclastogenesis (Seeman, 2002). The overall effect is that more bone is resorbed by the osteoclasts than is synthesized by the osteoblasts and a pronounced negative balance between bone resorption and formation eventually leads in net bone loss. However, the mechanical competence of bone is not dependent only on the absolute amount of bone but also on the trabecular microstructure (Parfitt *et al.*, 1983, Kleerekoper *et al.*, 1985). The bone loss results in thinning and perforation of bone trabeculae, increased cortical porosity and inefficient removal of microdamage (Eastell, 2003b). Osteoclastic penetration of trabecular plates and loss of connectivity greatly reduces structural integrity of bone (Seeman, 2002). Eventually, bone gets fragile and a fracture may occur as a result of a low energy trauma. In addition to postmenopausal osteoporosis, bone loss can be secondary to another identifiable medical condition or treatment. Secondary osteoporosis is often associated with disorders of the endocrine system such as hyperparathyroidism and hyperthyroidism (Khan and Bilezikian, 2000, Vestergaard and Mosekilde, 2003), or certain medications such as treatment with glucocorticoids (van Staa *et al.*, 2002).

The level of attainment of peak bone mass and the rate of age-related bone loss after menopause are two main determinants affecting the development of postmenopausal osteoporosis (Riggs and Melton, 1986, Riis *et al.*, 1996). Several risk factors have been identified including age, gender, and genetic factors. The peak bone mass achieved by the age of 20-30 years exhibits a wide range in the normal population and represents an important measure of predisposition to osteoporosis. The determinants of peak bone mass are primarily genetic but environmental factors such as diet, medication and physical activity may also contribute to bone mass (Peacock *et al.*, 2002). Body weight is also closely linked to bone mass (Reid, 2002). A single gene is unlikely to play a large role in a complex disease such as

osteoporosis but a polygenic model is more likely. Currently, more than 20 candidate genes have been shown to be associated with bone mass (Peacock *et al.*, 2002). Candidate genes that could play a role in the development of osteoporosis include type I collagen, OPG, estrogen receptor α, and vitamin D receptor (Ralston, 2002) as well as cytokines and growth factors such as BMPs (Styrkarsdottir *et al.*, 2003).

2.3.2 Osteoporotic fractures

Osteoporosis manifests clinically as fragility fractures which result from mild trauma acting on a skeleton that has reduced bone strength. The occurrence of one or often multiple fractures significantly reduces the quality of life of patients and fractures in the geriatric population also carry an increased risk of morbidity and mortality. It has been estimated that approximately 30 000 osteoporotic fractures occur in Finland annually (Alhava, 2004) and about one in three women over 50 and about one in ten men will have a fragility fracture in their lifetimes (Melton *et al.*, 1992). The incidence of fractures vary from one country to another but is among the highest in Scandinavia (Bacon *et al.*, 1996, Anonymous, 2002).

The most common sites for osteoporotic fractures are the hip, spine, and distal radius. Loss of cortical bone induces a disposition to fractures that occur at the hip and wrist whereas the rate of trabecular bone loss is associated especially with spinal osteoporotic fractures (Kanis, 2002). The most severe fracture type is the hip fracture because it has a major impact on quality of life and is significantly associated with increased morbidity and mortality (Baudoin *et al.*, 1996). Most hip fractures result from falling from standing height or less in individuals with reduced bone strength and the incidence increases exponentially with age. Although the majority of hip fractures appear to occur indoors, they tend to occur more frequently in winter (Cooper, 2003). The number of annual hip fractures in Finland is up to 8000 and the number has increased four-fold during the last 30 years (Alhava, 2004). In contrast, fractures engaging the vertebral column may be asymptomatic and the impact of a single vertebral fracture can be low to the individual. However, the effects of multiple vertebral fractures lead to back pain, limitations of physical activity, progressive kyphosis, and loss of height in a cumulative manner (Ismail *et al.*, 1999). Further it leads to reduction in functional capabilities and may promote social isolation and depression. The pain and fear of additional fractures further decrease physical activity and leads to an increasing risk of further fractures (Cooper, 2003). The incidence of wrist fractures increases linearly from 40 to 65 years in women and after that the rate of increase is less pronounced resulting in a plateau in female incidence. This pattern may be related to change in the pattern of falls with advancing age (Cooper, 2003). Wrist fractures almost always result from a fall on the outstretched arm. There is a winter peak in wrist fractures which is more pronounced than the peak of hip fractures and is more clearly related to a fall outdoors during periods of cold weather (Cooper, 2003). Fractures also tend to cluster in individuals. A previous fracture history is significantly associated with an increased risk of any fracture compared with individual without prior fracture (Ross *et al.*, 1991, Kanis *et al.*, 2004).

2.3.3 Management of osteoporosis

Intervention strategies for the management of osteoporosis and prevention of fractures have been designed with the aim to reduce bone turnover to levels found in premenopausals (Delmas, 2002, Stepan *et al.*, 2003). Estrogen replacement therapy was previously considered the treatment of choice for postmenopausal women since estrogens inhibit bone loss and bone turnover. The recently acknowledged risk of cancer and cardio-vascular diseases will make it less attractive as the first line therapy to prevent osteoporosis as the risks appear to exceed the

benefits (Rossouw *et al.*, 2002). Selective estrogen receptor modulators (SERMs) demonstrate tissue-specific pharmacology and function as an estrogen agonist in bone but as an antagonist in e.g. the breast and uterus. The SERM compound raloxifene has been shown to prevent bone loss and reduce vertebral fracture without stimulating the endometrium (Siris *et al.*, 2002). Bisphosphonates are analogs of pyrophosphate which are concentrated in bone and inhibit bone resorption by affecting osteoclasts via two distinct mechanisms depending on the compound. Non-nitrogen containing bisphosphonates (clodronate, etidronate) are metabolized within osteoclasts to form toxic ATP analogs that cause osteoclast apoptosis (Selander *et al.*, 1996). Nitrogen-containing compounds (alendronate, risedronate) impar osteoclast function by inhibiting farnesyl diphosphate synthase, an enzyme in the cholesterol biosynthesis pathway, thereby limiting the prenylation of small GTP-containing proteins (Watts, 2003). Bisphosphonates are currently considered a standard treatment for older women with osteoporosis (Eastell, 2003a). The calcitonin hormone, which binds to its receptor on osteoclasts and inhibits bone resorption, can also be used as an antiresorptive agent maintaining bone by a physiological mechanism (Silverman and Chesnut, 2003). It may be considered for those who fail to respond to bisphosphonate therapy or have acute fractures because it also has an analgesic effect (Eastell, 2003a). These antiresorptive treatments inhibit bone resorption and thus prevent the progression of bone loss. Excessive suppression of remodeling may, however, allow microdamage to accumulate and reduce some of the mechanical properties of bone (Mashiba *et al.*, 2000) and the long-term effects and safety of antiresorptive agents still requires further investigation. In addition, there is a need for an anabolic agent which would stimulate new bone formation. Promising results have been obtained with the parathyroid hormone (PTH) and a recombinant PTH fragment, teriparatide, which has been shown to act as anabolic agent when given intermittently and to reduce vertebral fractures (Neer *et al.*, 2001). Dietary supplementation with calcium and vitamin D is also able to reduce fracture rates (Chapuy *et al.*, 1992) and even more importantly, the effect of antiresorptive therapies and calcium/vitamin D supplementation appear to be additive (Nieves *et al.*, 1998) suggesting that an adequate intake of calcium and vitamin D should be considered an important component of any other therapy for osteoporosis. New additional therapeutics are likely to be developed and putative candidates include strontium ranelate (Reeve, 2003), inhibitors of the OPG/ RANKL/RANK system, cathepsin K or osteoclastic V-ATPase (Doggrell, 2003, Visentin *et al.*, 2000), lipid-lowering statins (Mundy *et al.*, 1999) or modulators of other recently identified pathways in bone cell control such as the one involving sclerostin (Warmington *et al.*, 2004).

Non-pharmacological intervention includes good nutrition with adequate calcium intake and improved physical activity, preferably exercise with high impact on bone (Cummings *et al.*, 1995, Delmas, 2002) as well as body weight maintenance (Reid, 2002). Furthermore, behaviours that promote bone loss such as smoking, high caffeine intake, or alcohol abuse should be avoided and factors promoting falls, such as poor visual function or excessive use of certain medications, should be managed (Cummings *et al.*, 1995). Reduction in the risk of falls and their impact should be considered especially in elderly individuals (Luukinen *et al.*, 1995, Kannus *et al.*, 2000, Delmas, 2002).

2.3.4 Bone mineral density in the evaluation of osteoporosis and fracture risk

2.3.4.1 Overview on techniques

Several non-invasive methods are available for the assessment of the skeleton for the diagnosis of osteoporosis and the evaluation of fracture risk (for review, see Blake and Fogelman, 2002, Kanis, 2002). *Dual energy X-ray absorptiometry (DX*A) has become the most

widely used method for measuring bone mineral density (BMD) (Kelly *et al.*, 1988). In BMD scanning the energy of X-ray beams passing through bones is absorbed and the non-absorbed energy is detected on the other side. The denser the bones are, the more energy is absorbed. Density is dependent on the bone mineral content which is the amount of mineral (calcium phosphate) in the specific site scanned. When divided by the scanned area it can be used to calculate a value for areal bone mineral density (g/cm^2) to reduce the variance among individuals. DXA uses two different X-ray energies which enables the measurement of densities of two different types of tissues, allowing an estimate for bone tissue absorption separately from that of soft tissues (Blake and Fogelman, 1997). A DXA measurement is usually performed in the spine (between vertebral bodies L2-L4) and hip (at several regions of interest) which are also common sites for fractures. The advantages of DXA include good precision, short scanning time and stable calibration (Blake and Fogelman, 1997). However, DXA gives information only in two dimensions rather than a true volumetric density. *Quantitative computed tomography (QCT)* provides true measure of three dimensional volumetric bone densities (g/cm^3). It is usually used for the measurement of trabecular bone in the vertebral bodies. Therefore, advantages of the method include high responsiveness of trabecular bone to aging and disease. Additionally, the technique avoids the effect of degenerative disease which gives artificially high values at the spine DXA. The cost of the equipment is perhaps the main drawback of the method. Because DXA and CT are available only at hospital-based facilities and are considered rather costly, smaller and low-cost devices for the scanning of peripheral skeleton have also been developed. *Peripheral DXA* uses the principles of conventional DXA but performs scans of the distal radius and calcaneus. Similarly, *peripheral QCT (pQCT)* are also available for measuring the forearm. The advantage of these methods is that they can be more easily utilized in minor health care facilities. However, the ability of peripheral measurements to predict spine and hip fractures is generally lower than for spine and hip BMD measurements (Marshall *et al.*, 1996). Also changes in forearm cortical bone are relatively small in response to treatment (Faulkner, 1998). A different approach for non-invasive assessment is *quantitative ultrasonometry (QUS)* which utilizes an ultrasound pulse instead of radiation and the heel as the measurement site. The ultrasound signal is attenuated as it is scattered and absorbed by the trabeculae in the heel and this attenuation is reduced in patients with osteoporosis because there are fewer trabeculae. Due to the low costs and portability of the equipment, QUS can be made more widely available than conventional methods (Gluer, 1997). However, there are no guidelines for QUS measurements yet and relatively poor precision makes it less suitable for monitoring response to treatment (Blake and Fogelman, 2002, Kanis, 2002). Probably the best means to evaluate the microarchitecture of bone tissue would still be the histologic evaluation of bone biopsy but such an invasive procedure can be recommended only for evaluating very unusual cases (Hammett-Stabler, 2004).

2.3.4.2 Measurement of bone mineral density

The BMD measurements are expressed in standard deviation units known as T-scores which indicate the difference between the ideal peak bone mass achieved by a young adult and the bone mass of the patient. The T-score is calculated by taking the difference between patient's BMD and the mean BMD of healthy young adults and the difference is expressed relative to the young adult population standard deviation (SD). In 1994, an expert panel of the World Health Organization (WHO) recommended that osteoporosis is defined using thresholds of BMD measured with DXA (Anonymous, 1994). According to the WHO guidelines, osteoporosis is designated at a bone density value at least 2.5 SDs below the mean value for young adults, i.e. the T-score is -2.5. The rationale for the definition is that it should define one third (1/3) of

Caucasian postmenopausal women into a category of osteoporosis as this approximates the lifetime risk of fracture for a 50-year old woman (Melton *et al.*, 1992). Severe or established osteoporosis uses the same threshold at -2.5 but in the presence of one or more fragility fracture (Table 1) (Kanis *et al.*, 1994, Kanis, 2002). Another way of expressing BMD is in Z-score units. Instead of comparing to the young adult mean, the Z-score is calculated by comparing to the mean BMD expected for a healthy normal subject matched for age, gender, and ethnic origin (Kanis, 2002).

Table 1. *The definition of osteoporosis according to WHO recommendations (Anonymous, 1994). T-scores refer to BMD measurements at the hip by DXA.*

Diagnosis	**Definition**
Normal	T-score ≥ -1.0
Osteopenia	-2.5 < T-score < -1.0
Osteoporosis	T-score ≤ -2.5
Osteoporosis (established)	T-score ≤ -2.5 and one or more fragility fractures

The spine and femur are often considered as the most important sites for DXA measurement because these are the sites of fractures that cause the greatest impairment on quality of life. The diagnostic use of T-scores in osteoporosis is reserved for the assessment of BMD at the hip using DXA and therefore, it could be considered the "golden standard" in terms of site (Kanis and Gluer, 2000). This does not mean that DXA at other sites is not useful but they may be used for risk assessment rather than for diagnosis. The spine can be considered an optimal measurement site because vertebral bodies are composed primarily of trabecular bone and thus, spine BMD has high sensitivity to changes during disease progression and therapy. The disadvantage of spine BMD is that the measurement is affected by degenerative changes in vertebra which occur with advancing age and lead to artificial elevation of BMD values (Kanis and Gluer, 2000). Generally, DXA scanning is done for both the hip and spine and thus both BMD values are usually available. Z-scores may be used in the long-term follow up of osteoporosis therapy.

The advantages of bone mass measurement include the non-invasiveness of the methodology and BMD as a true physical parameter for skeletal health. The major disadvantage is that BMD measurement is local and the assessment of BMD in a particular skeletal site only reflects events taking place on that site and thus, only in a fraction of the skeleton. Furthermore, BMD obtained as areal BMD provides information in only two dimensions and is not a true physiologic measurement. The measurement at one site is also a poor predictor of BMD at any other skeletal site as indicated by the correlation coefficients between BMDs (usually r values 0.6 - 0.7) (Blake and Fogelman, 2002). Diagnosis based on BMD also groups individuals into one homogenous group of osteoporotic patients on the basis of a single threshold value without giving information of the structural and biomechanical heterogeneity of bone fragility and the mechanisms responsible for it (Seeman, 2002).

2.3.4.3 Bone mineral density and the evaluation of fracture risk

There is general agreement that BMD is the most effective way of identifying patients at high risk for fracture. According to the Study of Osteoporotic Fractures, the risk of hip fracture, which is clinically the most important osteoporotic fracture, is best predicted by the measurement of BMD at the hip (Cummings *et al.*, 1993). In this study, women in the lowest

quartile of hip BMD had a ten fold higher risk for hip fracture than women in the highest quartile. There is a general agreement that hip BMD is the most reliable measurement for the prediction of hip fractures but for other fracture sites and all fractures together no differences between various BMD measurements have been established (Melton *et al.*, 1993, Marshall *et al.*, 1996, Blake and Fogelman, 2002). In the recently postmenopausal population the BMD measured at any site predicts any osteoporotic fractures with a risk of approximately 1.4-1.6 per SD decrease in BMD. The gradient is highest for the BMD measured at hip and its ability to predict hip fracture (2.6 per SD decrease in hip BMD) (Melton *et al.*, 1993, Marshall *et al.*, 1996). However, the absolute risk of fracture also depends on age and life expectancy as well as the relative risk at a given timepoint and it might be more informative to express the risk of fracture as a short-term absolute risk, such as a probability over a 10-year interval (Kanis, 2003).

The relationship between BMD and fracture risk is comparable with that between blood pressure and the risk of stroke and significantly better than that between serum concentration of cholesterol and risk of developing cardiovascular disease (Marshall *et al.*, 1996). As in the case for blood pressure, there in no threshold for BMD that discriminates absolutely between those who will or will not have a clinical event. It should be also noted that if BMD is in the normal range, there is no guarantee that a fracture will not occur but only that the risk is decreased. Osteoporotic fractures are multifactorial and in addition to low BMD depend on other issues such as accidents and the propensity to fall (Blake and Fogelman, 2002). Several risk factors such as age, sex, low body mass index, family history of hip fractures, and prior fragility fractures contribute to fracture probability independently of BMD and improvement of risk assessment can be achieved by using multiple risk factors and integrating them into risk assessment (Kanis, 2003). Variation in the bone remodeling rate has also become recognized as an important determinant of fracture risk (Recker *et al.*, 2004). Several fall-related factors may also contribute to the fracture risk, such as visual impairment, use of certain medication, and low physical activity (Cummings *et al.*, 1995). The National Osteoporosis Foundation in the United States currently recommends the evaluation of four predominant risk factors to be taken into consideration (Heinemann, 2000). These are likely to be involved in determining fracture risk independently of BMD and include maternal history of hip fracture, history of fragility fracture after 45 year of age, smoking, and low body weight.

2.4 Biochemical markers of bone turnover

Bone metabolism can be monitored by biochemical means using bone turnover markers. The analytes used as bone markers represent a variety of biochemical compounds. Markers may be excess products from bone formation or they can be degradation products produced during the breakdown of matrix components or they may reflect the enzymatic activity of the bone cells. They are also associated with different steps in bone formation or bone resorption. When bone metabolism is increased, both resorption and formation markers will increase and in this way they can be used in the assessment of bone turnover rate. For clinical purposes, markers are usually classified into those associated with bone formation and those associated with resorption according to the metabolic process they reflect (Fig.2) (for review, see Seibel, 2000, Seibel *et al.*, 2002, Hammett-Stabler, 2004).

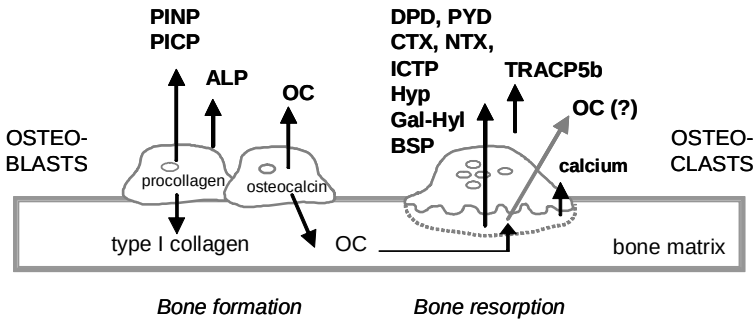

Figure 2*. The origin of biochemical markers of bone turnover.*

2.4.1 Bone formation markers

Propeptides of type I collagen. The C- and N-terminal ends of the procollagen I molecule are enzymatically cleaved during extracellular processing and fibril formation of collagen. The cleavage yields two relatively large extension peptides termed C-terminal propeptide (PICP) and N-terminal propeptide (PINP) produced in a stoichiometric ratio with collagen biosynthesis during bone formation. PICP is a globular protein with a molecular weight of ~100 kDa. It circulates as a single molecule and has a half-life of 6-8 min in serum, being cleared from circulation via mannose receptors in the liver. PINP is partly globular, partly helical 35-kDa protein which is eliminated from the circulation by scavenger receptors in the liver. Type I collagen is a component of several soft tissues and propeptides in the circulation may thus also arise from other sources than bone. However, other tissues are assumed to contribute very little to circulating propeptide levels because the rate of collagen turnover in nonskeletal tissues is significantly slower than in bone (Seibel *et al.*, 2002). Immunoassays have been developed to measure PICP and PINP in the blood and both markers are related to bone formation (Taubman *et al.*, 1974, Melkko *et al.*, 1990, Melkko *et al.*, 1996).

Alkaline phosphatase. Alkaline phosphatase (ALP) is a membrane-bound enzyme located on the outer cell surface. There are three tissue-specific genes (intestine, placenta, germ cell) and a tissue non-specific gene which is expressed in numerous tissues, including bone and liver (Henthorn, 1996). In bone, ALP is expressed on the surface of bone-forming osteoblasts. The enzyme may be cleaved off the membrane and released into the circulation and the enzymatic activity can thus be determined from serum samples (Seibel *et al.*, 2002). Total serum ALP activity is perhaps the most widely used marker of bone metabolism due to the wide availability of inexpensive methods for its detection (van Straalen *et al.*, 1991). It is cleared from blood slowly with a half-life of several days and the within day variation is estimated to be low. However, the total ALP pool in serum consists of several isoforms which originate from various tissues. In adults, approximately half of the total ALP is derived from the liver and the other half is derived from bone (Green *et al.*, 1971). Although bone and liver ALP share the same primary amino acid sequence, different isoforms exist due to post-translational modifications of the enzyme and both sialylation and glycosylation provide some uniqueness which allows distinguishing between bone and liver isoforms (Henthorn, 1996). The measurement of bone-specific ALP isoenzyme is currently preferred because of the higher specificity over total ALP (Woitge *et al.*, 1996). There are commercial kits available, which preferentially detect the bone isoform with the help of monoclonal antibodies (Garnero and Delmas, 1993, Gomez *et al.*,

1995). They are clear improvements over the total ALP technique but still exhibit some cross-reactivity with other alkaline phosphatases (Seibel, 2000).

Osteocalcin. Osteocalcin is a small bone-specific bone matrix protein produced primarily by osteoblastic cells during the late phases of bone formation (Hauschka *et al.*, 1989). The structure of osteocalcin is characterized by three Gla-residues which provide the protein with a high affinity to bone hydroxyapatite (Hoang *et al.*, 2003). Although osteocalcin is primarily deposited into the developing bone matrix, a small amount of it enters the circulation where it can be detected by immunoassays (Price *et al.*, 1980, Brown *et al.*, 1984). It has been also suggested that osteocalcin fragments may be released during bone resorption and contribute to the immunoreactive osteocalcin detected in serum and thus, it is unclear whether osteocalcin should be considered a marker of osteoblast activity or an indicator of bone matrix metabolism or turnover (Gundberg, 1998). Osteocalcin is rapidly degraded in serum and in addition to the intact molecule, osteocalcin fragments of various sizes exist in the circulation (Garnero *et al.*, 1994a). There are numerous commercial kits available for the measurement of human osteocalcin and levels of circulating osteocalcin have been used for assessing relative degrees of bone turnover in many clinical studies (Lee *et al.*, 2000). However, considerable inconsistency has been observed between different assays (Delmas *et al.*, 1990, Masters *et al.*, 1994). This may be related to the heterogeneity of osteocalcin in the circulation and the variability results from the differences in the ability of various assays to recognize these fragments. There is also no clear consensus regarding which osteocalcin peptide form provides the most optimal information (Gundberg, 1998). Osteocalcin and its measurement will be discussed in detail in the following chapters (*sections 2.5 – 2.9*).

2.4.2 Bone resorption markers

Pyridinoline and deoxypyridinoline. The structure of collagen molecules is stabilized by intra- and intermolecular covalent cross-linking located at both ends of the helical domain of collagen fibril. The main cross-link types are pyridinoline (PYD) and deoxypyridinoline (DPD). which are released into circulation during bone resorption, and subsequently excreted into the urine. PYD originates from cross-links between hydroxylysine residues whereas cross-linking between lysine residues is the source of DPD. Although type I collagen is a component of several other connective tissues, the amounts of PYD and DPD in serum and urine are mainly derived from bone which has a higher turnover rate compared to other collagen-containing tissues. Since the cross-links are formed only to mature collagen, PYD and DPD levels in serum and urine are not affected by degradation of newly synthesized collagen (Robins and Brady, 2002). Cross-links, either free or peptide-bound, were originally measured by HPLC technique. However, immunoassays have later been developed for DPD exclusively (Robins *et al.*, 1994) and for both pyridinium cross-links (Gomez *et al.*, 1996) and assays are also commercially available. In general, PYD is a more predominant cross-link in type I collagen and found in larger quantities but DPD is considered more bone-specific as it is present predominantly in bone-derived type I collagen (Eastell *et al.*, 1997).

Cross-linked collagen telopeptides NTX, CTX and ICTP. Instead of using the cross-links themselves as markers, the peptides derived from the cross-link regions of collagen can be measured. Immunoassays have been developed for both telopeptide regions of type I collagen known as the N-terminal telopeptide (NTX) (Hanson *et al.*, 1992) and C-terminal telopeptide (CTX) (Bonde *et al.*, 1994). Originally, telopeptides were measured from urine but recently assays have also been developed for serum samples (Clemens *et al.*, 1997). Several immunoassays are also commercially available for both urinary and serum NTX and CTX and a

point-of-care device has also been developed for urinary NTX (Seibel, 2000). The NTX assay recognizes the QYDGKGVG octapeptide sequence of cross-linked N-terminal telopeptide with no crossreactivity to free cross-links. The epitope for the CTX assay is an eight-amino-acid peptide EKAHDGGR located at the C-terminal segment of collagen chain and produced by the action of osteoclastic enzyme cathepsin K during bone resorption (Garnero *et al.*, 2003). Asp (D) residue in CTX can undergo isomerization (α/β) and racemization (L/D) giving rise to four forms of CTX detected in serum: αL, αD, βL, and βD (Cloos and Fledelius, 2000). The isomerization of Asp-residues occurs over extended periods of time and is associated with the aging of proteins. There are assays available for both urinary α-CTX (Bonde *et al.*, 1996) and β-CTX (Bonde *et al.*, 1994) as well as for serum β-CTX (Bonde *et al.*, 1997). The ratio of different forms changes as a function of the biological age of the protein so that the proportion of β and D forms increases with age in tissues (Cloos and Fledelius, 2000). Because each collagen molecule contains two α1(I) chain telopeptides, CTX can be found in α-α, α-β, and β-β forms. Currently, an assay recognizing only β-form and thus assaying the degradation of relatively old and mature bone is preferred and such an assay is available for both urine and serum (Christgau *et al.*, 1998, Rosenquist *et al.*, 1998). In addition to the short CTX fragment, a longer cross-linked C-terminal telopeptide segment can be measured (Risteli *et al.*, 1993). This immunoassay (known as ICTP or CTX-MMP) detects more extended fragments than CTX assay which are produced by degradation of collagen by matrix metalloproteinases instead of cathepsin K (Garnero *et al.*, 2003). The antigen detected by this assay is perhaps not applicable to evaluate physiologic resorption but may provide useful information when assessing pathological increases in bone degradation, such as those occurring in myeloma or metastatic bone diseases and in rheumatoid arthritis (Risteli *et al.*, 1993).

Tartrate-resistant acid phosphatase. Tartrate-resistant acid phosphatase (TRACP) belongs to the family of ubiquitously occurring acid phosphatases. Six isoenzymes are identified in human tissues out of which isoenzyme 5 is expressed in osteoclasts and macrophages (Cheung *et al.*, 1995). Furthermore, two isoforms for TRACP 5 have been identified which appear to be structurally similar, but TRACP5a contains sialic acid not found in TRACP5b (Lam *et al.*, 1981). Also, their pH optimum is different (Lam *et al.*, 1978). According to current knowledge, TRACP5a is derived from activated macrophages whereas TRACP5b is characteristic for osteoclasts and secreted during bone resorption (Janckila *et al.*, 2002). Total TRACP amount or activity in serum is associated with the bone resorption rate but lacks sufficient specificity to be used as a bone turnover marker (Cheung *et al.*, 1995, Halleen *et al.*, 1999a). Assays detecting active TRACP5b exclusively have been developed and the detection of the active form of TRACP5b in serum appears to be a useful method to assess osteoclast activity (Halleen *et al.*, 2000).

Other resorption markers. Additional indices of bone resorption include urinary hydroxyproline (Hyp), hydroxylysine glycosides (Gal-Hyl), and bone sialoprotein (BSP). Hydroxyproline constitutes 12-14% of the total amino acid content of type I collagen and is released as a result of collagen degradation during bone resorption. The majority of it is reabsorbed but about 10% is excreted into urine as free or peptide-bound forms. Hyp in urine can been measured using HPLC or colorimetric methods as an index of bone resorption (Deacon *et al.*, 1987). However, significant amounts of hydroxyproline in urine are derived from the diet and from the degradation of newly synthesized collagen and from collagens derived from tissues other than bone. Urinary hydroxyproline is therefore considered a non-specific index of collagen turnover and nowadays it has been largely replaced by more specific measures of bone resorption (Seibel, 2000). Other collagen degradation products are galatosyl

hydroxylysine and glycosyl-galactosyl-hydroxylysine residues (Moro *et al.*, 1984). They are formed as a result of post-translational modification of collagen, released into the circulation during collagen degradation, and considered to be relatively specific to bone collagen. Although they have potential as markers of bone resorption, they are not widely used due to lack of availability of simple assays (Moro *et al.*, 1988). An additional putative resorption marker is bone sialoprotein which accounts for a large proportion of non-collagenous bone matrix proteins. A small amount of bone sialoprotein is found in the circulation and, therefore, it may be a potential marker of bone turnover. It is assumed that serum bone sialoprotein mainly reflects resorption but the data are still limited concerning its use in clinical settings (Seibel *et al.*, 1996). The best use of bone sialoprotein may yet be in the evaluation of cancer patients as it is strongly upregulated by many tumors (Withold *et al.*, 1997). Uurinary calcium normalized to creatinine has earlier been used as a simple and inexpensive marker to assess skeletal loss but this test clearly lacks the diagnostic sensitivity and specificity needed to detect subtle changes in bone turnover (Hammett-Stabler, 2004).

The available markers provide information on the turnover rate in general but they fail to provide qualitative information. However, in addition to the physical bone mass (BMD) and the metabolic bone turnover rate (markers), bone fragility may also depend on the architecture and material properties of bone ("bone quality"). Currently, this cannot be readily measured but there is an increasing interest in finding markers which would also reflect the qualitative – not only quantitative - aspect of bone health. Putative candidates for such markers include e.g. the ratio of native and age-related forms of CTX (Garnero *et al.*, 2002, Garnero *et al.*, 2004b). The relative proportion of α and β isomer of CTX has been shown to contribute to mechanical properties of bone samples *in vitro* (Garnero *et al.*, 2004a). A relative increase in β-forms of CTX either measured in urine (Garnero *et al.*, 2002) or circulation (Garnero *et al.*, 2004b) has been shown to predict fractures independently of BMD and partly independently of bone turnover rate in elderly women. Another candidate is undercarboxylated osteocalcin (Szulc *et al.*, 1993). Increased proportion of undercarboxylated osteocalcin forms in the circulation appears to be related to an increased risk of fracture (Vergnaud *et al.*, 1997, Luukinen *et al.*, 2000) and the amount of carboxylated osteocalcin correlates significantly to bone quality determined by ultrasound (Sugiyama and Kawai, 2001). This suggests that the increased amount of undercarboxylated osteocalcin which is not able to bind to bone mineral might reduce the qualitative measures of bone. These are, however, preliminary observations and the putative biochemical indices of bone quality are not yet to be used on a routine basis.

2.4.3 Applications and limitations

Currently, the diagnostic tool in osteoporosis is the quantification of BMD by dual-energy x-ray absorptiometry. BMD allows diagnosis of osteoporosis according to WHO guidelines, the values change slowly providing a static measurement of skeletal status, and the measurement has low within-person variability but on the other hand, provide information only from a fraction of the skeleton (Looker *et al.*, 2000). Another approach to evaluate skeletal status involves the measurement of biochemical markers in blood or urine which reflect the total body skeletal metabolism. None of the current biochemical markers are used to diagnose osteoporosis since the variation is too great for diagnostic use in an individual patient. However, bone markers provide more dynamic and rapid measures of skeletal status and may be useful tools in monitoring osteoporosis therapy and in assessing the rate at which bone loss is occurring (Delmas *et al.*, 2000). Furthermore, there is increasing evidence that at least some markers could be applied for the determination of fracture risk or be useful in assessing disease severity

(Garnero, 2000). Therefore, these two approaches to assess skeleton may answer to different questions and supplement each other.

The efficacy of osteoporosis therapy is often monitored by the measurement of BMD. However, one or two years of successful treatment may be necessary before significant changes are observed in BMD using currently available techniques, making the BMD measurement less optimal for monitoring therapy (Cummings *et al.*, 2002). An alternative is to use bone markers to monitor changes in bone metabolism in response to treatment to assess its efficacy. The effects of drugs on bone markers are rapid with decrease seen generally within 1-3 months for the resorption markers and within 3-6 months for the formation markers (Delmas, 2000). Thus, bone markers may be helpful in monitoring especially within the first six months after initiation of the therapy when BMD changes are too small to be used clinically (Looker *et al.*, 2000). A potential intriguing clinical application of biochemical markers is in assessing fracture risk (Garnero, 2000, Garnero and Delmas, 2002). Identification of women at highest risk for developing osteoporotic fractures could be of great clinical importance in targeting women for preventative treatment. An increased bone remodeling rate increases skeletal fragility in women (Recker *et al.*, 2004) and variation in remodeling rates has become recognized as a powerful determinant of fracture risk (Heaney, 2003). The present experimental evidence indicates that increased levels of bone turnover markers, especially those reflecting bone resorption, are associated with increased rates of vertebral and hip fractures (Garnero *et al.*, 1996b). Prospective studies relating the bone formation markers for the prediction of fracture are more conflicting (Garnero, 2000). Furthermore, a combined approach using both bone turnover markers and BMD may improve fracture prediction in postmenopausal women and therefore, a potential use of the markers could be to enhance the value of BMD and other risk factors in fracture risk assessment (Johnell *et al.*, 2002). However, although some markers are independently associated with fractures in large epidemiologic studies it does not necessarily make them useful for individual patients. The major applications of bone turnover markers - monitoring of antiresorptive therapy, bone loss, and the prediction of fracture risk - will be discussed in more detail in *section 2.9.2.* The measurement of a marker may also be useful in other applications related to bone biology such as the management of patients with multiple myeloma, skeletal metastasis of breast and prostate carcinoma or other malignant bone disease (Demers *et al.*, 2000) but these will not be discussed in this review.

The disadvantages of markers, in addition to not being tools for diagnosis, include high level of within-person variability. Because marker levels are dynamic measures of bone metabolism in contrast to static measurement of prevailing bone density, markers are more easily affected by external factors. In many metabolic bone diseases, including osteoporosis, only subtle changes in bone marker levels are observed. However, the values can considerably vary in an individual over a short period of time only due to the normal variations in bone metabolism and excretion (Blumsohn and Eastell, 1997). Important controllable contributors to the variability in marker levels include circadian, seasonal, and menstrual fluctuations (Hannon and Eastell, 2000). Circadian variability has probably more impact on variation than any other factors. Almost all markers exhibit significant circadian patterns with highest concentrations during the night and early morning and falling during the day reaching lowest point in the afternoon and thus the precise timing of sample collection should be emphasized (Blumsohn *et al.*, 1994). Intra-individual variation within a day is particularly high for urinary resorption markers (Jensen *et al.*, 1997, Hannon and Eastell, 2000) which could be associated to the use of creatinine to normalize urinary marker values contributing a second source of variability to the result (Sebastian-Gambaro *et al.*, 1997). The variability is generally smaller for the resorption

markers measured from serum samples compared to urine samples and similar to the variation of the serum formation markers (Eastell *et al.*, 2000). The uncontrollable sources of preanalytical variability include age and gender, ethnicity, menopausal status, immobilization, concomitant disease (e.g. liver disease), use of certain drugs (e.g. corticosteroids), or the occurrence of a recent fracture (Hannon and Eastell, 2000). Fracture healing has a significant effect on levels of bone turnover markers which increase rapidly (Akesson *et al.*, 2005). Also bed rest increases bone resorption and the levels of biochemical markers are increased (Inoue *et al.*, 2000). Feeding has been demonstrated to suppress the levels of some bone turnover markers and sample collection in the fasting state is therefore likely to be preferable (Clowes *et al.*, 2002, Hannon *et al.*, 2004). The uncontrollable variability can be managed by using appropriate reference ranges and by studying the effects of different variables. The controllable sources can be minimized by standardizing the sampling time and sample handling (Seibel, 2000). The variability can also be reduced by measuring duplicate or even multiple samples or by performing a single measurement of pooled samples (Hannon *et al.*, 1998). Several markers are sensitive to thermodegradation, hemolysis and repeated freeze-thaw cycles. Therefore, in general, storage in several aliquots in a temperature of at least -70°C is recommended (Seibel, 2000). The preanalytical variability undoubtedly contributes to the conflicts found in the literature concerning the usefulness of markers and the variability will be even greater in clinical practice. It is, however, noteworthy that other commonly used markers such as serum cholesterol also demonstrate considerable within-person variability but it is still widely used clinically to assess risk for ischemic disease (Thompson and Pocock, 1990).

Although markers of bone formation are distinguished form indices of bone resorption for clinical purposes it should be recognized that bone formation and resorption are closely coupled and not independent of each other. Increases in either process will increase marker values and the interpretation of elevated marker values depends on which process predominates. It should also be borne in mind that some of these compounds may reflect at least to a certain extent both bone formation and bone resorption. Markers also originate from different steps in bone formation or resorption and different bone formation/resorption markers are thus not equivalent to each other and do not always have identical outcomes, although the overall trend may be similar. Changes in markers are usually not disease-specific but reflect changes in the skeletal metabolism independently of the cause which alters it. Furthermore, since most of the markers are also present in tissues other than bone they may be influenced by nonskeletal processes as well.

In summary, biochemical markers are not substitutes for the measurement of BMD, and at present, the diagnosis of osteoporosis cannot be based solely on the levels of biochemical markers. The analytic and biologic variability of bone markers can be significant and need to be considered when they are used (Seibel *et al.*, 2001) Despite the limitations, bone turnover markers are valuable tools in research applications of metabolic bone disease by providing dynamic information which is not obtained by a single measurement of BMD. Markers also have potential to be used in individual patients but recommendations for their use in clinical practice have not yet been elucidated. The ability to predict fractures and rapid kinetics in response to anti-resorptive therapy are perhaps the most appealing applications for markers and in these conditions they could add value to the assessment of BMD (Looker *et al.*, 2000). The recent guidelines recognize three major applications of bone marker testing which include 1) identification of individuals with increased bone turnover, 2) prediction of fracture risk in postmenopausal women and 3) the assessment of response to treatment of patients with osteoporosis or risk for it (NCCLS, 2004).

2.5 Osteocalcin – a bone-specific protein

2.5.1 Identification of osteocalcin as bone Gla protein

Amino acid γ-carboxyglutamate (Gla) was identified in bovine prothrombin as a post-translational modification of glutamate residues (Nelsestuen *et al.*, 1974, Stenflo *et al.*, 1974, Esmon *et al.*, 1975). The Gla residues were found to be necessary for the binding of prothrombin to insoluble salts such as calcium phosphate and to phospholipid vesicles in the presence of Ca^{2+} (Gitel *et al.*, 1973, Nelsestuen *et al.*, 1975). Subsequently, similar Gla residues were also searched for in other proteins where Gla might similarly facilitate association with membranes or crystal surfaces and calcified tissues represented an appealing system in which a Gla-containing protein might be found. In the middle of the 1970s, two research groups independently reported that bone contains a previously unknown protein rich in γ-carboxyglutamate. In their original publication, Hauschka and co-workers used direct isolation approach to demonstrate the presence of Gla from alkaline hydrolysates in protein fractions solubilized from chicken bone (Hauschka *et al.*, 1975). Since bone collagen was devoid of Gla, they suggested a role for a novel Gla-rich protein in the biology of mineralized tissues. An independent discovery of a similar Gla-containing protein in bovine bone extracts was published by Price and co-workers who reported the amino acid composition and sequence of the first 15 residues of this novel protein (Price *et al.*, 1976a). The complete primary structure of bovine "γ-carboxyglutamic acid-containing protein from bone" was published by the same authors later that year (Price *et al.*, 1976b). The protein was later abbreviated as bone Gla protein (BGP, BGLAP), or osteocalcin (OC). The sequence of purified human bone Gla protein was reported a few years later (Poser *et al.*, 1980). Human osteocalcin was found to exhibit a significant homology to its bovine counterpart with only four translational substitutions.

2.5.2 Genomic organization

The gene encoding human osteocalcin (*BGLAP*) was isolated using mouse and rat osteocalcin cDNA clones and found to consist of four exons of 64, 33, 70, and 130 bp, respectively, and three intronic sequences, each of them approximately 200 - 300 bp in length (Celeste *et al.*, 1986). Human osteocalcin is a single copy gene located at the distal arm of chromosome 1 (1q25-q31), telomeric to the α-spectrin gene (Puchacz *et al.*, 1989) (OMIM Mendelian Inheritance in Man database #112260, www.ncbi.nlm.nih.gov/entrez). Rat osteocalcin gene shares 77% sequence identity with human gene and spans approximately 1 kb of DNA (Yoon *et al.*, 1988, Lian *et al.*, 1989, Theofan *et al.*, 1989). In contrast to a single copy present in humans and rats, there are multiple copies of osteocalcin in the mouse genome (Rahman *et al.*, 1993, Desbois *et al.*, 1994). Mouse osteocalcin gene cluster contains three genes generated most likely by multiplication during evolution. Genes OG1 and OG2 are located physically close to each other and encode osteocalcin homologous to other mammalian species and with identical sequences, the only differences being in the promoter region (Desbois *et al.*, 1994). Third gene (ORG) encodes an osteocalcin-related protein expressed in the kidneys but not in bone and this gene product is also known as nephrocalcin. Despite variations in the organization of the osteocalcin loci, the two mouse osteocalcin genes exhibit the same modular organization as described in rat and human genes (Rahman *et al.*, 1993).

The structure of the human osteocalcin gene is conserved. At least to date, there are no clinical disorders or symptoms associated with mutations of the protein and no allelic variants reported for the coding region of the human gene. A one base pair substitution (C→T) has been described at the promoter region of human osteocalcin, at a position 198 nucleotides upstream

from exon 1 (Dohi *et al.*, 1998). This polymorphism can be defined with restriction fragment length polymorphism using restriction endonuclease HindIII and it has been proposed as a susceptibility locus for reduced BMD in postmenopausal women. The original study performed on 160 postmenopausal Japanese women suggested that subjects homozygous to the absence of HindIII site (HH) had the smallest BMDs and this genotype showed a significant effect for the prevalence of osteopenia (Dohi *et al*., 1998). Another study of more than 1000 Japanese women supported the osteocalcin gene and HindIII polymorphism as a quantitative trait locus underlying BMD variation (Yamada *et al.*, 2003) and the association of the polymorphism and hip BMD was reproduced in 630 subjects (Deng *et al*., 2002). However, no significant differences in spine or hip BMD across genotypes was found in a recent study on Chinese women (Mo *et al.*, 2004) and further investigations are needed to elucidate the role of this polymorphism. The genetic map has been refined by an identification of a novel microsatellite marker (D1S3737) tightly linked to osteocalcin gene. This microsatellite was used as a marker of genetic variation at the osteocalcin locus and one of the alleles was shown to be associated with the level of BMD in the postmenopausal age (Raymond *et al*., 1999).

2.5.3 Expression restricted to mature bone

Osteocalcin is not only present in bone tissue (Price *et al*., 1976b) but also synthesized in bone cell cultures (Nishimoto and Price, 1979) and the secreted protein is identical with the one isolated from bone (Nishimoto and Price, 1980). The expression of osteocalcin appears to be unique to bone tissue and predominantly limited to the cells of osteoblastic lineage. It is strongly expressed by mature osteoblasts (Ikeda *et al*., 1992, Kesterson *et al*., 1993) but also by osteocytes (Aarden *et al*., 1996, Mason *et al*., 1996) as well as hypertophic chondrocytes (Lian *et al.*, 1993), cementoblasts (Bronckers *et al.*, 1994), and odontoblasts (Gorter de Vries *et al.*, 1987, Papagerakis *et al.*, 2002). The intracellular distribution of osteocalcin in osteoblasts and odontoblasts resembles that of a secreted protein, being in the rough endoplasmic reticulum and Golgi apparatus (Camarda *et al*., 1987). There is also some evidence for low levels of osteocalcin expression in nonosteoid tissues (Fleet and Hock, 1994), such as brain (Kesterson *et al*., 1993), bone marrow megakaryocytes and peripheral blood platelets (Thiede *et al*., 1994), bone marrow stromal adipocytes (Benayahu *et al.*, 1997), and a subpopulation of vascular smooth muscle cells isolated from aortic media (Watson *et al*., 1994). The expression levels in bone are, however, several orders of magnitude higher (Fleet and Hock, 1994). Although osteocalcin messenger RNA has been detected in other tissues than bone, it appears to be processed properly to yield a functional protein only in the bone microenvironment suggesting the existence of putative bone-specific splicing factors (Jung *et al*., 2001). The two mouse osteocalcin genes OG1 and OG2 appear to be expressed at a different level. OG1 is the major (80%) transcript in mouse bone tissue *in vivo* whereas OG1 and OG2 are expressed at equal levels in osteoblastic cells *in vitro* (Yanai *et al.*, 2001).

The expression of osteocalcin appears to be stringently regulated in a developmental-stage-specific manner during differentiation of the osteoblast phenotype. Osteocalcin is not expressed during early stages of osteoblastic differentiation but it is prominently a marker of late, mature osteoblasts (Bellows *et al*., 1999). During the differentiation of fetal calvarial osteoblasts *in vitro,* osteocalcin expression increases up to 200-fold in cells producing a mineralizing matrix when compared to confluent osteoblasts in a non-mineralizing matrix (Lian *et al*., 1989). Osteocalcin appears in calcifying tissues approximately two weeks after mineral deposition, at the same time than the maturation of bone mineral to hydroxyapatite is thought to occur (Price *et al.*, 1981a) and is particularly present in mineralized bone matrix but not observed in

nonmineralized, newly formed osteoid (McKee *et al.*, 1992). Furthermore, the expression of osteocalcin is induced *in vitro* at the onset of mineralization, clearly after the expression of other osteoblastic markers such as alkaline phosphatase and type I collagen (Owen *et al.*, 1990a). Thus, the expression of osteocalcin is considered a specific feature of the late phases of bone formation.

At tissue level, staining for osteocalcin is predominantly seen in the bone matrix and to some extent in osteoblasts and osteocytes (Vermeulen *et al.*, 1989, Ohta *et al.*, 1991, Kasai *et al.*, 1994). Osteocalcin is associated especially with mineralized regions of extracellular matrices of bone and cartilage (Groot *et al.*, 1986). In normal lamellar human bone, osteocalcin is detected along the lamellar bone matrix in fine granular deposits, and under pathological conditions the provisory woven bone is devoid of osteocalcin (Vermeulen *et al.*, 1989). Intense, but regionally variable, staining for osteocalcin can be seen in mineralized bone matrix and in small mineralization loci dispersed throughout the osteoid while unmineralized osteoid shows weak to moderate staining indicating that osteocalcin synthesis is low (McKee *et al.*, 1992). Osteocalcin expression is concentrated at the mineralization front i.e. just ahead of the mineralized matrix both *in vitro* (Nefussi *et al.*, 1997) and *in vivo* (Kasai *et al.*, 1994). Pattern of osteocalcin distribution within osteons changes with age and gender (Ingram *et al.*, 1994) and weaker expression of osteocalcin is seen in newborn bone compared to the adult (Ikeda *et al.*, 1992). In humans, cortical bone contains approximately 30 times more osteocalcin than trabecular bone, probably reflecting the differences in age and maturity of these two bone types (Ninomiya *et al.*, 1990). Osteocalcin is also detected in dental tissues (Camarda *et al.*, 1987, Papagerakis *et al.*, 2002) and in the hypertrophic zone of growth plate cartilage throughout the calcified cartilage matrix (McKee *et al.*, 1992) but not in the primary spongiosa near the growth plate (Ikeda *et al.*, 1992). Increased expression of osteocalcin is seen with the onset of development of the hypertrophic phenotype also *in vitro* (Lian *et al.*, 1993). Interestingly, staining for osteocalcin is also seen at the cell borders of osteoclasts and the bone margins of resorption cavities, probably indicating osteocalcin released from bone matrix during resorption (Vermeulen *et al.*, 1989). In pathological circumstances, osteocalcin has been found in sites of ectopic calcification of cardiovascular tissues (Lian *et al.*, 1976, Levy *et al.*, 1983).

2.5.4 Regulation of expression

Various promoter elements lying less than a kilobase 5' to the transcription initiation site contribute to basal expression and osteoblast specificity (Kerner *et al.*, 1989, Lian *et al.*, 1996). Due to osteoblast-specific expression, the osteocalcin promoter has been used as a model in the elucidation of the transcriptional control of osteoblast differentiation and function (Stein *et al.*, 1997, Schinke and Karsenty, 2002). The osteocalcin promoter has also served as a tool to direct the expression of various peptides, such as growth hormone (Baker *et al.*, 1992) and TGFβ (Erlebacher and Derynck, 1996) to the bone microenvironment in transgenic mouse models. Recently, it has also been used to generate conditional gene ablation exclusively in the cells of osteoblast lineage by targeting the expression of Cre recombinase enzyme into osteoblasts via osteocalcin promoter (Dacquin *et al.*, 2002, Castro *et al.*, 2003)

Osteoblast-specific and developmental expression: Runx2 and other transcription factors

Two osteoblast-specific cis-acting elements, OSE1 and OSE2 are located between -74 and -47 and between -146 and -132 of the mouse osteocalcin promoter, respectively (Ducy and Karsenty, 1995). OSE2 element (also known as OC box II) (Lian *et al.*, 1996) is a binding site for protein produced by the Runx2 gene, the expression of which is strictly restricted to the cells

of the osteoblast lineage (Ducy *et al.*, 1997). Runx2, also known as Cbfa1, belongs to the Runt family of transcription factors (Ogawa *et al.*, 1993). It controls the lineage commitment for osteogenesis and has an indispensable role in osteoblast differentiation, maturation, and bone formation (Komori *et al.*, 1997). In addition to osteocalcin promoter, functional OSE2-like elements have been identified in the promoter regions of genes that are expressed at relatively high levels in osteoblasts such as type I collagen, osteopontin and bone sialoprotein (Ducy *et al.*, 1997). Runx2 is also a regulator of osteoblast function, not merely differentiation (Ducy *et al.*, 1999). The presence of three Runx2 motifs in rat osteocalcin promoter, all of them required for maximal basal expression, suggests a multifunctional role for Runx2 factors not only as simple transcriptional transactivators but also as factors facilitating modifications in promoter architecture (Javed *et al.*, 1999). It is, however, noteworthy that osteoblast-specific transcription of osteocalcin occurs even in the absence of Runx sites (Hoffmann *et al.*, 1996) and on the other hand, Runx proteins are present in immature proliferating osteoblasts in which the osteocalcin gene is not activated (Banerjee *et al.*, 1997) suggesting a role for other regulatory factors in the regulation of osteocalcin expression. The other cis-acting element in mouse promoter, OSE1, is regulated by a osteoblast-specific factor Osf1 (Schinke and Karsenty, 1999). Osf1 is hypothesized to act downstream of Runx2 and be required solely for osteocalcin expression, since Osf1 binding site is not present in the promoter of other extracellular matrix encoding genes. In contrast to OSE2 which controls osteocalcin expression in both human and mouse, OSE1 is not identical in the human and mouse osteocalcin promoter. Human OSE1 site does not bind Osf1 but confers a weak binding site for Sp1 class of universal transcription factors (Yeung *et al.*, 2002). The serum levels of osteocalcin are significantly lower in humans than in mice (Hauschka *et al.*, 1989) and this could be explained by the absence of an additional regulatory factor such as Osf1 in the regulation of human osteocalcin. Furthermore, a novel bone-specific zinc finger-containing transcription factor Osterix was recently identified (Nakashima *et al.*, 2002). Osterix is required for the differentiation of preosteoblasts to functional osteoblasts but it is not needed in the early stages of commitment to osteblastic lineage. The expression of osteocalcin is absent in Osterix null embryos (Nakashima *et al.*, 2002) and the expression of osteocalcin appears to be synchronous with that of Osterix mRNA (Igarashi *et al.*, 2004) indicating that Osterix may be involved in the regulation of osteocalcin expression.

The binding sites for Msx1 and Msx2, two mammalian homologues of the *Drosophila* muscle segment homeobox gene, are located in mouse and rat osteocalcin promoters (Hoffmann *et al.*, 1994, Towler *et al.*, 1994). Msx2 acts as a transcriptional repressor of osteocalcin gene expression *in vitro* (Towler *et al.*, 1994) and the expression of osteocalcin is strongly reduced in Msx2-deficient mice (Satokata *et al.*, 2000). Msx2 is an important regulator of craniofacial bone development and it maintains the osteoblast in the proliferative stage through inhibition of their terminal differentiation (and osteocalcin expression) also during endochondral bone formation (Satokata *et al.*, 2000). Another homeodomain transcription factor, Dlx5, a homologue of distal-less in *Drosophila*, induces osteocalcin expression six-fold in a calvaria-derived cell line (Miyama *et al.*, 1999) whereas it leads to decreased osteocalcin promoter activity in the long bone-derived osteosarcoma cell line (Ryoo *et al.*, 1997). Dlx5 appears to regulate osteocalcin promoter in calvarial osteoblasts at least in part by blocking the Msx2-mediated repression of basal promoter activity (Newberry *et al.*, 1998). The sequence-specific interactions of both homeodomain transcription factors occur at a homeodomain consensus binding site overlapping a transcriptional element known as OC box I (Hoffmann *et al.*, 1996, Ryoo *et al.*, 1997).

The effects of vitamin D and glucocorticoids

The rate of osteocalcin protein synthesis in human and rat bone cells *in vitro* is dramatically upregulated by 1,25-dihydroxyvitamin D_3 (vitamin D) (Price and Baukol, 1980, Beresford *et al.*, 1984, Lian *et al.*, 1985). Furthermore, rachitic animals with vitamin D deficiency demonstrate osteomalacia with wide osteoids and up to 50% reduction in bone osteocalcin content (Lian *et al.*, 1987). The stimulation of gene transcription by vitamin D depends on binding of vitamin D receptor and retinoid X receptor heterodimers (VDR/RXR) or VDR homodimers to specific vitamin D-responsive elements (VDREs) within the human and rat osteocalcin promoters (MacDonald *et al.*, 1993). The functional VDRE in human osteocalcin promoter is a 21 base pair element at position -509/-489 (Kerner *et al.*, 1989) and supports a 10-15 -fold stimulated transcription by vitamin D (Yoon *et al.*, 1988, Lian *et al.*, 1989, Mahonen *et al.*, 1990). Interestingly, although osteogenic signals that regulate mouse and human osteocalcin are usually common, the mouse gene is resistant to induction by vitamin D (Clemens *et al.*, 1997, Sims *et al.*, 1997, Thomas *et al.*, 2000). Furthermore, osteocalcin mRNA is not regulated by vitamin D in nonosteoid tissues which have low basal expression of osteocalcin (Fleet and Hock, 1994). The treatment of rat osteosarcoma cells with vitamin D prolongs the half-life of osteocalcin messenger RNA up to four-fold suggesting that the stabilization of RNA may also participate in the upregulation of osteocalcin by vitamin D (Mosavin and Mellon, 1996)

VDRE is contiguous to a site for activator protein 1 (AP-1) (Schule *et al.*, 1990, Goldberg *et al.*, 1996) and the vitamin D -induced promoter activity is dramatically reduced in the absence of an intact AP-1 site, suggesting a functional synergism between VDR and AP-1-related proteins (Ozono *et al.*, 1990, Aslam *et al.*, 1999). AP-1 is a heterodimeric complex of members of the Fos (e.g. c-Fos, FosB, Fra-1 and Fra-2) and Jun (e.g. c-Jun, JunB, and JunD) families of transcription factors (Karin *et al.*, 1997). The expression of c-Fos and c-Jun is upregulated during the proliferative period of osteoblast development while others such as Fra-1 and Fra-2 appear to be needed for osteoblast differentiation and matrix mineralization (McCabe *et al.*, 1995, McCabe *et al.*, 1996). It is probable that in proliferating osteoblasts, c-Fos and c-Jun heterodimer suppresses osteocalcin promoter activity (Schule *et al.*, 1990), while the association of Fra-2 and JunD in the post-proliferative osteoblasts facilitates VDR/RXR binding to the promoter and induces its activity (McCabe *et al.*, 1996, Lian *et al.*, 1998). In addition to the VDRE associated site which influences the vitamin D-enhanced expression, there is also another AP-1 site close to the homeodomain consensus binding site which may affect the basal level of transcription (Owen *et al.*, 1990b).

The activity of human osteocalcin promoter is repressed by glucocorticoids (Beresford *et al.*, 1984, Morrison *et al.*, 1989). Glucocorticoids directly affect the expression of osteocalcin at the transcriptional level by binding to the glucocorticoid receptors (GR) and subsequently to specific recognition sequences in the proximal promoter leading to a repression of osteocalcin gene to up to 40% of basal levels (Morrison *et al.*, 1989). Glucocorticoid receptor binding element (GRE) resides in osteocalcin promoter in close proximity and partly overlapping with the TATA box, a core promoter element (Strömstedt *et al.*, 1991). The competition or interference of GR and TATA-binding transcription factors at overlapping sites and the disruption of preinitiation complex by GR could thus explain the conditional repression of osteocalcin gene by glucocorticoids (Meyer *et al.*, 1997) and reflect the rapid repression of serum osteocalcin concentrations noted after short-term treatment with glucocorticoids (Godschalk and Downs, 1988, Prummel *et al.*, 1991) and in syndromes of glucocorticoid excess (Osella *et al.*, 1997). Sequence-specific binding of GR to other promoter regions has also been

demonstrated (Heinrichs *et al.*, 1993) and the regulation by glucocorticoids may involve integrated activities of multiple, independent binding sites (Morrison and Eisman, 1993).

Further modulation of expression

The proximal promoter of the rat osteocalcin gene contains a CCAAT/enhancer-binding protein (C/EBP) element in close proximity to the Runx2 binding site. C/EBP and Runx2 factors physically interact together in a synergistic manner to enhance osteocalcin transcription up to 35-40-fold in a cell culture system (Gutierrez *et al.*, 2002). The osteocalcin gene also contains an intragenic silencer directed against basal transcription (Frenkel *et al.*, 1994). The binding sequence overlaps the first exon and first intron but the molecular mechanism as well as the physiological role of this silencer element awaits further investigations. Furthermore, osteocalcin may not be regulated only at the promoter level but also via tissue-specific RNA splicing. Only bone tissues appear to efficiently splice osteocalcin messenger RNA whereas intron retention occurs in non-osteoid tissues (Jung *et al.*, 2001). Osteocalcin is also regulated by several growth factors and cytokines although it is often unclear whether the factor(s) regulates the expression of the osteocalcin gene or affects its synthesis and secretion. Osteocalcin expression is activated by basic fibroblast growth factor (bFGF) (Schedlich *et al.*, 1994) and down-regulated by tumour necrosis factor α (TNFα) (Li and Stashenko, 1993). TGFβ also inhibits osteocalcin gene expression at least in part through transcriptional control (Noda, 1989, Staal *et al.*, 1998) while insulin-like growth factor (IGF) dose-dependently induces osteocalcin production (Staal *et al.*, 1998). Recently, a thyroid response element has also been identified which mediates stimulation of osteocalcin expression by triiodo-thyronine (T3) (Varga *et al.*, 2003). Conflicting results exist for the parathyroid hormone (PTH), which has been shown to both activate (Yu and Chandrasekhar, 1997) and repress (Lajeunesse *et al.*, 1991) osteocalcin expression.

2.6 Structure and properties of osteocalcin

2.6.1 Biosynthesis of the polypeptide chain

Human osteocalcin is synthesized as a prepromolecule (molecular weight ~11000) consisting of a 23-residue leader sequence with a signal peptide, a 28-residue propeptide, and a 49 residue mature protein (Celeste *et al.*, 1986). The hydrophobic leader sequence targets the protein for secretion and is cleaved by a signal peptidase co-translationally. After cleavage the pro-osteocalcin is γ-carboxylated because the pro-region contains a γ-carboxylation recognition site homologous to vitamin K-dependent blood coagulation factors (Pan and Price, 1985). Subsequently, the propeptide is removed intracellularly and the mature protein with a molecular weight of approximately 5900 is secreted from the cell (Gundberg and Clough, 1992), although secretion of propeptide has also been found in some cell culture studies (Hosoda *et al.*, 1993). The propeptide of rat and mouse osteocalcin is only 26 residues in length compared to 28 residues in human and bovine protein which have additional Gly and Ala at positions -19 and -18 (Kiefer *et al.*, 1990). The primary structure from N to C terminus is

$$Y^{1}LYQWLGAPV\ PYPDPL\mathbf{E}PRR\ \mathbf{E}VC\mathbf{E}LNPDCD\ ELADHIGFQE\ AYRRFYGPV^{49}$$

in which glutamic acid residues (**E**) in positions 17, 21 and 24 are the ones to be carboxylated into Gla residues (Poser *et al.*, 1980). However, human osteocalcin usually contains only two residues of Gla instead of three found in several other species. The glutamic acid residue at position 17 is only 9% γ-carboxylated in human osteocalcin whereas positions 21 and 24 are fully carboxylated (Poser *et al.*, 1980). The incomplete carboxylation probably reflects partial γ-

carboxylation during biosynthesis rather than decarboxylation in bone because the same degree of decarboxylation is found in bones in which the *in situ* age of the bone matrix differs substantially such as fetal and adult bones (Cairns and Price, 1994). The primary structure of osteocalcin is further characterized by a proline-rich segment with prolines at positions 9, 11, 13, 15, and 18. Proline at position 9 is converted to 4-hydroxylproline in most species such as bovine (Price *et al.*, 1976a) but not in humans (Poser *et al.*, 1980). Two arginine-arginine bonds (R-R) at positions 19-20 and 43-44, respectively, introduce putative sites for proteolytic cleavage by trypsin (Garnero *et al.*, 1994a).

In addition to human (Poser *et al.*, 1980, Celeste *et al.*, 1986), osteocalcin has been isolated from numerous mammalian species including macaque monkey (Hauschka *et al.*, 1982), rat (Pan and Price, 1985), mouse (Desbois *et al.*, 1994), cow (Price *et al.*, 1976b, Kiefer *et al.*, 1990), horse (Carstanjen *et al.*, 2002), pig (Hoang *et al.*, 2003), rabbit (Virdi *et al.*, 1991), dog (Colombo *et al.*, 1993), cat (Shimomura *et al.*, 1984), sheep (Mende *et al.*, 1984) and goat and wallaby (Huq *et al.*, 1984) as well as avian species such as chicken (Carr *et al.*, 1981) and emu (Huq *et al.*, 1987) and also frog *Xenopus laevis* (Cancela *et al.*, 1995). The primary structure of osteocalcin is highly conserved among vertebrates. The homology is strongest in the Gla-containing central domain. All three glutamic acid residues subject to γ-carboxylation at positions 17, 21, and 24 are completely conserved as well as are the neighboring residues. The evolutionary conservation of primary structure within this domain can be correlated with the ability of all vertebrate osteocalcins to bind to bone mineral. The sequence preservation has also been demonstrated by extracting osteocalcin from fossil bones (Huq *et al.*, 1985, Ulrich *et al.*, 1987, Nielsen-Marsh *et al.*, 2002). Bony fish also contain osteocalcin (Price *et al.*, 1977, Nishimoto *et al.*, 1992, Cancela *et al.*, 1995, Nishimoto *et al.*, 2003). The common ancestor of mammals and birds (*Tetrapoda*) and bony fish (*Teleostei*) evolved bone over 200 million years ago and the comparison of these two orders has provided insight into the evolution of osteocalcin. The Gla region including the two cysteines is highly conserved but the *Teleostei* and *Tetrapoda* have evolved unique sequences near C-terminus (Nishimoto *et al.*, 2003). A truncated N-terminus and extended C-terminus are conserved among *Teleostei* which are lacking the RFYGPV C-terminal sequence common in *Tetrapoda.* A multiple alignment of fifteen selected species, including also two bony fish, is shown in Fig.3.

```
                *         *         *         *         *
human     YLYQWLGAPVPYPDPLEPRREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
macaque   YLYQWLGAPAPYPDPLEPKREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
bovine    YLDHWLGAPAPYPDPLEPKREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
rat       YLNNGLGAPAPYPDPLEPHREVCELNPNCDELADHIGFQDAYKRIYGTTV- 50
mouse     ----YLGASVPSPDPLEPTRQQCELNPACDELSDQYGLKTAYKRIYGITI- 46
horse     YLDHWLGAPAPYPDPLEPRREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
bison     YLDHGLGAPAPYPDPLEPKREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
dog       YLDSGLGAPVPYPDPLEPKREVCELNPNCDELADHIGFQEAYQRFYGPV-- 49
cat       YLAPGLGAPAPYPDPLEPKREICELNPDCDELADHIGFQDAYRRFYGTV-- 49
rabbit    QLINGQGAPAPYPDPLEPKREVCELNPDCDELADQVGLQDAYQRFYGPV-- 49
chicken   HYAQDSGVAGAPPNPLEAQREVCELSPDCDELADQIGFQEAYRRFYGPV-- 49
emu       SFAVGS-SYGAAPDPLEAQREVCELNPDCDELADHIGFQEAYRRFYGPV-- 48
frog      SYGNNVGQGAAVGSPLESQREVCELNPDCDELADHIGFQEAYRRFYGPV-- 49
bluegill ------AAGELTLTQLESLREVCEANLACEDMMDAQGIIAAYTAYYGPIPY 45
sparus   ------AAGQLSLTQLESLREVCELNLACEHMMDTEGIIAAYTAYYGPIPY 45
```

Figure 3. *Multiple alignment of osteocalcin coding sequence from selected species with conserved regions shown in black.* Pro^9 is hydroxylated into 4-hydroxyproline in macaque, bovine, rat, horse, bison, cat, and rabbit osteocalcin (not shown). Two sequences are from *Teleostei* fish (bluegill, *Lepomis macrochirus* and sparus, *Sparus aurata*). The sequences were obtained from UniProt / Swiss-Prot entries (www.ebi.uniprot/org) and the alignment was made with Clustal W 1.82. The GeneDoc program was used for visualization of conserved regions (black, 100%; dark grey 80%; light grey, 60%).

2.6.2 Post-translational modifications

The structure of osteocalcin is characterized by the presence of three residues of calcium-binding amino acid γ-carboxyglutamic acid (Gla) which are formed post-translationally from glutamic acid in a vitamin K-dependent manner (Poser *et al.*, 1980). Osteocalcin is a member of a larger family of proteins called Gla proteins or vitamin K dependent proteins (VKD). This group of proteins requires modification of specific glutamic acid residues to γ-carboxyglutamate in order to function properly. The best known members of this family are perhaps several plasma proteins involved in blood coagulation such as prothrombin, blood coagulation factors X, IX and VII, and proteins C, S and Z (Oldenburg and Schwaab, 2001). Other members include the two proteins occurring in calcified tissues, osteocalcin and matrix Gla protein (Hauschka *et al.*, 1989) and growth arrest protein 6 (Gas6) which is expressed in serum-starved cells and many human tissues and probably involved in the regulation of a protease cascade relevant for growth regulation (Manfioletti *et al.*, 1993) and several proline-rich transmembrane Gla proteins of unknown function expressed in broad as well as variable distribution in fetal and adult tissues (Kulman *et al.*, 1997, Kulman *et al.*, 2001). The non-mammalian members of the family include e.g. neuroactive peptides conantokin-G and –T purified from the venom of the fish-hunting cone snail (Haack *et al.*, 1990, Prorok *et al.*, 1996). The structure of Gla containing proteins share very little homology, even at the γ-carboxylated region. However, the propeptide sequences show substantial sequence similarity suggesting that this common structural feature residing in propeptide is recognized by the carboxylation system. A pentapeptide consensus sequence Asn/Gln-Arg-X-Arg/Lys-Arg is found in most propeptides of carboxylated proteins (Pan and Price, 1985). Interestingly, matrix Gla protein has no propeptide but there is a region in the mature protein (residues +15 to +30) that is homologous to the propeptide of other vitamin K dependent proteins (Price *et al.*, 1987). However, recent findings suggest that osteocalcin is recognized in a different mechanism than other vitamin K dependent proteins since osteocalcin lacking propeptide is carboxylated normally (Houben *et al.*, 2002). The propeptide of osteocalcin differs from that of other vitamin K-dependent proteins at one highly conserved residue lowering the affinity of carboxylase enzyme and, instead of propeptide, osteocalcin seems to be recognized at two specific regions flanking the Gla domain (regions spanning residues 1-12 and 26-39) (Houben *et al.*, 2002). Neither of these has structural homology with the propeptide region. An additional common structure residing in the Gla-containing region of vitamin K-dependent vertebrate proteins is the sequence Glu-X-X-X-Glu-X-Cys (EXXXEXC) (Price *et al.*, 1987). However, the EXXXEXC consensus sequence does not contribute to the recognition since small peptides containing this sequence remain uncarboxylated (Houben *et al.*, 2002).

During carboxylation reaction, a second carboxyl group is added to specific glutamic acid residues to yield Gla residues. The vitamin K-dependent γ-carboxylation system is a multicomponent system of proteins. The enzyme responsible for the reaction is vitamin K dependent γ-glutamyl carboxylase. The enzymatic activity is located in the luminal side of

rough endoplasmic reticulum suggesting that γ-carboxylation is an early event in biosynthesis of carboxylated proteins (Carlisle and Suttie, 1980). The reduced hydroquinone form of vitamin K (KH_2) is an active cofactor for carboxylase and concomitant with carboxylation, hydroquinone KH_2 is converted to vitamin K epoxide (Wallin *et al.*, 2002). The oxidation of hydroquinone to epoxide provides the energy required for the carboxylation of Glu residues into Gla (Vermeer, 1990). Because the amount of vitamin K in the human diet is limited, vitamin K epoxide must be reduced back to KH_2 to prevent its depletion. Coumarin derivatives, such as warfarin, are potent inhibitors of γ-carboxylation. They are widely used for the treatment and prophylaxis of thrombo-embolic diseases due to their effect on blood coagulation factors and they function by inhibiting vitamin K epoxide reductase which is needed for the regeneration of vitamin K (Li *et al.*, 2004). Accordingly, the Gla content of osteocalcin is also strongly affected by coumarin derivatives (Menon *et al.*, 1987, van Haarlem *et al.*, 1988).

Osteocalcin may become post-translationally glycated to the N-terminal tyrosine with one glucose per osteocalcin molecule (Gundberg *et al.*, 1986). Glycation is a process in which glucose reacts non-enzymatically with free amino groups on proteins to form a stable adduct distinct from N- and O-linked glycosylation. Glycation, or nonenzymatic glycosylation, is perhaps best known as the major cause of heterogeneity in human hemoglobin (Garlick *et al.*, 1983). Other long-lived proteins of connective tissue such as collagens, lens crysallins, and nerve proteins are also known to be modified by glycation, the degree of which increases with age and elevated glycation may play a role in e.g. complications of diabetes and in normal aging (Monnier *et al.*, 1984). There is a strong correlation between the amount of glycated osteocalcin and age both in bovine as well as human bone specimens; however, the greatest relationship exists in the older subset (Gundberg *et al.*, 1986). Osteocalcin molecules remain deposited in the skeleton and are only replaced with newly synthesized material during bone remodeling. Therefore, the extent of glycation in osteocalcin could depend on the age of the bone site and could hypothetically also play some role in bone diseases (Gundberg *et al.*, 1986). Furthermore, an age-dependent accumulation of D-aspartic acid has been demonstrated in bone osteocalcin (Ritz *et al.*, 1996), similar to the one observed in CTX (Cloos and Fledelius, 2000). The content of D-aspartic acid may be used as a measure of the age of bone osteocalcin and hence, the age of the entire organism (Ritz *et al.*, 1996).

2.6.3 Binding to calcium

Osteocalcin has a property to bind calcium (Hauschka and Gallop, 1977). Circular dichroism and ^1H NMR studies indicate that in the absence of Ca^{2+} osteocalcin exists primarily in random coil (Hauschka and Carr, 1982) or in extended, unstructured conformation except for the turn required by the disulphide bridge Cys^{23}-Cys^{29} (Atkinson *et al.*, 1995). Due to the unstructured properties, a single structure for apo-osteocalcin has not been solved (Dowd *et al.*, 2003). The addition of millimolar levels of Ca^{2+} induces an alpha-helical structure. In the original study by Hauschka and Carr, the presence of physiological levels of Ca^{2+} resulted in a significant increase in alpha-helical structure, from 8% in apo-osteocalcin to 38% after addition of Ca^{2+} (Hauschka and Carr, 1982). Studies on bovine osteocalcin on solution also indicated a transition to a folded state on the addition of physiological concentrations of Ca^{2+} with an increase in helical structure from 19% to 31% (Atkinson *et al.*, 1995). Titration with Ca^{2+} indicated that three moles of calcium per one mole of bovine osteocalcin is responsible for the conformational change. The three calcium ions are coordinated to the three Gla residues within each osteocalcin molecule and the oxygen atoms in side chains as well as water molecules are involved in coordination (Dowd *et al.*, 2003). Ca^{2+} mediates a structural transition also in other

Gla proteins such as prothrombin in which the three-dimensional structure of the Gla-rich domain is induced and stabilized by metal ions (Furie and Furie, 1979). However, binding of calcium to osteocalcin is not associated with a clear transition from a disordered to an ordered structure as in the Gla domain of prothrombin (Soriano-Garcia *et al.*, 1992). This property of osteocalcin may be associated with the potential to bind Ca^{2+} on a crystal surface. The structure might be induced in the presence of hydroxyapatite and flexibility could thus have a functional value (Atkinson *et al.*, 1995).

2.6.5 Folding and three-dimensional conformation

The empirical modelling of calcium-bound osteocalcin by Hauschka and Carr predicted two antiparallel alpha-helical domains "the Gla helix" (positions 18-25) and "the Asp-Glu helix" (positions 30-41) and a beta-turn between them (positions 26-29) forcing the alpha-helical segments into close antiparallel approximation (Hauschka and Carr, 1982) (Fig.4a). Other beta turns were predicted at position 12-15 and a beta sheet structure was predicted at the C-terminal regions from 42 to 49 resulting in an accessible C-terminus. Furthermore, six to seven residues at both N and C termini were predicted to be in a beta sheet structure and in close proximity to each other. This model served as a structure for osteocalcin for two decades. Major progress was made in 2003, when two groups independently provided evidence for the three dimensional structure of calcium-bound osteocalcin using 1H NMR spectroscopy for bovine osteocalcin (Dowd *et al.*, 2003) or, just a few months later, X-ray crystallography for porcine osteocalcin (Hoang *et al.*, 2003). The structure is in reasonable agreement with some of the secondary structural elements predicted by the model of Hauschka and Carr but is also clearly different from the predicted one. According to current knowledge, the structure of osteocalcin consists of an unstructured N-terminus from residues 1-15 and a structured region from residues 16-49 which contain three regions of secondary structure (Dowd *et al.*, 2003). Since the N-terminal region can not be confined to a fixed orientation with respect to the rest of the molecule, it is oriented differently in each model. Furthermore, the N-terminal region does not contain any areas of secondary structure although some interactions between hydrophobic side chains of Leu^2 and Trp^5 can be observed. The N-terminal 12 residues were also excluded from the three-dimensional model of X-ray crystallography because of the disordered organization (Hoang *et al.*, 2003). The hydrophobic side chains in the C-terminal region interact with the side chains in the middle part of the molecule forming a loop of residues 16-49. Thus, the N- and C-termini are not involved in beta-sheet formation as previously speculated. The current model does not support a beta-turn within residues 26-29 so the two helical regions are not antiparallel. The tertiary structure of molecule is formed by various long range and hydrophobic side chain interactions which serve to stabilize the tertiary structure (Dowd *et al.*, 2003). Similarly, the X-ray crystallization studies supported a topology consisting from three alpha-helices and a short extended C-terminal strand (Hoang *et al.*, 2003). The first two helices are connected by a type III turn from Asn^{26} to Cys^{29} stabilized by the disulphide bridge and the three helices compose a tight globular structure comprising a previously unknown fold. This unique fold has been identified in the model for porcine osteocalcin (Hoang *et al.*, 2003) as well as the model for bovine osteocalcin (Dowd *et al.*, 2003) and the fold appears unique as compared to other calcium binding proteins as well as Gla containing blood coagulation factors. One unique feature is that the calcium addition induces a folded structure with calcium coordinating Gla residues on the same face of the helical region as surface exposed (Fig.4b). In contrast, the blood coagulation factors have the Gla residues reoriented inward with calcium addition and most of them are not surface-exposed. Furthermore, the global fold of e.g. factor IX and

prothrombin consist of N-terminal loop next to C-terminal core while osteocalcin, although having a C-terminal core, has an unstructured and flexible N-terminus (Dowd *et al*., 2003).

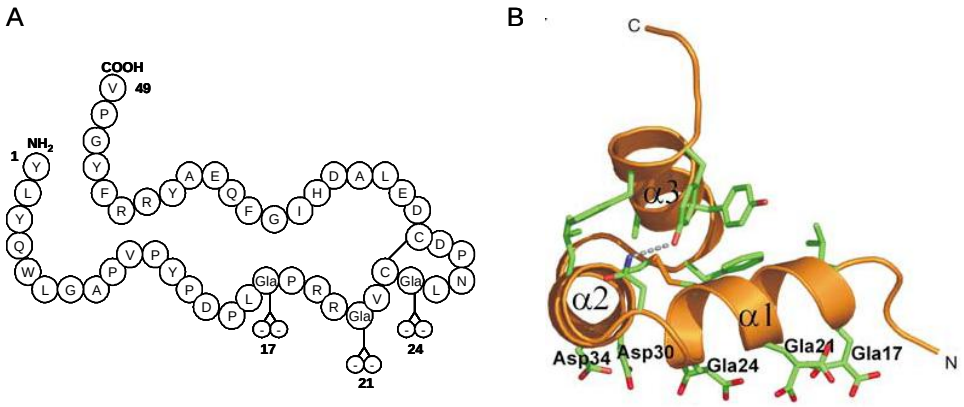

Figure 4. *Structure of osteocalcin.* A) A schematic diagram of osteocalcin modified from the empirical model of Hauschka and Carr (1982) and adapted from the original publication **I**. B) A ribbon representation of osteocalcin based on the crystal structure reported by Hoang *et al*. (2003).

2.6.6 Binding of properly folded osteocalcin to bone mineral

Osteocalcin binds strongly and selectively to the mineral component of bone, hydroxyapatite (Price *et al*., 1976a), and the ability is dependent on the properly folded Gla-containing conformation (Poser and Price, 1979) which may be understood in terms of transition to the alpha-helical structure. The most recent data demonstrate that the calculated distances of the Gla side chains have the best correlation with the intercalcium distance of 9.43Å which fits to the unit cell dimensions of the hydroxyapatite crystal (Dowd *et al*., 2003). The coordinated calcium atoms are aligned with calcium atoms in the mineral and coordinated calcium atoms can fill in vacant positions in the crystal lattice. All three Gla residues implicated in hydroxyapatite binding are located on the same surface of the first helix and together with Asp^{30} from helix 2 they coordinate five Ca^{2+} ions in a complex network of ionic bonds with extensive bridging (Hoang *et al.*, 2003). The crystal structure finally allows the position of five bound calcium ions to be "seen" on the protein surface and the binding is not between positive calcium ions and negative Gla carboxyl groups as previously thought but calcium is coordinated to oxygen atoms in side chains in an elaborate network of ionic bonds (Hoang *et al.*, 2003). Importantly, the calcium ions coordinated by the Gla residues have a periodic order similar to a crystalline lattice and the molecular surface defined by the calcium ions appears to be the one expressed in synthetic hydroxyapatite (Onuma *et al.*, 1995). In summary, the structure contains a negatively charged protein surface that interlocks exactly with the position of positively charged calcium ions in a hydroxyapatite crystal lattice. The recognition of crystal lattices is important in many biological proteins including inhibition of ice crystal growth and the development of teeth, bone and shells, and the binding of osteocalcin provides insight into interactions involved in biomineral recognition. Perhaps the best characterized protein-crystal recognition is the interaction of antifreeze proteins (AFP) with ice crystals (Davies *et al.*, 2002). AFPs protect fish from freezing when the temperature in their surroundings falls below the freezing point of their body fluids. AFPs circulate in fish body fluids and function by absorbing into the surface of growing ice crystals preventing crystal growth. The shape-complementarities between the flat ice-binding surface of AFP and the ice-crystal are the primary determinants for

binding specificity (Yang *et al.*, 1998). The surface complementarities between osteocalcin and hydroxyapatite suggest that osteocalcin has similar selective binding characteristics towards bone mineral.

In the bone binding orientation, the C-terminus of osteocalcin extends outward (Dowd *et al.*, 2003, Hoang *et al.*, 2003) making it accessible to proteases (Fig.4b). Plasmin has been shown to cleave osteocalcin at this region between residues 43 and 44 to produce a large 1-43 fragment and a small C-terminal pentapeptide. Interestingly, the plasmin-mediated cleavage of hydroxyapatite-bound osteocalcin releases both osteocalcin cleavage products into solution (Novak *et al.*, 1997). The degradation of the most N-terminal sequence of rat osteocalcin (residues 1 to 7) by cathepsins also decreases the calcium-binding capacity of osteocalcin leading to detachment of truncated protein forms from bone mineral (Koboyashi *et al.*, 1998). The conformation of intact protein thus appears to be essential for interaction with hydroxyapatite and fragments of osteocalcin are not able to bind to bone mineral as efficiently. The crucial role of Gla-residues in the coordination of calcium ions (Hoang *et al.*, 2003) suggests that also the loss of specific Gla-residues may render the protein biologically inactive and thus, decarboxylated osteocalcin could detach from the bone matrix. In warfarin-administered rats, osteocalcin lacking γ-carboxylation is no longer able to bind to hydroxyapatite (Price *et al.*, 1981c) and is not accumulated in bone matrix but continuously circulating in serum (Price and Williamson, 1981b). Furthermore, decarboxylated molecule is cleared from the circulation more rapidly (Hauschka *et al.*, 1989). It has been suggested that the *in vivo* decarboxylation might represent natural aging of the protein and subsequently those molecules which have been in the bone matrix the longest time would be more extensively decarboxylated and finally removed and replaced by newly synthesized molecules (Poser *et al.*, 1980). This has resulted in attempts to measure undercarboxylated osteocalcin in circulation as a putative index of poor "bone quality" and a susceptibility factor for fractures (Vergnaud *et al.*, 1997, Luukinen *et al.*, 2000).

2.7 Putative functions of osteocalcin

The abundance of osteocalcin in the mineralized matrix and its well conserved amino acid sequence emphasize the importance of osteocalcin in bone. One could argue that osteocalcin must play some yet unidentified important role in bone biology. However, its biological function has remained obscure. Because the size of osteocalcin is only 49 residues, it has been hypothesized that it does not necessarily possess an enzymatic function or play a role in bone structure. Most hormones and chemotactic factors have a low molecular weight and osteocalcin might thus have such an informational role in skeletal homeostasis (Nishimoto and Price, 1980).

2.7.1 Role in mineralization

Osteocalcin binds strongly to hydroxyapatite (Price *et al.*, 1976b) with Gla side chains interacting with the mineral (Poser and Price, 1979). It is localized in the mineralized matrix of bone (McKee *et al.*, 1992) and appears in newly developing bone approximately at the same time as the initial mineral phase matures into hydroxyapatite (Price *et al.*, 1981a). There is also a significant positive correlation between the concentration of osteocalcin and the degree of mineralization (Roy *et al.*, 2001). These data suggest that osteocalcin may play a role in the maturation of bone mineral. Osteocalcin is a potent inhibitor of hydroxyapatite-seeded crystal growth *in vitro*, and decarboxylation significantly reduces inhibitory activity (Boskey *et al.*, 1985, Romberg *et al.*, 1986). Osteocalcin apparently inhibits hydroxyapatite crystal formation by delaying the nucleation rather than altering the amount of hydroxyapatite formed (Hunter *et*

al., 1996). Thus, the role of osteocalcin may be related to the prevention of excessive mineralization by slowing down the crystal growth. By analogy to antifreeze proteins, the binding of osteocalcin into hydroxyapatite could directly modulate hydroxyapatite crystal morphology and growth (Hoang *et al*., 2003). It is also possible that the soluble protein, not the bone matrix bound, may be the more important component of osteocalcin function and the equilibrium between soluble protein and the one bound to mineral may be important for the maintenance of bone mineralization (Dowd *et al*., 2003).

A model to study the osteocalcin function *in vivo* was initially generated by inducing osteocalcin ablation by treating rats with warfarin (Price and Williamson, 1981b). Rats were maintained on a ratio of warfarin to vitamin K which prevents γ-carboxylation of osteocalcin but circumvents the bleeding problems. Bone osteocalcin levels were only 2% of the control levels but no abnormalities in any structural parameter such as bone size, morphology, bone mineral content, or mineralization in bones were noted. Furthermore, osteocalcin deficiency had no effect on fracture repair. There is, however, a possibility that osteocalcin level must be reduced below 2% of normal levels before clear abnormalities appear. Rats treated for long term with warfarin developed excessive mineralization of the bone growth plate with premature growth plate closure and were osteopenic, indicating a role for osteocalcin (or some other γ-carboxylated protein) in regulating the quantity of mineral also *in vivo* (Price *et al.*, 1982).

The complete ablation of osteocalcin from bone and circulation was achieved by generating osteocalcin-deficient mice by simultaneously deleting both mouse OG1 and OG2 genes (Ducy *et al*., 1996). The absence of osteocalcin did not affect bone mineralization. Both mineral apposition rate and bone mineral content was indistinguishable in wild-type and osteocalcin-deficient bones (Ducy *et al.*, 1996). A conditional knockout for osteocalcin ablation specifically in osteoblastic cells was generated with a promoter fragment of the α1(I) collagen and failed to show any metabolic or histological abnormalities or defects in mineralization (Murshed *et al.*, 2004). However, when osteocalcin knockout mice were studied using a more sensitive assay of mineralization (Fourier transform infrared microspectroscopy), differences in mineralization in the cortical bone of elderly knockout animals were found (Boskey *et al.*, 1998). In the knockouts, cortical bone has characteristics indistinguishable from the trabecular bone whereas in the control animals the characteristics of these two bone types differ from each other. Both trabecular and cortical bone in osteocalcin-deficient mice have parameters indicative of lower maturity and resemble the trabecular bone in control animals. The mineral crystals in cortical bones of wild-type animals are larger and more perfect than those in wild-type trabecular bones, and those in both the cortical and trabecular bones of the knockout. In wild-type ovariectomized animals, there is a gradual increase in the crystal size and maturity across the cortical bone from periosteum to endosteum. Such increases in crystal maturity are not observed in osteocalcin-deficient animals. The apparent impaired maturation of cortical bone in osteocalcin-deficient bone implies a role for osteocalcin in mineral maturation and maintaining the optimal crystal size. Since the amount of mineral does not differ, the mineralization process itself is not altered. Changes in trabecular mineral may be less striking because osteocalcin is less abundant in trabecular bone compared to cortical bone (Ninomiya *et al*., 1990).

The effect of osteocalcin on extracellular mineralization of nonosseous tissues has been studied on mice deficient in MGP which develop massive and lethal calcification of the arteries (Luo *et al*., 1997). The transgenic expression of osteocalcin in MGP-deficient mice is not able to rescue the mice from the mineralization of aorta demonstrating that unlike MGP, osteocalcin

cannot inhibit mineralization in arteries (Murshed *et al.*, 2004). Thus, despite the Gla residues, osteocalcin is not an inhibitor of extracellular mineralization of nonosseous tissues *in vivo*.

2.7.2 Limiting factor for bone formation

In mice lacking functional osteocalcin genes bone density is increased, suggesting a role for osteocalcin in limiting osteoblastic bone formation in mouse (Ducy *et al.*, 1996). Osteocalcin deficient mice are normal at birth, viable and fertile and have no skeletal-patterning defects or ectopic bone formation. Although osteocalcin does not appear to be required for embryonic development in the mouse, it plays a role in postnatal bone physiology since abnormalities of bone remodeling become noticeable in 6-month old animals. The cortical thickness and density as well as the amount of trabecular bone is increased in mutant animals compared to the wild-type littermates. The increase in bone tissue has a beneficial effect on the biomechanical properties of the bones demonstrated by an increase in the failure load. The increase in bone mass is due to an increase in the rate of bone-matrix deposition shown by histomorphometry. There is no increase in the number of osteoblasts, indicating that each osteoblast is laying down more matrix. This suggests that osteocalcin functions to limit bone formation while bone resorption and mineralization are unimpaired. The increased bone formation rate in osteocalcin-deficient animals could also be one reason for the impaired mineral maturation (Boskey *et al.*, 1998) resulting in an increased number of sites for initial mineral deposition and a decrease in the time available for crystal maturation. Because osteocytes express high levels of osteocalcin (Aarden *et al.*, 1996), it is also possible that osteocytes function differently in knockout mice. In summary, the increased bone formation in osteocalcin-deficient mice suggests that osteocalcin produced by mature osteoblasts during late phases of osteoblastic differentiation negatively regulates the bone matrix production rate by autocrine and/or paracrine manner, thus inhibiting excessive bone formation. By assuring that too much bone is not produced, osteocalcin may play a role in the control of bone remodeling. Osteocalcin produced by osteocytes could inhibit excessive bone formation in the vicinity of osteocytes. Thus, it could participate in the maintenance of the osteocytic lacunae and canaliculi thereby ensuring the diffusion of oxygen, nutrients and waste products through the lacuno-canalicular system (Mikuni-Takagaki *et al.*, 1995, Aarden *et al.*, 1996). Osteocalcin may bind to a specific receptor on osteoblasts to fulfill this function. Interestingly, conditionally immortalized human osteoblast cell lines respond transiently and dose-dependently to osteocalcin treatment *in vitro* (Bodine and Komm, 1999). The response is at least in part mediated by a signal transduction pathway involving the release of calcium from intracellular stores. The putative osteocalcin receptor is coupled to G-proteins (guanine nucleotide-dependent regulatory protein) and specifically, to inhibitory G-proteins (Bodine and Komm, 1999). The G-protein-coupled receptors, like PTH-1 receptor, signal through phospholipase C and adenylate cyclase and the response to osteocalcin can be inhibited by interference of these pathways. Treatment with osteocalcin results in 40% reduction in cellular alkaline phosphatase activity in 48h in a dose-dependent manner. The putative receptor has not been identified and the molecular cloning and characterization of the receptor is required in order to verify its existence. Furthermore, the *in vitro* studies on osteoblasts derived from osteocalcin-deficient mice would be important to elucidate the role of osteocalcin ablation at the cellular level.

2.7.3 Recruitment and differentiation of osteoclasts

Osteocalcin has been implicated as a chemotactic factor for cells of osteoclastic lineage in early *in vitro* studies (Mundy *et al.*, 1978, Mundy and Poser, 1983). Mononuclear cells from peripheral human blood known to be related to osteoclast precursor cells respond to a gradient

of osteocalcin in a dose-dependent manner (Malone *et al.*, 1982). Osteoclast-like cells obtained from giant cell tumors of bone adhere to osteocalcin-coated surfaces and show a significant migration towards osteocalcin gradient demonstrating the chemotactic activity of osteocalcin (Chenu *et al.*, 1994). The cell adhesion does not require Gla residues and is lost after treatment with vitamin D which promotes differentiation suggesting that only less mature osteoclasts respond to osteocalcin. Osteocalcin-deficient bone particles (from rats treated with warfarin) implanted subcutaneously in normal rats show impaired cellular recruitment and differentiation of osteoclast-like cells and reduced resorption compared to implanted control bones (Lian *et al.*, 1984). Defective degradation by human monocytes has also been demonstrated *in vitro* (Lian *et al.*, 1986). Normal and osteocalcin-deficient particles are resorbed to a different extent even when adjacent within the same implant (DeFranco *et al.*, 1991). When implanted particles of synthetic crystalline apatite alone and particles of apatite with osteocalcin were compared, the osteocalcin-containing particles generated more multinucleated cells than apatite alone particles and were more efficiently resorbed (Glowacki *et al.*, 1991). Thus, osteocalcin may act as a signal from the bone matrix for recruitment and adhesion of osteoclasts to the sites of active bone remodeling.

It is also possible that osteocalcin or osteocalcin fragments released from bone may activate monocytes to differentiate resulting in an increased number of active bone-resorbing cells. Osteocalcin has been shown to enhance the development of TRACP-positive multinucleated cells from mouse bone marrow without affecting the survival or proliferation indicating that osteocalcin may also promote osteoclastic differentiation (Liggett *et al.*, 1994). Furthermore, when an EDTA extract prepared from bovine bone was added to calvarial cell cultures, the cells acquired an ability to resorb bone (Ishida and Amano, 2004). The activity responsible for the induction had a sequence corresponding to residues 7-36 in bovine osteocalcin indicating that a fragment of osteocalcin, putatively released from the matrix during resorption is able to induce osteoclast differentiation. In contrast to earlier findings (Liggett *et al.*, 1994), the stimulatory action of osteocalcin fragment occurred in later stages of osteoclast differentiation. although it was not able to enhance the resorption activity of fully mature cells (Ishida and Amano, 2004). Despite the *in vitro* data on osteocalcin and osteoclast function, the *in vivo* studies have, however, provided very little evidence for the role of osteocalcin in regulating osteoclast activity (Ducy *et al.*, 1996). Histomorphometric analysis of osteocalcin-deficient mice revealed an increase in osteoclast number and surface (Ducy *et al.*, 1996). Since bone mass is increased in these animals despite the increased osteoclast number, the osteoclasts might be functioning poorly in the mutant mice. However, the expected decreases in bone density as well as in biomechanical properties in the ovariectomized animals demonstrate that bone resorption occurs normally in osteocalcin-deficient mice.

2.7.4 Additional aspects

Although osteocalcin has been shown to recruit osteoclast precursor cells and to mediate also attachment of osteoclast-like cells to bone matrix (Lian *et al.*, 1986), there are no reports of cell attachment domains in osteocalcin itself. Instead, osteopontin contains a RGD-sequence which is able to mediate cell attachment via the vitronectin receptor (Butler, 1989). Osteocalcin forms a complex with osteopontin and the binding is of high affinity as well as specific and stable (Ritter *et al.*, 1992). In theory, osteocalcin-osteopontin complex could both recruit osteoclast to a site of bone resorption and mediate the attachment of cells to the region. A reversible complex formation has been demonstrated also between osteocalcin and type I collagen (Prigodich and Vesely, 1997).

Osteopontin is a substrate for transglutaminase activity, a reaction that can produce complexes between proteins *in vivo* (Prince *et al.*, 1991). Tissue transglutaminase catalyzes the formation of high molecular mass complexes of proteins with covalent cross-link bonds stable and resistant to proteolysis (Aeschlimann and Paulsson, 1994). Transglutaminase is thought to participate in matrix cross-linking before the tissue undergoes calcification and therefore, it might be involved in the initiation and regulation of mineralization processes (Aeschlimann *et al.*, 1993). Transglutaminase has three substrates in bone, osteopontin, bone sialoprotein, and α2HS-glycoprotein, and large protein aggregates are formed of them in the presence of the enzyme (Kaartinen *et al.*, 2002). Interestingly, osteocalcin reduces the formation of transglutaminase-catalyzed high molecular mass complexes of osteopontin most likely by competing with the enzyme for the binding site of protein substrate by a sequence homology to tissue transglutaminase (Kaartinen *et al.*, 1997). The elements required for the inhibition are located in the unstructured N-terminus within the first 13 N-terminal residues and Gla-residues are not essential for the inhibition. Residues 14-25 may to some extent contribute to the binding affinity. The cross-linking products such as osteopontin complexes could be involved in the initiation of calcification suggesting a novel potential role for osteocalcin in the mineralization process (Kaartinen *et al.*, 1997).

Osteocalcin has also been speculated to function in hemostasis because osteocalcin messenger RNA has been detected in platelets (Thiede *et al.*, 1994). Platelet action is important for fracture repair and the release of substances such as osteocalcin by platelets at sites of new matrix deposition could stimulate bone turnover by activating osteoclast precursors. Osteocalcin can also inhibit the activation of prothrombin by competition for membrane binding sites with Factor Va, and is able to chelate calcium ions and might thus act as an anticoagulant within bone or contribute to blood coagulation (Gendreau *et al.*, 1989). Additional biological activities for osteocalcin have been attributed to the peptide from the C-terminal region, which is easily released from the intact molecule by degradation by plasmin (Novak *et al.*, 1997). Oxytocin-hormone inhibits osteosarcoma tumor cell growth and the C-terminal hexapeptide of osteocalcin is able to reverse this inhibition via a sequence similarity to the oxytocin receptor (Novak *et al.*, 2000). The ability of tumor cells to grow in bone may be in part due to the release of osteocalcin peptide from bone matrix which in turn acts as a potent antagonist of oxytocin binding to its receptor.

Taken together, the *in vitro* experiments emphasize a dual role for osteocalcin. It is assumed to directly participate in the maturation of mineralization and in the promotion of bone resorption. The gene knockout experiments have demonstrated that mice lacking osteocalcin develop bone of increased mass and strength, further implying the importance of osteocalcin in bone as a regulator cellular functions and/or matrix turnover.

2.8 Osteocalcin in the circulation and clearance from the blood

2.8.1 Origin of circulating osteocalcin

In vitro data suggest that osteocalcin is synthesized primarily by bone-forming osteoblasts (Nishimoto and Price, 1980, Beresford *et al.*, 1984). The majority of biosynthetic osteocalcin is incorporated into the extracellular matrix of newly synthesized bone, but a fraction of molecules enters the blood stream where they can be detected (Price and Nishimoto, 1980). It has been estimated that approximately 10-40% of newly synthesized osteocalcin is released into serum (Blumsohn *et al.*, 1995). Because of the osteoblastic origin, osteocalcin measured in serum samples has been widely used as a biochemical marker of bone formation (Hauschka *et al.*,

1989, Power and Fottrell, 1991, Lee *et al.*, 2000). However, most synthesized osteocalcin is incorporated into bone matrix where it remains until removed by bone resorption. Bone matrix osteocalcin could, in theory, be released during bone resorption in an immunoreactive form and increase plasma osteocalcin levels. Thus, circulating osteocalcin could reflect bone formation, bone resorption of a combination of both (Fig.5).

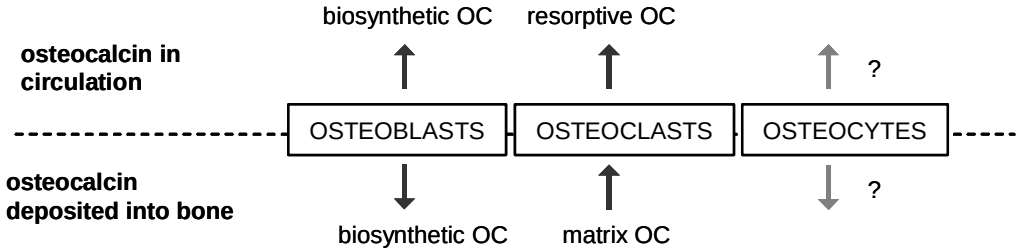

***Figure 5**. Possible sources of osteocalcin (OC) in circulation.* The newly synthesized (biosynthetic) OC is partly incorporated into the matrix and partly released into the circulation. OC embedded in bone matrix is released during bone resorption (resorptive OC) but it is not precisely known how extensively OC is degraded during the resorption process. Osteocytes express OC but their contribution to the circulating levels of OC is unknown.

The initial reports focusing on the origin of circulating osteocalcin suggested that it is derived from the protein synthesized by osteoblasts and not bound to hydroxyapatite. In a series of experiments Price and co-workers (Price and Williamson, 1981b, Price *et al.*, 1981c) injected rats with warfarin to introduce a reduction in the γ-carboxylation of newly synthesized osteocalcin. γ-carboxylation status was assessed by measuring binding to hydroxyapatite. Within 4 hours of injecting rats with warfarin, the degree of γ-carboxylation in serum osteocalcin was reduced from 95% to 10%. In contrast, matrix-bound osteocalcin was found to be fully carboxylated and possess the ability to bind bone mineral. This demonstrated that serum osteocalcin can be different from matrix-bound osteocalcin and thus, virtually all circulating osteocalcin was concluded to arise from cellular synthesis and not from release from bone matrix (Price *et al.*, 1981c). Alternatively, rats chronically treated with warfarin in which osteocalcin content in bone was only 2% of the control animals were used (Price and Williamson, 1981b). The osteocalcin levels in bone required over 30 days to return to normal when a warfarin-maintained rat was taken off the warfarin protocol. When vitamin K, an antagonist for warfarin, was administered to warfarin-maintained animals, the γ-carboxylation status of serum osteocalcin increased within a few hours and reached the control levels by 15 hours (Price *et al.*, 1981c). The return to normal serum levels was considered to reflect new cellular synthesis since the osteocalcin level in matrix in these animals was low. The experiments have, however, some limitations. The determination of carboxylation relied on hydroxyapatite binding and partially γ-carboxylated osteocalcin species may be present which do not bind hydroxyapatite and are counted as non-carboxylated. Furthermore, the osteocalcin levels were measured by the original radioimmunoassay (Price *et al.*, 1980) which most likely detects only intact osteocalcin and not truncated osteocalcin fragments (Baumgrass *et al.*, 1997). However, several further studies also state that the osteoblast is the sole source for circulating osteocalcin. Intravenous 24h infusion of PTH was found to significantly decrease serum osteocalcin concentration (Riggs *et al.*, 1986). Since infusion of PTH results in acute increase in bone resorption, it was suggested that osteocalcin reflects largely, if not exclusively,

bone formation and osteocalcin must be degraded into fragments during bone resorption which are not detectable by immunoassays. Significant correlation between serum osteocalcin and histomorphometric parameters of bone formation, but not of bone resorption, has also been detected (Brown *et al.*, 1984, Delmas *et al.*, 1985). Furthermore, a correlation between serum osteocalcin and bone mineralization rate was observed with radiocalcium kinetics while such a correlation could not be demonstrated with parameters of resorption (Charles *et al.*, 1985).

These early studies demonstrate that osteocalcin detected in circulation is not derived from bone matrix but originates exclusively from biosynthesis in osteoblasts. Later studies on patients with different metabolic bone diseases have, however, suggested that not all circulating osteocalcin fragments are derived from the metabolism of osteocalcin but also from osteocalcin embedded in bone (Gundberg and Weinstein, 1986, Taylor *et al.*, 1990, Gundberg *et al.*, 1991) and the potential contribution of resorptive osteocalcin to the circulating osteocalcin pool has been discussed (Chen *et al.*, 1996, Christenson, 1997, Fohr *et al.*, 2003). There is some evidence that both intact and fragmented osteocalcin molecules are released during bone resorption *in vitro* and these fragments are detectable by immunoassays (Kurihara *et al.*, 1998, original publication **IV** Ivaska *et al.*, 2004). Osteocalcin has also been demonstrated inside bone-resorbing osteoclasts cultured on bone slices *in vitro* (Salo *et al.*, 1997). Osteocalcin was detected by immunostaining in the resorption lacunae and released via the transcytotic route of osteoclasts. Whether these osteocalcin molecules are also released *in vivo* and contribute to the circulating osteocalcin pool is not known. It is also important to note that present studies have not been able to consider the possibility of osteocalcin derived from osteocytes. Osteocytes express osteocalcin (Aarden *et al.*, 1996) and could thus further contribute to the circulating levels (Fig.5).

2.8.2 Heterogeneity of osteocalcin in circulation

Serum osteocalcin is a heterogeneous pool of structurally different molecular forms. The most important contributor to the heterogeneity is the presence of various fragments in addition to the intact molecule (Gundberg *et al.*, 1985b). Osteocalcin fragments have been found in the serum of healthy adults (Taylor *et al.*, 1990, Garnero *et al.*, 1992, Diaz Diego *et al.*, 1998) as well as serum from patients with chronic renal failure (Gundberg and Weinstein, 1986, Taylor *et al.*, 1990), or Paget's disease (Taylor *et al.*, 1990). It has also been hypothesized that different bone pathologies could have different patterns of immunoreactive forms of osteocalcin (Taylor *et al.*, 1990). Garnero and co-workers utilized monoclonal antibodies for different regions of osteocalcin in order to clarify the proteolytic forms of circulating osteocalcin (Garnero *et al.*, 1994a). They hypothesized that the most susceptible sites to proteolysis would be the Arg-Arg bonds at position 19-20 and 43-44 leading to generation of putative osteocalcin peptides 1-43, 44-49, 1-19, 20-49, and 20-43. Four pools of fragments were detected in the serum of normal adults. The fragments were "N-terminal" 1-19, "midfragment" 20-43, "N-terminal midfragment" 1-43, and "C-terminal midfragment" 20-49 on the basis of the putative cleavage sites. The Arg^{19}-Arg^{20} bond was less sensitive to proteolytic cleavage than the Arg^{43}-Arg^{44} bond and fragments resulting from the cleavage between Arg^{19}-Arg^{20} were detected only in minute amounts. Thus, intact osteocalcin and N-terminal midfragment 1-43 represent the main circulating osteocalcin species in normal adults and approximately one third (36%) of circulating consisted of intact osteocalcin, one third (30%) of osteocalcin fragments 1-43, and one third (34%) of smaller fragments (Garnero *et al.*, 1994a). The results were, however, not based on direct sequencing of fragments. The authors were also not able to exclude the possibility of fragments resulting from cleavage at other sites than Arg-Arg bond.

The origin of heterogeneous circulating fragments is unclear. In addition to circulation, N-terminal midfragment 1-43 was also detected in the supernatant of osteoblastic cells (Garnero *et al.*, 1992). Therefore, it must be secreted by osteoblastic cells after intracellular processing of intact protein or generated from proteolytic degradation in the culture medium or circulation. Intact osteocalcin is rapidly degraded *in vitro* generating mainly large N-terminal midfragments, supporting the latter alterative. Furthermore, circulating levels of 1-43 were not altered after acute treatment with bisphosphonate indicating that it is not derived from bone resorption (Garnero *et al.*, 1994a). Osteoblast itself has been shown to be the origin of some osteocalcin peptides truncated at the N-terminus (Gundberg, 2001). It is also possible that some of the fragments are created during bone resorption. Gundberg and co-workers detected at least five additional fragments in patients with renal diseases which were not detected in healthy adults (Gundberg and Weinstein, 1986). The fragments were speculated to be released into the circulation during osteoclastic resorption of bone matrix and were presumably C-terminal. Furthermore, the authors found significant correlations between serum osteocalcin fragments and parameters of both bone formation and bone resorption. More recently, a direct demonstration of the release of immunoreactive osteocalcin fragments during bone resorption *in vitro* has been reported (Kurihara *et al.*, 1998). The fragments were, however, predominantly N-terminal. Some heterogeneity in the N-terminus has, however, been demonstrated also earlier, when two assays specific for intact osteocalcin with different antibody for the N-terminus resulted in different absolute values (Deftos *et al.*, 1992)

There have been several attempts to identify the proteases responsible for the production of osteocalcin fragments found in circulation. The presence of the plasminogen activator system on the surface of osteoblastic cells could be responsible for fragmentation of osteocalcin during biosynthesis. Plasmin cleaves intact osteocalcin *in vitro* both in solution and when bound to hydroxyapatite at a single site producing an N-terminal midfragment 1-43 and a C-terminal fragment 44-49 (Novak *et al.*, 1997). It is thus capable of producing the N-terminal midfragment 1-43 found in supernatants of osteoblast cultures and in circulation (Garnero *et al.*, 1994a). Cathepsins represent perhaps even more attractive candidates, especially for the putative resorptive fragments. Cathepsins are a heterogeneous group of lysosomal proteinases with rather broad substrate specificity (Turk *et al.*, 2001). Cathepsin L is a powerful lysosomal enzyme with endopeptidase activity and cathepsins S and T share similar properties, while cathepsins B and H show more exopeptidase activity. Several cathepsins are expressed in bone (Uusitalo *et al.*, 2000) and have been suggested to play an important role in bone resorption (Delaisse *et al.*, 1984). Cathepsin K is predominantly expressed in osteoclasts (Inaoka *et al.*, 1995, Drake *et al.*, 1996) and considered the predominant cathepsin in bone resorption and the major protease responsible for organic bone matrix degradation (Gelb *et al.*, 1996, Inui *et al.*, 1997). The cathepsins expressed in bone during resorption might contribute to generation of osteocalcin fragments (Page *et al.*, 1993, Baumgrass *et al.*, 1997, Koboyashi *et al.*, 1998). There are relatively few bonds in human osteocalcin that are cleaved by cathepsins (Baumgrass *et al.*, 1997). The N-terminal Gly^{7}-Ala^{8} bond is a sensitive site for cathepsins L, H, S and B. Furthermore, there are three additional sensitive sites between residues 41 and 45: Arg^{43}-Arg^{44} for cathepsins L, Arg^{44}-Phe^{45} for cathepsin B, and Ala^{41}-Tyr^{42} for cathepsin D. The middle region of osteocalcin appears resistant, probably due to the structural properties of the molecule and inaccessibility to the proteases (Hauschka and Carr, 1982). Degradation of rat osteocalcin by cathepsin L or B results in rather similar fragmentation with intact middle portion consisting of residues 8-39, 8-43, or 8-44, accompanied by generation of small N- and C-terminal peptides (Koboyashi *et al.*, 1998). Similar degradation has also been reported by cathepsin K (Gundberg

et al., 2002). Proteolysis of γ-carboxylated human osteocalcin by cathepsin K results in major fragments of residues 8-33 and 8-35 and minor N- and C-terminal peptides 1-3, 4-7, and 45-49. Fragments resulting from proteolytic action of cathepsins would thus be truncated at both N- and C-terminus.

In addition, microheterogeneity of the intact osteocalcin (residues 1-49) has been reported *in vitro*. Osteocalcin purified from bovine bone contained four forms of intact osteocalcin with identical molecular weight but a different isoelectric point (Delmas *et al.*, 1984). Accordingly, several immunoreactive osteocalcin forms with different molecular weight but with immunoreactivity similar to intact osteocalcin were found in osteoblast culture medium (Diaz Diego *et al.*, 1998). The origin of the microheterogeneity of intact osteocalcin is unknown but might be related to different rates of γ-carboxylation (Poser *et al.*, 1980, Cairns and Price, 1994) or glycation (Gundberg *et al.*, 1986). The presence of such forms in circulation is unclear. Additional heterogeneity might result from the presence of multimers or complexes in circulation. At the concentrations used in structural studies, the majority of osteocalcin appeared to be dimeric. In the crystal structure the calcium ions were sandwiched between two protein molecules (Hoang *et al.*, 2003) and was also found in NMR studies, with the dimer interface on the calcium-binding surface (Dowd *et al.*, 2003). Although the dimer predominates in experimental models, the *in vivo* concentration of circulating osteocalcin is in the nanomolar range, which would indicate it is all monomeric (Dowd *et al.*, 2003, Hoang *et al.*, 2003).

2.8.3 Metabolism of osteocalcin

Osteocalcin is rapidly cleared from the circulation. The clearance of radiolabeled osteocalcin after single injection into rats demonstrated that the serum half life of administrated protein was less than 5 minutes (Price *et al.*, 1981c). Most of the cleared radioactivity was recovered in the kidney which appears to be the major clearance mechanism for serum osteocalcin. The role of the kidney was further emphasized by a seven-fold elevation in plasma osteocalcin in rats after nephrectomy (Price *et al.*, 1981c). Kidney tissue homogenates degraded labeled osteocalcin by 60% in 20 minutes indicating that renal degradation may be a more predominant mechanism for clearance than renal excretion (Farrugia and Melick, 1986). Although osteocalcin clearance occurs predominantly in the kidney (Price *et al.*, 1981c) some metabolism is also seen in the liver but the degradation of the protein is clearly slower (Farrugia and Melick, 1986). Furthermore, osteocalcin levels are elevated in patients with renal failure but not in patients with liver disease (Price *et al.*, 1980). Either the uptake of osteocalcin by the kidney and liver cells differ or that the cells that degrade osteocalcin are not affected by liver disease. After two days of injections, only negligible label from injected osteocalcin was found in circulation and the label had entered the organs (Price *et al.*, 1981c). The remaining label was removed more slowly with a half-life of 6 days and it appeared to be predominantly accumulated in the skulls and long bones, revealing bone tissue as an additional organ for clearance of serum osteocalcin. Thermally decarboxylated osteocalcin accumulated with a substantially lower rate suggesting that the accumulation to bone was dependent on Gla residues. A significantly longer half-life of 91 minutes for osteocalcin has been reported in sheep after cessation of constant infusion of labelled osteocalcin (Melick *et al.*, 1988). This may, however, be related to different half-lifes of protein metabolized in kidney and the one entering bone Also the lung is a possible site for osteocalcin catabolism. Serum osteocalcin measured from the pulmonary and radial artery of human patients undergoing aorto-coronary bypass demonstrated that the proteolytic activity of pulmonary vessel endothelium involves about 5% of circulating osteocalcin (Aliberti *et al.*, 2000). Osteocalcin is known to be sensitive

to several common proteases such as trypsin, plasmin, and cathepsins (*section 2.8.2*) which could promote the metabolism in the vascular system. Intact osteocalcin is also rapidly degraded by serum proteases *in vitro* and the degradation can be prevented by protease inhibitors (Garnero *et al.*, 1994a). Osteocalcin may, however, be protected from degradation by a larger molecular weight carrier(s) in the circulation but such a complex has not yet been identified.

Since the main route for clearance is the glomerular filtration in the kidney, remnants of osteocalcin should be excreted into urine. This was first suggested when Gla residues were detected in urine samples (Gundberg *et al.*, 1983). However, Gla is found also in other proteins and urinary Gla may not be derived exclusively from osteocalcin. Taylor and co-workers were the first to demonstrate the presence of immunoreactive osteocalcin fragments in urine (Taylor *et al.*, 1990). Urine did not contain intact osteocalcin and thus, only assays recognizing osteocalcin fragments were applicable for detection. They characterized urine osteocalcin by comparing the HPLC profiles of osteocalcin isolated from the serum and urine of healthy individuals and patients with renal disease or Paget's disease. Normal serum did not contain peaks that corresponded to those found in urine suggesting that breakdown of intact osteocalcin molecules is a normal step in the metabolism and clearance of osteocalcin. However, serum from patients with renal failure contained fragments that corresponded to those found in normal urine. This indicated that the production of some of the urinary fragments occurred before renal clearance and was not a result of it and these fragments were not cleared from serum due to renal impairment. Serum sample obtained from a patient with Paget's disease also contained fragments that corresponded to some of those found in urine and in addition, "a Paget's disease specific peak" was seen in both serum and urine samples. Direct isolation and characterization methods have subsequently been used to clarify the identity of urine osteocalcin. The predominant urine osteocalcin fragments isolated from the urine sample of a pubertal boy and detectable by two-site assays spanned residues Leu^{6}-Asp^{30} and Gly^{7}-Asp^{30} (Matikainen *et al.*, 1999). Trace amounts of intact osteocalcin were detected in the solid phase of urine associated with epithelial cells or crystals of calcium oxalate. Some years later a structure for osteocalcin fragment isolated from Pagetic urine was resolved sequenced as Asp^{14}-Asp^{28} (Srivastava *et al.*, 2002). Furthermore, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-MS) was applied to elucidate the molecular weight of osteocalcin fragments in pooled urine samples collected either from healthy premenopausal women, postmenopausal women or postmenopausal women after 30-day-treatment with alendronate. The observed molecular weight (1921) fitted to the theoretical mass of osteocalcin fragment Asp^{14}-Asp^{28} identified from Pagetic urine (1914) and to the epitope mapping of the antiserum, suggesting that this fragment was a predominant fragment of osteocalcin in urine. The peak was suppressed in women receiving alendronate leading to the conclusion that this predominant urine fragment might be of resorptive origin (Srivastava *et al.*, 2002). The composition of urine osteocalcin may, however, be more complex (original publication **I** Ivaska *et al.*, 2003) which will be discussed in detail in *Results and Discussion (section 5.1.1*).

2.9 Osteocalcin as a diagnostic tool

2.9.1 Measurement of osteocalcin

The first assays for osteocalcin were directed against bovine osteocalcin due to enhanced stability and availability compared to human protein as well as high conservation of osteocalcin between species. The very first assay for the measurement of osteocalcin in serum was described in 1980 and it was a radioimmunoassay (RIA) based on rabbit polyclonal antibody

against bovine osteocalcin (Price and Nishimoto, 1980). The assay was not dependent on the degree of carboxylation or native conformation of osteocalcin but tryptic peptide fragments were not immunoreactive. The first enzyme immunoassay (EIA) was introduced in 1986 (Tanaka *et al.*, 1986). Quantitative bone histomorphometry and calcium kinetics studies demonstrated that serum osteocalcin levels correlated with bone formation rate but not with parameters of bone resorption and validated osteocalcin as a marker of bone formation (Brown *et al.*, 1984, Charles *et al.*, 1985, Delmas *et al.*, 1985, Eastell *et al.*, 1988, Wand *et al.*, 1992). Already the early clinical studies suggested osteocalcin as a potential marker of bone pathophysiology and elevated concentrations were reported in patients with variety of metabolic bone diseases including primary hyperparathyroidism, Paget's disease, cancer metastases involving bone (Price *et al.*, 1980, Deftos *et al.*, 1982), postmenopausal osteoporosis (Delmas *et al.*, 1983b, Brown *et al.*, 1984), and aging (Delmas *et al.*, 1983a). In the 1980s, the biochemical tests used in the assessment of bone metabolism were mostly the measurement of total ALP in blood and hydroxyproline in urine. Therefore, the assays for bone-specific osteocalcin provided major advantages and raised high hopes over the rather unspecific analytes (Price *et al.*, 1980).

The differences between osteocalcin assays were first reported in the mid 1980s (Gundberg *et al.*, 1985b) demonstrating that one assay cannot be substituted for another. This was confirmed by a series of studies demonstrating marked differences between different osteocalcin assays (Delmas *et al.*, 1990, Deftos *et al.*, 1992, Diaz Diego *et al.*, 1994, Masters *et al.*, 1994). The results of different assays could not be compared even after attempts to normalize the values against groups of healthy adults measured with the same assays. The presence of multiple immunoreactive osteocalcin fragments in the circulation has definitely potential to introduce discrepancies in serum osteocalcin levels measured by different immunoassays which would depend upon the epitope specificity of the particular immunoassay used. Furthermore, the specificity and cross-reactivity of the assays are usually poorly defined and the specificity of assays may even differ from that claimed by the manufacturers (Colford *et al.*, 1997, Souberbielle *et al.*, 1997). The divergent results obtained by different assays may also be due to cross-reactivity of antibodies to other molecules, especially at lower concentrations of analyte. When a well-defined reference material is lacking, some of the differences observed between assays and laboratories could be due, in part, to the origin, purity, and estimated concentration of standard material. There is not even clear consensus which fragment would be of greatest use as a calibrator and whether the standard should be isolated from human bone, produced by chemical synthesis, or as a recombinant protein (Houben *et al.*, 1997). The lack of international standards for osteocalcin has clearly limited the development of commercial assays (Lee *et al.*, 2000). However, the use of a common reference material is unlikely to solve the discordant results until the differences between antibodies in terms of epitope specificity and reactivity with multiple forms in serum are addressed (Delmas *et al.*, 1990).

Intact osteocalcin is markedly unstable *in vitro*, introducing an additional level of complexity to osteocalcin assays (Banfi and Daverio, 1994, Garnero *et al.*, 1994a, Käkönen *et al.*, 2000). Osteocalcin in serum samples is degraded within a few hours at room temperature after sampling and also to some extent at +4°C or as a result of repeated freeze-thaw cycles (Power and Fottrell, 1991, Diaz Diego *et al.*, 1994, Käkönen *et al.*, 2000). Long-term storage in aliquots in temperatures below -70°C can be recommended to reduce the loss of immunoreactivity. It can also be prevented to certain extent by antiproteolytic agents (Banfi and Daverio, 1994). Degradation of intact osteocalcin results in generation of N-terminal midfragment 1-43. The measurement of this fragment alone or in a combination of intact osteocalcin by specific assays has proved to be advantageous over intact osteocalcin in terms of

improved stability of immunoreactivity during storage (Garnero *et al.*, 1992, Garnero *et al.*, 1994a, Rosenquist *et al.*, 1995). However, not all assays measure intact osteocalcin and N-terminal midfragment equivalently but may overestimate the amount of fragments (Hellman *et al.*, 1996). There has been an attempt to overcome the heterogeneity of circulating osteocalcin by selecting an assay specific for intact protein as an index of osteoblast activity but this has not led to improved clinical performance and has encountered stability problems (Colford *et al.*, 1999). Osteocalcin is also sensitive to haemolysis (Tracy *et al.*, 1990) and the general considerations of biological variability of markers, including the high level of within-person variability are valid for serum osteocalcin as well (*section 2.4.3*). Circulating osteocalcin demonstrates a circadian rhythm peaking late at night and early morning and dropping during late morning and early afternoon (Gundberg *et al.*, 1985a) and is influenced by defects in renal function (Delmas *et al.*, 1983c, Melick *et al.*, 1985, Gundberg and Weinstein, 1986). Furthermore, genetic factors may contribute on the variability of serum osteocalcin. Twin studies have suggested that genetic factors may account as much as 82% of the total variance in circulating osteocalcin levels (Kelly *et al.*, 1991); although lower estimates (37%) have been reported in others (Garnero *et al.*, 1996a). Genome-wide scannings have identified two regions in chromosomes 16 and 20 as putative sites influencing circulating osteocalcin levels (Mitchell *et al.*, 2000). A candidate gene study demonstrated an association of vitamin D receptor gene polymorphisms with serum osteocalcin levels (Morrison *et al.*, 1992) although other studies have failed to replicate this finding (Garnero *et al.*, 1995, Garnero *et al.*, 1996c, McClure *et al.*, 1997).

Currently there are numerous immunoassays available in RIA, EIA, immunoradiometric assay (IRMA), and enzyme-linked immunoassay (ELISA) formats (Gundberg, 1998, Lee *et al.*, 2000, Seibel, 2000, Seibel *et al.*, 2002). The first generation RIA assays have mostly been replaced by more convenient second generation ELISA assays employing monoclonal antibodies with characterized specificity in order to reduce the problems related to variability. Many of the two-site assays measure the intact form of osteocalcin (Deftos *et al.*, 1992, Garnero *et al.*, 1992, Hosoda *et al.*, 1992, Jaouhari *et al.*, 1992, Monaghan *et al.*, 1993, Parviainen *et al.*, 1994, Hellman *et al.*, 1996) but in addition, several assays detect shorter proteolytic fragments, especially the N-terminal midfragment (Garnero *et al.*, 1994a, Eguchi *et al.*, 1995, Rosenquist *et al.*, 1995, Hellman *et al.*, 1996). The increased interest in osteocalcin also led to the development of commercial assay kits (for review, see Gundberg, 2001). Automated methods for clinical purposes are available on e.g. Roche Elecsys and Nichols Advantage systems. However, there is no definitive reference method for the measurement of osteocalcin in serum. The detection of intact osteocalcin or its fragments is an important issue in the choice of osteocalcin assays to be used and the potential contribution of osteoclastic resorption to immunoreactive species detected by certain assays has to be considered. However, in many cases the exact antibody specificity is still unknown and the uncharacterized methods should be used with caution. Current understanding suggests that measurement of intact osteocalcin together with large N-terminal midfragment can give an accurate assessment of bone turnover (Garnero *et al.*, 1994a, Rosenquist *et al.*, 1995, Gundberg, 1998).

Serum osteocalcin was, soon after its discovery, considered a promising bone-specific analyte. Thus, there is a large number of studies of variable design published in peer-reviewed journals in which osteocalcin concentration has been measured in serum samples. Accordingly, there are several reviews available concerning the use of osteocalcin assays in various disease states (Gundberg, 1998, Watts, 1999, Lee *et al.*, 2000, Seibel, 2000). The following sections will focus on three major applications: the estimation of bone loss, monitoring the efficacy of

antiresorptive therapy, and the prediction of fracture risk. It should be appreciated that due to variations in assay performance and antigens, the direct comparison of results between clinical studies should be done with caution. Although technically the same analyte is used, the results from studies using different assays are not necessarily comparable. As there are no assays commercially available for urine osteocalcin measurement, the results of in-house assays for urine osteocalcin will be discussed in *Results and Discussion.*

2.9.2 Evaluating the rate of bone loss

Cross-sectional studies have demonstrated a significant relationship between bone turnover markers and BMD at different skeletal sites (Mazzuoli *et al.*, 1985, Stepan *et al.*, 1987, Melton *et al.*, 1997) and the correlation becomes stronger with advancing age (Garnero *et al.*, 1996c). However, the relationship between a single measurement of marker and a single measurement of BMD is not sufficient to allow for estimating BMD level on the basis of marker measurement, especially in individual patients (Melton *et al.*, 1997). Thus, biochemical markers are not substitutes for the measurement of BMD and prediction of bone mass and therefore, the diagnosis of osteoporosis cannot be based on the levels of biochemical markers of bone turnover. The measurement of a marker may, however, be useful in determining whether the bone loss in accelerated. The mean values of bone resorption and formation markers are typically higher in postmenopausal women compared to premenopausals and the increase in resorption markers precedes by a few months the increase in formation markers (Stepan, 2000). The increase is sustained decades after menopause (Garnero *et al.*, 1996c). The concentrations of bone markers are typically greater in conditions of high turnover demonstrating an association with rapid bone loss (Ross and Knowlton, 1998). Biochemical markers reflect the whole-body rates of bone resorption and formation and therefore, markers may provide a more representative index of the overall skeletal bone loss than the measurement of changes in bone mass.

Longitudinal evaluation of bone mineral content (BMC) values in early postmenopausal women has suggested that one third of women lose significant amounts of bone rapidly (“fast losers”) compared to the two thirds who lose bone more slowly (“slow losers”) (Christiansen *et al.*, 1987). The fast losers appeared to have elevated concentrations of markers compared to the slow losers (Riis *et al.*, 1996) and markers correlated with changes in BMC for 12-15 years after menopause (Christiansen and Riis, 1990). Thus, bone marker levels may be helpful in the prediction of future bone loss rate but at the moment, it is not possible to use a single measurement of bone marker to predict the absolute rate of bone loss in an individual (Rogers *et al.*, 2000). Significantly elevated marker levels in postmenopausal women could rather be considered a risk factor for rapid bone loss in the coming years. Alternatively, the magnitude of change in markers in the immediate postmenopausal period might be beneficial in identifying fast bone losers (Seibel *et al.*, 2002). More prospective studies are required to address the relationship of baseline marker levels and rates of future bone loss assessed by serial BMD or BMC measurements. One early long-term prospective study indicated that the early postmenopausal women classified as fast losers on the basis of markers lost significantly more bone during a 12-year follow-up than women classified as slow losers, the total bone loss being 26.6% vs. 16.6%, respectively (Hansen *et al.*, 1991). However, this study utilized early, rather non-specific markers of bone turnover such as urinary calcium, urinary hydroxyproline and serum total ALP.

Correlation between serum osteocalcin measurement at the baseline and subsequent rate of bone loss has been addressed in a number of studies with shorter follow-up times (reviewed in

Stepan, 2000). A significant association has consistently been demonstrated for serum osteocalcin and the rate of bone loss in distal radius. Serum osteocalcin correlated significantly with bone loss assessed by serial measurement of BMD at 4-month intervals in the forearm in 84 peri-and postmenopausal women followed for 2-3 years (R value from -0.32 to -0.47) (Slemenda *et al.*, 1987). In a larger cohort of 305 postmenopausal women aged 50-88 years serum osteocalcin together with other markers (urinary CTX and NTX, serum CTX and PINP) measured at the baseline correlated with the rate of bone loss at the radius measured annually over 4 years (R value -0.45) (Garnero *et al.*, 1999a). Each women was also classified as a low or high bone turnover subject using the mean value of premenopausal women +2SD as a cut-off. Women with baseline marker values indicating high bone remodeling rate lost bone two to six times faster, depending on the marker, over the next four years than women with baseline marker values in the premenopausal range. The loss of bone in the low osteocalcin group during 4 years was ~0.5% whereas in the high osteocalcin group it was nearly 2.5%. The association of serum osteocalcin to vertebral bone loss is more inconsistent (Stepan, 2000), although a strong relationship between baseline serum osteocalcin and rates of spinal bone loss has been demonstrated in some studies (Yoshimura *et al.*, 1999, Rogers *et al.*, 2000, Bruyere *et al.*, 2003). The association to bone loss in the hip is questionable since the prospective studies have demonstrated only modest or no correlation for serum osteocalcin to bone loss at the total femur or femoral neck (Bauer *et al.*, 1999, Dennison *et al.*, 1999, Yoshimura *et al.*, 1999, Stepan *et al.*, 2004). Osteocalcin measurement is currently no able to predict a change in hip BMD accurately enough in an individual. As the rate of bone loss differs between various skeletal sites and different studies have utilized different body sites and follow-up times, more prospective studies are clearly needed in order to confirm the correlation between markers and bone loss. Currently, there is no clear consensus if markers provide benefit in identifying fast losers (Seibel *et al.*, 2002).

2.9.3 Monitoring the efficacy of antiresorptive treatment

The aim of osteoporosis therapy is to reduce the occurrence of fragility fractures. This is difficult to assess because the incidence of fractures is low and the lack of fractures after certain treatment period in an individual patient does not necessarily imply that the treatment is effective. Prolonged use of the drug, if little or no benefit is achieved, is undesirable given their costs and potential complications. On the other hand, feedback on the effectiveness could improve the compliance to the medication (for review, see Delmas, 2000).

BMD measurement is often used to estimate treatment efficacy. Measurements are performed at the initiation of the treatment and after one or two years and the potential antiresorptive agents used in postmenopausal osteoporosis induce a significant increase in BMD from baseline during this period (Eastell, 1998, Delmas, 2000). The magnitude of response varies according to the drug and skeletal site and the largest decreases are seen at sites which contain high amounts of trabecular bone such as the vertebral column (Eastell, 1998). Alendronate, a potent bisphophonate drug, induces a 3-4% increase in spine BMD at 2 years and 8-9% increase at 3 years (Liberman *et al.*, 1995, Hosking *et al.*, 1998). Other drugs such as raloxifene produce changes of smaller magnitude which are more challenging to detect with BMD (Delmas *et al.*, 1997). Densitometry is unlikely to be useful to detect changes before one year of therapy because the changes are to be within the variability of the technique (Delmas *et al.*, 2000).

An alternative is to use bone markers to monitor rapid changes in bone metabolism in response to treatment. Markers appear to decrease in response to hormonal therapy, SERMS,

and bisphosphonates and the marker levels reach a drug- and dose-dependent plateau level within months (Delmas, 2000). The short-term (3-6 months) decrease in bone markers is strongly related to the long-term increase in BMD in women receiving antiresorptive therapy and markers could be used especially within the first six months after initiation of the therapy when BMD changes are too small to be used clinically (Looker *et al.*, 2000). In individual patients the interpretation of change in marker level is, however, complicated by the large within-person variability of markers. Despite the variability, the signal-to-noise ratio for markers appears to be better at 3 to 6 months than for BMD at 1 or 2 years (Ravn *et al.*, 1999, Seibel *et al.*, 2002). Whether combination of different bone markers will add to the usefulness of single measurements remains to be evaluated.

A marked decrease in serum osteocalcin has been associated with a positive response in BMD in a number of studies. Hormone replacement therapy induces a rapid decrease in resorption markers already after 2 weeks reaching a plateau within 3-6 months (Johansen *et al.*, 1988). The decease in formation markers is delayed and plateau is reached within 6-12 months. A significant reduction in serum osteocalcin is seen after three months of hormone replacement therapy suggesting serial determination of serum osteocalcin useful in the determination of the effect of estrogen therapy (Johansen *et al.*, 1988; Riis *et al.*, 1995, Garnero *et al.*, 1999b). Accordingly, the reduction of bone turnover by alendronate rapidly decreases serum osteocalcin levels in postmenopausal women (Harris *et al.*, 1993, Garnero *et al.*, 1994b) and changes in serum osteocalcin correlate well with changes in spinal or femoral BMD (Ravn *et al.*, 1999). Similar responses are also observed in treatment with another bisphosphonate, ibandronate (Ravn *et al.*, 1996, Delmas *et al.*, 2004).

The ability of a marker to detect responses is not only dependent on the magnitude of response to therapy but also on the variability. Even if the response of a treatment group is statistically significant, it is not useful clinically unless the change in an individual patient is greater than would be expected on the basis of variability. The identification of responders to therapy can be improved by calculating a least significant change (reference change value). It is the minimum change in the quantity that would imply a true biological response and it defines a threshold for marker change as the change greater than the precision error in a single individual (Hannon *et al.*, 1998, Eastell *et al.*, 2000, Hannon *et al.*, 2004). Unfortunately, markers were not the primary interest in most clinical drug therapy trials and the studies are still inconsistent regarding the ability of change in the marker for predicting the magnitude of change in BMD from the baseline level (Looker *et al.*, 2000). Furthermore, there have been only few studies relating the short-term changes in osteocalcin with therapy to the reduction in risk of a new fracture. Such association has recently been reported at least for raloxifene- (Sarkar *et al.*, 2004) and ibandronate-induced (Delmas *et al.*, 2004) changes in serum osteocalcin.

2.9.4 Estimating the risk of osteoporotic fractures

An important aspect in assessing the clinical utility of markers is their ability to predict fracture risk. Identification of women at high risk could be of great clinical importance in targeting women for preventative treatment. The relationship between markers and fracture risk has been investigated in retrospective studies comparing marker levels in controls and in patients with fractures. In patients with very recent osteoporotic fracture serum osteocalcin levels have been found to be 20-30% lower compared to the controls (Akesson *et al.*, 1993, Akesson *et al.*, 1995). The changes in bone turnover in retrospective studies may, however, be due to acute changes related to the trauma and to changes in bone metabolism occurring after the fracture. The measurement of marker levels at the baseline before the occurrence of

fractures and relating them to subsequent risk of fractures is perhaps a more valid methodology (Garnero and Delmas, 2002). However, prospectively designed studies need to include a considerable number of individuals and extend over long-enough periods of follow-up to collect enough fractures, which are relatively rare clinical events, in order to have statistical power. Fracture risk should also be assessed separately for different skeletal sites due to potential heterogeneity in the pathogenesis of different fractures (Garnero, 2000). The predictive ability may also differ for fractures representing senile osteoporosis affecting mainly the cortical bone compared to postmenopausal osteoporosis engaging predominantly the trabecular bone.

There is a limited number of adequate prospective studies focusing on the utility of biochemical markers in prediction of fracture risk (Akesson *et al.*, 1995, van Daele *et al.*, 1996, Garnero *et al.*, 1996b, Chapurlat *et al.*, 2000, Garnero *et al.*, 2000, Luukinen *et al.*, 2000, Ross *et al.*, 2000, Bruyere *et al.*, 2003, Gerdhem *et al.*, 2004). The French EPIDOS study was a population-based cohort of 7598 healthy women more than 75 years of age with a mean follow up of 22 months (Garnero *et al.*, 1996b). The Study of Osteoporotic Fractures (SOF) included 295 women for a follow-up period of mean 3.3 years (Bauer *et al.*, 1999). The OFELY study consisted of 435 healthy postmenopausal women (age 50-89) followed prospectively for an average of 5 years (Garnero *et al.*, 2000). The Hawaii Osteoporosis Study (HOS) consisted of 512 community-dwelling postmenopausal women with average follow-up of 2.7 years (mean age 69 years) (Ross *et al.*, 2000). In the Osteoporosis Prospective Risk Assessment (OPRA) study 1044 women, all 75-years of age, were followed prospectively for fractures for 3 - 6.5 years (original publication **III** Gerdhem *et al.*, 2004). Other prospectively designed studies include the Swedish study of 328 women aged 40-80 years (Akesson *et al.*, 1995) and the nested case-control analyses from the Rotterdam Elderly Study in the Netherlands, a prospective cohort study involving over 10000 women aged 55 or over (Hofman *et al.*, 1991, van Daele *et al.*, 1996). In the Belgian study, the 603 subjects (age 50-80 years) were members of the placebo arm in a bisphosphonate intervention trial and were followed prospectively for 3 years (Bruyere *et al.*, 2003). Most studies still continue to obtain longer follow-up times.

Serum osteocalcin has been evaluated in most prospective studies listed above, although different assay formats have been used. Results demonstrate no or very low association between serum osteocalcin and future fracture risk. In the EPIDOS study (109 hip fracture cases and 292 controls) serum markers of bone formation, including total serum osteocalcin and bone-specific ALP, were not associated with an increased hip fracture risk during a 2-year follow-up (Garnero *et al.*, 1996b). This was reproduced in the SOF study which had 150 hip fracture cases and showed no significant relationship between levels of serum osteocalcin and hip fractures (Bauer *et al.*, 1999). The OFELY study focused on the vertebral and peripheral fractures and demonstrated that increased levels of serum osteocalcin were associated with increased vertebral and peripheral fracture risk independent of BMD but the association was not statistically significant (Garnero *et al.*, 2000). In the OPRA study, none of the four different assays for serum osteocalcin tested was able to predict prospective fractures at hip (41 fractures), vertebra (49), or any skeletal site (178) during the mean follow-up time of 4.6 years (original publication **III** Gerdhem *et al.*, 2004). A moderate association to vertebral fractures has been demonstrated by using short-term (three months) changes in serum osteocalcin instead of baseline values (Bruyere *et al.*, 2003). The relative risk of having a new vertebral fracture (71 cases) over 3 years was 0.31 (0.15-0.65, 95% CI) when the subject was in the lowest compared with the highest quartile for changes in serum osteocalcin. The relative risk was non-significant for baseline serum osteocalcin values.

The only prospective study to assess urine osteocalcin in prediction of fracture risk is the OPRA study which will be discussed in *Results and Discussion.*

The levels of serum undercarboxylated osteocalcin above the premenopausal range have been associated with 2-3 fold increase in the risk of hip fracture in a cohort of 195 elderly institutionalized women followed for 3 years (Szulc *et al.*, 1993) and in the EPIDOS study of elderly healthy women (104 fractures and 255 non-fractured controls) (Vergnaud *et al.*, 1997). The ratio between carboxylated and total osteocalcin, an indirect index of undercarboxylated osteocalcin, has also been associated with increased fracture risk in home-dwelling women and men, especially those older than 80 years (Luukinen *et al.*, 2000). Impaired γ-carboxylation may be an indirect index of both vitamin D and K deficiency in the elderly.

In contrast to serum osteocalcin and other markers of bone formation, very consistent data has been reported on the relationship between resorption markers and risk of fracture in most large prospective studies (van Daele *et al.*, 1996, Garnero *et al.*, 1996b, Chapurlat *et al.*, 2000, Garnero *et al.*, 2000, Gerdhem *et al.*, 2004). These studies indicate that resorption markers may predict vertebral and peripheral fractures in women aged 50-80 years and hip fractures in the very elderly (Garnero, 2000). In spite of the promising results, bone mass estimated by densitometry is still regarded as the best predictor of osteoporotic fractures (Melton *et al.*, 1993, Garnero, 2000).

3 AIMS OF THE STUDY

At the initiation of the present study, the levels of circulating osteocalcin had already been used for years as a marker of bone formation (Lee *et al.*, 2000). The discordant results obtained from different assays and the instability and heterogeneity of osteocalcin in circulation had, however, limited the widespread usage of serum osteocalcin assays in clinical applications (Delmas *et al.*, 1990). The presence of immunodetectable osteocalcin in urine samples had also been reported (Taylor *et al.*, 1990) and the molecular structure of a predominant urinary osteocalcin fragment had been published by our research group (Matikainen *et al.*, 1999).

The access to a selection of carefully characterized monoclonal antibodies against various epitopes on osteocalcin (Hellman *et al.*, 1996) prompted us to utilize these antibodies as tools to investigate the structure of urine osteocalcin and its role in bone metabolism in more detail. During the course of the study we also aimed to better understand the contribution of different bone cells on circulating osteocalcin. For this purpose, immunoassays were modified for *in vitro* applications and used to study osteocalcin in osteoclast cultures. The following specific aims were set for the study:

- Identification of urinary osteocalcin fragments in urine samples obtained from individuals belonging to different age groups.
- Development and validation of immunoassays for the detection of various molecular forms of urinary osteocalcin.
- Evaluation of urinary osteocalcin a marker of bone metabolism in clinical settings related to bone biology, including monitoring the efficacy of osteoporosis and prediction of fracture risk.
- Evaluation of osteocalcin in bone cell cultures in order to clarify the contribution of each type of bone cell in serum and urinary osteocalcin levels.

4 MATERIALS AND METHODS

4.1 Subjects and sample collection

4.1.1 Urine samples for isolation of osteocalcin (I)

Urine samples were collected from five healthy volunteers, two males aged 4 and 8 years and three females aged 24, 33, and 75 years. Also, one sample was obtained as a pool from four females aged 12 - 16 years (an equal amount of osteocalcin from each individual was used). Samples were obtained as the first morning void and stored at -70°C until analyzed. Subjects did not receive any medication affecting bone metabolism.

4.1.2 Pre- and postmenopausal women (II)

Urine samples were collected as second morning void from 91 healthy women, frozen at –70 °C, and stored at –20 °C. Women were classified into pre- and postmenopausal groups according to menstrual status and the postmenopausal group was further divided into subjects with or without hormone replacement therapy (HRT). The premenopausal group consisted of 58 women (median age 41 years, interquartile range 36 - 45), the postmenopausal group without HRT of 20 women (54 years, interquartile range 52 - 60), and the postmenopausal groups on HRT of 13 women (57.5 years, interquartile range 56 - 59).

4.1.3 Intervention with alendronate (unpublished)

A questionnaire was sent to a random population sample of 3000 women from a cohort born from 1942 to 1947 living in the city of Tampere, Finland. Two hundred and sixty-three women were invited to a screening examination, and 164 of them were selected for the study. Inclusion criteria were no previous bone fractures, postmenopausal for 1-5 years, no current or previous use of estrogen, corticosteroids, bisphosphonates or other drugs affecting bone metabolism, no current or previous illnesses affecting bone metabolism, no contraindication to alendronate, femoral neck BMD no more than 2.5 SD below the young normal women's reference value as determined by DXA, and FSH level greater than 30 IU/L. (Uusi-Rasi *et al.*, 2003). The study was a one-year double-blind randomized placebo-controlled intervention trial with two experimental groups; one receiving 5 mg of alendronate (Fosamax, Merck & Co, USA) daily and the other receiving a placebo (placebo pills similar to the effective ones donated by Merck & Co). All subjects in both groups also received a daily supplement of calcium carbonate (630 mg) and vitamin D (200 IU = 5 µg; Citracal + D, Mission Pharmacal, USA). Originally, the study contained the following four experimental groups: 1) alendronate + exercise; 2) alendronate; 3) placebo + exercise; 4) placebo. Because exercise had no effect on any of the bone markers (Uusi-Rasi *et al.*, 2003) groups 1 and 2 were combined into one group receiving alendronate, and groups 3 and 4 into one group receiving the placebo. Baseline urine and serum samples were collected before the start of the intervention. The follow-up samples were collected at 3, 6, and 12 months. 24-h urine collections were performed after 12 h fasting together with serum samples, and all samples were stored at –70 °C until analysis. Alltogether 152 women had both serum and urine sampled analyzed for osteocalcin, 76 in the placebo group (median age 52.5 years, interquartile range 51.3 - 55.0) and 76 in the alendronate group (median age 53.4 years, interquartile range 51.9 - 54.9).

4.1.4 Elderly women (I, II, III)

1044 women, all 75-years old, were randomly selected from the population files of the city of Malmö, Sweden during 1995-1999 to enter the Osteoporosis Prospective Risk Assessment

study (OPRA). Inclusion was made by sending an invitation letter to 1604 women the week following the 75th birthday of each woman. Illness or unwillingness was given as reasons for non-participation in 152 and 376 women, respectively. Despite several attempts, 32 women were not reached. In four women neither urine nor serum was available and these were excluded from this study. No exclusion criteria were used. Urine samples were obtained as first morning void and stored at -80°C. Non-fasting serum samples were obtained between 08.00 and 13.00 and stored at -80°C. Urine was available in 1019 women and serum in 1024 women.

All clinical studies were approved by the local ethics committee and performed in accordance with the Declaration of Helsinki. Also, an informed consent was obtained for all subjects taking part to the studies.

4.2 Immunoassays for osteocalcin (I-IV)

4.2.1 Antibodies, calibration, and reagents

Monoclonal antibodies (MAbs) used in this study have been described in detail previously (Hellman *et al.*, 1996). Briefly, MAb 3G8 requires the full-length molecule for recognition; MAbs 8H12 and 6F9 bind to residues in region 7-19 and MAbs 2H9 and 3H8 have an epitope on the residues spanning positions 20-43. In addition, MAb 3H8 favors the Gla-containing forms of osteocalcin with 8% cross-reactivity towards non-carboxylated osteocalcin. The MAbs were raised either against bovine osteocalcin (3G8 and 3H8) or a fusion protein consisting of glutathione-S-transferase and human osteocalcin (6F9, 8H12 and 2H9). MAb 6F9 is specific to human osteocalcin, while other MAbs recognize also bovine osteocalcin. Furthermore, MAbs 3H8, 2H9, and 8H12 cross-react with rat osteocalcin. Antibodies were either biotinylated with 50-fold molar excess of biotin-isothiocyanate or labeled with 200-fold molar excess of europium(III) chelate as described previously (Hellman *et al.*, 1996). Synthetic peptide of human osteocalcin amino acids 1-49 (Gla at positions 17, 21, and 24) was purchased from Advanced Chemtech and biotinylated with 30-fold excess of biotin-isothiocyanate. The reaction was carried out in 50 mM $NaHCO_3$-Na_2CO_3 buffer, pH 9.8 at +4°C for 5 hours. Buffer exchange was performed with NAP-5 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 15mM NaN_3, pH 7.75.

Synthetic peptide of human osteocalcin amino acids 1-43 (Gla at positions 17, 21, and 24, Advanced Chemtech) was used as a calibrator for urine osteocalcin assays and synthetic peptide corresponding to intact human osteocalcin of residues 1-49 (Gla at positions 17, 21, and 24) from the same manufacturer was used as a calibrator in serum and cell culture assays. Calibrators were diluted in 50 mM Tris, 150 mM NaCl, 5 g/l NaN_3, pH 7.75 buffer containing 10 g/l bovine serum albumin and stored in aliquots at -70°C. At the beginning of the study (I), pubertal urine diluted in Assay buffer was used as a calibrator and osteocalcin concentrations in dilutions were determined with 6F9/3H8, or U-MidOC assay (*section 4.2.2*).

Streptavidin-coated plates were purchased from Innotrac Diagnostics, Turku, Finland. Assay buffer, Delfia Wash Solution, Delfia Enhancement solution, and Victor2 were from PerkinElmer Life Sciences / Wallac, Finland.

4.2.2 Immunoassay procedures

Two-site assays for urine osteocalcin (Fig.6A). 400 ng of bio-6F9 (U-MidOC) or bio-2H9 (U-LongOC) in 50 µl of Assay buffer was added to the wells of streptavidin-coated microtiter plates, incubated with continuous shaking at room temperature (22°C) for 30 minutes, and washed twice with Delfia Wash Solution. The samples or calibrators (10 µl) were added,

followed by 100 ng of Eu-labeled 3H8 (U-MidOC) or 200 ng of Eu-labeled 2H9 (U-LongOC) in 50 µl of Assay buffer containing 5 mmol/l EDTA. After two hours shaking at room temperature (22°C), the plates were washed six times and 200 µl of Delfia Enhancement Solution was added to each well. After 30 minutes shaking, time-resolved fluorescence was measured using a Victor2 Multilabel Counter.

Competitive assay for urine osteocalcin (Fig.6B). Streptavidin-coated microtiter plates were pre-washed once and coated with bio-hOC (1.7 ng per well) in 50 µl of Assay buffer for 30 minutes in continuous shaking. Plates were washed four times and samples or calibrators (30 µl) were added together with 2 ng of Eu-labeled 3H8 in 100 µl of Assay buffer. Incubation was carried out for one hour after which the wells were washed four times. 200 µl of Delfia Enhancement Solution was added to each well and the time-resolved fluorescence was detected after 30 minutes shaking. The initial trial for competitive immunodetection (I) utilized 7.5 ng of bio-hOC per well and 1h incubation. Furthermore, all washing steps were performed six times.

Immunoassays for osteocalcin in cell culture medium (Fig.6C). MAb 3G8 or 8H12 were used as biotinylated capture antibodies and MAb 2H9 or 3H8 as europium-labeled tracer antibodies resulting in three different two-site combinations: 3G8/2H9 (I-OC, for intact OC), 8H12/2H9 (M-OC, for the majority of OC), and 8H12/3H8 (T-OC, for total OC). Samples or calibrators (10 µl of each) were added to the wells of streptavidin-coated plates. A mixture containing 100 ng of bio-MAb and 100 ng of Eu-MAb in 50 µl of Assay Buffer containing 5 mmol/l EDTA was added to each well. After two hours of shaking at room temperature (22°C), the plates were washed six times with Delfia Wash Solution and 200 µl of Delfia Enhancement Solution was added to each well. After 30 minutes of shaking, time-resolved fluorescence was measured using the Victor Multilabel Counter.

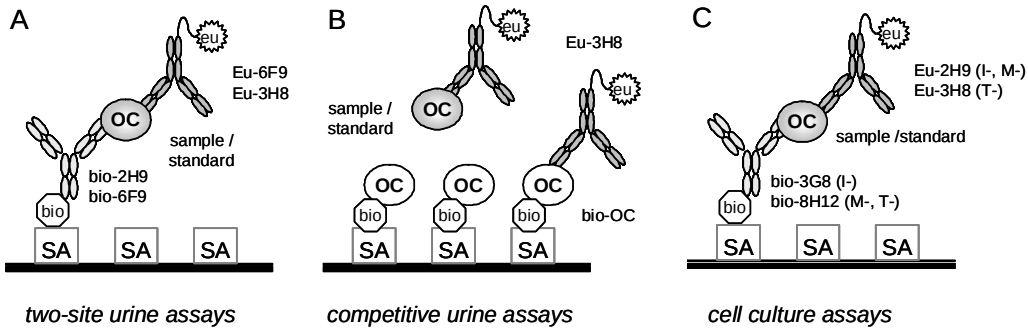

***Figure 6**. The principles of osteocalcin immunoassays.* A) Urinary assays U-MidOC and U-LongOC were two-site sandwich immunoassays based on biotinylated capture-antibody (MAb) (bio-2H9, -6F9) and Eu-labeled tracer-MAb (Eu-6F9, -3H8). Assays were performed in two steps: first bio-MAb was immobilized on streptavidin-coated plate (SA) and then, the analye (OC) and tracer were added. B) Urinary assay U-TotalOC was a competitive immunoassay based on biotinylated osteocalcin (bio-OC) immobilized on SA-plates. The tracer (Eu-3H8) was added simultaneously with the analyte (OC), and the bound bio-OC and OC in solution competed for binding to the tracer. C) The same principle as in A) was applied to I-OC, M-OC and T-OC assays for cell culture samples but bio-MAb, OC and Eu-MAb were all added in one step simultaneously.

4.2.3 Characterization of immunoassays

Specificity of urine osteocalcin assays towards different fragments. The ability of assays to recognize different molecular forms of osteocalcin present in human urine was determined using osteocalcin fragments isolated and fractionated from pre-pubertal urine. Sample was collected as the first morning void from an 11-year-old boy and stored at -70°C. The total urine osteocalcin was isolated and fractionated as will be described in *section 4.3*. Briefly, urine sample was extracted with a solid phase column followed by immunoaffinity chromatography. Different molecular forms were separated by fractionation on a high-performance liquid chromatography column and collected fractions were analyzed for osteocalcin with all three assays. To determine the molecular masses and sequences of isolated fragments, fractions containing osteocalcin were analyzed by mass spectrometry and N-terminal sequencing as will be described.

Analytical characteristics of urine osteocalcin assays. The calibration series covered the range from 0.3 to 90 ng/ml (U-MidOC), from 0.04 to 30 ng/ml (U-LongOC), or from 35 to 840 ng/ml (U-TotalOC). The ranges were appropriate to measure osteocalcin concentrations in clinical urine samples. Imprecision was determined using control samples which were prepared by diluting a urine sample (obtained as the first morning void from an 8-year-old boy) in a zero calibrator in two concentrations, low and high (respective OC concentrations in U-MidOC 9.1 and 44.7 ng/ml, in U-LongOC 0.08 and 0.55 ng/mll and in U-TotalOC 125 and 380 ng/ml). Within-run imprecision was defined as the variation of twelve replicate determinations. Total imprecision was determined by calculating the variation for control samples from 15 separate assays (25 for the competitive assay). The detection limit (limit of the blank) was determined using calibrator free of analyte (zero calibrator) and defined as the concentration corresponding to a signal two SD above (two-site assays) or below (competitive assay) the mean of zero calibrator measured in twelve replicates in a single assay. Linearity of dilution was evaluated by diluting three urine samples into zero calibrator in 2-, 4-, and 8-fold dilutions and calculating for each dilution as a percentage of the value obtained from the diluted sample to the value obtained from undiluted sample. Recovery of added analyte was tested by adding a known amount of calibrator peptide to three individual urine samples containing a known amount of osteocalcin and calculated as the percentage of the added analyte recovered. The exact amounts of added analyte were assayed simultaneously (79 ng/ml and 19 ng/ml for U-MidOC, 10.5 ng/ml and 2.4 ng/ml for U-LongOC and 219 ng/ml and 75 ng/ml for U-TotalOC) and the recoveries were calculated as a percentage of added analyte recovered. For stability studies, fresh first morning void urine was collected from three healthy females and stored at +4°C until delivered to the laboratory within 2 hours after collection. Samples were stored in aliquots either at +4°C or room temperature for 0h, 2h, 4h, 8h, 1 day, 3 days, or 5 days. After incubation, samples were frozen and stored at -70°C until measured simultaneously.

Seasonal and circadian variation of urine osteocalcin. Seasonal variation was evaluated from the cohort of elderly women described above (*section 4.1.4*). Urine samples were collected throughout all seasons and the number of samples collected each month, from January to December, was 96, 118, 111, 127, 83, 53, 32, 50, 88, 104, 112, and 69, respectively. Circadian variation was evaluated in nine healthy young females (median age 24.5 years, interquartile range 24 - 26) following their daily routines who collected all urine voids during one day at 6:00 (first morning void), 9:00, 12:00, 15:00, 18:00, 21:00, and 24:00 and the following morning at 6:00. First morning voids were first stored at +4°C and frozen at -70°C within two

hours. The samples collected during the daytime were immediately stored at -70°C and samples collected in the evening were initially stored at -20°C and then at -70°C the following day.

Analytical characteristics of cell culture assays. The calibration series of all three assays covered a range from 0.4 to 59 ng/ml. Analytical detection limits were defined as the concentration corresponding to a signal 3 standard deviations above the mean value of 12 determinations of the zero calibrator and were set for the I-OC, M-OC, and T-OC assays as 0.02 ng/ml, 0.06 ng/ml, and 0.50 ng/ml, respectively. The within-assay and between-assay imprecisions were determined using a control sample prepared from fetal bovine serum and found to be less than 10% (N = 12).

4.3 Characterization of osteocalcin fragments (I, II, IV)

4.3.1 Purification of osteocalcin from urine and cell culture medium

Urine samples from 4, 8, 12-16, 24, 33, and 75-year-old individuals (I) and an 11-year-old boy (II) were centrifuged for 30 min at 10 000g, filtrated (Sterivex-HV 0.45 μm, Millipore, USA), and extracted using solid phase extraction cartridges (Sep-Pak Plus C18, Millipore) primed with acetonitrile. 40% acetonitrile was used to elute adsorbed material, fractions containing osteocalcin were pooled and acetonitrile was removed by evaporation (HetoVac, Heto, Denmark). Osteocalcin fragments in the extracted material were isolated by immunoaffinity chromatography. The gel matrix was prepared according to the instructions of the Affi-Gel Hz Immunoaffinity Kit (Bio-Rad Laboratories, USA) and equal amounts of MAbs 6F9, 2H9, and 3H8 were immobilized (1 mg MAb mixture per 1 ml matrix). Extracted material was loaded onto columns (2 ml) and the gel was washed with 0.1M Na-phosphate buffer (pH 7), 0.5M NaCl, followed by 0.1M Na-phosphate buffer (pH 7), 0.3M NaCl, and finally 0.1M Na-phosphate buffer (pH 7) and 0.1% trifluoroacetic acid (TFA) was used for elution. All isolation steps were performed at room temperature (22°C). A control sample was prepared by adding synthetic peptide corresponding to intact osteocalcin 1-49 (50 μg, from Advanced Chemtech) into urine (100 ml) depleted of osteocalcin with immunoaffinity chromatography and the sample was subjected to isolation and fractionation steps.

For cell culture experiments (IV, unpublished), four samples were prepared. First, culture medium (4 ml) was collected from rat osteoclasts cultured for 5 days with 10 nM PTH. Second, a medium sample was collected from rat osteoblasts cultured for 15 days in the presence of 1,25-dihydroxyvitamin D_3. Furthermore, supernatant from bone slices incubated with HCl at +4°C for 24 hours was collected. Osteocalcin was also isolated from fetal bovine serum using immunoaffinity chromatography. Equal amounts of MAbs 8H12, 2H9, and 3H8 (1 mg MAb mixture per 1 ml matrix) were coupled to a gel matrix (Affi-Gel 10, Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Fetal bovine serum (14 ml) was mixed with the coupled matrix (1 ml) in an end-over-end rotator for one hour at +4°C and centrifuged for 10 min at 1000 rpm. The matrix was washed two times with phosphate buffered saline (PBS) and osteocalcin eluted with 0.5 M glycine-HCl (pH 2.5) in an end-over-end rotator for 15 min at +4°C. All samples (the culture mediums and the supernatant as such and the osteocalcin isolated from the serum) were extracted in solid phase extraction cartridges (Sep-Pak Plus C18, Millipore) using 40 % acetonitrile for elution as described above.

4.3.2 Analytical protein techniques

1.0-3.7 μg isolated urine osteocalcin (I, II) was fractionated on a Vydac C4 reverse phase high performance liquid chromatography (RP-HPLC) column (2.1 mm x 150 mm) equipped

with a Vydac C4 guard column (both from The Sep/a/ra/tions Group, USA) at flow rate 150 µl / min using the following acetonitrile gradient for elution: 2-30% B (0-63 minutes) (A = 0.1% trifluoroacetic acid/water and B = 0.08% trifluoroacetic acid/acetonitrile), 30-60% B (63-95 minutes) and 60-80 % B (95-105 minutes). The detection wavelength was 276 nm and peak fractions were collected manually for further characterization. In another run, 50µl fractions were collected for immunochemical detection. The material extracted from osteoclast and osteoblast cultures, HCl-treated bone slices, or fetal bovine serum (IV) was fractionated on a similar RP-HPLC but the solvent gradient used was 2-30% B (0-23 minutes), 30-60% B (23-45 minutes), 60-80 % B (45-65 minutes), 80% B (65-70 minutes), and 80%-2% B (70-75 minutes) with a flow rate of 150 µl/minute. Fractions (50 µl) were collected during elution, diluted in Assay Buffer and analyzed for osteocalcin with immunoassays.

HPLC fractions were evaporated to dryness in HetoVac and reconstituted with 30 µl of 60% acetonitrile in 0.1% TFA. 1 µl samples were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 60% acetonitrile and 0.1% TFA) containing calibration mixture 2 from Sequazyme™ Peptide Mass Standards Kit (Perseptive Biosystems, USA). Samples were analyzed in the positive ion reflector mode by matrix assisted laser desorption ionization time-of-flight mass spectrometry instrument (MALDI-TOF, Voyager-DE Pro, Perseptive Biosystems) according to the manufacturer's instructions. A linear mode was used for control sample with synthetic osteocalcin 1-49. Amino-terminal amino acid sequence analyses of selected fractions were performed with an Applied Biosystems (USA) model 477A protein sequencer equipped with an on-line Applied Biosystems model 120 A phenylthiohydantoin amino acid analyzer.

4.4 Evaluation of clinical studies

4.4.1 Measurement of bone mass (I-III)

Bone mineral content (BMC) and areal bone mineral density (aBMD) of the femoral neck and lumbar spine (LII – LIV) were assessed by DXA-technique (Lunar® DPX-L, USA). Out of 1040 women enrolled to the OPRA study, a result was obtained from the hip (femoral neck) in 951, from the lumbar spine in 974 and from the total body in 931 women.

4.4.2 Measurements of biochemical markers of bone turnover (I-III)

Serum bone specific alkaline phosphatase (S-BoneALP), was determined using the Metra BAP immunoassay (Quidel Corporation, USA). Serum intact osteocalcin (S-IntactOC, or S-OC [1-49]), serum total osteocalcin (S-TotalOC), and serum total carboxylated osteocalcin (S-GlaOC, or S-cOC) were determined using two-site in-house assays described previously (Käkönen *et al.*, 2000), with an intra- and inter-assay CV of less than 5% and 8%, respectively, for all assays. Briefly, assays are one-step sandwich assays based on monoclonal antibodies and reagents described above (*section 4.2.1*) as follows: 3G8/2H9 (S-IntactOC), 2H9/6F9 (S-TotalOC), and 6F9/3H8 (S-GlaOC) and synthetic osteocalcin peptide 1-49 was used as a calibrator. Serum osteocalcin was also determined using the Elecsys N-MID Osteocalcin immunoassay (S-TotalOC N-MID) from Roche Diagnostics (Germany) which detects both intact and N-terminal midfragment of osteocalcin. Serum C-terminal cross-linked telopeptides of type I collagen (S-CTX) were determined using the Elecsys β-CrossLaps immunoassay from Roche Diagnostics and serum tartrate resistant acid phosphatase type 5b (S-TRACP5b) with a solid-phase, immunofixed, enzyme activity assay as described earlier (Halleen *et al.*, 2000) (intra-assay CV 1.8%, inter-assay CV 2.2%). Metra DPD immunoassay (Quidel Corporation) was used to measure urinary concentrations of deoxypyridinoline (U-DPD). Urine osteocalcin

(U-OC) was analyzed using U-MidOC, U-LongOC, and U-TotalOC assays. Urinary creatinine was determined in accordance with the kinetic Jaffé reaction. All bone marker measurements were performed in duplicates and all urine measurements were corrected for urinary excretion of creatinine by dividing the concentration of bone marker with the concentration of creatinine (expressed as marker/crea) before further analysis.

4.4.3 Fractures (III)

The mean follow-up time was 4.6 years with a minimum of 3 years and a maximum of 6.5 years. All prospectively sustained clinical symptomatic fractures were ascertained by two means. The files of the Department of Radiology were searched at the Malmö University Hospital, which is the only hospital serving the city. Furthermore, at prospective follow-up visits, at 1, 3, and 5 years, all women were asked whether they had sustained any fracture since the previous visit or not, and the information was confirmed against files. In case of death after the baseline inclusion, this date was registered by means of the Swedish national population register.

4.5 Cell cultures (IV)

4.5.1 Reagents for cell culture

α-modified minimum essential medium (α-MEM), fetal bovine serum (FBS), 1M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) solution, and antibiotics (penicillin, streptomycin) were purchased from Gibco BRL, USA. Macrophage colony-stimulating factor (M-CSF) was purchased from R&D Systems and the receptor activator of nuclear factor kappa B ligand (RANKL) and tumor necrosis factor alpha (TNFα) were from Peprotech, UK. Dexamethasone, parathyroid hormone (PTH), bafilomycin A1 (BafA1), trans-epoxysuccinyl-L-leucylamido-[4-guanidino]butane (E64), and 1,25$(OH)_2$dihydroxy vitamin D_3 (vitamin D) were purchased from Sigma. Ascorbic acid was purchased from Merck and sodium beta-glycerophosphate from Fluka Chemie. Fetal bovine serum used in the rat osteoclast cultures was depleted of bovine osteocalcin prior to use. Equal amounts of MAbs 8H12, 2H9, and 3H8 (1 mg MAb mixture per 1 ml matrix) were coupled to a gel matrix (Affi-Gel 10, Bio-Rad Laboratories, USA) according to the manufacturer's instructions using sterile reagents. FBS (14 ml) was mixed with the coupled matrix (1 ml) in an end-over-end rotator for one hour at +4°C and centrifuged for 10 min at 1000 rpm. The supernatant, i.e. osteocalcin-depleted FBS was collected and stored at -20°C.

4.5.2 Osteoclast cultures

A mixed rodent bone cell population was cultured on bovine bone slices as described in detail previously (Lakkakorpi *et al.*, 1989) and originally introduced by Boyde and co-workers and Chambers and co-workers (Boyde *et al.*, 1984, Chambers *et al.*, 1984). Osteoclasts were mechanically isolated from the long bones of one-day old Sprague-Dawley rats and allowed to attach to devitalized slices of bovine cortical bone (thickness approx. 0.15 mm) for 30 min, after which the non-adherent cells were washed away. Osteoclasts were cultured on 24-well plates in α-MEM (1 ml / well) supplemented with 10% osteocalcin-depleted FBS, 20 mM HEPES, and 100 U / ml penicillin, 100 μg / ml streptomycin for 3 - 5 days at +37°C and 5% CO_2. Controls consisting either of bone slices alone or mixed bone cell population plated on glass cover slips were included in each experiment. PTH (10 nM), BafA1 (3 nM), and E64 (50 μM) were added at the beginning of the culture when indicated and medium samples (30 - 50 μl / well) were collected daily and stored at -20°C until analyzed. Additionally, in order to study the release of

the inorganic matrix in the absence of osteoclasts, some bovine bone slices were exposed to 0.6 M HCl at +4°C for 24 hours.

Human osteoclasts were induced to differentiate from peripheral blood mononuclear cells as published elsewhere (Hentunen and Väänänen, 2001). Briefly, mononuclear cells were isolated from human peripheral blood using the Ficoll-Paque™ technique (Pharmacia Biotech). The cells were washed four times with PBS and 100 000 cells / bone slice were allowed to adhere for 2 hours. The non-adherent cells were washed away and the adhered monocytes cultured in α-MEM supplemented with 10% regular FBS, 20 mM HEPES, antibiotics, 10 ng/ml of M-CSF, 20 ng/ml of RANKL, 10 ng/ml of TNFα, and 10^{-8} M dexamethasone for 12 days. Half of the medium was replaced with fresh medium containing two-fold concentrations of cytokines every four days.

4.5.3 Osteoblast cultures

Osteoblasts were induced from rat bone marrow cells as described elsewhere (Qu *et al.*, 1998). Briefly, bone marrow cells were collected from 4 week-old Sprague-Dawley rats and plated in 260 ml tissue culture flasks at a cell density of 7 - $8*10^7$ cells/flask. The cells were grown in αMEM supplemented with 13% FBS, 20mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml ascorbic acid, and 10 mM Na-β-glycerophosphate at +37°C and 5% CO_2. After 7 days of primary culture with half of the medium changed once, the preosteoblasts were collected by scraping or trypsin-EDTA treatment, and subcultured on 24-well plates at a cell density of 5000 cells/cm^2 in the presence of vitamin D (10^{-8}M) and cultured for up to two weeks. Osteocalcin-depleted FBS prepared as described above was used in the subculture and half of the medium was changed every three or four days.

4.5.4 Evaluation of cell cultures

Cells were fixed with 3% paraformaldehyde for 20 min and stained for TRACP enzyme activity with the reagents for the cytologic demonstration of leukocyte acid phosphatase (kit 387-A, Sigma diagnostics). The nuclei were visualized by staining with Hoechst 33258 (Sigma) and the TRACP-positive multinucleated cells (at least 3 nuclei) were counted as osteoclasts. The organic bone matrix components were visualized by labeling bone slices with fluorescein. Bone slices were incubated in a bicarbonate solution (pH 8.3) containing a succimidyl ester of carboxyfluorescein for two hours with gentle stirring and then washed with PBS before usage. Rat osteoclasts were cultured on labeled bone slices for 48 hours, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min on ice, and washed once with 2% bovine serum albumin in PBS (BSA-PBS). The anti-osteocalcin MAb 3H8 (1000 ng / bone slice) diluted in 0.5% BSA-PBS was added and incubated for 45 minutes at room temperature. After washing with BSA-PBS, the cells were incubated with the Alexa647-conjugated anti-mouse antibody and TRITC-phalloidin in BSA-PBS for 45 minutes at room temperature. After washing, the cells were examined with a Leica TCS-SP confocal laser scanning microscope equipped with an Argon-Krypton laser (Leica Microsystems, Germany). Fluorescein-labeled samples were visualized using a 495-530 nm filter, TRITC-labeled samples using a 580-630 nm filter, and Alexa647-labeled samples using a 660-740 nm filter. The confocal images of triple staining were acquired by a sequential scanning method, i.e. all channels were scanned separately to avoid overlapping. The Alexa647-labelled goat anti-mouse antibody, TRITC-labelled phalloidin, and succimidyl ester of carboxyfluorescein were purchased from Molecular Probes (USA).

Osteocalcin immunoassays I-OC, M-OC, and T-OC and a competitive osteocalcin ELISA (Rat-MID Osteocalcin Assay, Nordic Biosciences, Denmark) were used to measure osteocalcin concentration in the medium. Osteocalcin was also determined from the supernatants collected from the HCl-treated bone slices which were also analyzed for calcium using the Calcium Roche/Hitachi reagents from Roche Diagnostics. The amount of degraded bone matrix was assayed by measuring CTX from the medium (CrossLaps for Culture, Nordic Biosciences). The activity of TRACP5b in the medium was analyzed as described (Alatalo *et al.*, 2000).

4.6 Statistical analyses (I-IV)

All results from urine osteocalcin measurements were normalized for urine creatinine before analyses. The Statistical Analysis System Enterprise Guide 2 program (SAS 8.2, SAS Institute, USA) was used in all analyses, except for the fracture prediction study, which was performed with Statistica 6.1 (Statsoft Inc OK, USA). All results from clinical samples are presented as median with interquartile range and results from cell culture experiments as the mean with standard error of mean unless otherwise stated. A p value of less than 0.05 was considered statistically significant.

The Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variance were applied for all markers and in the case of abnormal distributions, statistical calculations were done only after logarithmic transformation or with non-parametric tests if logarithmic transformation did not result in normal distribution. Comparisons between the groups were made with one-way ANOVA with Tukey's or Dunnett's post-hoc adjustment when neede. Non-parametric Wilcoxon's test followed by Bonferroni adjustment was used if distributions were not normal. The comparison of baseline levels of aBMD and bone markers between women with and those without prospective fractures (III) was performed with the Student´s t-test (after logarithmic transformations if distribution was not normal). Pearson correlation (III) or non-parametric Spearman correlation (I, II) was used for correlation analysis.

In a logistic regression model the fracture predictive ability of a bone marker was calculated using two different thresholds. Women with a bone marker value in the highest quartile were compared to all others. In addition, the predictive ability was tested with or without correction with aBMD of the femoral neck or lumbar spine. Separate calculations were made for women sustaining any prospective fracture, hip fracture, clinical vertebral fracture or multiple fractures (two or more fractures). In addition, Kaplan-Meier survival analysis with log rank testing of differences between groups was applied for all markers with the objective of comparing the predictive ability for fracture.

In the alendronate trial, the one-sided least significant change (LSC) at $p < 0.05$ was determined for each marker based on their analytical and biological variability using the equation: $LSC = 2.33 \times \sqrt{(CVa2 + CVi2)}$ (Hannon *et al.*, 2004). The analytical variability (CVa) of each marker was determined as the mean coefficient of variation of all duplicated measurements performed in the alendronate study. Biological variability (CVi) was determined as the mean of the changes observed at 3 months compared with baseline in the placebo group. Specificity was determined as the percentage of non-responders in the placebo group, and sensitivity as the percentage of responders in the alendronate group.

5 RESULTS AND DISCUSSION

5.1 Characterization of osteocalcin fragments (I, IV)

5.1.1 Osteocalcin in urine consists of multiple midmolecule fragments

HPLC fractionation of osteocalcin isolated from human urine revealed several immunoreactive fractions and two distinct elution profiles. Multiple proteolytic forms of osteocalcin were identified in fractions by MALDI-MS and sequencing. The fragments identified in all samples evaluated had most often Gly^{7} in their N-terminus but also fragments with Leu^{6} and Pro^{9} in the N-termini were found. The C-terminus of fragments was more heterogeneous and located around residue 30. The monoisotopic masses of the six prominent ions detected in all samples were between 2564 and 2992. The peak with the highest intensity was seen at 2808 and spanned residues 7-31 (Gly^{7}-Glu^{31}). Other fragments identified spanned residues 7-29, 7-32, 7-33, 6-29, and 9-31. The HPLC fractions producing these ions were recognized by two-site immunoassay 6F9/3H8 (later named U-MidOC).

Three additional ions of molecular weight 2127, 2240, and 2733 were present in the samples of children and young adults. These additional fragments started with residue Asp^{14} and spanned residues 14-31, 14-32, and 14-37, respectively, the first one being the most pronounced. The two smallest ones (2127 and 2240) eluted in the area where an additional peak of competitive hOC/3H8 assay (later named U-TotalOC) was observed while the largest ion (2733, Asp^{14}-Gly^{37}) was observed in the two-site detectable region together with other ions of higher molecular weight (from 2564 to 2992). An additional fragment identified in one urine sample only and producing an ion with molecular weight 1914.86 likely represents osteocalcin fragment spanning residues Leu^{16}-Glu^{31} (theoretical molecular weight 1914.81). All theoretical molecular masses were calculated without Gla-modification since it has been reported that γ-carboxylation is destroyed in MALDI-MS and is not included in the molecular masses of observed ions (Prorok *et al.*, 1996, Kalume *et al.*, 2000). The identification of fragments was confirmed using N-terminal sequencing and the results are summarized in Table 2.

Table 2. *Osteocalcin fragments identified in human urine samples with MALDI-MS analysis.* Plus sign (+) indicates that the fragment was detected in the sample and minus sign (-) that it was not detected. Monoisotopic masses without γ-carboxylation are shown (a). nd = not determined.

Osteocalcin fragment	Length (residues)	Theoretical mass (M+H+)[a]	Age of subjects						N-terminal sequencing
			4	8	12–16	24	33	75	
6 – 29	24	2677.26	+	-	+	+	+	+	LGAPVPY
7 – 29	23	2564.17	+	+	+	+	+	+	nd
7 – 31	25	2808.24	+	+	+	+	+	+	GAPVPYPD
7 – 32	26	2921.33	+	+	+	+	+	+	GAPVPYPDPL
7 – 33	27	2992.36	+	+	+	+	+	+	nd
9 – 31	23	2680.18	+	+	+	+	+	+	XVPYPXP
14 - 31	18	2126.89	-	+	-	+	-	-	DPLXPRR
14 - 32	19	2239.98	-	+	+	+	-	-	nd
14 - 37	24	2733.20	-	+	+	+	-	-	nd
16 - 31	16	1914.81	-	+	-	-	-	-	nd

Altogether, ten novel proteolytic forms of osteocalcin were identified in the urine samples of healthy volunteers. Osteocalcin in urine appeared to be a complex mixture and consisted of peptides less than thirty residues in length from the middle region of the molecule. No intact

osteocalcin was detected. Fragments differed from each other by only a small number of residues. The proteolytic forms of urine osteocalcin can be classified into two main categories depending on their N-terminus, to more extended fragments starting from Gly^{7} (or Leu^{6} or Pro^{9}) and to more truncated ones with Asp^{14} (or Leu^{16}) at the N-terminus.

Most urine osteocalcin fragments common to all samples had Gly^{7} at the N-terminus (Fig.7). The peptide bond between Leu^{6} and Gly^{7} appears to be susceptible to proteolysis *in vivo* although the protease(s) responsible for cleavage has not been identified. The removal of the N-terminal region of osteocalcin has also been detected in osteocalcin isolated from bone when osteocalcin fragments Gly^{7}-Asn^{26}, Gly^{7}-Asp^{34}, and Gly^{7}-Ile^{36} were sequenced from EDTA extracts of bovine bone powder (Ishida and Amano, 2004). Osteocalcin fragments lacking the first six residues and starting from Gly^{7} have been detected in urine also previously when a urine osteocalcin fragment detected by two-site immunoassays was reported to consist of residues Gly^{7}-Asp^{30} (Matikainen *et al.*, 1999). Fragments starting from residue Gly^{7} have also been suggested to exist in serum (Jaouhari *et al.*, 1992). The origin of Gly^{7} at the N-terminus is interesting since although several proteases such as plasmin, trypsin, and cathepsin have been reported to cleave osteocalcin into smaller fragments *in vitro* (Garnero *et al.*, 1994a, Baumgrass *et al.*, 1997, Novak *et al.*, 1997) there are no reports on proteases cleaving osteocalcin at peptide bond Leu^{6}-Gly^{7}. An interesting difference between urine samples was observed in regard to the presence of fragments starting from Asp^{14}. Fragments spanning residues Asp^{14}-Glu^{31}, Asp^{14}-Leu^{32}, and Asp^{14}-Gly^{37} were detected in the samples of 8 and 24-year-old individuals and in the pool of 12-16-year-olds. Asp^{14}- fragments could be produced in high quantities especially during e.g. periods of high bone turnover and gaining of bone mass, which might be typical to these subjects. The cleavage site between Pro^{13} and Asp^{14} has recently been reported in urine osteocalcin isolated from a patient with Paget's disease which consisted of the fragment Asp^{14}-Asp^{28} (Srivastava *et al.*, 2002). The protease(s) involved in generation of the Asp^{14} terminus is not known.

Tyr^{1}-Leu^{2}-Tyr^{3}-Gln^{4}-Trp^{5}-**Leu^{6}-Gly^{7}**-Ala^{8}-**Pro^{9}**-Val^{10}-Pro^{11}-Tyr^{12}-Pro^{13}-**Asp^{14}**-Pro^{15}-**Leu^{16}**-Gla^{17}-Pro^{18}-Arg^{19}-Arg^{20}-Gla^{21}-Val^{22}-Cys^{23}-Gla^{24}-Leu^{25}-Asn^{26}-Pro^{27}-Asp^{28}-$\underline{Cys}^{29}$-Asp^{30}-$\underline{Glu}^{31}$-$\underline{Leu}^{32}$-$\underline{Ala}^{33}$-Asp^{34}-His^{35}-Ile^{36}-$\underline{Gly}^{37}$-Phe^{38}-Gln^{39}-Glu^{40}-Ala^{41}-Tyr^{42}-Arg^{43}-Arg^{44}-Phe^{45}-Tyr^{46}-Gly^{47}-Pro^{48}-Val^{49}

Figure 7*. Amino acid sequence of human osteocalcin.* Urinary fragments consist of the middle region of the molecule. Residues found in the N-terminus of fragments are shown in **bold** and residues located at the C-terminus are underlined.

The information on γ-carboxylation of urine osteocalcin fragments was not directly available due to decarboxylation of samples in MALDI-MS. However, it has been previously reported that MAb 3H8 used in U-MidOC and U-TotalOC assays prefers binding to γ-carboxylated osteocalcin forms with only 9% cross-reactivity towards non-carboxylated osteocalcin (Hellman *et al.*, 1996). Thus, the majority of urinary fragments must be at least partially γ-carboxylated. Furthermore, Gla residues are not extracted in N-terminal sequencing which results in blank chromatograms at positions occupying γ-carboxylation (Cairns and Price, 1994). A blank chromatogram was obtained in the sequencing of Asp^{14} fragments at putative Gla position 17 (data not shown) suggesting γ-carboxylation at this site. Other Gla-containing proteins such as prothrombin has been reported to exist in urine almost entirely in γ-carboxylated forms (Buchholz *et al.*, 1999) indicating that Gla is not readily destroyed during renal processing.

Urinary osteocalcin peptides consisted of 16 - 27 amino acid residues from the middle region of the molecule. There were no fragments identified containing the most N- or C-terminal sequences. The middle region of the molecule has been suggested to be the most stable part of the molecule due to the structural properties of the molecule (Hauschka and Carr, 1982). The middle region of osteocalcin contains regions which adopt an α-helical conformation (Dowd *et al.*, 2003) and a disulphide bond between Cys^{23} and Cys^{29} further stabilizes the midmolecule structure. The Cys residues involved in disulfide bridge formation were included in all the urine fragments identified. Midmolecule fragments also contained all three Gla residues, the most conserved region between species. The compact structure with stabilizing features is likely to be responsible for the resistance against the glomerular filtration and degradation and further processing of urine compared to the extremes of the molecule. This could be explained by the relatively few sites susceptible to proteolysis which reside at the terminal regions of the molecule, extending outwards from the more compactly folded midmolecule region. The first fifteen N-terminal residues do not contain any areas of secondary structure making the N-terminus rather unstructured and more accessible to proteases (Dowd *et al.*, 2003). This is in contrast to the rest of the molecules as segment Leu^{16}-Val^{49} contains three regions of secondary structure which fold into an "osteocalcin fold" (Hoang *et al.*, 2003). Osteocalcin isolated from human bone also demonstrates the instability of the N-terminus, as it frequently contains osteocalcin peptide Ala^{8}-Val^{49}, assumed to result from endogenous proteolytic action in bone (Taylor *et al.*, 1988). In addition to the N-terminus, most C-terminal regions appear to be readily accessible to proteases. In the bone-binding orientation, the C-terminus extends outward rendering it more susceptible to proteolytic cleavage than the mineral bound surface of the molecule (Dowd *et al.*, 2003, Hoang *et al.*, 2003). Plasmin (Novak *et al.*, 1997) and trypsin (Garnero *et al.*, 1994a) are among the proteases that have been reported to cleave osteocalcin between Arg^{43} and Arg^{44} to produce a large Tyr^{1}-Arg^{43} fragment and a small C-terminal pentapeptide. Cathepsins can also process both terminal sequences. At the N-terminus cathepsins readily cleave osteocalcin between residues Gly^{7} and Ala^{8} and there are four cleavage sites at the C-terminus, between residues Gln^{39}-Glu^{40}, Ala^{41}-Tyr^{42}, Arg^{43}-Arg^{44}, and Arg^{44}-Phe^{45}, depending on cathepsin subtype, while the midmolecule region is resistant to cathepsin-mediated proteolysis (Baumgrass *et al.*, 1997, Koboyashi *et al.*, 1998).

The length of osteocalcin fragments was very consistent between different samples and fragments spanning residues Gly^{7}-Cys^{29}, Gly^{7}-Glu^{31}, Gly^{7}-Leu^{32}, Gly^{7}-Ala^{33}, and Pro^{9}-Glu^{31} were found in all the samples we evaluated. Also, fragment Leu^{6}-Cys^{29} is probably common to all samples, although we failed to detect it in one subject. The similarity between different samples suggests that the fragments are not results of the random degradation processes, but that the fragments result from similar degradation cascades in all individuals studied, probably due to the stability of the middle region of the molecule. Urine may, of course, contain shorter osteocalcin fragments, which were not detected with the antibodies used in this study and remain to be characterized. In theory, the N-terminal and C-terminal ends of osteocalcin could be released concomitantly with the processing of midmolecule fragments. The antibodies used in this study covered three epitopes on the osteocalcin sequence but because an antibody for the most C-terminal sequence was lacking, short C-terminal fragments may have escaped the isolation step. Although such short fragments may be generated *in vivo*, they are probably easily degraded and do not survive the processing of urine as well as the more stable midmolecule structures and thus, their existence in urine in large quantities is unlikely. The same applies to short N-terminal fragments. Furthermore, the confirmation of short urinary fragments to ones

derived from osteocalcin may not be exclusive. Therefore, the shortest N- or C-terminal peptides are unlikely to contribute to the immunoreactivity of osteocalcin assays.

5.1.2 Insights into the origin of urinary fragments

In addition to intact osteocalcin, several osteocalcin fragments have been detected in human serum (Gundberg and Weinstein, 1986, Taylor *et al.*, 1990). Circulating fragments might be produced in osteoblasts during biosynthesis (Garnero *et al.*, 1994a) or they might be released from bone matrix by osteoclastic bone resorption (Gundberg and Weinstein, 1986). Catabolic degradation may also occur in the circulation or peripheral organs (Price *et al.*, 1981c, Farrugia and Melick, 1986, Aliberti *et al.*, 2000) providing a third source for fragments. For further studies it would be important to understand whether the multiple midmolecule osteocalcin fragments identified in urine reflect bone formation or resorption or bone turnover in general.

Osteocalcin molecules of resorptive origin would be of clinical interest but the circulating resorptive fragments may be masked by *de novo* synthesized intact osteocalcin which is inevitably recognized by most immunoassays in addition to the shorter fragment. Fragmentation of intact osteocalcin may also result in structurally similar forms than the putative resorptive fragments due to relatively few sites susceptible to proteolysis (Baumgrass *et al.*, 1997). The detection of small osteocalcin fragments of resorptive origin may be further limited because all assays do not necessarily pick up small fragments for measurement due to antibody specificity or the proportion of these fragments may be low. Bone hydroxyapatite is able to absorb intact osteocalcin molecules but not fragments (Price *et al.*, 1976a, Novak *et al.*, 1997) suggesting that intact osteocalcin may traffic also back to the skeleton. Circulating intact osteocalcin which is not adsorbed to hydroxyapatite as well as large osteocalcin fragments are likely to be degraded by proteases in the circulation or peripheral organs while smaller osteocalcin fragments containing an intact midmolecule region would be more resistant to degradation and accumulate into urine as a result of renal filtration. Therefore, urine could potentially offer a predominant source for osteocalcin fragments of resorptive origin and measurement of fragments may be feasible in urine due to absence of intact osteocalcin and large fragments such as N-terminal midfragment Tyr^{1}-Arg^{43}.

The generation of proteolytic fragments of osteocalcin may in principle occur at several stages of the life cycle of the molecule. Cathepsin K (Gundberg *et al.*, 2002), the predominant proteolytic enzyme in bone matrix degradation, as well as other cathepsins (Baumgrass *et al.*, 1997, Koboyashi *et al.*, 1998) cleave human osteocalcin at the N-terminus between Gly^{7}-Ala^{8} and putative resorptive fragments should have Ala^{8} at the N-terminus. This suggests that urine fragments starting from Gly^{7} cannot be derived from a cathepsin-like action on osteocalcin and these fragments are not directly derived from breakdown of bone matrix by cathepsins. This does not, however, exclude the possibility that Gly^{7}- fragments could be released from resorption. The activity of cathepsins may not be a requirement for the detachment of osteocalcin from bone matrix as the cleavage of even a short hexapeptide segment from the C-terminus is enough to detach the remaining osteocalcin from bone matrix *in vitro* (Novak *et al.*, 1997). Thus, resorptive fragments do not necessarily have to be the ones starting with Ala^{8}. Since intact osteocalcin is not detected in urine, the Gly^{7}- fragments could also represent putative degradation products of intact osteocalcin. In general, Leu^{6}-Gly^{7} is a very susceptible site to proteolysis (Jaouhari *et al.*, 1992, Matikainen *et al.*, 1999, Ishida and Amano, 2004).

In contrast, fragments starting from Asp^{14} could be derived from cathepsin degradation products and it is tempting to speculate that these fragments would originate from cathepsin-

mediated bone resorption. The putative resorptive origin of Asp^{14}- fragments is supported by the preliminary observation that the measurement of fragment Asp^{14}-Asp^{28} in patients treated with alendronate correlated to the rate of bone resorption assessed with urinary or circulating levels of NTX and CTX (Srivastava *et al.*, 2002). Although cathepsins originally cleave osteocalcin at Gly^{7}-Ala^{8} bond, the resulting fragments may be further degraded at the N-terminal unstructured region of osteocalcin. Since the midmolecule region is able to resist degradation due to its compact structure, fragments truncated further than Asp^{14} (or Leu^{16}) might not be produced in large quantities. With regard to C-terminus, it appears to be easily degradaded by proteases but the degradation does not exceed the region around position 30, probably due to steric hindrances of the midmolecule fold. These are all, of course, only mechanistic assumptions and require further investigation.

5.1.3 Evidence for circulating osteocalcin fragments

In order to clarify the origin of urinary fragments it would be informative to carry out similar characterization on circulating osteocalcin. However, the concentration of osteocalcin in serum is less than 10 ng/ml when measured with the immunoassays used in this study (Käkönen *et al.*, 2000). For mass spectrometric analyses and sequencing similar to the ones performed on isolated urine osteocalcin, one would need more than one microgram of isolated serum osteocalcin and thus, several hundred milliliters of serum which is not trivial to obtain from a single subject. Although the concentration in urine (without creatinine correction) was not substantially higher, approximately 4 - 18 ng/ml (~100 ng/ml in the young boys, unpublished), it was easy to collect and pool several urine voids in order to obtain enough osteocalcin for isolation purposes (one to eight voids per subject). This volume of collected urine ranged from ~200 ml (young boys) to ~1500 ml (75-year-old woman) depending on the osteocalcin concentration. Due to the difficulties encountered in the collection of adequate amounts of human serum, we addressed this issue by performing the isolation and characterization on commercially available fetal bovine serum. This sample material was easily available in large enough quantities and contained high amounts of osteocalcin due to fetal origin.

The elution profile of bovine serum osteocalcin was heterogeneous and consisted of at least four main peaks. The MALDI-MS analysis of the four peaks revealed several prominent ions with molecular weights between 2713 and 5720. One predominant form was identified as intact osteocalcin Tyr^{1}-Val^{49} on the basis of both immunoreactivity (I-OC assay) and size (observed mass 5720, theoretical 5719) and another one as osteocalcin fragment Ala^{8}-Ala^{33} according to mass spectrometric data (observed mass 2896, theoretical 2897). Other forms identified in the bovine serum were osteocalcin fragments Ala^{8}-Glu^{31}, Ala^{8}-Gln^{39}, and Ala^{8}-Val^{49}. Serum may, of course, contain additional shorter osteocalcin fragments, which remain to be characterized. It has been suggested that circulating human osteocalcin consists of intact osteocalcin (one third), N-terminal midfragments of Tyr^{1}-Arg^{43} (one third) and smaller, unidentified fragments (one third) (Garnero *et al.*, 1994a). We detected both intact osteocalcin as well as four different fragments in bovine serum but were unable to demonstrate the presence of N-terminal midfragment Tyr^{1}-Arg^{43}. However, the N-terminal midfragment has never been directly sequenced but the sequence of this fragment is proposed on the basis of tryptic site at residue Arg^{43}-Arg^{44} (Garnero *et al.*, 1994a).

Cathepsins cleave osteocalcin between residues Gly^{7} and Ala^{8} and circulating osteocalcin fragments starting from Ala^{8} could thus putatively result from the degradation by cathepsins (Baumgrass *et al.*, 1997, Gundberg *et al.*, 2002). When intact osteocalcin is subjected to degradation by cathepsin K *in vitro*, an osteocalcin fragment Ala^{8}-Ala^{33} has been detected as

one of the main proteolytic product, the other one being Ala^{8}-His^{35} (Gundberg *et al.*, 2002). Interestingly, the fragment is identical to the one found in bovine serum in this study. Furthermore, two of the C-termini found in serum fragments (Glu^{31} and Ala^{33}) were identical to the C-termini of urinary fragments, suggesting common proteolytic cascades for their generation. However, the results obtained from the characterization of fetal bovine osteocalcin cannot directly be applied to human serum. Although the primary sequence is highly conserved among all vertebrates, there are five amino acid substitutions in bovine protein compared to the human counterpart. These reside in the N-terminal region and include change of Tyr^{3} and Gln^{4} in human to Asp^{3} and His^{4} in bovine. Also Arg^{20} is substituted in bovine osteocalcin to Lys^{20}. More importantly, Pro^{9} in human osteocalcin is converted to hydroxyproline in bovine protein and the neighboring Val^{10} is replaced by Ala^{10}. The latter substitutions reside in the near vicinity of the observed N-terminal cleavage sites (Leu^{6}-Gly^{7}, Gly^{7}-Ala^{8}) and the differences may have an effect on the proteolytic cleavage at this region and subsequently to the fragments generated. Furthermore, the molecular fragments generated during fetal life in bovine species and their relative proportions can be different from those of adult humans and the collection and storage conditions of the commercial serum prior to purchase are not known.

5.2 Osteocalcin assays (I, II)

The isolation and characterization of fragments from urine samples is a laborious task and cannot be performed on a routine basis to evaluate fragments in an individual patient. In order to clarify the clinical and biological significance of urine osteocalcin, more convenient methods are clearly needed. Immunochemical detection of fragments with specific antibodies can be applied for this purpose. At the moment, there are no commercial methods available for the measurement of osteocalcin in urine samples. The development of such assays has probably been limited by the low concentration of osteocalcin in urine and the inability of antibodies to detect small urinary fragments. The original finding of immunoreactive osteocalcin in urine (Taylor *et al.*, 1990) was based on an in-house RIA utilizing a polyclonal guinea pig antibody against osteocalcin purified from human bone (Taylor *et al.*, 1988). Osteocalcin in 24h urine collections measured with midmolecule human RIA correlated with total ALP and osteocalcin in serum indicating an association to bone metabolism. Later, the same group reported a second RIA involving human osteocalcin midmolecule peptide Tyr^{12}-Asp^{30} as a radioiodinated tracer and a polyclonal chicken antibody against synthetic osteocalcin peptide Tyr^{12}-Ile^{36} (Srivastava *et al.*, 2002). With this midmolecule RIA it was demonstrated that urine osteocalcin levels were increased in postmenopausal women and reduced in response to alendronate after one month of treatment. In-house two-site IFMAs employing monoclonal murine antibodies for various epitopes of human osteocalcin have also been used to measure osteocalcin in urine (Matikainen *et al.*, 1999). Urine osteocalcin measured with these assays was increased in postmenopausal women and decreased in women receiving hormone replacement therapy and ten fold higher values were observed in pubertal samples.

5.2.1 The specificity of assays

In order to choose appropriate antibodies for the assays, the exact primary structure of urinary osteocalcin fragments has to be known, since the structure dictates which epitopes are represented within the fragments. The characterization of urinary osteocalcin fragments resulted in detailed information on ten fragments and the knowledge on the detailed peptide structure of urine osteocalcin made it feasible to develop specific immunoassays for their measurement. Urine osteocalcin fragments were rather small consisting of 16 - 27 residues. There are not many binding epitopes present in such small fragments and not all antibodies available are able

to recognize these short peptides but the midmolecular structure of fragments allowed detection by immunoassays developed against the midmolecule epitopes. MAbs for three distinct epitopes on osteocalcin were available for the development of immunoassays in this study. One set of MAbs recognized region Arg^{20}-Arg^{43}, another set was for region Gly^{7}-Arg^{19}, and one antibody specific for intact osteocalcin was also available (Hellman *et al.*, 1996). For two-site assays, two antibodies with no overlapping epitopes were selected. These included MAb 6H9 which binds to residues Gly^{7}-Arg^{19} and MAbs 2H9 or 3H8 which recognize fragment Arg^{20}-Arg^{43}resulting in combinations 2H9/6F9 (later named U-LongOC) and 6F9/3H8 (later named U-MidOC). 3H8 prefers Gla-containing forms with only 9% cross-reactivity to non-Gla-containing osteocalcin (Hellman *et al.*, 1996) and therefore, U-MidOC detects primarily γ-carboxylated urine osteocalcin fragments. MAb 3H8 was selected for the competitive assay (later named U-TotalOC) because it has an epitope on midmolecule fragment Arg^{20}-Arg^{43} and therefore, it should also be able to detect the shortest fragments in urine.

Three immunoassays for the measurement of osteocalcin fragments in human urine (U-OC) were developed and characterized. Assays U-MidOC and U-LongOC were two-site assays while assay U-TotalOC was a competitive one. The ability of assays to recognize different naturally occurring molecular forms of urine osteocalcin was verified with HPLC fractionation and MALDI-MS analysis. Pre-pubertal urine contained two major pools of immunoreactive osteocalcin (peak I and a double peak II+III) eluting at distinct times during fractionation (original publication **II** Ivaska *et al.*, 2005 Figure 2). According to N-terminal sequencing and MALDI-MS, the first pool (peak I) consisted of fragments starting predominantly from residue Asp^{14}. In contrast, the second peak II+III contained fragments with more extended N-terminus starting from residue Gly^{7} or Pro^{9}. The identity of fragments was similar to the ones characterized earlier (*section 5.1.1*). The most predominant fragment in the characterization studies and detected in all samples was the one of residues Gly^{7}-Glu^{31}. It was detected in fractions positive for U-MidOC and represents the main fragment detected with two-site approach. Fragments of residues Gly^{7}-Leu^{32} and Gly^{7}-Ala^{33} were present in very small amounts and identified from fractions positive for two-site U-LongOC, being the only fragments recognized by this assay. Fragments, which started with residue Asp^{14}, were not detected by the two-site assays. They were, however, clearly recognized by the competitive U-TotalOC assay, which inevitably also detected fragments with Gly^{7} at the N-terminus. We were unable to separate fragments Leu^{6}-Cys^{29}, Gly^{7}-Cys^{29}, and Pro^{9}-Glu^{31} into individual fractions but instead, some of the main fragment immunoreactivity (Gly^{7}-Glu^{31}) co-eluted with them. In theory, at least the competitive assay should be able to pick also these fragments for measurement. In summary, the immunoassays had unique specificity towards different, naturally occurring urinary osteocalcin fragments. U-TotalOC assay detected fragments present in all three peaks, therefore reflecting the total concentration of immunoreactive urinary osteocalcin. Two-site assay U-MidOC recognized only fragments present in peaks II and III and failed to detect more truncated fragments present in peak I. Two-site assay U-LongOC was able to detect only the longest fragments present in peak III but not as effectively as U-MidOC.

The specificity of different assays also provided new insights into the epitopes of MAbs. Mab 6F9 has been reported to recognize fragment Gly^{7}-Arg^{19} (Hellman *et al.*, 1996). The U-MidOC assay utilizing MAb 6F9 was able to recognize fragments starting from residues Leu^{6}, Gly^{7}, and probably also Pro^{9} but not fragments starting from residues Asp^{14}. This narrows the epitope region to residues Gly^{7}-Pro^{13}. In this heptapeptide epitope, the human sequence is GAPVPYP and the corresponding bovine sequence GAHypAPYP. Te only differences are the substitutions of Pro^{9} to Hyp^{9} and Val^{10} to Ala^{10}. These residues must be crucial for the binding

since MAb 6F9 is specific to human osteocalcin and fails to bind bovine protein (Hellman *et al.*, 1996). MAb 2H9 has been reported to recognize fragment Arg^{20}-Arg^{43}. U-LongOC assay involving Mab 2H9 was not able to detect fragment Gly^{7}-Glu^{31} and other fragments in peak II but did bind to fragments only one or two residues longer in peak III, namely fragments Gly^{7}-Leu^{32} and Gly^{7}-Ala^{33}. Thus, the epitope of Mab 2H9 must involve the area around position 30 and at least Leu^{32} appears to be required. MAb 3H8 has also been reported to recognize the same Arg^{20}-Arg^{43} region with the additional requirement of γ-carboxylation. Two of the Gla residues in human osteocalcin are located in this sequence at positions Gla^{21} and Gla^{24} (Cairns and Price, 1994) and the epitope of Mab 3H8 probably involves binding to this area. Assays U-MidOC and U-TotalOC which utilize 3H8 were able to detect osteocalcin fragments truncated from the C-terminus up to the residue Glu^{31}. The binding region must thus be between Arg^{20} and Glu^{31}. In this region, the sequence of human and bovine osteocalcin is identical.

5.2.2 Analytical considerations

Two sandwich assays and the competitive assay were all based on synthetic osteocalcin peptide as a calibrator and carefully characterized MAbs (Hellman *et al.*, 1996), some of which were already used in the assays of Matikainen and co-workers (Matikainen *et al.*, 1999). Assay procedures were easy to perform and the detection limits and dynamic ranges were adequate to allow measurement of urine samples, with the exception of urine samples which were too dilute and therefore excluded from the evaluations. The within-run variations calculated for low and high controls, respectively, were 2.7% and 1.7% (U-MidOC), 9.1% and 6.2% (U-LongOC), and 9.7% and 18.3% (U-TotalOC). The total variation (within-run and between-run) in low and high controls, respectively, was in U-MidOC 11.6% and 6.1%, in U-LongOC 13.6% and 10.9% and in U-TotalOC 26.7% and 23.1%. Detection limits were 0.17 ng/ml (U-MidOC), 0.03 ng/ml (U-LongOC), and 28 ng/ml (U-TotalOC). Linearity and recovery of added analyte were adequate. The variations in competitive U-TotalOC assay were greater than for two-site assays. The total variation (over 20%) was observed mainly for the control samples and the standard curve of U-TotalOC with synthetic osteocalcin was highly reproducible with overlapping standard curves and within-assay CV-%s less than 10%. This may partly be due to the control samples which were prepared by diluting urine collected from an 8-year-old boy and containing very high concentration of osteocalcin. The molecular fragments generated in this setting and their relative proportions can be different from those of postmenopausal and older subjects and a pre- or postmenopausal undiluted adult urine sample will probably serve as a more appropriate model for control samples in the future. The value of urine osteocalcin in clinical settings could be improved by reducing the analytical variability.

Urinary osteocalcin seemed to tolerate well short-term storage at 4°C or even at room temperature prior to freezing. The immunoreactivity detected by two-site assays U-LongOC and U-MidOC did not differ from the initial value when urine samples were stored at either 4°C or 22°C for up to five days. Concentrations measured by the competitive U-TotalOC assay were slightly decreased during incubation at both 4°C and 22°C and the decrease was less pronounced at 22°C – presumably due to higher variation. In serum, a large proportion of osteocalcin is intact molecules which are easily degraded. This results in the loss of immunoreactivity noted within a few hours when samples are stored at room temperature or after repeated freeze-thaw cycles (Diaz Diego *et al.*, 1994, Käkönen *et al.*, 2000). Therefore, the sample collection and handling has to be carefully controlled when osteocalcin is measured in serum samples (Banfi and Daverio, 1994). The improved stability of urine osteocalcin *in vitro* - at least when measured with two-site assays - facilitates the collection and storage requirements

of urine samples and makes urinary osteocalcin an attractive analyte compared to serum osteocalcin. However, in addition to variation during the storage, urinary osteocalcin may also exhibit variability resulting from sample handling immediately after collection. Although the collection and storage of urine samples for stability study was carefully controlled, we were unable to address the pre-storage variability and it remains to be evaluated.

There are a number of possible advantages in the measurement of osteocalcin in urine compared to circulation. Urine samples can be collected easily without the help of health care professionals. Samples could be even collected at home and mailed by the patient to the laboratory which has already been tested for urinary NTX and DPD measurements (Worsfold *et al.*, 2004). The problems related to the instability after sampling appear to be less pronounced for urinary osteocalcin which is presumably an end-product of fragmentation. The short-term changes, such as those induced by feeding, are also likely to be less dramatic in urinary analytes because they provide an integrated measure of turnover over the collection period (Clowes *et al.*, 2002). Furthermore, osteocalcin is a bone-specific analyte with no or very little expression in non-skeletal tissues which presumably also increases the specificity of assays. The bone-specificity together with simple and non-invasive collection and storage requirements for spot urine samples make urine osteocalcin an attractive candidate for a new bone turnover marker. Urine as a sample material is also intriguing because it has been traditionally used for measurement of resorption markers and not markers of osteoblastic origin. Furthermore, there is a wide selection of already available assays for the measurement of serum osteocalcin which might be applicable to the measurement of osteocalcin concentrations in urine without need for large modifications and resources on the assay development.

Correspondingly, there are some limitations associated with urinary assays, such as the requirement for normalization to urinary creatinine. It is needed for all urinary parameters to correct the variations in urine dilution between different voids. The use of creatinine to normalize urinary marker values contributes a second source of variability to the results and, due to creatinine correction, urinary markers may be affected by additional factors such as alterations in muscle mass. In general, urine-based assays tend to show higher preanalytical variability than serum assays (Hannon and Eastell, 2000). Furthermore, urine excretion rate demonstrates circadian variability with peak excretion in the afternoon (Bollen *et al.*, 1995) as well as some day-to-day variation and may contribute to the overall variability of the urinary markers (Sebastian-Gambaro *et al.*, 1997). The urinary excretion of osteocalcin fragments may also be altered in diseases affecting renal function. Serum concentration of osteocalcin, and especially the amount of fragments, is increased in renal diseases, suggesting that the excretion of the fragments via kidneys is reduced (Gundberg and Weinstein, 1986). A gradual age-related renal impairment may further influence the results, particularly when creatinine clearance is substantially impaired (Delmas *et al.*, 1983c).

5.2.3 Biological variation of urinary osteocalcin

A circadian rhythm similar to the one reported for serum osteocalcin was observed for urinary osteocalcin with all three assays. Values were highest during the night and early morning and decreased in the afternoon. This was followed by an increase later in the evening and night. This emphasizes the importance of regulating the time of the urine sample taken for measuring osteocalcin for clinical investigations. In this study, urine samples collected as the 1st morning void, the 2nd morning void, or as 24h collections were tested and all were found suitable sample materials for urinary osteocalcin assays. The absolute concentration of osteocalcin was highest and thus most easily measurable in the 1st morning void which can be

recommended as a sample material for further studies. Furthermore, it is rather difficult to obtain and verify 24h urine collections making the collection of spot urines more practical.

The diurnal variation of urinary osteocalcin resembled the one reported for serum osteocalcin, suggesting that the diurnal variation reflects a true variation in bone metabolism and is not the result of changes in glomerular filtration rates. Circulating osteocalcin levels demonstrate a circadian rhythm characterized by a decline during the morning to a low around noon. This is followed by a gradual rise which peaks after midnight. The amplitude of variation in serum osteocalcin in usually less than 20% but varies depending on the assays (Gundberg *et al.*, 1985a, Nielsen *et al.*, 1990). Our results with all three urine osteocalcin assays were consistent with the rhythm of serum osteocalcin but the difference between the highest and lowest values was larger, approximately 40%, than the one reported for serum osteocalcin. Pronounced diurnal variation has also been observed for resorption markers such as urinary NTX (Bollen *et al.*, 1995) and serum CTX (Qvist *et al.*, 2002). The magnitude of variation is higher for collagen degradation markers than for bone formation markers, being approximately 40% for the cross-linked telopeptides and free cross-links and 10-20% for serum osteocalcin and ALP (Schlemmer *et al.*, 1992, Greenspan *et al.*, 1997, Qvist *et al.*, 2002). The fluctuations are assumed to reflect true differences in bone resorption frequency. The daily rhythm of PTH secretion or calcium intake is likely to be an important determinant of this rhythm, since the nocturnal increase in bone resorption can be suppressed by calcium supplementation in the evening (Blumsohn *et al.*, 1994).

No major seasonal variation was observed n urinary osteocalcin. Variation was evaluated in the cross-sectional study of elderly women by comparing results obtained from different individuals sampled in different months throughout the year. The number of subjects in each month was different, being smallest in July (N=32) and largest in April (N=127). We did not detect any major seasonal variation in urinar osteocalcin. There was a small but statistically significant tendency for higher values in late autumn and winter compared to the rest of the year when osteocalcin was measured with U-MidOC ($p=0.02$, nonparametric Wilcoxon's test) which might be related to the summertime increase in sunlight and vitamin D. Two other assays U-LongOC and U-TotalOC demonstrated no significant variability ($p=0.25$ and $p=0.93$, respectively), probably due to lower sensitivities of these assays to detect minor changes. Nevertheless, the seasonal effects appeared to be rather small in comparison with other sources of measurement noise and not of sufficient magnitude to confound the use of urinary osteocalcin. The effect of sampling season on urinary osteocalcin has not been previously investigated but there have been attempts to clarify the seasonal variation in circulating levels of osteocalcin. Minor seasonal fluctuations with concentrations declining from January to July and then increasing to a peak in winter have been reported for serum osteocalcin (Thomsen *et al.*, 1989). However, other studies have failed to demonstrate any important wintertime increase (Overgaard *et al.*, 1988, Blumsohn *et al.*, 2003) and suggested that only a borderline summertime increase, if any, may occur (Blumsohn *et al.*, 2003).

5.3 Urinary osteocalcin as a marker of bone metabolism

5.3.1 Correlation with bone mass and bone turnover rate (I, II)

The ability of urinary osteocalcin to reflect bone metabolism and to serve as a marker of bone turnover was evaluated in the cohort of elderly women. In this cohort, urinary osteocalcin correlated significantly with bone turnover rate assessed by serum markers of bone turnover used in the study, namely S-CTX, S-TRACP5b, S-BoneALP, and S-TotalOC ($p<0.0001$ for all

correlations). Urinary osteocalcin measured with U-MidOC and U-LongOC had a strong correlation to bone resorption marker S-CTX, the Spearman's rank correlation being 0.51 (0.46-0.56, 95% confidence interval, CI) for both assays. Assays also had a strong correlation to S-TRACP5b, with r value 0.44 (0.39-0.49) and 0.41 (0.41-0.46), respectively. Interestingly, the correlation of these assays to S-BoneALP appeared to be less pronounced and the r values were 0.38 (0.32-0.43) and 0.36 (0.30-0.41), respectively. Thus, the correlation of urinary osteocalcin to serum bone formation marker (S-BoneALP) was significantly lower, without overlapping confidence limits, than the correlation to bone resorption marker (S-CTX). Correlation coefficients of U-TotalOC and bone markers were slightly smaller being 0.38 (0.33-0.43) for S-CTX, 0.38 (0.33-0.43) for S-TRACP5b, and only marginally smaller 0.30 (0.24-0.36) for S-BoneALP, a marker of bone formation. Furthermore, U-MidOC and U-LongOC had a strong correlation to serum osteocalcin (R=0.57 and 0.55, respectively) while the association of U-TotalOC and serum osteocalcin (R=0.30) was lower and equal to the one of U-TotalOC and S-BoneALP (R=0.30). The association to other markers of bone turnover strongly suggests that the measurement of urinary osteocalcin provides information on the skeletal turnover rate.

Connection to skeletal metabolism was further supported by the association between bone mass evaluated by densitometry and bone metabolism assessed by urinary osteocalcin. The individual values of osteocalcin had a statistically significant negative correlation to bone mineral content (BMC) measured at total body, spine, or hip ($p<0.0001$ for all associations). To study the association in more detail, women were classified into quartiles according to bone turnover rate (1 representing the lowest and 4 the highest quartile for urinary osteocalcin). There was a significant negative association between total body BMC and urinary osteocalcin and the individuals in high turnover quartiles had clearly lower BMC-values ($p<0.0001$ for all assays, Table 3.). In women classified as high turnover subjects according to urinary osteocalcin, the total body BMC was up to 10% smaller than in women classified as low turnover subjects. The results were similar with all three urinary assays.

Table 3. *Women with high bone turnover assessed by urinary osteocalcin (U-OC) have lower bone mass than low turnover subjects.* Bone mineral content (BMC) of total boy in U-OC quartile groups is shown as the percentage of the values observed in lowest U-OC quartile (1) (median with interquartile range).

	Total body bone mineral content in quartiles of urinary osteocalcin % of the lowest quartile		
Quartile	**U-MidOC quartiles**	**U-LongOC quartiles**	**U-TotalOC quartiles**
1 (lowest U-OC)	100% (89.5-111.9)	100% (87.7-109-5)	100% (90.8-111.7)
2	99.3% (88.7-108.0)	96.5% (87.4-105.9)	100.0% (89.6-110.8)
3	94.4% (86.4-105.5)	91.5% (81.6-101.6)	98.3% (86.7-106.1)
4 (highest U-OC)	90.7% (79.1-100.6)	87.6% (77.3-98.6)	90.9% (80.5 – 102.2)

In addition to the association to BMC, we also evaluated the association with bone mineral density assessed at femoral neck (aBMD). The percentage of osteoporotic subjects (T-score <-2.5) was greater (~39-42%) in high turnover quartile compared to low turnover quartile (~22-26%) ($p<0.001$). However, it should be recognized that bone turnover markers reflect the total body skeletal metabolism and thus, it may not be physiologically correct to try to relate biochemical markers such as urine osteocalcin to BMD. Furthermore, instead of single bone mass measurements, it would be more informative to evaluate correlation of long-term changes in BMC (or BMD) with baseline measurements of urinary osteocalcin or short-term changes in it (or turnover in general) in order to better understand the correlation between bone markers

and bone mass. This would demonstrate whether urinary osteocalcin is useful in predicting future bone loss and identifying subjects loosing bone at accelerated rate. The data concerning the long-term changes in spine and hip BMD in this cohort were not available at the time this study was conducted and this remains a topic for further studies. A correlation between baseline serum osteocalcin and subsequent rate of bone loss has been addressed and serum osteocalcin can be used to estimate future bone loss to some extent, at least when BMD is measured at the radius (reviewed in Stepan, 2000).

5.3.2 Monitoring anti-resorptive therapy (II, unpublished)

Urinary osteocalcin measured with any of the three assays was increased in postmenopausal women compared to the group of premenopausals (Fig.8). Especially the total concentration (U-TotalOC) was significantly higher in postmenopausals than premenopausals, reflecting the accelerated bone turnover after menopause ($p = 0.0051$). A similar trend was also observed with assays detecting only longer Gly^7- fragments (U-MidOC, U-LongOC) but without reaching statistical significance (p values 0.16 and 0.09, respectively). When bone turnover was suppressed with hormone replacement therapy (HRT), urinary osteocalcin values measured with all assays declined to levels observed in premenopausal. The p values for the difference between postmenopausal women with and without HRT were 0.0003, 0.019, and 0.0048 for U-MidOC, U-LongOC, and U-TotalOC, respectively. The values in postmenopausals with HRT were indistinguishable from the premenopausals ($p>0.05$ for all assays). This suggests that urinary osteocalcin can be a candidate marker for monitoring the efficacy of treatments.

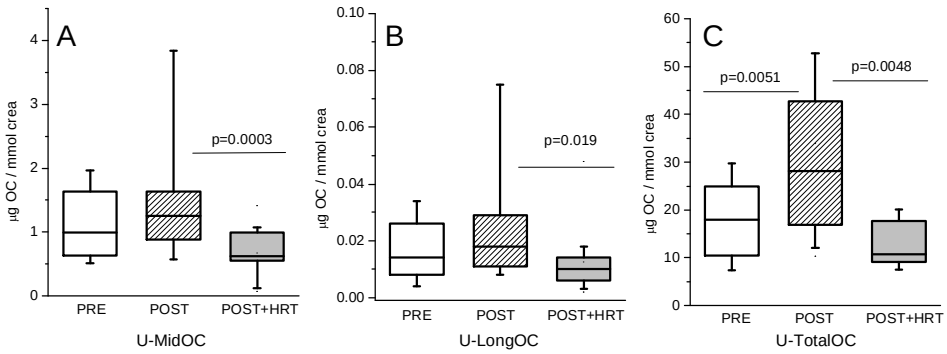

***Figure 8**. Urinary osteocalcin is reduced in response to hormone replacement therapy (HRT).* Box-and-whiskers plot of urinary osteocalcin values in groups of pre- (white) and postmenopausal women (hatched) and postmenopausal women with HRT (gray) measured with (A) U-MidOC, (B) U-LongOC, and (C) U-TotalOC. The horizontal lines represent the 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles. All statistical calculations were performed after logarithmic transformation and one-way ANOVA with Tukey's post-hoc test used in multiple comparisons.

Similar suppression in bone turnover markers was observed in response to treatment with alendronate (Fig.9). Both urinary and serum osteocalcin levels decreased significantly in the alendronate groups when compared to the baseline values ($p<0.001$). After three months, U-MidOC, U-LongOC, and U-TotalOC values were decreased to the level of 41.5% (median, interquartile range 32.4 - 59.3), 33.7% (25.8 - 53.0), and 59.2% (41.7 - 81.5) of the baseline values in the alendronate group. In the placebo group, the levels were 75.4% (61.0 - 89.2), 70.3% (51.3 - 91.7), and 99.3 % (79.3 - 133.0), respectively. Urinary osteocalcin levels remained unchanged during the remaining follow-up in the alendronate group with minor

fluctuations seen in the placebo (p values > 0.05 for all comparisons). There was also a significant difference between the placebo and alendronate group in all osteocalcin measurements during the entire follow-up period (p<0.001 for all assays in each timepoint). The decrease in serum osteocalcin was not of the same magnitude than in urine. After three months, serum osteocalcin concentration was 69.4% (58.9 - 81.2) of the baseline values in the alendronate group and 88.4% (79.2 - 105.6) in the placebo group. In contrast to urinary osteocalcin, there was a tendency for serum osteocalcin values to further decrease in the alendronate group from 3 months timepoint to 6 months (p=0.016), although it was not statistically significant after Tukey's adjustment (p=0.067). Such a declining trend from 3 to 6 months could not be detected for any of the urinary assays (p values after Tukey's adjustment 0.73 - 0.92). In conclusion, urinary osteocalcin decreases substantially in patients treated with alendronate, emphasizing it as an alternative candidate as a marker for rapid monitoring of anti-resorptive therapy. However, earlier timepoints would have been valuable in order to clarify the short-term kinetics more accurately.

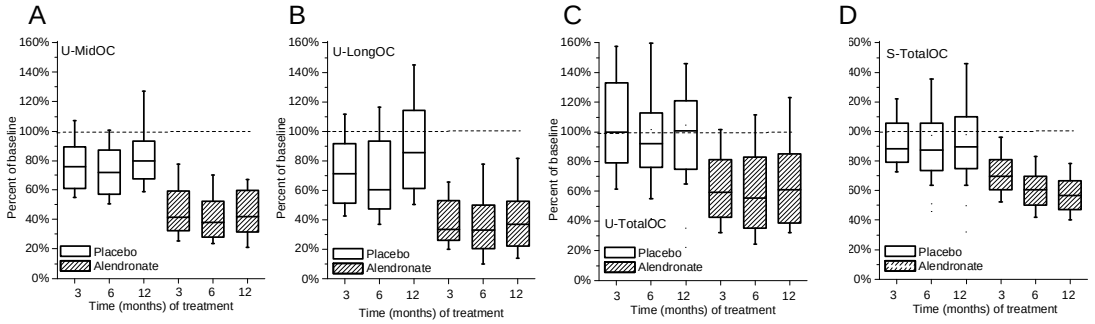

Figure 9. *Urinary osteocalcin is reduced in response to treatment with alendronate*. Box-and-whiskers plot of A) U-MidOC, B) U-LongOC, C) U-TotalOC, and D) S-TotalOC concentration after three, six and twelve months in groups receiving placebo (white) or alendronate (hatched). Results are shown as percentage (%) of baseline values and the horizontal lines represent the 10th, 25th, 50th, 75th, and 90th percentiles.

When the effect of calcium and vitamin D supplementation seen in the placebo group was taken into account, the specific net decrease in urinary osteocalcin due to alendronate was 34 - 40% at three-month timepoint depending on the assay. The difference was largest with U-TotalOC and statistically significant for all assays (p<0.001). However, even if the response in the treatment group is significant when compared to the placebo, this is not useful clinically unless the change in an individual patient is greater that would be expected due to normal variability of the marker, including both analytical and biological factors. The calculation of least significant change (LSC) for markers helps in the identification of responders to therapy. LSC is the minimum change between two successive results in an individual that would imply a real biological response. It defines a threshold as the change in marker greater than the precision error of the method in a single individual (Hannon *et al.*, 1998, Eastell *et al.*, 2000, Hannon *et al.*, 2004). The LSC-values for U-MidOC, U-LongOC, and U-TotalOC assays were 54%, 63%, and 51%, respectively, implying that a substantial decrease in urinary osteocalcin has to be detected before the result can be considered a true biological response to antiresorptive agent. Due to the high LSCs, the specificities were high (92.9 – 100%) but the sensitivities rather low (50.8 – 59.3%). Large LSCs were mostly due to high analytical variability (CVa) for U-

TotalOC and a large reduction in the placebo group (CVi) for U-MidOC and U-LongOC. LSC for serum osteocalcin (S-TotalOC) was substantially smaller (18%) and the assay was less specific (68.6%) and equally sensitive (59.3%) compared to urinary assays. Table 4 summarizes the analytical variability (CVa), biological variability (CVi), least significant change (LSC), specificity, and sensitivity of each assay calculated at three-month timepoint.

***Table 4**. The sensitivity and specificity of urinary and serum osteocalcin assays to detect the response to alendronate after three months of treatment.*

	U-MidOC	U-LongOC	U-TotalOC	S-TotalOC
CVa	7.4 %	8.0 %	15.9 %	5.0 %
CVi	21.8 %	25.8 %	-7.0 %	5.9 %
LSC (%)	53.5 %	63.0 %	40.5 %	17.9 %
Sensitivity	59.3 %	57.6 %	50.8 %	59.3 %
Specificity	100.0 %	100.0 %	92.9 %	68.6 %

There were a small number of individuals in the alendronate group in which osteocalcin levels increased (instead of decreasing) from baseline values after 3 months of treatment. It was not verified by other means whether these individuals (1 - 8 depending on the assay) did not respond to therapy or whether the markers were not able to detect the change in bone metabolism. Furthermore, the question about the utility of markers in monitoring the response in individual patients as opposed to carefully controlled research studies remains to be elucidated. Nevertheless, urinary osteocalcin has potential to be used in monitoring the early responses to alendronate therapy and may, therefore, also improve patient adherence to therapy by providing positive feedback to the patient.

5.3.3 Fracture prediction (III)

In the cohort of elderly women, 231 fractures were sustained in 178 women during a 3 - 6.5 year follow-up period (mean 4.6 years). Forty women sustained a hip fracture, 49 a vertebral fracture, and 37 multiple fractures (two or more fractures of any kind). The baseline marker levels were first compared between those women who sustained a fracture during the follow-up and those women who did not. Marker levels were consistently higher in women who sustained at least one fracture and the difference reached statistical significance for four markers (S-IntactOC, S-TRACP5b, S-CTX and U-LongOC). The marker levels were also elevated in subjects with multiple fractures but the levels were not significantly different from the subjects without any fracture. Similarly, five of the markers were elevated in women with a hip fracture, but none reached statistical significance. In contrast, all ten markers evaluated in the study, both formation and resorption markers were significantly elevated in women who later experienced a vertebral fracture during the follow up.

The relationship between markers and fracture risk can be studied in a linear manner (per SD increase) or by using cut-off values for markers. Large prospective studies collectively indicate that the relationship between fractures and markers is not directly proportional suggesting that bone turnover has to exceed a certain threshold to become deleterious to bone and be associated with increased fracture risk (Garnero, 2000). Different cut-offs have been used such as the median, two upper tertiles, the highest quartile, or the upper limit of the premenopausal range, but there is no clear consensus which is the most adequate method. We assessed the predictive ability by measuring the biochemical markers at the initiation of the study and monitoring the occurrence of fractures in the study population prospectively. Women

were classified into quartiles according to the baseline marker levels and women within the highest quartile were compared to all other women (i.e. women in three other quartiles) in their susceptibility to sustain a fracture during the follow-up period.

The odds ratios for prospective fractures were studied separately for each marker and they are summarized in Fig.10. When women within the highest quartile of a bone marker were compared with all others, S-TRACP5b and U-LongOC predicted the occurrence of fracture of any type with odds ratios (OR) 1.55 and 1.53, respectively ($p<0.05$). Both urinary osteocalcin assays UMidOC and U-LongOC as well as resorption markers S-TRACP5b and S-CTX predicted vertebral fracture (OR 2.28, 2.75, 2.71 and 1.94, respectively, all $p<0.05$) but there was no predictive ability for hip fracture with any of the markers investigated. In general, elevated levels of bone formation markers S-BoneALP and different assays for serum osteocalcin as well as resorption marker U-DPD did not predict any fractures. The statistical calculations were performed as an "intention to predict fracture" analysis and deaths were not excluded. For comparison, the conventional Kaplan-Meier survival analysis was performed which also considers deaths during the follow-up period. The predictive ability of markers with this method was essentially the same. The log rank test for at least one fracture was significant for U-MidOC ($p=0.03$), U-LongOC ($p=0.01$), and S-TRACP5b ($p=0.02$). The p values for other markers in the log rank test were between 0.17 and 0.86. Only two of the urinary osteocalcin assays were available at the initiation of the study and thus, information on the predictive ability of U-TotalOC assay was not obtained.

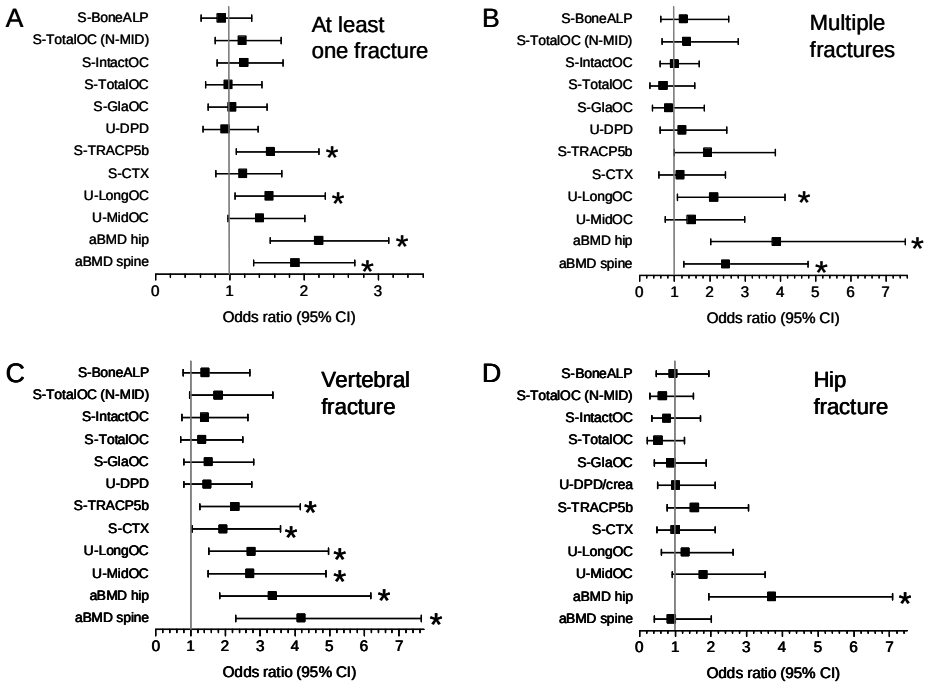

Figure 10. *Odds ratios with 95% confidence intervals for sustaining prospective fracture at A) any skeletal site, B) multiple sites, C) spine or D) hip*. Comparisons were made between women belonging to the highest quartile of bone turnover with all other women (or the lowest quartile for BMD in the femoral neck or lumbar spine). Statistically significant odds ratios are indicated with asterisks and the vertical line represents odd ratio of 1.0.

In this cohort, resorption markers had a stronger association to fractures than formation markers. This has also been observed previously (reviewed in Garnero, 2000, Garnero and Delmas, 2002). Urinary osteocalcin and two resorption markers S-TRACP5b and S-CTX were able to predict vertebral fractures but none of the markers investigated had predictive ability for hip fracture. This may be due to the larger amount of trabecular bone in the vertebra compared to the hip and therefore, a comparably higher metabolic activity.

Biochemical markers have several appealing properties to be potentially useful in fracture prediction, such as their ability to reflect the whole-body turnover instead of a fraction of the skeleton. They may also be more easily accessible than densitometry in a clinical situation. However, the risk estimated by densitometry is still higher than the risk increase seen with bone markers (Delmas *et al.*, 2000, Garnero, 2000). Also in our cohort, the odds ratios for prospective fractures were generally higher for BMD measurement than for any marker. The odds ratios for fractures varied from 0.90 to 4.19 for subjects in the lowest quartile BMD when compared with all other subjects, depending on the fracture type and the site of densitometric measurement. They were all statistically significant with the exception of lumbar spine BMD, which was not statistically significant in predicting hip fractures.

Interestingly, the predictive value of some biochemical markers remained significant after adjusting for aBMD. The odds ratios for predicting vertebral fractures were 2.02 and 2.25 for U-MidOC and U-LongOC, respectively after adjusting for lumbar spine BMD ($p<0.05$). Accordingly, the odds ratios for vertebral fractures were 2.15 and 2.21 for U-LongOC and S-TRACP5b, respectively after adjusting for femoral neck BMD ($p<0.05$). This suggests that markers reflect bone fragility independently of bone mass. Apparently, increased bone resorption increases bone fragility, not only by reducing BMD due to increased turnover, but also by inducing microarchitectural deterioration to bone tissue. The loss of connectivity and bone microarchitecture are presumambly major components of bone strength and are not necessarily detected by measuring bone mass (Seeman, 2002).

Since the turnover rate (mainly resorption) is able to predict fractures independently of BMD, the combination of markers and BMD could improve the risk assessment in an individual (Riis *et al.*, 1996, Looker *et al.*, 2000). In the EPIDOS study, the age-adjusted relative risk of hip fracture in women with both low BMD (T-score < -2.5) and high urinary CTX (highest quartile) was 4.8 when compared to the general population. In contrast, the relative risk in women with low BMD or high urinary CTX alone was 2.8 and 2.3, respectively. The addition of history of previous fragility fracture to the assessment increased the relative risk of fracture to 5.8. In the OFELY study, women with either low BMD or high serum CTX alone had 39% and 25% probability of fracture over the 5-year follow-up period while in women with both low BMD and high S-CTX the probability was 55% (Garnero *et al.*, 2000). In our study, the combination of aBMD and bone markers (U-MidOC, U-LongOC, S-TRACP5b, S-CTX) further enhancde the ability to predict fractures. In women with both low BMD (lowest quartile) and high urinary osteocalcin (highest quartile) the odds ratios for prospective fracture ranged from 1.98 to 4.05 depending on the fracture type and assay ($p<0.05$). However, the number of women fulfilling these criteria was limited making the confidence limits broader.

The study was population-based and the analyses were calculated for all women who participated at baseline with blood and urine sampling. The statistical calculations were performed as an “intention to predict fracture analysis” and therefore deaths were not excluded.

Nevertheless, the outcome of the log rank testing (prediction of at least one fracture) was essentially the same than when deaths were not taken into account. All women included in the study were of the same age (75 years at baseline) and thus, correction for age, that may mask actual differences, was unnecessary. Fracture data was searched for all participants and all fractures were objectively verified and no exclusions were made because of "lost to follow-up time" or unwillingness to continue. Correspondingly, there are some limitations. A subject was considered having a clinical symptomatic vertebral fracture if a spine radiograph had been undertaken due to back pain and a vertebral fracture had been identified. The possibility that an older vertebral deformity has been misclassified as a new fracture cannot be excluded, since baseline radiographs were not available. Furthermore, unknown previous fractures and their healing may interfere with baseline bone marker levels (Delmas *et al.*, 2000).

In conclusion, urinary osteocalcin (U-MidOC, U-LongOC) was predictive for forthcoming fractures, in particular those engaging trabecular bone such as vertebral fractures. Both assays were also predictive for vertebral fractures after correction for BMD and a combination of BMD and markers appeared to be able to improve fracture prediction. There are a number of studies supporting a role for bone turnover markers in prediction of fracture risk and in identifying women with high turnover (reviewed in Garnero *et al.*, 2000, Garnero and Delmas, 2002). However, markers are continuous risk factors and there is a considerable overlap in marker levels in women who will sustain a fracture and in women who will not. Whether there is a clinical role for the bone markers in the assessment of fracture risk in individual patients remains obscure.

5.3.4 Distinct properties of urinary and serum osteocalcin

The clinical properties of urine osteocalcin (U-OC) assays appear to be slightly different from the one of serum osteocalcin (S-OC) assays. Several pieces of evidence suggest that U-OC may be more associated with the resorption rate than the rate of bone formation, although it still needs further verification.

1) U-OC measured with U-MidOC and U-LongOC was predictive for fractures in the large prospective study of elderly women. The odds ratios for sustaining a clinical vertebral fracture were 2.71 (1.50 - 4.89, 95% CI) for U-MidOC and 2.75 (1.52 - 4.96) for U-LongOC. Together with resorption markers S-CTX and S-TRACP5b, these two U-OC assays were the only bone turnover markers evaluated which were statistically significant and the odds ratios of U-OC were comparable to the ones of S-CTX (1.94, 1.05 - 3.58) and S-TRACP5b (2.28, 1.26 - 4.15). Interestingly, none of the S-OC assays was predictive for vertebral fractures (odds ratios 1.80, 0.97 - 3.36; 1.41, 0.75 - 2.64; 1.33, 0.71 - 2.50 and 1.51, 0.81 - 2.81). This clearly emphasizes distinct clinical properties for S-OC and U-OC assays.

2) The kinetics of U-OC assays was slightly different from the kinetics of S-OC assays in the alendronate intervention trial. U-OC levels reached a plateau after three months of treatment but in contrast, there was a further decline in S-OC levels for up to six months. However, earlier timepoints would have been valuable in order to clarify the short-term kinetics more accurately. Since anti-resorptive agents primarily affect osteoclasts, a reduction is more rapidly seen in resorption markers and a decrease in formation markers is detected somewhat later due to the coupling of these two processes (Delmas, 2000). This may suggest that U-OC is more associated to bone resorption than bone formation.

3) In the cohort of elderly women, the correlation of U-OC assays was more pronounced to the well-known resorption marker S-CTX than to bone formation marker S-BoneALP.

Correlation coefficients of U-OCs tended to be also higher for another resorption marker, S-TRACP5b, than for S-BoneALP but the confidence limits were slightly overlapping.

A discordance between the measurements of serum osteocalcin and urinary excretion of its breakdown product (Gla) has been observed in patients with Paget's disease (Gundberg *et al.*, 1983) which is characterized by high bone turnover with increased number and appositional rate of osteoblasts seen in the affected sites with no net bone loss (Meunier *et al.*, 1980). More recently, Åkesson and co-workers monitored patients after tibia osteotomy (N=14) and demonstrated that U-OC values had short-term kinetics similar to the resorption markers such as U-DPD and distinct from S-OC (Akesson *et al.*, 2005). Different kinetics for S-OC and U-OC has been also seen in osteoporotic women after treatment with alendronate (N=19) (Srivastava *et al.*, 2002). Furthermore, the alendronate-induced changes in U-OC - but not in S-OC - were correlated to changes in resorption markers (U-NTX, U-CTX, and S-CTX) suggesting putatively a resorptive origin for U-OC (Srivastava *et al.*, 2002).

Intact osteocalcin and large fragments are degraded in the circulation and peripheral organs (Farrugia and Melick, 1986) while smaller fragments appear to be more resistant to degradation and accumulate into urine. Because of the rapid clearance from the circulation (Price *et al.*, 1981c), serum osteocalcin could reflect more acute changes in bone metabolism, while some of fragments accumulating into urine might serve more as an index of basal bone turnover and long-term changes because they provide an integrated measure over the collection period. Furthermore, urine could offer a better source for osteocalcin fragments of resorptive origin which are inevitably masked in serum by the large quantities of newly synthesized intact osteocalcin and resulting fragments making the detection of these fragments in circulation a difficult task. Thus, urinary osteocalcin may reflect different aspects of bone turnover than serum osteocalcin and offer an additional method for monitoring bone metabolism.

5.4 Implications on osteocalcin released from bone resorption (IV)

5.4.1 Immunoreactive osteocalcin in osteoclast cultures

It is well-documented that osteoblasts produce osteocalcin which is used as a marker of bone formation (*reviewed in sections 2.8 - 2.9*). In order to understand the contribution of each type of bone cell to the circulating osteocalcin levels we studied whether osteocalcin detectable by immunoassays is released from bone matrix during bone degradation.

Immunoreactive osteocalcin was clearly released into the medium during osteoclastic resorption both in the rat and human *in vitro* models investigated. Osteocalcin was detected with assays measuring either intact osteocalcin exclusively (I-OC) or assays detecting fragments in addition to intact molecules (M-OC and T-OC). The release of osteocalcin was increased in a time-dependent manner. It also increased in the presence of PTH, a known stimulator of bone resorption, and was almost undetectable when BafA1, a potent inhibitor of V-ATPase and bone resorption, was present in the cultures. In addition to the immunoassays, osteocalcin was demonstrated inside bone-resorbing osteoclasts by immunostaining with an antibody against a midmolecular epitope (MAb 3H8, epitope in the fragment Arg^{20}-Arg^{43}). The intracellular osteocalcin was predominantly located in the same vesicles as the endocytosed bone matrix labeled with fluorescein. Although the labeling of bone with fluorescein has probably produced small amounts of labeled osteocalcin which can be responsible for some of the co-localization, this does not invalidate the conclusion that osteocalcin can be detected inside osteoclasts in the very same compartments as the proteins originally present in the bone matrix. In addition to intracellular vesicles, osteocalcin was also observed at the bottom of the

resorption lacunae, but not on intact bone surface. The immunoassay results and microscopic data together strongly support a concept for the release of immunodetectable osteocalcin during bone resorption.

The amount of osteocalcin in medium correlated with the concentration of CTX in both human and rat osteoclast cultures with r values 0.93 and 0.95, respectively ($p<0.0001$). This indicates that osteocalcin detected by the immunoassays is derived from degraded matrix and can thus be used as an index of bone resorption *in vitro*. Various assays detecting different molecular forms of osteocalcin can be used since the correlation with CTX was similar with all osteocalcin assays tested (r values from 0.902 to 0.934). The correlation coefficient was highest for T-OC assay but also highly significant for other assays I-OC, M-OC, and commercial osteocalcin ELISA. The same assays were also useful in monitoring osteocalcin production in osteoblast cultures (Ivaska *et al.*, 2002). Thus, the very same osteocalcin assays can be applied to monitor both *de novo* synthesized osteocalcin in osteoblast cultures, as well as the bone matrix-derived osteocalcin in osteoclast cultures and in addition to the conventional use as a marker of bone formation, osteocalcin can be used as a marker of bone resorption *in vitro*.

Whether resorptive osteocalcin molecules are also released *in vivo* and contribute to the circulating osteocalcin pool is not known, but in theory, part of osteocalcin found in the blood may also be derived from osteocalcin embedded in the bone matrix and released during bone degradation. Circulating osteocalcin has been indicated to originate exclusively from biosynthesis in osteoblasts and not from the breakdown of bone matrix (Price *et al.*, 1981c, Brown *et al.*, 1984, Charles *et al.*, 1985). However, the early studies utilized rather insensitive first generation RIAs. The molecular forms they detected were rarely known and it is likely that many assays detected only intact osteocalcin, not fragments. For instance, the original RIA (Price and Nishimoto, 1980) fails to detect osteocalcin after cathepsin-mediated cleavages in region Ala^{41}-Phe^{45} (Baumgrass *et al.*, 1997). Later, some clinical studies on different metabolic bone diseases have suggested that not all circulating osteocalcin fragments are derived from the metabolism of osteocalcin in circulation and peripheral organs but also from osteocalcin embedded in bone (Gundberg and Weinstein, 1986, Taylor *et al.*, 1990, Gundberg *et al.*, 1991). Considering the high variability in the performance of different osteocalcin assays, it is not surprising that conflicting results have been obtained depending on the selection of the assay.

5.4.2 Resorptive osteocalcin contains both intact and fragmented molecules

The heterogeneity of released osteocalcin was determined using reverse-phase HPLC (Fig.11.) and several molecular forms, including intact molecule, were identified in the culture medium. The fractionation of osteocalcin from osteoclast culture medium resulted in one predominant peak which was detectable by all three assays I-OC, M-OC, and T-OC and two minor peaks. The molecular forms eluting in the smaller peaks could not be identified due to the small amount of protein, but on the basis of earlier elution time and lack of immunoreactivity in I-OC assay they most likely represent osteocalcin fragments. Only one single peak containing intact osteocalcin (positive for I-OC assay) was observed when osteocalcin released by HCl treatment was fractionated. A similar profile with one twin peak was observed in the medium of rat osteoblasts. Since the intact-specific assays I-OC does not recognize osteocalcin of rat origin, we were unable to test whether osteocalcin released from osteoblasts was intact but, according to other studies (Gundberg and Clough, 1992, Garnero *et al.*, 1994a, Diaz Diego *et al.*, 1998), it seems likely. The elution profile of bovine serum osteocalcin was more heterogeneous and consisted of at least four main peaks (*see section 5.1.3*).

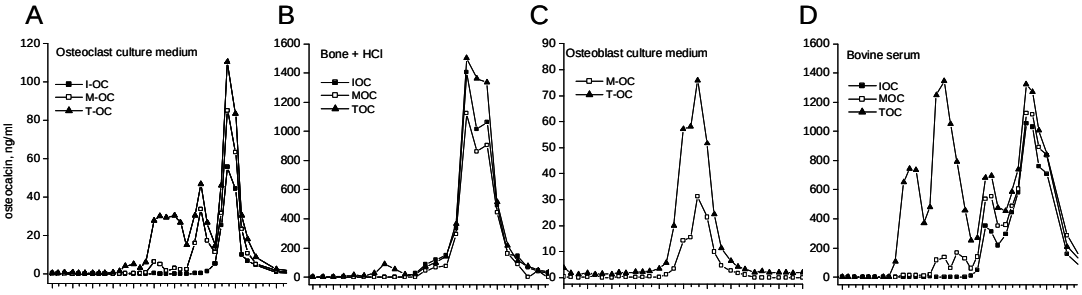

Figure 11. *HPLC fractionation of osteocalcin.* A) Rat osteoclasts on bovine bone + PTH (7 days). B) Bovine bone + HCL (without cells). C) Rat osteoblasts + vitamin D (15 days). D) Osteocalcin isolated from fetal bovine serum. Fractions were measured with I-OC (solid squares), M-OC (open squares), and T-OC (triangles) assays.

The correlation between CTX and intact osteocalcin (*see section 5.4.1*) already indicated that part of osteocalcin was able to escape complete degradation in osteoclasts and was released from resorption as intact, unfragmented molecules. This was further supported by the HPLC fractionation of osteocalcin from osteoclast culture medium, since the largest peak in the elution profile was positive for the I-OC assay and consisted of intact osteocalcin molecules. Kurihara and co-workers used an assay for the N-terminal epitope of osteocalcin to detect osteocalcin in the supernatant of bone particle-derived osteoclasts cultured on bone slices (Kurihara *et al.*, 1998). Although the N-terminal fragment was clearly the predominant form, a small amount of intact osteocalcin was also detected.

Osteoclast culture medium contained a large amount of intact osteocalcin and minor amounts of osteocalcin fragments, represented by the two smaller peaks in elution profile (Fig.11A.). Interestingly, these two peaks eluted approximately in same position respect to intact osteocalcin as the two fragments observed in serum osteocalcin (Fig.11D.). Therefore, osteoclast culture medium may contain fragments similar to those present in serum, although the comparison of detailed structures was not possible. Because the elution profiles of osteocalcin isolated from the culture medium and serum had similarities, resorptive osteocalcin could be able to contribute to the circulating pool of both osteocalcin fragments and intact molecules. Osteocalcin is, however, further metabolized in the circulation and peripheral organs making it difficult to draw conclusions on the basis of *in vitro* studies only. Furthermore, the results were obtained with isolated rat osteoclasts cultured on bovine bone, an unnatural substrate, and they do not necessarily apply as such to human *in vivo* situation. If these resorptive forms are released also *in vivo* and contribute to the circulating osteocalcin pool, the circulating osteocalcin should be considered an indicator of bone turnover rather than a marker of bone formation.

5.4.3 Osteocalcin may escape from extensive degradation during resorption

5.4.3.1 Overview on resorption

Activation of osteoclasts leads to attachment on a target matrix and generation of an isolated microenvironment in the subosteoclastic space between the cell and the bone surface (Silver *et al.*, 1988). During this process, changes in the organization of the cytoskeleton and vesicle transportation occur which lead to the polarization of the cell (Väänänen *et al.*, 2000). As a result, vesicles from the endocytic and biosynthetic pathway are directed to the bone-facing surface of the cell and fused to the plasma membrane facing bone leading to the formation of ruffled border membrane due to expanded membrane area (Baron *et al.*, 1988, Palokangas *et*

al., 1997). This is followed by the release of proteases such as cathepsin K (Littlewood-Evans *et al.*, 1997) and secretion of hydrochloric acid by V-ATPase to the bone surface (Väänänen *et al.*, 1990). Before proteolytic enzymes can reach and degrade collagenous bone matrix, tightly packed hydroxyapatite crystals must be dissolved. The secretion of HCl into resorption lacuna results in a decrease in pH which solubilizes the mineral phase of the bone (Väänänen and Zhao, 2002). After solubilization, proteolytic enzymes degrade the organic bone matrix, although the detailed sequence of events is still unclear. The major protease responsible for the organic matrix degradation is the lysosomal proteinase cathepsin K which functions in acidic pH environment provided by acid secretion (Bromme *et al.*, 1996, Inui *et al.*, 1997). As a result of the acid and protease secretion, bone matrix under the osteoclast is digested and a resorption lacuna is formed.

The resorption lacuna underneath the ruffled border with all degradative components is tightly separated from the surrounding by a sealing zone which mediates a tight attachment to bone matrix and has been suggested to inhibit free diffusion of molecules (Väänänen and Horton, 1995). Matrix degradation products are removed from the resorption lacuna in a controlled manner by internalization to the resorbing cell. The area behind the low pH zone consists of a collagenous gel that further restricts solute diffusion from below the osteoclast (Stenbeck and Horton, 2000). Receptors for the uptake of degradation products into transcytotic route are not conclusively elucidated but $\alpha_v\beta_3$ integrin has been suggested as a putative candidate due to its affinity to denatured type I collagen (Helfrich *et al.*, 1996). The matrix uptake occurs at the central area of ruffled border while the fusion of secretory vesicles occurs at the peripheral zone (Mulari *et al.*, 2003). The internalization of degraded matrix is followed by transcytosis through the cell and finally, exocytosis to the intercellular space via the functional secretory domain at the top of the cell (Salo *et al.*, 1996, Nesbitt and Horton, 1997, Salo *et al.*, 1997). Further destruction of internalized matrix components during transcytosis may be facilitated by other factors such as TRACP. It is found in the transcytotic pathway together with cathepsin K and generates reactive oxygen species, probably upon activation by cathepsin K (Vääräniemi *et al.*, 2004). The reactive oxygen species, in turn, are capable of degrading collagen and needed to finalize the degradation of the matrix components during transcytosis (Halleen *et al.*, 1999b).

5.4.3.2 Resorption and matrix osteocalcin

The treatment of osteoclast cultures with two inhibitors of bone resorption, BafA1 and E64, resulted in distinct responses in osteocalcin and the well-established resorption marker CTX. The inhibition of acidification by BafA1 resulted in a complete inhibition in the release of CTX and in all molecular forms of osteocalcin. When the proteolytic activity of cathepsins was inhibited with E64, a nearly complete inhibition in the release of CTX was again evident, indicating that the osteoclasts were unable to degrade the collagenous matrix. However, E64 did not have such a profound effect on the release of osteocalcin, suggesting that part of osteocalcin is liberated from the matrix via a cathepsin-independent pathway. Strikingly, the release of intact osteocalcin was almost unaffected by E64. At least the detachment of intact molecules does not seem to require the activity of cathepsins. One alternative is that the different molecular forms are released during different stages of osteoclastic bone resorption. According to HPLC, the osteocalcin released during the dissolution of mineral consisted almost exclusively of intact molecules which may indicate that the demineralization, and not cathepsin-mediated degradation, is the main source of intact osteocalcin during bone resorption.

Bone matrix targeted for resorption contains a large amount of osteocalcin. Osteocalcin is the second most common bone matrix protein after type I collagen and it has been estimated that it constitutes up to 2% of the total dry weight of bone (Hauschka *et al.*, 1975, Price *et al.*, 1976a). During bone resorption, osteocalcin embedded in the matrix is most likely exposed to the proteolytic microenvironment which could lead to degradation of matrix-bound osteocalcin into small fragments during resorption. However, the experimental results on the release of intact osteocalcin molecules and large fragments during resorption *in vitro* are not in agreement with this assumption. Structural properties of the osteocalcin molecule could provide some explanations together with the assumption on the release of fragments and intact molecules via different routes – the other mediated by cathepsins and the other by acidification - and it is tempting to hypothesize the following:

Osteocalcin binds strongly to the mineral component of bone, hydroxyapatite, (Price *et al.*, 1976a). All three Gla residues are implicated in binding and they form a negatively charged protein surface that interlocks exactly to the positively charged calcium ions in the hydroxyapatite crystal lattice (Hoang *et al.*, 2003). Osteocalcin released during dissolution of mineral *in vitro* consisted almost exclusively of intact molecules. Thus, the acidification alone is able to detach osteocalcin from matrix and the acidic environment does not lead to extensive fragmentation. When mineral is dissolved and its component ions released from hydroxyapatite, osteocalcin bound to calcium via Gla residues may be concomitantly released as intact molecules independent of the proteolytic pathways. The surrounding of resorbing osteoclasts as well as the resorption lacunae presumably contains high local calcium levels, which could protect osteocalcin against proteolysis (Novak *et al.*, 1997). Also the Gla residues and the fold they introduce appear to protect osteocalcin from degradation since Gla-containing core region is not sensitive to cathepsins (Baumgrass *et al.*, 1997). The midmolecule region of decarboxylated osteocalcin is also more susceptible to proteolysis by cathepsin K than the fully carboxylated form (Gundberg *et al.*, 2002). There is also a possibility that osteocalcin or part of it (and calcium) detached during mineral dissolution leaks from the sealing zone and does not enter the degradative intracellular pathway. Although the sealing zone has been considered to inhibit free diffusion of molecules (Väänänen and Horton, 1995), there is some evidence that negatively charged molecules with a molecular weight of less than 10 000 diffuse freely under the sealing zone (Stenbeck and Horton, 2000).

After demineralization, proteolytic enzymes will cleave collagen and other matrix proteins into smaller fragments. At this step, osteocalcin which was not mobilized during matrix dissolution will be susceptible to degradation by proteases, especially cathepsins, resulting in the generation of osteocalcin fragments. Because only intact osteocalcin is able to interact with hydroxyapatite (Novak *et al.*, 1997), the proteolytic attack will lead to a detachment of fragmented osteocalcin molecules from matrix. These fragments may be transported via transcytosis together with other matrix components (Salo *et al.*, 1997). Since the middle portion of osteocalcin is resistant to cathepsin-mediated proteolysis (Baumgrass *et al.*, 1997, Koboyashi *et al.*, 1998, Gundberg *et al.*, 2002) osteocalcin would be released as relatively large midmolecule fragments and contribute to the heterogeneity observed in resorptive osteocalcin.

A simplified overview on the origin and metabolism of osteocalcin in circulation and urine based on the literature and findings of this study is presented in Fig.12.

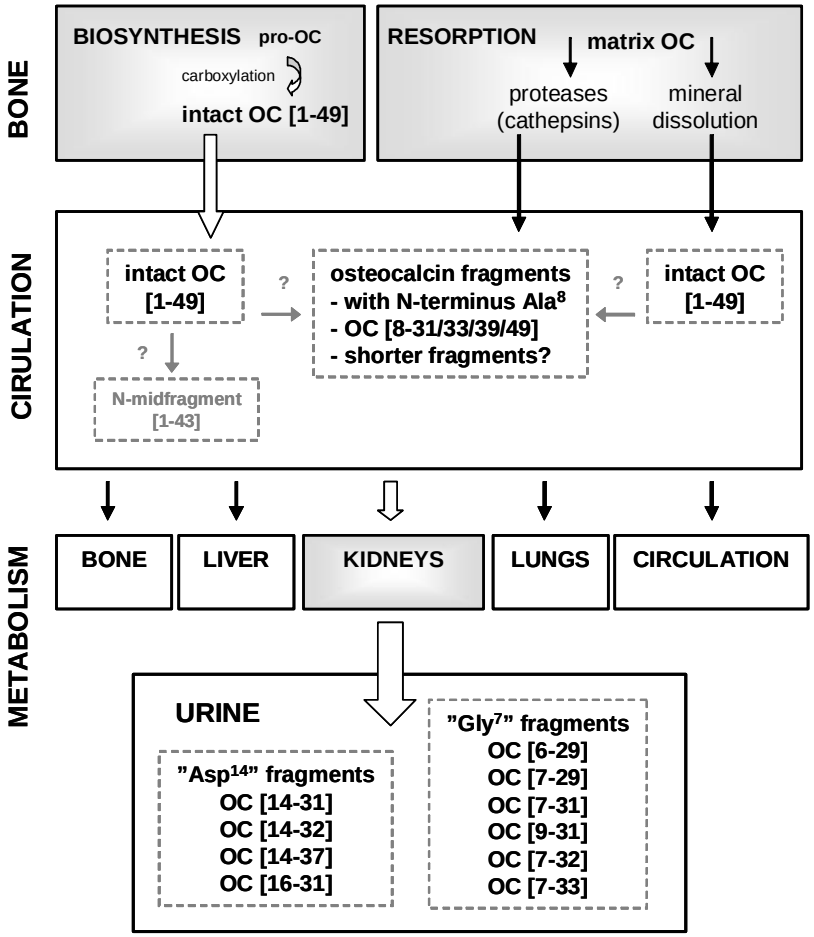

Figure 12*. A simplified overview on the origin and heterogeneity of osteocalcin in the circulation and urine and possible routes for metabolic clearance*. See text for details.

6 CONCLUDING REMARKS

The research we carried out indicates that osteocalcin is excreted into urine as a mixture of fragments from the middle region of the molecule. Urinary fragments can be classified into at least two categories according to size. Fragments are large and abundant enough to be detected with conventional immunoassays making it appealing to study the role of urinary osteocalcin as an alternative method to assess bone metabolism. In this study, three immunoassays for the detection of different molecular forms of urinary osteocalcin were developed and characterized. Emphasis was put on evaluating them in clinical settings related to osteoporosis and the analysis of the clinical data demonstrated urinary osteocalcin as a valid marker for the assessment of bone metabolism. Urinary osteocalcin detected with any of the assays is associated with bone turnover rate making it an attractive candidate for monitoring events in bone metabolism. The results also demonstrate that it is a promising new marker for prediction of future fractures, in particular vertebral fractures. The clinical utility needs, however, to be verified in forthcoming long-term evaluations. Interestingly, the clinical performance of the urinary assays appeared to be distinct from the one of serum osteocalcin assays. Osteocalcin in urine may reflect different aspects of bone turnover than serum osteocalcin and be more associated with resorption than formation. Thus, it offers an additional method for monitoring bone metabolism and may have potential applications in diagnostics related to disorders of bone metabolism. It may be a valuable clinical tool either independently or in combination with other biochemical markers of bone turnover or bone densitometric measurements.

It is well-documented that osteoblasts produce osteocalcin which is used as a marker of bone formation. The results presented in this work suggest that osteocalcin is also released during bone resorption as well as being produced by osteoblasts during bone formation. A part of osteocalcin pool escapes the proteolytic degradation during bone resorption and is released both as intact molecules and large midmolecule fragments *in vitro*. Therefore, a fraction of osteocalcin in the circulation may actually be derived from bone resorption although it is difficult to estimate the relative proportion of serum osteocalcin which would come from each process in a clinical setting.

Assays for serum osteocalcin are widely used to assess bone formation rate in patients. However, circulating osteocalcin contains several molecular forms which are differently detected by various assays making the comparison of results inappropriate. The results presented in this study provide novel insights into the complexity and heterogeneity of osteocalcin. The potential contribution of osteoclastic resorption to immunoreactive species detected by certain assays has to be considered and serum osteocalcin should preferentially be considered a marker of bone turnover in general, instead of a pure marker of bone formation. The possibility to measure osteocalcin fragments in urine expands the utility by providing specific methods to assess bone metabolism in a sample material which can be collected conveniently and demonstrates improved stability of osteocalcin compared to serum. Interestingly, urinary osteocalcin seems to reflect different aspects of bone metabolism than serum osteocalcin and therefore, it adds to the information obtained by serum measurements. This can be clearly seen in the ability of urinary osteocalcin to predict fractures.

In conclusion, the measurement of osteocalcin in urine clearly offers an additional method to assess bone turnover and has potential applications in diagnostics related to bone diseases. In particular, it may be used for monitoring the efficacy of anti-resorptive therapy and for the estimation of risk for osteoporotic fractures.

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Kaisa Ivaska

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