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TARTRATE-RESISTANT ACID PHOSPHATASE 5b: A SERUM MARKER OF BONE RESORPTION

by

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ABSTRACT

Bone is a physiologically dynamic tissue being constantly regenerated throughout life as a consequence of bone turnover by bone-resorbing osteoclasts and bone-forming osteoblasts. In certain bone diseases, such as osteoporosis, the imbalance in bone turnover leads to bone loss and increased fracture risk. Measurement of bone mineral density (BMD) predicts the risk of fracture, but also biochemical markers of bone metabolism have been suggested to be suitable for prediction of fractures and monitoring the efficacy of antiresorptive treatment.

Tartrate-resistant acid phosphatase 5b (TRACP 5b) is an enzyme released from osteoclasts into the circulation, from where it can be detected kinetically or immunologically. Conventional assays for serum total TRACP were spectrophotometric and suffered from interference by other acid phosphatases and non-osteoclastic TRACP 5a isoform. Our aim was to develop novel immunoassays for osteoclastic TRACP 5b.

Serum TRACP 5b levels were elevated in individuals with high bone turnover, such as children, postmenopausal women, patients with osteoporosis, Paget's disease and breast cancer patients with bone metastases. As expected, hormone replacement therapy (HRT) in postmenopausal women decreased the levels of serum TRACP 5b. Surprisingly, the highest TRACP 5b levels were observed in individuals with rare autosomal dominant osteopetrosis type II (ADO2), which is characterized by high BMD and fracture risk with simultaneously elevated levels of deficient osteoclasts. In ADO2 patients, elevated levels of serum TRACP 5b were associated with high fracture frequency. It is likely that serum TRACP 5b reflects the number of inactive osteoclasts in ADO2. Similar results supporting the hypothesis that TRACP 5b would reflect the number of osteoclasts instead of their activity were observed with cultured osteoclasts and in animal models.

Novel TRACP 5b immunoassays may prove to be of value either as independent or combinatory tools with other bone metabolic markers and BMD measurements in clinical practice and bone research.

Keywords: Bone, osteoclast, TRACP 5b, osteoporosis

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TIIVISTELMÄ

Luukudos uusiutuu läpi elämän luunsyöjäsolujen eli osteoklastien hajottaessa vanhaa luuta ja luuta muodostavien solujen eli osteoblastien tuottaessa uutta luuta. Useat luusairaudet, myös luukato eli osteoporoosi, ovat seurausta tasapainon järkkymisestä luun uudelleenmuodostuksessa. Tämä epätasapaino saa aikaan luumassan vähenemisen ja luuston mikrorakenteen heikentymisen, jotka yhdessä lisäävät murtumariskiä. Luuntiheysmittaukset ennustavat murtumariskiä riskiryhmässä oleville, mutta myös uudet verenkierrosta mitattavat biokemialliset merkkiaineet soveltuvat potilaiden hoidon seurantaan ja murtumariskin arviointiin.

Tartraatille resistentti hapan fosfataasi 5b (TRACP 5b) on osteoklasteista verenkiertoon vapautuva entsyymi, jota voidaan mitata kineettisesti tai immunologisesti. Perinteiset menetelmät mittaavat TRACP 5b:n lisäksi muita happamia fosfataaseja sekä TRACP 5a:ta, joka ei ole osteoklasteista peräisin. Tavoitteenamme oli kehittää uusi spesifinen immunomääritysmenetelmä osteoklasti-peräiselle TRACP 5b:lle.

Seerumin TRACP 5b on koholla kiihtyneen luun hajotuksen vuoksi lapsilla ja postmenopausaalisilla naisilla. Samoin luun hajotukseen vaikuttavissa taudeissa, kuten osteoporoosissa, Pagetin taudissa ja luuhun levinneessä rintasyövässä kohonneet seerumin TRACP 5b -tasot heijastavat lisääntynyttä luun hajotusta. Odotetusti seerumin TRACP 5b reagoi nopeasti luun hajotusta vähentävään hormonikorvaushoitoon. Korkeimmat seerumin TRACP 5b -tasot havaitsimme yllättäen osteopetroosi-potilailla, joiden luumassa on koholla vähentyneen luun hajotuksen vuoksi. Tyypin 2 autosomaalista dominanttia osteopetroosia (ADO2) sairastavien potilaiden osteoklastit eivät toimi normaalisti, mitä elimistö pyrkii kompensoimaan lisäämällä luunsyöjä-solujen lukumäärää. Luun hajotukseen kykenemättömät osteoklastit erittävät kuitenkin edelleen TRACP 5b:tä verenkiertoon, mikä selittää kohonneet entsyymiarvot ADO2 -potilailla. Seerumin TRACP 5b:llä havaittiin selvä yhteys lisääntyneeseen murtuma-riskiin ADO2 -potilailla. Soluviljely- ja eläinmalleissa havaittiin myös yhteys eritetyn TRACP 5b:n ja osteoklastien lukumäärän välillä, mikä vahvistaa ADO2 -potilailla tehtyä havaintoa.

Uusilla TRACP 5b -immunomääritysmenetelmillä tullee olemaan tulevaisuudessa käyttösovelluksia sekä kliinisissä että luun perusbiologisissa tutkimuksissa.

Avainsanat: Luu, osteoklasti, TRACP 5b, osteoporoosi

CONTENTS

ABBREVIATIONS

AcP	acid phosphatase
ADO(2)	autosomal dominant osteopetrosis (type II)
ALP	alkaline phosphatase
BC	breast cancer
BMD	bone mineral density
BMP	bone morphogenetic protein
bp	base pair
BRU	bone-remodelling unit
BSP	bone sialoprotein
CAII	carbonic anhydrase II
CK-BB	BB isoform of creatine kinase
ClCN7	chloride channel 7
CTX	crosslinked C-telopeptide of type I collagen
CV	coefficient of variation
DPD	deoxypyridinoline
DXA	dual-energy X-ray absorptiometry
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FSD	functional secretory domain
HCL	hairy cell leukemia
HF	hepatic dysfunction
HHM	humoral hypercalcemia of malignancy
HPLC	high-performance liquid chromatography
HRE	hemin-responsive elements
HRT	hormone replacement therapy
ICTP	cross-linked C-telopeptide of type I collagen
IGF	insulin-like growth factors
IL	interleukin
kb	kilo base
kD	kilo dalton
LAP	lysosomal acid phosphatase
LSC	least significant change
MAb	monoclonal antibody
M-CSF	macrophage-colony stimulating factor
MiTF	microphthalmia transcription factor
MM	multiple myeloma
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
4-NPP	4-nitrophenyl phosphate
NTX	crosslinked N-telopeptide of type I collagen
OC	osteocalcin
OPG	osteoprotegerin

ORC	orchidectomy
OVX	ovariectomy
PAGE	polyacrylamide gel electrophoresis
PAP	purple acid phosphatase
PDB	Protein Data Bank
PICP	C-terminal propeptide of type I collagen
PINP	N-terminal propeptide of type I collagen
PMA	phorbol 12-myristate 13-acetate
PP	protein phosphatase
PTH	parathyroid hormone
PTHrP	PTH-related peptide
PTPase	protein tyrosine phosphatase
PYD	pyridinoline
(p)QCT	(peripheral) quantitative computed tomography
QUS	quantitative ultrasound
RA	rheumatoid arthritis
RANK	receptor for activator of nuclear factor-κB
RANKL	RANK ligand
RB	ruffled border
RF	renal failure
RIA	radioimmunoassay
RT	room temperature
ROS	reactive oxygen species
SD	standard deviation
SERM	selective estrogen receptor modulator
tsp	transcription start point
TRACP	tartrate-resistant acid phosphatase
TF	transcription factor
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
USFDA	U.S. Food and Drug Administration
UTR	untranslated region
V-ATPase	vacuolar-type proton adenosine triphosphatase
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Halleen JM, Alatalo SL, Suominen H, Cheng S, Janckila AJ, Väänänen HK. Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. 2000 J Bone Miner Res. 15(7): 1337-45.

II Alatalo SL, Halleen JM, Hentunen TA, Mönkkönen J, Väänänen HK. Rapid screening method for osteoclast differentiation in vitro that measures tartrate-resistant acid phosphatase 5b activity secreted into the culture medium. 2000 Clin Chem. 46(11): 1751-4.

III Halleen JM, Alatalo SL, Janckila AJ, Woitge HW, Seibel MJ, Väänänen HK. Serum tartrate-resistant acid phosphatase 5b is a specific and sensitive marker of bone resorption. 2001 Clin Chem. 47(3): 597-600.

IV Alatalo SL, Peng Z, Janckila AJ, Kaija H, Vihko P, Väänänen HK, Halleen JM. A novel immunoassay for the determination of tartrate-resistant acid phosphatase 5b from rat serum. 2003 J Bone Miner Res. 18(1): 134-9.

V Alatalo SL, Ivaska KK, Waguespack SG, Econs MJ, Väänänen HK, Halleen JM. Osteoclast-derived serum tartrate-resistant acid phosphatase 5b in Albers-Schönberg disease (type II autosomal dominant osteopetrosis). 2004 Clin Chem. 50(5): 883-90.

1. INTRODUCTION

The skeleton is remodelled throughout the lifetime to fulfill the criteria needed for its supporting, protective and metabolic functions. The cells responsible for bone degradation and formation are called osteoclasts and osteoblasts, respectively. The normal physiological bone remodelling process is tightly coupled, meaning that the amount of bone removed by osteoclasts is replaced by new bone formed by osteoblasts. Alterations in remodelling and uncoupling of bone resorption and formation are responsible for most metabolic bone diseases such as osteoporosis, myeloma and breast and prostate cancers with bone metastases (Mundy 1999).

The most widely used method for assessing bone density is dual-energy X-ray absorptiometry (DXA), since it has high precision, short scan times, low radiation and stable calibration (Blake and Fogelman 2002). Determination of bone mineral density (BMD) from the hip by DXA has been selected as the tool to diagnose osteoporosis (WHO 1994). In contrast to densitometric measurements of bone mass and structure from a particular site of the skeleton, biochemical markers of bone metabolism measured from serum or urine are helpful tools in detecting the dynamics of the metabolic imbalance in the whole skeleton. Biochemical markers of bone metabolism are non-invasive, relatively inexpensive, generally available, can measure changes in bone metabolism over short intervals, and can be assessed repetitively. For clinical purposes, markers of bone metabolism can be classified into three classes: 1) bone formation markers, released during bone formation by osteoblasts; 2) bone resorption markers released from bone matrix or from osteoclasts during bone resorption; and 3) bone turnover markers, released during bone formation and resorption. Clearly, measurement of bone metabolic markers does not substitute for BMD measurements, but they provide excellent adjunctive tools in the management of patients with osteoporosis and other high bone turnover diseases (Seibel *et al.,* 2002).

Tartrate-resistant acid phosphatase (TRACP; EC 3.1.3.2) was discovered from human leukocytes and named as type 5 acid phosphatase (AcP) according to the electrophoretic mobility in native polyacrylamide gel (Li *et al.,* 1970a, b). Physiologically, TRACP expression is found in cells of the mononuclear phagocyte system, most abundantly in osteoclasts, alveolar macrophages and dendritic cells (Efstratiadis and Moss 1985b; Andersson and Marks 1989; Yaziji *et al.,* 1995). In serum, TRACP exists in two differentially glycosylated forms, sialylated TRACP 5a and non-sialylated TRACP 5b, which are products of the same gene (Lam *et al.,* 1978a). Of these, TRACP 5b originates exclusively from osteoclasts and TRACP 5a from activated macrophages and dendritic cells (Janckila *et al.,* 2002b). Therefore, serum TRACP 5b has been suggested to be an excellent marker of bone resorption (Minkin 1982; Chambers *et al.,* 1987; Janckila *et al.,* 2001b; Janckila *et al.,* 2002b). Several kinetic and immunological assays have been developed for circulating total TRACP activity or protein, but they lack both specificity and sensitivity for osteoclastic TRACP 5b (Lau *et al.,* 1987b; Chamberlain *et al.,* 1995; Cheung *et al.,* 1995). However, these total TRACP assays show that the levels of serum TRACP are elevated in patients with skeletal diseases, such as osteoporosis, hyperparathyroidism, Paget's disease and in breast and prostate cancer patients with bone metastases (Lau *et*

al., 1987b; Scarnecchia *et al.,* 1991). TRACP 5b is unique among bone resorption markers, since it is a product of resorbing osteoclasts, while other bone resorption markers are released from bone matrix during osteoclastic bone degradation.

The present study aimed at developing a novel immunoassay for circulating osteoclastic TRACP 5b, and at investigating the use of this assay in different bone diseases. In addition to human TRACP 5b assays, immunoassays were developed for rat serum TRACP 5b and TRACP 5b secreted from cultured osteoclasts. The validity of these assays was studied using *in vivo* and *in vitro* approaches.

2. REVIEW OF THE LITERATURE

2.1 Bone

Bone has developed during evolution to be mechanically strong, but at the same time light and elastic to fulfill all the criteria needed for distinct functions. Bone tissue has three main functions: 1) mechanical support and the site of muscle attachment for locomotion, 2) protective, for vital organs and bone marrow; and 3) metabolic, as a reserve of ions, especially calcium and phosphate, for the maintenance of serum homeostasis. Bone is a physiologically dynamic and unique tissue being mineralized and constantly regenerated throughout life as a consequence of bone turnover.

Bones can be developed directly via intramembranous ossification or indirectly via endochondral ossification through a cartilage model. Anatomically, two types of bones can be distinguished in the skeleton: flat bones such as skull bones, scapula, mandible and ilium, and long bones such as the tibia, femur and humerus. Long bones have a cylindrical tube in the middle called a diaphysis, which is connected via epiphyseal plates to the ends of the bones, called epiphyses. The external and internal surfaces of bones are lined with osteogenic cells, which form the external periosteum and the internal endosteum. Morphologically, there are also two forms of bone: compact or cortical bone, forming the outer thick and dense layer of bone, and cancellous or trabecular bone inside the cortical layer (Marks and Odgren 2002). Both cortical and trabecular bone are formed from the same cells and matrix elements, but they are structurally and functionally different. Cortical bone constitutes approximately 90% of the skeleton and it is highly calcified, having mainly a mechanical and protective function, while trabecular bone has a large area facing the bone marrow, serving a metabolic function.

2.1.1 Bone matrix

Biochemically, bone tissue is composed of abundant mineralized extracellular matrix and functionally distinct cell populations, osteoblasts, osteocytes and osteoclasts, which maintain the structural, biochemical, and mechanical integrity of the extracellular matrix. Chemically, inorganic minerals form two thirds of bone tissue and organic bone components the remaining one third. Inorganic minerals are almost exclusively crystals of calcium phosphate called hydroxyapatite.

The main protein in bone matrix (~90%) is a triple-helical type I collagen, which is responsible for the strength of the tissue. Type I collagen is characterized by a Gly-X-Y repeating triplet (where X is usually proline), and by several post-translational modifications including hydroxylation of certain prolyl or lysyl residues, glycosylation of certain lysyl or hydroxylysyl residues, and formation of intra- and inter-molecular covalent crosslinks (Rossert and de Crombrugghe 2002). Bone consists predominantly of type I collagen, but trace amounts of other types of collagens (type III, V and X) are also present in bone matrix. Proteoglycans and hyaluronan are macromolecules

containing acidic polysaccharide side chains, binding water molecules and forming an amorphous gel for other proteins and minerals to be embedded into bone matrix.

Noncollagenous proteins compose 10-15% of the total bone protein content of bone matrix. Virtually all of the bone matrix proteins are modified post-translationally to contain either N- or O-linked oligosaccharides, being glycoproteins. The most abundant glycoproteins that most likely play major structural and metabolic roles are alkaline phosphatase (ALP), osteonectin and tetranectin (Robey 2002). Bone matrix is also rich in proteins containing a cell-attachment consensus sequence RGD (Arg-Gly-Asp). In addition to type I collagen, fibronectin, thrombospondin, vitronectin, fibrillin, osteopontin, and bone sialoprotein (BSP) also all contain RGD-sequence and may therefore mediate cell attachment to the bone surface (Robey 2002). Gamma-carboxyglutamic acid (Gla) –containing proteins; matrix-gla-protein (MGB), osteocalcin (also called bone-gla-protein (BGP)), and protein S are post-translationally modified by vitamin K-dependent γ-carboxylases (Ducy and Karsenty 2002). They are calcium-binding proteins, which may function in the regulation of mineralization of bone matrix. The physiological roles for individual bone proteins are not well defined. However, they may participate not only in forming the structure of mineralized matrix, but also in the control of osteoblastic and osteoclastic metabolism.

2.1.2 Bone cells

Bone cells form a quantitatively small proportion of organic bone tissue. However, they have a very important function in maintaining the structure and function of the extracellular matrix.

2.1.2.1 Osteoblasts

Osteoblasts, bone-forming cells, originate from mesenchymal stem cells (MSC) found in bone marrow and the periosteum. Stromal osteoprogenitor cells contribute to maintaining the osteoblast population and bone mass; osteoblasts that synthesize the bone matrix; osteocytes, organized throughout the mineralized bone matrix; and the protective bone-surface lining cells (Lian *et al.,* 1999). Osteoblasts are always found as clusters of cubical cells lining the layer of bone matrix they have produced, called osteoid. Osteoblasts produce and secrete bone matrix proteins, type I collagen and noncollagenous proteins as osteoid towards the mineralizing front of the tissue. The osteoid is calcified approximately within ten days, becoming a mature bone matrix. Osteoblasts are ultrastructurally characterized by an extremely well developed rough endoplasmic reticulum, a large nucleus, and an enlarged Golgi complex due to a high level of protein synthesis. Active osteoblasts are highly enriched with alkaline phosphatase, BSP, osteopontin and osteocalcin. When new bone is formed, some osteoblasts are trapped into the newly synthesized bone matrix, becoming osteocytes.

Different cytokines, growth factors and hormones mediate the commitment of mesenchymal stem cells to osteoblast lineage, as well as influence the growth and differentiation of osteoprogenitors. Fibroblast growth factors (FGFs), transforming growth factor β (TGFβ), and bone morphogenetic proteins (BMPs) are potent

osteoinductive factors at the early stages of osteoprogenitor differentiation and proliferation. Key regulators of osteoblast differentiation and maturation also include parathyroid hormone (PTH), PTH-related peptide (PTHrP), insulin-like growth factors (IGFs), glucocorticoids, vitamin D, and estrogen (Aubin and Triffitt 2002). In addition to the bone formation process, osteoblasts actively regulate the differentiation and activity of bone-resorbing cells, osteoclasts.

2.1.2.2 Osteocytes

Osteocytes are the most abundant cells in mature bone and have the potential to live as long as the organism itself. However, research and understanding of osteocyte biology is very narrow, because of the remote location of the cells in the mineralized tissue (Nijweide *et al.,* 2002). Osteocytes are developed from osteoblasts, which are trapped in the newly formed bone matrix during bone formation. Osteocytes remain embedded in the bone matrix in small osteocytic lacunae, producing high amounts of osteocalcin, osteonectin and osteopontin, but little alkaline phosphatase (Nijweide *et al.,* 2002). The transformation from an osteoblast to an osteocyte takes about three days (Palumbo 1986), but the exact mechanism for the switch from an osteoblast to an osteocyte is still poorly understood. Cell polarity is maintained so that the nucleus remains in proximity to the vasculature, but the cell shape is changed from rounded osteoblast to a more dendritic-shaped osteocyte. An osteocyte will develop numerous thin and long processes rich in microfilaments, connecting the cell to the neighboring osteocytes and also other cells at the bone surface. These processes form a network of thin canaliculi permeating the entire bone matrix. The canaliculi form a metabolically and electrically active network connected via gap-junctions (Knothe Tate *et al.,* 2004). This unique three-dimensional organization of osteocytes throughout the bone tissue suggests an important function for osteocytes in the metabolism and maintenance of bone. It has been hypothesized that osteocytes may work as a mechanosensory organ of bone, sensing the stress and loading by an unknown mechanism (Nijweide *et al.,* 2002). Also, osteocytes have been shown to respond to fluid flow *in vitro*, strengthening the hypothesis of the mechanosensing role. Osteocytes may also regulate other bone cells, osteoblasts and osteoclasts, and participate in calcium homeostasis (Nijweide *et al.,* 2002).

The knowledge of osteocyte biology is increasing due to osteocyte-like cell lines published within the last decade. Previously, primary cells were the only source for osteocytes, being limited both in cell number and viability (van der Plas and Nijweide 1992). Human preosteocytic cell line (HOB-01-C1) became available in 1996 (Bodine *et al.,* 1996) and murine long bone osteocyte-like cell line MLO-Y4 in 1997 (Kato *et al.,* 1997).

2.1.2.3 Osteoclasts

Osteoclasts are the principle bone-resorbing cells forming the smallest proportion of the bone cells. During skeletal growth, osteoclasts are needed for the resorption of calcified cartilage and modelling of growing bone. In adult bone, osteoclasts fulfil the

requirements of calcium homeostasis and normal remodelling (Väänänen and Zhao 2002). The characteristics of osteoclasts include multinuclearity, TRACP activity, expression of calcitonin and vitronectin receptors (αvβ3), and the ability to resorb bone, dentin and cartilage (Suda *et al.,* 1992).

Osteoclasts are derived from hematopoietic cells of the monocyte-macrophage lineage (Walker 1973; Takahashi *et al.,* 2002b). The differentiation pathway of osteoclasts is common with macrophages until the final differentiation steps. Previously, *in vitro* maturation of osteoclast precursors into mature osteoclasts was revealed in co-cultures with marrow stromal cells or osteoblasts (Udagawa *et al.,* 1990). Later, it became clear that these accessory cells express the two molecules that are essential and sufficient to promote osteoclastogenesis: macrophage-colony stimulating factor (M-CSF) and receptor for activator of nuclear factor kappa B ligand (RANKL), which is also known as osteoprotegerin ligand (OPGL) or TRANCE (Fuller *et al.,* 1998; Lacey *et al.,* 1998). M-CSF promotes the proliferation of osteoclast precursors, and RANKL binds to its receptor RANK expressed in osteoclast precursors, stimulating them to commit the osteoclast phenotype (Fig. 1). A soluble decoy receptor, osteoprotegerin (OPG), binds to RANKL and blocks its interaction with RANK, inhibiting osteoclast development (Simonet *et al.,* 1997). Several other cytokines and hormones regulate the differentiation of osteoclasts, most commonly by affecting the RANK-RANKL-OPG system. Stimulatory factors include e.g. PTH, 1,25-dihydroxyvitamin D_3, tumour necrosis factor α (TNFα), and interleukins (ILs) 1, 6, 11, 15 and 17 (Alsina *et al.,* 1996). ILs 4, 10, 12, 13 and 18, as well as estrogen, TGFβ, interferon γ and calcitonin inhibit osteoclastogenesis (Roodman 2001).

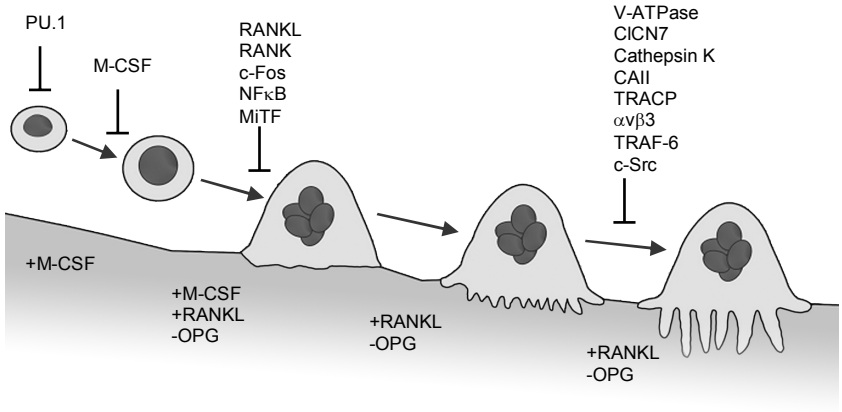

Figure 1. Osteoclasts originate from hematopoietic cells of the monocyte-macrophage lineage. M-CSF[1] and RANKL have a central role in osteoclastogenesis as positive regulators, whereas OPG can bind and neutralize RANKL, being a negative regulator for both the differentiation and function of osteoclasts.[2] The single-gene mutations that block osteoclastogenesis and activation of osteoclasts in the different stages of osteoclast differentiation are shown above.

[1] M-CSF, macrophage-colony stimulating factor; RANK(L), receptor for activator of nuclear factor kappa B (ligand); OPG, osteoprotegerin; NFκB, nuclear factor kappa B; MiTF, microphthalmia transcription factor; V-ATPase, vacuolar-type proton adenosine triphosphatase; ClCN7, chloride channel 7; CAII, carbonic anhydrase II; TRACP, tartrate-resistant acid phosphatase; αvβ3, vitronectin receptor; TRAF-6, tumor necrosis factor receptor-associated factor 6.

[2] Modified from (Boyle *et al.,* 2003; Zaidi *et al.,* 2003).

Mononuclear preosteoclasts are fused into multinucleated osteoclasts and guided by thus far unknown mechanisms to bone surfaces that will be resorbed. The strongest candidates for the regulation of determination for bone resorption sites are osteocytes and bone lining cells, which may have both negative and positive effects on osteoclast differentiation, recruitment and attachment (Heino *et al.,* 2002). Nevertheless, after the determination of the resorption site, osteoclasts go through a sequence of cellular events called the resorption cycle (Väänänen 1996). At the beginning of the resorption cycle, osteoclasts attach to the bone surface via at least four distinct integrin extracellular matrix receptors: $\alpha_v\beta_3$ (a classical vitronectin receptor), $\alpha_v\beta_5$, $\alpha_2\beta_1$ (collagen receptor) and $\alpha_v\beta_1$, which bind to a variety of extracellular proteins such as vitronectin, collagen, osteopontin and BSP (Nesbitt *et al.,* 1993). The sealing zone structure formed attaches osteoclasts tightly onto the bone surface creating a microenvironment between the plasma membrane of the osteoclast and the bone surface. Simultaneously, osteoclasts become highly polarized with three different membrane domains: 1) the sealing zone, 2) the ruffled border (RB) facing the bone surface, and 3) the basolateral membrane facing the bone marrow. In addition to these domains, a fourth, structurally and functionally distinct domain has been observed in resorbing osteoclasts, namely a functional secretory domain (FSD), which is located on the basal membrane (Fig. 2) (Salo *et al.,* 1996; Nesbitt and Horton 1997; Salo *et al.,* 1997).

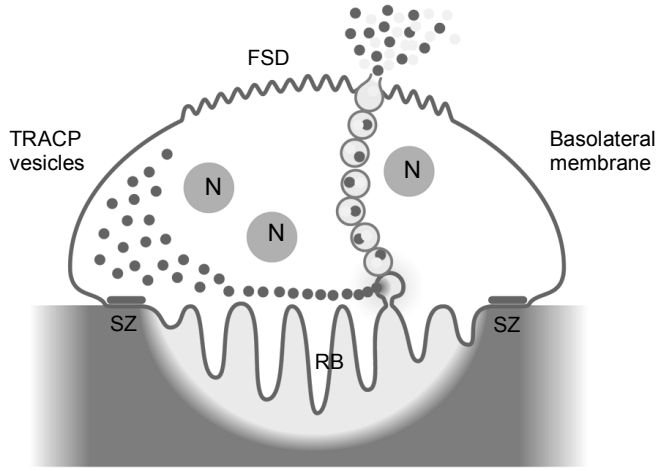

Figure 2. A resorbing osteoclast has four distinct membrane domains: the sealing zone (SZ), ruffled border (RB), basolateral membrane and the functional secretory domain (FSD). Bone degradation products are removed via the transcytotic pathway, where TRACP may participate in the final degradation of bone matrix components. (N = nucleus)

The actual degradation of bone matrix takes place in a sealed area between the osteoclast and the bone matrix, known as the resorption lacuna. The RB is formed by a rapid fusion of acidic intracellular vesicles (Palokangas *et al.,* 1997), acidified mainly by carbonic anhydrase II (CAII). The low pH of resorption lacunae is also mediated by the V-type ATPase proton pump (V-ATPase), which pumps protons into the

resorption lacunae (Blair *et al.,* 1989; Väänänen *et al.,* 1990). The secretion of anions, mainly chloride, balances the proton pumping through the RB (Blair and Schlesinger 1990). Low pH in the resorption lacunae dissolves hydroxyapatite, exposing bone matrix proteins for lysosomal proteases secreted through the RB. Two enzymes, cathepsin K and matrix metalloproteinase 9 (MMP-9) are mainly responsible for the degradation of demineralised bone matrix in resorption lacunae (Everts *et al.,* 1998; Xia *et al.,* 1999). Bone degradation products are removed from the resorptive area by internalisation and transportation through the resorbing osteoclast in transcytotic vesicles into the FSD, from where they are released into the circulation (Nesbitt and Horton 1997; Salo *et al.,* 1997). Further degradation of matrix molecules may happen during transcytosis. TRACP has been shown to be localised in transcytotic vesicles and being able to destroy collagen and other proteins by a highly destructive reactive oxygen species (ROS) (Halleen *et al.,* 1999b), suggesting a function for TRACP in the final degradation of matrix molecules during transcytosis.

2.1.3 Bone remodelling

Mature skeleton is continuously broken down and reformed by the coordinated actions of osteoclasts and osteoblasts on trabecular bone surfaces and in Haversian systems. This turnover is called bone remodelling. Approximately 10% of the whole skeleton is under remodelling at each point in time. The major reason for remodelling is to enable the bones to adapt to mechanical stress (Ott 2002). Remodelling also allows bone to repair microdamage and maintain its strength. Finally, remodelling is an important component of mineral metabolism.

Remodelling occurs throughout the skeleton in focal and discrete packets called bone-remodelling units (BRUs) (Takahashi *et al.,* 1964). Each BRU is geographically and chronologically separated from other packets, suggesting that activation of the sequence is locally controlled in the bone microenvironment. The initial step before the actual remodelling is the focal attraction of preosteoclasts to the quiescent bone surface. The factors that determine the site for remodelling are not known, but there is increasing evidence that microdamage in bone and osteocyte apoptosis are preceding events for remodelling a site (Verborgt *et al.,* 2000). The remodelling cycle can be divided into four different phases over time: 1) activation of osteoclast precursors and osteoclastic bone resorption; 2) reversal, with apoptosis of osteoclasts and smoothing of erosion cavities by mononuclear cells; 3) migration and differentiation of osteoblasts and formation of osteoid bone matrix; and 4) mineralization of newly formed osteoid (Fig 3). The whole remodelling cycle is estimated to last from months to years. Bone resorption is completed within a few days or weeks, but the formation of the same amount of mineralised new bone takes months, explaining why formation surfaces on bone are more abundant than resorption surfaces (Mundy 1999). Activation frequency describes the frequency of osteoclastic activation on bone surfaces. Normally, an activation occurs every 10-15 seconds, leading to millions of BRUs in the skeleton simultaneously (Parfitt *et al.,* 1987). The activation frequency is increased by PTH, thyroid hormones and calcitriol, whereas gonadal steroids and calcitonin inhibit the activation (Kanis 1994).

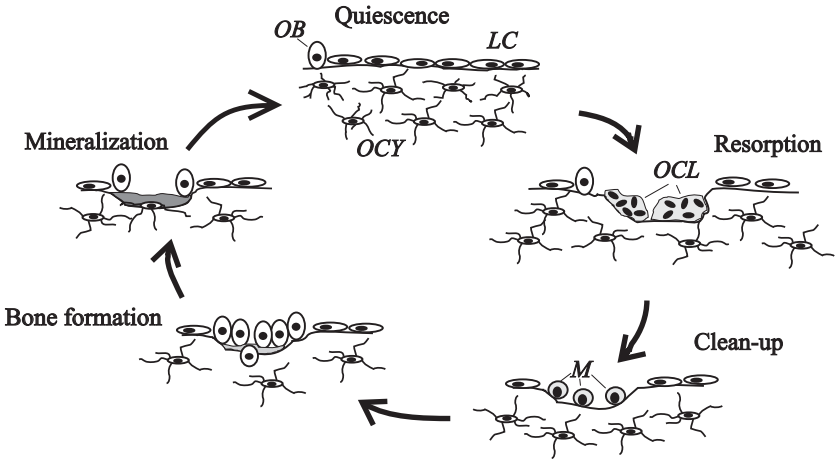

Figure 3. The bone remodelling cycle is started with bone resorption by osteoclasts (OCL), followed by smoothing of erosion cavities by mononuclear cells (M), continued with formation of new osteoid by osteoblasts (OB), and finally mineralization of newly formed osteoid. During bone formation some osteoblasts are captured into the osteoid and they become osteocytes (OCY). In the resting phase newly formed bone is lined with bone lining cells (LC).

In normal physiological conditions, the amount of bone removed is replaced during formation, and this phenomenon is called coupling. The cellular and humoral mechanisms responsible for mediating the coupling process are still not clear. It has been suggested that growth factors such as TGFβ, insulin-like growth factors (IGFs), and FGFs released during bone resorption could recruit preosteoblasts into the resorption site and activate bone formation (Howard *et al.,* 1981; Parfitt 2000). A recent study introduced an idea that TRACP could be a coupling factor between osteoclasts and osteoblasts (Sheu *et al.,* 2003). Alterations in remodelling and uncoupling of bone resorption and formation are responsible for most metabolic diseases such as osteoporosis, myeloma and breast and prostate cancers with bone metastases (Mundy 1999).

2.2 Metabolic and malignant bone diseases

2.2.1 Osteoporosis

Osteoporosis, or porous bone, is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures (Anonymous 1993). The amount of bone increases during growth, reaches a peak bone mass at the age of 20-30, starts to decrease in the late 30s and exhibits an accelerated loss in women after menopause (Heaney *et al.,* 2000). The loss of bone also happens in men, only later, mainly after 70 years of age. The determinants of peak bone mass are primarily genetic (Cooper 1999), with the contribution of diet, calcium intake, and exercise during childhood and

puberty (Nieves *et al.,* 1995). The rate of bone loss during adulthood is important from a therapeutic and medical perspective, since the majority of osteoporotic individuals are elderly patients seen many years after reaching peak bone mass (Rodan *et al.,* 2002). Four out of five osteoporotic individuals are women, which is due to the higher bone loss and longer lives of women than men (Cummings and Melton 2002).

Osteoporosis is often called a silent disease because bone loss occurs without symptoms. The disease is clinically recognized by the occurrence of characteristic low-trauma fractures, the best documented of these being hip, vertebral and wrist fractures. Hip fractures have become the international barometer of osteoporosis since they are strongly related to low BMD, cost more to repair and cause more disability than any other type of osteoporotic fracture. Also patients with hip fracture are almost always treated in hospital and are therefore easier to count and compare from country to country (Cummings and Melton 2002). In Finland, the incidence of hip fractures is over 7000, whereas worldwide the numbers are as high as 1.7 million annually. Hip fractures are the most serious type of osteoporotic fractures because of the high frequency of mortality (15-20%) within the first year after trauma (Kanis 1994). The epidemiology of vertebral fractures is less well established because there is no universally accepted definition and a substantial proportion of these fractures escape clinical diagnosis (Cummings and Melton 2002). It has been estimated that only one third of all vertebral deformities come to medical attention and an even smaller proportion is treated in hospital. Vertebral fractures may be asymptomatic or the symptoms may be like normal back pain. However, loss of height or spinal deformities such as kyphosis give reason to suspect vertebral fracture. Mortality is not as strongly associated with vertebral fractures as with hip fractures. Nevertheless, fractures occurring in the spine and the forearm are associated with significant morbidity, affecting everyday life. The incidence of fracture increases exponentially with age after 50 years, and the incidence is markedly higher for women than for men. The overall lifetime risk for any type of osteoporotic fracture has been estimated to be about 40% for women and 13% for men at the age of 50 years (Kanis 1994).

The financial and health related costs of osteoporosis are difficult to assess because they include for example acute hospital care, loss of working days, chronic care in the home or nursing homes and medication. However, the estimation of costs of osteoporotic fractures in the United States is $17-20 billion (approximately €14-17 billion; population of 250 million; NIH Osteoporosis and bone-related diseases resource center: www.osteo.org) and in the United Kingdom £1.7 billion (approximately €2.5 billion; population of 50 million) annually (Christodoulou and Cooper 2003). The vast majority of these costs are attributable to hip fractures because the patients need to be treated in hospital and home for a long time. Costs will increase in the future because the number of individuals aged 65 years or more is increasing from the present 323 million up to 1555 million by the year 2050. These demographic changes are expected to increase the number of hip fractures throughout the world from 1.7 million in 1990 to 6.3 million in 2050 (Kanis 1994; Christodoulou and Cooper 2003). The number may be even higher since the frequency of hip fractures is increasing 1-3% per year in most areas of the world (Gullberg *et al.,* 1997).

2.2.1.1 Diagnosis

The diagnosis of osteoporosis is based on the measurement of BMD at the hip, most commonly with DXA. In 1994, the WHO defined four diagnostic categories for osteoporosis for women as shown in Table 1 (Kanis *et al.,* 1994; WHO 1994). The International Osteoporosis Foundation modified the definition in 2000 (Kanis and Gluer 2000).

Table 1: Diagnostic criteria for the osteoporosis determined from the hip with DXA.

	DEFINITION	**T score value**
Normal	BMD < 1 SD below the young adult mean	≥ -1
Osteopenia	BMD > 1 SD below the young adult mean, but < 2.5 SD below this value	< -1 and > -2.5
Osteoporosis	BMD > 2.5 SD below the young adult mean	≤ -2.5
Severe osteoporosis	BMD > 2.5 SD below the young adult mean in the presence of one or more fragility fractures	≤ -2.5 + Fracture

Because the clinical consequence of osteoporosis is a fracture, it is therefore important to be able to predict the likelihood of fractures. Several studies have shown that the risk of fracture approximately doubles for each SD reduction in BMD (Kanis 2002). However, normal BMD does not guarantee that fracture will not occur – only that the risk is reduced. And conversely, low BMD only predicts that fractures are more likely. This low sensitivity is one of the reasons why widespread population-based screening is not recommended in women at menopause (WHO 1994). Fracture risk assessment may be improved by taking into account other risk factors and also by measuring biochemical markers of bone turnover, which have independent value in fracture risk prediction (Garnero and Delmas 2002; Johnell *et al.,* 2002; Eastell *et al.,* 2003). The identification of the high-risk individuals is important to precisely treat those individuals with expensive treatments.

2.2.1.2 Risk factors

Osteoporosis is a multifactorial disease having several risk factors. One of the most important risk factors is reduced BMD, which has been shown to be negatively associated with fractures. Other skeletal risk factors are low body mass index (BMI < 21), previous fractures of the patient or their close relatives, and imbalance in bone turnover leading to thinning and even loss of trabecular connectivity (Seeman 2002). Advanced age and female gender are important risk factors for osteoporotic fractures. Estrogen deficiency as a result of surgery or menopause, and also amenorrhoea and anorexia nervosa are major risk factors in women. Primary hypogonadism is associated with low BMD also in men. Lifestyle also has a strong effect on the development of osteoporosis. Low calcium and vitamin D intake, bed rest, and physical inactivity are all risks for osteoporosis. Smoking has effects on the body

weight and estrogen metabolism in women, whereas excessive use of alcohol has effects on protein and calcium metabolism, mobility, gonadal function, coordination, and simply on falls. Other causes for osteoporosis are several diseases such as hyperparathyroidism, chronic renal failure, and myeloma. Use of certain medications, such as corticosteroids may also induce osteoporosis. Finally, low peak bone mass achieved as a young adult is a clear risk factor for having osteoporosis later on (Kanis 1994; Christodoulou and Cooper 2003).

2.2.1.3 Prevention and treatment

The aim of the prevention and treatment of osteoporosis is to prevent the occurrence of fractures. As mentioned previously, high-risk individuals need to be identified for proper treatment. Nevertheless, lifestyle changes may be enough for individuals not at the high risk for osteoporosis and fractures (Delmas 2002). Proper diet, including adequate calcium and vitamin D intake, physical activity, avoiding smoking and heavy alcohol consumption would diminish the frequency of osteoporosis. Moreover, the risk of falling should be reduced for elderly people by e.g. improving lightning, correcting the deficits in vision and hearing, avoiding sedative drugs and slippery floors, and using hip protectors. Indeed, recent studies demonstrate that hip protectors can half the risk of hip fracture even among frequent fallers (Kannus *et al.,* 2000).

In patients with low BMD and other risk factors for osteoporosis, pharmacological treatments need to be offered. The majority of the drugs developed against bone diseases affect via the inhibition of bone resorption instead of increasing bone formation. The most important treatments include bisphosphonates, hormone replacement therapy (HRT) with estrogen alone or in a combination with progestin, the selective estrogen receptor modulators (SERMs) raloxifene and tamoxifen, calcitonin and the only bone forming agent at the moment, a fragment of PTH known as teriparatide (Delmas 2002). The antifracture efficacy of all these agents is clearly better for vertebral fractures than for non-vertebral (hip) fractures. The best antifracture efficacy for hip fractures has been observed with alendronate, risedronate and teriparatide as reviewed in a recent article (Delmas 2002). HRT was previously offered as the first choice for early menopausal women for menopausal symptoms and fracture prevention. However, a recent large-scale trial by the Women's Health Initiative (WHI) demonstrated several non-skeletal risks such as increased frequency for coronary heart disease and invasive breast cancer, which exceeded the benefits in fracture prevention (Rossouw *et al.,* 2002). The decision of when, how and by which treatment osteoporotic patients should be treated must be carefully evaluated and based on the assessment of the fracture risk and on the efficacy and side-effects of drugs.

The growing knowledge of the pathophysiology of osteoporosis and other bone diseases offers a possibility to develop new therapeutic agents for these diseases. Antiresorptive agents are still primary therapeutic objectives due to the elevated bone resorption in most of the bone diseases. The new antiresorptive agents include e.g. more selective SERMs, members of the TNF receptor/ligand family (RANK-RANKL-OPG), and inhibitors for cathepsin K, $\alpha_v\beta_3$ integrin receptor and osteoclast-specific V-

ATPase (Rodan and Martin 2000). However, stimulators of bone formation would be a valuable adjunct therapy for patients receiving inhibitors of bone resorption. In addition to PTH and its fragments, a novel agent strontium ranelate has been shown to increase bone formation and reduce fracture risk (Meunier *et al.,* 2002). Other possible stimulators for bone formation are e.g. statins, fluoride, and different growth factors such as IGF-1, TGFβ, FGFs and BMPs. Important factors for new therapeutics of bone diseases are selectivity for bone, optimised benefit-to-risk ratio, and convenience in use (Rodan and Martin 2000).

2.2.2 Osteopetrosis

Osteopetrosis is a heterogeneous group of genetic diseases all of which have increased skeletal mass and density due to impaired bone resorption (de Vernejoul and Benichou 2001). Human osteopetrosis has been categorized into infantile malignant, autosomal recessive osteopetrosis, and adult type I and type II autosomal dominant osteopetrosis (ADO) based on their clinical features. Three different genes, all playing a role in the acidification process of the resorption lacunae during osteoclastic bone resorption, have been identified in human osteopetrosis. Mutations in intracellular CAII result in a relatively mild autosomal recessive osteopetrosis with fractures, renal tubular acidosis, and cerebral calcification (Sly *et al.,* 1983). The severe, malignant form of osteopetrosis is due to the loss-of-function mutations in the transmembrane 116 kD subunit of the V-ATPase proton pump (Frattini *et al.,* 2000; Kornak *et al.,* 2000). Some patients with severe malignant osteopetrosis have a loss-of-function mutation in the chloride channel 7 (ClCN7) gene (Kornak *et al.,* 2001), whereas the milder ADO type II, also called Albers-Schönberg disease is caused by missense mutations in the same ClCN7 gene (Cleiren *et al.,* 2001). While some animal models of osteopetrosis are caused by a defect in osteoclast differentiation, this is, at present, not the case for any human form (Janssens and Van Hul 2002).

In addition to impaired bone resorption, increased skeletal mass may also be due to elevated bone formation or imbalance between bone resorption and formation. These diseases are however not called osteopetrotic but preferably osteosclerotic, due to different molecular and genetic mechanisms underlying the diseases (Janssens and Van Hul 2002). One example of osteosclerotic diseases is pycnodysostosis, which results in cathepsin K deficiency (Gelb *et al.,* 1996).

2.2.3 Metastatic bone diseases

Skeletal malignancies have multiple effects on the skeleton, all of which are mediated by tumour cells altering the behaviour of bone cells and causing disruptions or enhancement of the normal bone remodelling process. Among the common skeletal syndromes caused by tumours are osteolytic and osteoblastic bone metastases, myeloma bone disease, and humoral hypercalcemia of malignancy (HHM) (Mundy *et al.,* 2002).

Breast and prostate cancers and, to a lesser extend, thyroid, kidney, and lung cancers all show a strong propensity to metastasize to bone. Bone metastases usually

indicate progressive disease and are associated with severe pain, pathological fractures, and profound morbidity (Mundy *et al.,* 2002). Only 5-10% of all metastatic bone lesions are osteoblastic, and the most notable example of osteosclerotic metastases is prostate cancer. Primary prostate cancer cells produce regulatory factors such as TGFβ and BMPs that enhance the activity of osteoblasts to proliferate and differentiate to form bone nodules (Charhon *et al.,* 1983). Other cancers, especially breast and lung cancers develop osteolytic bone lesions that are most likely osteoclast-mediated. The factors responsible for osteoclast activation include e.g. PTH-related peptide (PTHrP), ILs 1 and 6, prostaglandins and TGFβ (Powell *et al.,* 1991; Boyce *et al.,* 1999).

Extensive bone destruction, including bone pain, fractures and hypercalcemia are the major clinical hallmarks of multiple myeloma (MM) (Callander and Roodman 2001; Mundy *et al.,* 2002). The bone lesions in myeloma are multiple, discrete lytic lesions, which occur at the site of deposits or nests of myeloma cells without evidence of new bone formation (Mundy and Bertolini 1986). Bone destruction is mediated through normal osteoclasts that respond to TNFβ, RANKL, IL-1, PTHrP, TNFα and macrophage inflammatory protein-1-alpha (MIP-1α), released by myeloma cells in the microenvironment of osteoclasts (Mundy *et al.,* 1974; Callander and Roodman 2001). HHM is also characterized by increased osteoclastic bone resorption, primarily due to the elevated tumour-derived PTHrP (Moseley *et al.,* 1987). Simultaneously bone formation is decreased, which differentiates HHM from primary hyperparathyroidism, having otherwise similar symptoms (Stewart 2002).

2.2.4 Animal models for bone diseases

The principle use of animal models for bone diseases is to provide efficient experimental environments in which bone cells function within their intermediary organizations of modelling and remodelling (Kimmel 2002). Animal models provide insight into the *in vivo* skeletal events, and also improve understanding of the pathogenesis, prevention and treatment of osteoporosis and other bone diseases. The U.S. Food and Drug Administration (USFDA) has published guidance for animal models for osteoporosis (USFDA 1994). Since estrogen-deficiency is the most common cause of osteoporosis, ovariectomized animals have been selected as the preferred animal models. The guidelines suggest measuring endpoints, such as bone density, biochemical markers of bone metabolism, bone strength and bone turnover by histomorphometry. Requirements for new anti-osteoporotic drugs include anti-fracture efficacy, which has to be proven with one small (rat) and one large animal (e.g. dog, ewe, or primate) (USFDA 1994).

Several knock-out and over-expressing mouse models, as well as congenitally osteoporotic or osteopetrotic mice have elucidated the role of individual genes and basic mechanisms at the cellular level in bone (Boyle *et al.,* 2003). Furthermore, development of breast and prostate cancer mouse models has provided important information about the invasion and migration of cancer cells and development of bone metastases.

2.3 Measurement of bone quality and quantity

The most important reasons for measuring bone quality or quantity are the assessment of fracture risk and response to treatment. Measurement of BMD has become a gold standard due to the WHO's proposed guidelines for the diagnosis of osteoporosis (WHO 1994). At present, there are several techniques available for the determination of bone mass (Table 2). All these techniques are used for the measurement of relatively small changes in BMD (only few percents) that are easily masked by errors caused by the variability (precision CV 1-5%) of the techniques. In order to obtain reliable results, measurements have to be repeated after several months or years. Thus, these techniques are not suitable for short-term follow-up of antiresorptive treatment or detection of early changes in BMD for example in the beginning of menopause.

Table 2. Summary of different bone densitometry techniques[1]

Technique[2]	Region of interest	Units reported	Precision (%CV)	Radiation exposure (μSv)
DXA	Spine, hip, whole body	BMD (g/cm^2)	1-2	1-10
pDXA	Radius, calcaneus	BMD (g/cm^2)	1-2	0,1-1
SXA	Radius, calcaneus	BMD (g/cm^2)	1-3	1
QCT	Spine	BMD (g/cm^3)	2-4	50-500
pQCT	Radius	BMD (g/cm^3)	1-2	1-3
RA	Phalanx	BMD (g/cm^2)	1-2	5-10
QUS	Calcaneus, tibia, multi-site	BUA (dB/MHz), SOS (m/s)	1-5	None

[1] Modified from (Blake and Fogelman 2002; Cummings *et al.,* 2002).

[2] DXA, dual-energy X-ray absorptiometry; pDXA, peripheral DXA; SXA, single-energy X-ray absorptiometry; QCT, quantitative computed tomography; pQCT, peripheral QCT; RA, radiographic absorptiometry; QUS, quantitative ultrasound; BUA, broadband ultrasonic attenuation; SOS, speed of sound.

Over the past decade, dual-energy X-ray absorptiometry (DXA) has established itself as the most widely used method of measuring BMD because of its advantages of high precision, short scan times, low radiation and stable calibration (Blake and Fogelman 2002). DXA measurements can be taken from the most important sites regarding the fracture risk, namely spine and hip. The measurement of hip BMD has been shown to be the most reliable way of evaluating the risk of hip fracture (Cummings *et al.,* 1993; Marshall *et al.,* 1996), and it has been therefore selected for the site of the diagnosis of osteoporosis (Kanis and Gluer 2000). The fundamental principle behind DXA is the measurement of the transmission through the body of X-rays of two different photon emissions. Results of DXA measurements are areal densities of two different types of tissue, bone mineral (hydroxyapatite) and soft

tissue. In contrast to DXA, quantitative computed tomography (QCT) has the advantage that it determines the true three-dimensional volumetric bone density instead of areal BMD. Also, QCT permits isolated measurement of trabecular bone density instead of total BMD. QCT is normally applied to measure the trabecular bone in vertebral bodies of spine. Vertebral bodies consist mainly of the metabolically active trabecular bone, and therefore the spine has been regarded as the optimum site for monitoring response to treatment (Eastell 1998). The disadvantages of QCT measurements are the relatively high dose of radiation and the cost of the equipment (Blake and Fogelman 2002).

Since both DXA and QCT equipments are relatively large and expensive, there is clearly a need for smaller and lower-cost devices. Peripheral DXA (pDXA) and peripheral QCT (pQCT) are an alternative choice to measure BMD from the forearm or heel. Peripheral tests normally cost much less than central measurements, and also the dose of radiation is much lower (Gluer *et al.,* 1997). However, these tests are less predictive and less accurate than BMD at the hip or spine for fractures of the hip or spine (Marshall *et al.,* 1996). Further, there is a lot of variation from one type of device to the other. Radiographic absorptiometry (RA) is an additional peripheral technique that compares the density of proximal phalanges to that of a wedge of aluminium with known densities placed on the film alongside the hand (Yang *et al.,* 1994). The characteristics and costs of RA are similar to those of other peripheral measurements. The main advantage of RA is its potential for general use based on the widespread availability of conventional film radiography (Blake and Fogelman 2002).

Quantitative ultrasound (QUS) for measuring the peripheral skeleton has raised considerable interest in recent years. Most of the QUS devices use the heel as the measurement site. The technique uses an ultrasound pulse that is scattered and absorbed by trabeculae. Bone density and structure can be assessed quantitatively by the speed of sound (SOS), the pattern of absorption of different wavelengths of sound, called broadband ultrasound attenuation (BUA), or calculations derived from these parameters (Kaufman and Einhorn 1993; Gluer 1997). The major advantage of QUS is that it does not use ionising radiation, and also the instrumentation is relatively inexpensive and designed to be portable. However, relatively poor precision and variability in the exact anatomical site of measurement of the heel have decreased the acceptance of the technique.

2.4 Biochemical markers of bone metabolism

In contrast to densitometric measurements of bone mass and structure from a particular site of the skeleton, biochemical markers of bone metabolism measured from serum or urine are helpful tools in detecting the dynamics of metabolic imbalance in the whole skeleton. Biochemical markers of bone metabolism are non-invasive, relatively inexpensive, generally available, can measure changes in bone metabolism over short intervals, and can be assessed repetitively. Clearly, measurement of bone metabolic markers is not a substitute for bone mass measurement. However, they provide excellent adjunctive tools in patient management (Seibel *et al.,* 2002).

Novel biochemical markers of bone metabolism have been developed over the past 20 years that are more specific for bone tissue and more sensitive for detecting abnormalities in bone metabolism than conventional ones. From the biochemical perspective, various bone markers are products of osteoblasts or osteoclasts, or alternatively components released during the formation or breakdown of the organic bone matrix. For clinical purposes, bone metabolic markers can be classified into three categories: 1) Bone formation markers, released during bone formation by osteoblasts; 2) Bone resorption markers released from bone matrix or from osteoclasts during bone resorption; and 3) Bone turnover markers, released during bone formation and resorption (Fig. 4; Table 3). Most of these markers are present in tissues other than bone and may therefore be influenced by nonskeletal processes as well. Furthermore, changes in the biochemical markers of bone metabolism are usually not disease-specific, but reflect alterations in the skeletal metabolism independently of the underlying cause [reviewed in (Calvo *et al.,* 1996; Delmas *et al.,* 2000; Seibel 2000; Seibel *et al.,* 2002)].

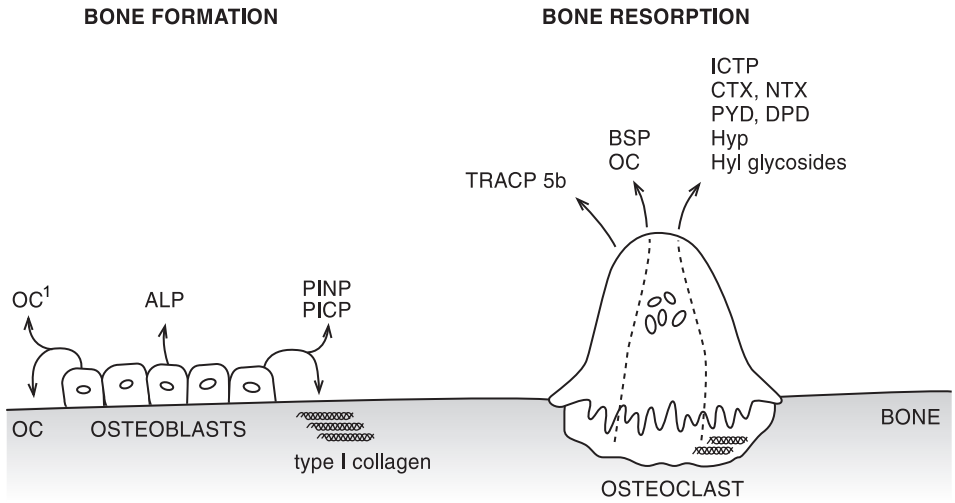

Figure 4. Biochemical markers of bone metabolism released during bone formation by osteoblasts and during bone resorption by osteoclasts.

[1]OC, osteocalcin; ALP, alkaline phosphatase; PINP or PICP, N- or C-terminal propeptide of type I collagen; TRACP 5b, tartrate-resistant acid phosphatase 5b; BSP, bone sialoprotein; ICTP (CTX-MMP), cross-linked C-telopeptide of type I collagen; CTX or NTX, C- or N-telopeptide of type I collagen crosslinks; PYD, pyridinoline; DPD, deoxypyridinoline; Hyp, hydroxyproline; Hyl glycosides, hydroxylysine glycosides.

Table 3: Biochemical markers of bone metabolism [modified from (Delmas *et al.,* 2000; Seibel 2000; Seibel *et al.,* 2002)].

MARKER	Abbreviation	Analyte	Method	Comments
1) Formation markers:				
Alkaline phosphatase				
Total alkaline phosphatase	Total ALP	Serum	Colorimetric	From bone, liver and other tissues
Bone specific alkaline phosphatase	Bone ALP	Serum	Colorimetric, electrophoretic, precipitation, IRMA[1], EIA	Some assays cross-react with liver ALP up to 20%
Type I collagen propeptides				
N-terminal propeptide of type I collagen	PINP	Serum	RIA, ELISA	Assays for monomer, trimer, intact or total PINP
C-terminal propeptide of type I collagen	PICP	Serum	RIA, ELISA	
2) Resorption markers:				
Hydroxyproline	Hyp	Urine	Colorimetric, HPLC	Total, free or peptide-bound
Hydroxylysine glycosides	Gal-Hyl, Glc-Gal-Hyl	Serum or urine	HPLC	
Free and total pyridinolines	PYD	Serum or urine	HPLC, ELISA, RIA, EIA	Total, free or peptide-bound
Free and total deoxypyridinoline	DPD	Urine	HPLC, ELISA, RIA, EIA	Total, free or peptide-bound
Type I collagen telopeptides				
Cross-linked C-telopeptide of type I collagen	ICTP (CTX-MMP)	Serum	RIA	MMP-derived
C-telopeptide of type I collagen crosslinks	CTX	Serum or urine	ELISA, RIA	Cathepsin K-derived; α- and β-CTx; β-isomerized unless otherwise specified
N-telopeptide of type I collagen crosslinks	NTX	Serum or urine	ELISA, RIA	
Bone sialoprotein	BSP	Serum	RIA, ELISA	
Tartrate-resistant acid phosphatase 5b	TRACP 5b	Serum or plasma	Colorimetric, RIA, ELISA	TRACP 5b from osteoclasts, TRACP 5a from activated macrophages and dendritic cells
3) Turnover markers:				
Osteocalcin or bone Gla-protein	OC			
Total osteocalcin	Total OC	Serum or urine	RIA, ELISA	Intact + N-mid fragment
Intact osteocalcin	Intact OC (1-49)	Serum	RIA, ELISA	
N-mid fragment of osteocalcin	N-mid OC (1-43)	Serum or urine	RIA, ELISA	
Undercarboxylated osteocalcin	OC	Serum	RIA, ELISA	

[1]IRMA, immunoradiometric assay; EIA, enzyme immunoassay; RIA, radio immunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography.

2.4.1 Bone formation markers

Bone formation markers are direct or indirect products of active osteoblasts during different phases of osteoblast development, reflecting different aspects of osteoblast function and bone formation. All present bone formation markers are measured from serum or plasma.

Alkaline phosphatase (ALP) is a ubiquitous enzyme attached via a glycosyl-phosphatidylinositol-link (GPI-link) to the extracellular surface of cell membranes (Low and Saltiel 1988). It is thought to have an essential role in mineralization (Whyte 1994). The total ALP serum pool consists of several isoforms that originate from various tissues such as the liver, bone, intestine, spleen, kidney and the placenta. In adults with normal liver function, approximately 50% of the total ALP activity in serum is derived from the liver, whereas 50% arises from bone (Green *et al.,* 1971). Thus, measurement of total ALP does not provide specific information on bone formation. Several techniques have been developed to differentiate the two main isoforms of circulating ALP (liver and bone), including heat denaturation, electrophoresis, precipitation, selective inhibition and, more recently, immunoassays (Mazda and Gyure 1988; Farley *et al.,* 1989; Chamberlain *et al.,* 1992; Garnero and Delmas 1993; England *et al.,* 1994; Gomez *et al.,* 1995). However, like all the other techniques, immunoassays also show some cross-reactivity between bone and liver ALP (15-20%) (Garnero and Delmas 1993). Therefore, in subjects with high liver ALP, results of bone ALP measurements may be artificially high, leading to false positive results.

Propeptides of type I collagen. The major synthetic product of osteoblasts is type I collagen, the most abundant protein in bone matrix. Type I collagen is synthesized as a procollagen form, having a short signal sequence and terminal extension propeptides: the amino-terminal propeptide (PINP) and the carboxy-terminal propeptide (PICP) (Prockop *et al.,* 1979b; Vuorio and de Crombrugghe 1990). These globular propeptides are cleaved from a newly formed procollagen and are partly released into the circulation (Fig. 5). Since both PINP and PICP are generated from newly synthesized collagen in a stoichiometric fashion, they are considered quantitative measures of newly formed type I collagen. Several immunoassays have been developed for both PINP (Ebeling *et al.,* 1992; Linkhart *et al.,* 1993; Melkko *et al.,* 1996; Orum *et al.,* 1996) and PICP (Taubman *et al.,* 1974; Simon *et al.,* 1984; Melkko *et al.,* 1990; Pedersen and Bonde 1994). Different assays for PINP detect monomeric, trimeric or intact N-terminal propeptide of type I collagen, giving substantially different results. The advantage of the PINP and PICP assays is the thermostability of the propeptides, but at the same time the disadvantage is the lack for specificity because of the propeptides of type I collagen released from tissues other than bone.

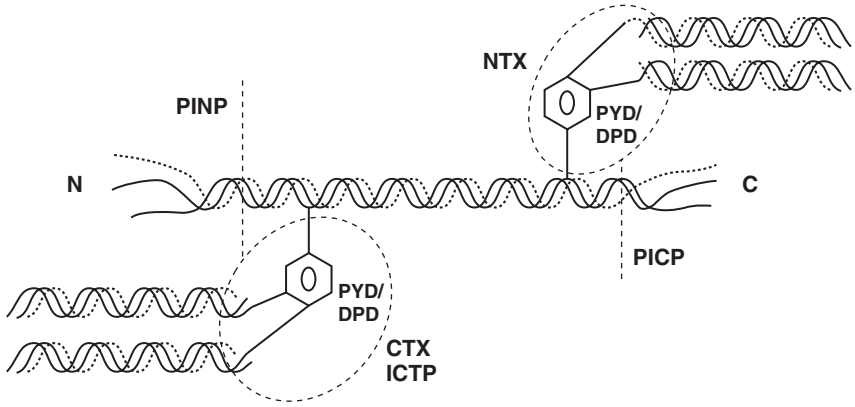

Figure 5: Biochemical markers of bone metabolism related to type I collagen. The amino-terminal propeptide (PINP) and the carboxy-terminal propeptide (PICP) are markers of bone formation. Markers for bone resorption include hydroxypyridinium crosslinks of collagen: pyridinoline (PYD) and deoxypyridinoline (DPD) and crosslinked C- and N-terminal telopeptides of type I collagen crosslinks (CTX, ICTP (=CTX-MMP) and NTX).

2.4.2 Bone resorption markers

Except for osteoclast-derived TRACP 5b, all other bone resorption markers are degradation products of bone matrix collagen or noncollagenous proteins such as bone sialoprotein. Conventionally all assays were confined to urine, but nowadays most of the assays are also available for serum measurements.

Hydroxyproline (Hyp) is formed intracellularly from the post-translational hydroxylation of proline. It constitutes 12-14% of the total amino acid content of mature collagen. Only 10% of Hyp released during bone resorption reaches the urine in free or peptide-bound forms (Lowry *et al.,* 1985). Urinary Hyp may be detected either as free or peptide-bound Hyp by colorimetric or high-performance liquid chromatographic (HPLC) methods (Kivirikko 1970; Deacon *et al.,* 1987). Significant amounts of Hyp are also derived from the degradation of newly synthesized collagens, from collagens of tissues other than bone, and from the diet (Prockop *et al.,* 1979a, b; Bienkowski 1984). Thus, Hyp is considered a nonspecific index of collagen turnover and it has largely been replaced by more specific techniques.

Hydroxylysine-glycosides (Hyl-glycosides) are integral parts of bone collagen and occur in two forms: glucosyl-galactosyl-hydroxylysine (Glc-Gal-Hyl) and galactosyl-hydroxylysine (Gal-Hyl) (Cunningham *et al.,* 1967). Both components are released into the circulation during bone resorption and may be measured in urine by HPLC (Krane *et al.,* 1977; Moro *et al.,* 1984). The advantage of hydroxylysine-glycosides over hydroxyproline is that the glycosylated forms are not metabolized and are not influenced by dietary components (Segrest and Cunningham 1970). Moreover, Glc-

Gal-Hyl is present in bone and skin, while Gal-Hyl is more specific for bone. Although these hydroxylysine-glycosides are potential markers of bone resorption (Moro *et al.,* 1988), their major disadvantage has been the absence of a convenient immunoassay format. However, a novel immunoassay for serum Gal-Hyl has been published recently showing a good correlation with urinary measurements (Al-Dehaimi *et al.,* 1999).

Pyridinoline (PYD) and *deoxypyridinoline* (DPD) are hydroxypyridinium crosslinks of collagen, formed during the extracellular maturation of fibrillar collagens. PYD and DPD are trifunctional crosslinks that bridge several collagen peptides and mechanically stabilize the collagen molecule (Fujimoto *et al.,* 1978). These hydroxypyridinium crosslinks are released only upon the degradation, not from newly synthesized collagens or from dietary sources (Fig. 5). In addition, PYD and DPD show a high specificity for skeletal tissues: PYD is found in cartilage, bone, ligaments and vessels, and DPD almost exclusively in bone and dentin (Eyre *et al.,* 1988). Thus, their levels strictly reflect the degradation of mature crosslinked collagen and the excretion of these crosslinks have been shown to correlate with histomorphometric or dynamic parameters of bone resorption (Delmas *et al.,* 1991; Eastell *et al.,* 1997). Released crosslinks are secreted into the urine (Gunja-Smith and Boucek 1981), where they are present both as free moieties (about 40%) and peptide-bound (about 60%) forms (Abbiati *et al.,* 1993). Both PYD and DPD can be measured by a reverse-phase ion-paired HPLC technique (Eyre *et al.,* 1984; Black *et al.,* 1988) and by immunoassays (Robins 1982; Delmas *et al.,* 1993; Seyedin *et al.,* 1993; Robins *et al.,* 1994).

Crosslinked C-telopeptide of type I collagen (ICTP) is a carboxy-terminal phenylalanine-rich crosslink of type I collagen (Fig. 5). The first peptide assay against crosslinked C-telopeptide was a radioimmunoassay (RIA) called ICTP, in which the antigenic determinant is a trivalent crosslink, created by matrix metalloproteinases (MMPs) (Risteli *et al.,* 1993). Because of the collagenolytic pathway of ICTP fragment, the assay is also called CTX-MMP. Treatment with cathepsin K cleaves the telopeptide structure between the phenylalanine-rich region and the crosslink, abolishing recognition of the ICTP assay (Sassi *et al.,* 2000). Serum levels of ICTP are not elevated in postmenopausal women as with other bone resorption markers (Koizumi *et al.,* 2003). Instead, the assay appears to be sensitive for pathological bone resorption as seen in MM and metastatic bone diseases (Abildgaard *et al.,* 1997; Jakob *et al.,* 2002; Keskikuru *et al.,* 2002; Sassi *et al.,* 2003).

Crosslinked C- and N-terminal telopeptides of type I collagen (CTX and NTX) are released during bone resorption, and they can be detected from serum and urine (Fig. 5). The first generation CTX assays were urinary measurements. The enzyme-linked immunosorbent assay (ELISA) for CTX (Bonde *et al.,* 1994) detected C-telopeptide of type I collagen containing an isoaspartyl (=β-aspartyl) peptide bond in its L-enantiomeric form (Fledelius *et al.,* 1997). The assay was abbreviated as β-CTX. The βL-aspartyl is a transformed aspartyl residue and is considered to result mainly from

the aging of extracellular proteins. The antibody used in the β-CTX assay was raised against synthetic octapeptide (EKAH-βD-GGR), and only one peptide strand is needed for immunoreactivity. At the same time, a radioimmunoassay (RIA) for CTX (α-CTX) was developed detecting the non-isomerized octapeptide (EKAH-αD-GGR) (Bonde *et al.,* 1996).

A novel sandwich type ELISA was developed for the measurement of β-CTX from serum. This assay uses two monoclonal antibodies that recognize only structures containing a crosslink and two β-isomerized peptides with the same sequence as shown for the urinary assay. In contrast to MMP-derived ICTP fragment of C-telopeptide, CTX is released through the cathepsin K degradation pathway (Garnero *et al.,* 2003). It is likely that these distinct collagenolytic pathways creating ICTP and CTX fragments of type I collagen explain why the abundance of CTX and ICTP in circulation varies in different bone pathologies. Currently, serum β-CTX is the most widely used crosslink assay, and unless otherwise stated, CTX refers to β-CTX.

Immunoassays have also been developed for the measurement of urine and serum crosslinked N-telopeptides (NTX) (Hanson *et al.,* 1992; Clemens *et al.,* 1997). The monoclonal antibody specifically recognizes the crosslinked α2 chain of type I collagen with sequence QYDGKGVG. More recently, point of care devices have been developed for several of the telopeptide markers, and in early 2000, such a device for NTX was approved in the USA for clinical use. These devices provide rapid results, but have a higher degree of variability compared with traditional assays.

Bone sialoprotein (BSP) accounts for 5-10% of the noncollagenous proteins in bone matrix (Fisher *et al.,* 1983). Intact BSP contains an RGD integrin recognition sequence and it is thought to play an important role in cell-matrix adhesion processes and in the supramolecular organization of the extracellular matrix of mineralized tissues (Ross *et al.,* 1993; Hunter and Goldberg 1994; Fujisawa *et al.,* 1995). Immunoassays have been developed for the measurement of immunoreactive BSP in serum (Saxne *et al.,* 1995; Karmatschek *et al.,* 1997). Serum BSP mainly reflects bone resorption, but data are lacking to assess the utility of this bone resorption marker (Seibel *et al.,* 1996).

TRACP 5b is a unique bone resorption marker, because it is the only marker released from resorbing osteoclasts, instead of being a bone matrix degradation product. In circulation TRACP exists in two isoforms named TRACP 5a and 5b, of which TRACP 5b is specifically derived from osteoclasts, and TRACP 5a from activated macrophages and dendritic cells (Janckila *et al.,* 2002a; Janckila *et al.,* 2002b). Several kinetic assays and immunoassays have been developed for the detection of total TRACP activity or mass, but these assays have lacked the appropriate specificity and sensitivity for osteoclast-derived TRACP 5b (Lam *et al.,* 1982; Whitaker *et al.,* 1989; Jalovaara *et al.,* 1990; Kraenzlin *et al.,* 1990; Scarnecchia *et al.,* 1991; Chamberlain *et al.,* 1995; Cheung *et al.,* 1995; Nakanishi *et al.,* 1998; Halleen *et al.,* 1999a).

2.4.3 Bone turnover markers

Osteocalcin (OC), formerly called 'bone gla-protein' (BGP), is a small, noncollagenous, hydroxyapatite binding protein synthesized by osteoblasts. It contains three vitamin-K dependent gamma-carboxyglutamic acid (Gla) residues responsible for the calcium binding properties of the protein (Hauschka *et al.,* 1989). The three-dimensional structure of OC was determined very recently, showing an optimal binding for bone matrix (Hoang *et al.,* 2003). The precise biological function of the protein is unknown, but it has been suggested that OC may have a role in the process of osteoid mineralization, since the protein is expressed mainly during this phase of bone formation. OC deficient mice have an unexpected phenotype with increased cortical and trabecular thickness, and mechanically more stable bones than wild type mice (Ducy *et al.,* 1996).

OC is considered to be a specific marker of osteoblast function, as its level in circulation correlates with bone formation rate (Brown *et al.,* 1984). Nevertheless, OC is not only released by active osteoblasts, but also during bone resorption from bone matrix by osteoclasts, and therefore OC is more properly a marker of bone turnover rather than bone formation (Kurihara *et al.,* 1998; Ivaska *et al.,* 2004). In circulation, OC is present as an intact molecule and as OC fragments of various sizes. Conventional immunoassays detect various OC fragments, while the novel immunoassays measure separately either intact OC (residues 1-49) or N-terminal fragments (residues 1-43) or both with the total OC assays. In practice, there are numerous OC immunoassays available using different antibodies and detecting OC fragments of various sizes (Seibel *et al.,* 2002). Therefore, it should be kept in mind that the results are not necessarily readily comparable using different OC assays (Diaz Diego *et al.,* 1994; Colford *et al.,* 1997). Traditionally, OC has been measured from serum only, but nowadays fragments of OC can also be measured from urine (Taylor *et al.,* 1990; Srivastava *et al.,* 2002b; Ivaska *et al.,* 2003).

2.4.4 General considerations of bone metabolic markers

Like all chemical analytes, markers of bone metabolism have their specific technical and analytical limitations. Some of the markers are sensitive to thermodegradation, UV radiation, hemolysis or other ambient influences. In order to obtain meaningful results, sampling, sample handling and storing should be strictly standardized to keep the components stable and to provide reproducible conditions for their measurement. Also, the assays need to be standardized, and each laboratory should establish its own age- and gender-based reference ranges. Advantages and disadvantages of bone metabolic markers are summarized in Table 4.

Table 4. Advantages and disadvantages of bone metabolic markers

Advantages	Disadvantages
Noninvasive	Technical limitations
Easy to repeat	Instability during storage
Large variation across the menopause	Variation between assay - precision, accuracy
Assessment of overall bone turnover	Lack of internationally agreed standards
Investigation of the pathogenesis	Diurnal variation
Identification of 'fast bone losers'	Variation in metabolism and clearance
Early monitoring of therapy	Impossible to localize the disturbance in the skeleton
Low seasonal variation	

In order to obtain reliable results, the pre-analytical and analytical variability of bone turnover markers must be taken into account. There are several sources for pre-analytical variability, which can be classified into two broad categories: 1) uncontrollable factors, such as age, gender, menopausal status, renal function, pregnancy and lactation, drugs, disease, immobility or recent fracture, and 2) controllable factors, such as circardian, menstrual, seasonal, exercise or diet effects [reviewed by (Hannon and Eastell 2000)]. Of these, the most important factors to be taken into account are age, gender, menopausal status for women, and circardian rhythm (Qvist *et al.,* 2002). Other important factors are fractures, drugs, diseases, immobility, fasting status and exercise, all of which cause variability for the biochemical markers (Clowes *et al.,* 2002). The effects of uncontrollable factors cannot be altered, but they can be accounted for by using appropriate age- and gender-specific reference ranges and by using the information from published reports to allow for other factors such as disease or recent fracture. The effects of controllable factors can be minimized by standardizing the timing and conditions of sample collection. However, despite minimizing the sources of pre-analytical variability, there will always remain some endogenous day-to-day variability, which varies between different markers. In general, assays using serum or plasma as an analyte are preferred over urinary-based measurements, because they have lower variability than urinary assays (Fall *et al.,* 2000; Takahashi *et al.,* 2002a). Nowadays the analytical precision of different markers of bone turnover is generally good.

2.4.5 Use of bone metabolic markers

Bone metabolic markers are valuable diagnostic tools in metabolic bone diseases with high bone turnover, such as Paget's disease and primary and secondary hyperparathyroidism (Seibel *et al.,* 2002). However, these markers are not suitable for the diagnosis of osteoporosis, since it is a condition where subtle modifications of bone turnover can lead to a substantial loss of bone mass after a long period of time (Seibel *et al.,* 2002). Nevertheless, markers of bone metabolism do have a clinical value in the management of osteoporotic patients. Markers of bone metabolism may

predict bone loss and fracture risk in postmenopausal women, and can be used for monitoring the efficacy of antiresorptive therapy.

Markers of bone metabolism reflect the whole-body rates of bone formation and resorption and may therefore reliably predict the imbalance in bone turnover and the rate of bone loss (Stepan 2000). This would provide an easy and inexpensive method of identifying individuals at risk for developing osteoporosis. Several prospective studies have shown the association between the markers and the bone loss rate in the distal forearm, calcaneus and hip (Christiansen *et al.,* 1987, 1990; Dresner-Pollak *et al.,* 1996; Ross and Knowlton 1998; Garnero *et al.,* 1999). However, this association has not been shown constantly and therefore further studies are needed to confirm the predictive value of bone metabolic markers. A high rate of bone turnover may not be associated only with bone loss but also with a disruption of the trabecular network and connectivity, and therefore increased risk of fractures. The prospective data reviewed clearly indicates that increased levels of bone metabolic markers, especially markers of bone resorption, are associated with an increased risk of vertebral, non-vertebral and hip fracture (Garnero 2000). The combination of BMD and bone metabolic markers will further increase the prediction of fracture risk (Garnero *et al.,* 2000; Johnell *et al.,* 2002).

Similar to most chronic diseases, monitoring the efficacy of treatment of osteoporosis is a challenge. Measurement of BMD by DXA has been accepted as a method for monitoring the efficacy of the treatment. However, the relatively small changes in bone mass (up to 6-7%) subsequent to therapy are difficult to reliably differentiate from the precision errors of the machine (1-2%) (Delmas 2000). Furthermore, it normally takes at least 1 to 2 years to reach significant changes in BMD to detect responders to treatment. Therefore, biochemical markers of bone metabolism will provide an alternative way to assess efficacy of treatment after a short period of time. Several studies have shown that a change in bone marker after 3 to 6 months correlates with a change in BMD at 1 to 3 years (Bjarnason and Christiansen 2000; Greenspan *et al.,* 2000; Halleen *et al.,* 2002; Eastell *et al.,* 2003). Additionally, changes in bone markers (20-100%) are much higher than changes in BMD (6-7%) subsequent to therapy, which gives better "signal-to-noise ratio" for markers (Delmas *et al.,* 2000). Thus far there is no perfect way to identify responders and non-responders to therapy. One approach is to consider the least significant change (LSC) of the bone marker, which is determined from the variability of the marker multiplied by a constant to obtain the change (two-tailed) that can be considered significant ($p<0.05$) based on single measurements at baseline and follow-up (Hannon *et al.,* 1998).

$$LSC = 1.96 \times (2)^{0.5} \times [(CVi)^2 + (CVa)^2]^{0.5}$$

CVi = intraindividual variability

CVa = analytical variability

2.5 Tartrate-resistant acid phosphatase (TRACP)

2.5.1 History and nomenclature

TRACP belongs to the acid phosphatases (EC 3.1.3.2) sharing catalytic activity towards phosphoesters in an acidic environment. In 1970 Li and co-workers reported the results of studies of human leukocyte acid phosphatases, and found at least seven distinct isoenzymes 0, 1, 2, 3a, 3b, 4 and 5, numbered in order of their increasing electrophoretic mobility towards the cathode (Li *et al.,* 1970a, b). ”Band 5” acid phosphatase (AcP 5) or type-5 AcP was the only one resistant to L(+)-tartrate inhibition, from which the name tartrate-resistant acid phosphatase (TRACP) derives. There are other tartrate-resistant AcPs present in platelets and red blood cells, but they are not basic, and are therefore not demonstrable in an acidic environment. Hence, TRACP specifically refers to type-5 AcP demonstrated on acidic acrylamide gels. AcPs from platelets and erythrocytes clearly differ from AcP 5 by their molecular weights, substrate specificities, electrophoretic mobilities and antigenic properties (Fenton and Richardson 1967; Sorenson 1972). Previously, the commonly used abbreviation for type-5 AcP was TRAP, but following a recommendation from the nomenclature committee an additional ‘C’ was added to TRACP as a separation from alkaline phosphatases (APs) (Delmas *et al.,* 2000).

The members of TRACP family are also known as purple acid phosphatases (PAPs) because of the bound metal ions in their active sites giving an intense purple color. As early as 1954 Sundararajan and Sarma were able to partially purify a bovine splenic phosphatase with an acidic pH optimum and a purple or violet color (Sundararajan and Sarma 1954). Twenty years later, in 1974, Schlosnagle and colleagues were able to purify a basic, progesterone-induced, intrauterine glycoprotein with a purple color from uterine fluids of pigs (Schlosnagle *et al.,* 1974), which had acid phosphatase activity. A few years later this enzyme was named as uteroferrin, because of the proposed role in iron transport from maternal to fetal circulation (Buhi *et al.,* 1979).

2.5.2 Isolation of mammalian TRACP enzymes

TRACP enzymes have been purified from many mammalian sources, including pig uterine fluid (Schlosnagle *et al.,* 1974), bovine (Davis *et al.,* 1981; Orlando *et al.,* 1993) and rat (Hara *et al.,* 1984) spleen; bovine bone (Lau *et al.,* 1987a); the spleens of patients affected with hairy cell leukemia (Lam and Yam 1977; Ketcham *et al.,* 1985) and Gaucher’s disease (Robinson and Glew 1980a); human (Allen *et al.,* 1989; Hayman *et al.,* 1989; Halleen *et al.,* 1996) and rat (Anderson and Toverud 1986; Ek-Rylander *et al.,* 1991) bone; and human lungs (Efstratiadis and Moss 1985a) and placenta (Ketcham *et al.,* 1989). When TRACP enzymes were purified from distinct sources they were shown to exist either in monomeric approximately 30-35 kD proteins or in two subunits, sized approximately 16 and 20-25 kD, connected by a covalent bond. For a long time this discrepancy remained enigmatic, until in the 1990s several research groups were able to demonstrate an exposed, highly antigenic,

protease sensitive loop structure in the sequence of TRACP (Orlando *et al.,* 1993; Ljusberg *et al.,* 1999; Funhoff *et al.,* 2001a). After cleavage of this loop, the enzyme was turned into two subunits connected by a disulfide bridge between two conserved cysteine residues.

2.5.3 Expression of TRACP

Physiologically, TRACP expression is found in cells of the mononuclear phagocyte system, most abundantly in osteoclasts, alveolar macrophages and dendritic cells (Efstratiadis and Moss 1985b; Andersson and Marks 1989; Yaziji *et al.,* 1995). High TRACP activity has been demonstrated in adult mice at tissue level in bone, colon, liver, spleen, and thymus, with lower amounts in brain, kidney, lung, skin, and stomach and also with trace amounts in heart, muscle, and testis (Angel *et al.,* 2000; Hayman *et al.,* 2000). TRACP activity in other than bone tissue is primarily due to the wide distribution of TRACP positive dendritic cells and activated macrophages (Hayman *et al.,* 2000). Unstimulated monocytes in bone marrow or peripheral blood are TRACP negative (Janckila *et al.,* 1978; Radzun *et al.,* 1983). Lång and co-workers observed that in the rat nervous system, TRACP activity and mRNA expression were localized into small ganglion cells of the trigeminal ganglion, in α-motor neurons of the ventral spinal cord and in Purkinje cells of the cerebellum (Lång *et al.,* 2001).

In human tissues, TRACP mRNA is expressed abundantly in the spleen, liver, colon, lung, small intestine, kidney, stomach, testis, placenta, lymph node, thymus, peripheral blood leukocyte and bone marrow (Hayman *et al.,* 2001). Enzymatically active protein has been detected by cytochemistry and by immunohistochemistry in bone, spleen, lung, skin, colon, stomach, and the ileum (Yaziji *et al.,* 1995; Hayman *et al.,* 2001). At a cellular level, TRACP activity has also been detected in specialized macrophage progeny, namely in Kupffer cells of the liver and pulmonary alveolar macrophages as well as in dendritic cells (Efstratiadis and Moss 1985b; Yaziji *et al.,* 1995; Janckila *et al.,* 2003). Some researchers have also found TRACP in osteoblasts and osteocytes, but the intensity of the staining has been lower than in osteoclasts (Bianco *et al.,* 1988; Yamamoto and Nagai 1998). In some other studies osteoblasts and osteocytes have been totally negative for TRACP (Clark *et al.,* 1989; Reinholt *et al.,* 1990). Analysis of human placenta at the cellular level revealed TRACP activity in decidual cells and syncytiotrophoblasts (Janckila *et al.,* 1996).

Pathologically, TRACP expression is elevated in diseases such as hairy cell leukaemia, Gaucher's disease and osteoclastoma (Yam *et al.,* 1971; Robinson and Glew 1980b; Hayman *et al.,* 1989), hence TRACP is clinically used as a marker enzyme for these diseases. Elevated TRACP activities have also been demonstrated in situations with increased osteoclastic activity such as hyperparathyroidism, Paget's disease, inflamed synovial joints, postmenopausal women, osteoporosis and various cancers with bone metastases (Lau *et al.,* 1987b; Chamberlain *et al.,* 1995).

2.5.4 Subcellular localization of TRACP in osteoclasts and macrophages

In studies using electron microscopy or separation of subcellular granules by density gradient centrifugation followed by cytochemical or immunocytochemical staining, TRACP was localized to lysosomes, lysosome-like organelles of the Golgi complex and microsomes in spleen cells of HCL, rat liver macrophages, rat bone osteoclasts and bovine spleen histiocytes (Katayama *et al.,* 1972; Lam *et al.,* 1976; Andersson *et al.,* 1986; Schindelmeiser *et al.,* 1987; Clark *et al.,* 1989; Vincent and Averill 1990a). Within the lysosome-like organelles, TRACP activity was restricted to the inner membrane surface (Clark *et al.,* 1989), which is consistent with the sequence data, suggesting a putative N-terminal lysosomal leader sequence in the TRACP gene (Lord *et al.,* 1990).

Several studies have been made to localize TRACP in osteoclasts. Before immunohistochemical detection, TRACP activity was detected close to the RB membrane with different non-specific substrates such as β-glycerophosphate and adenosine triphosphate (Miller 1985; Lindunger *et al.,* 1990). However, TRACP is not able to hydrolyse β-glycerophosphate (Antanaitis and Aisen 1983), and the activity detected with this substrate was most probably tartrate-sensitive lysosomal AcP (LAP) activity. Also, adenosine triphosphate is a better substrate for V-ATPase located in the RB membrane than for TRACP (Blair *et al.,* 1989; Väänänen *et al.,* 1990). Immunohistochemical detection has revealed more reliable results, showing intracellular vesicular staining for TRACP with no positive reaction at the RB (Clark *et al.,* 1989; Halleen *et al.,* 1999b). However, one study using immunogold technique demonstrated intensive TRACP staining at the RB membrane and intracellularly in vesicular structures (Reinholt *et al.,* 1990).

Colocalization studies have been made in osteoclasts and in alveolar macrophages, and no or only partial co-localization has been observed with TRACP and known lysosomal markers, lysosome-associated membrane protein 1 and the cation-independent mannose-6-phosphate receptor (Räisänen *et al.,* 2001). Instead, TRACP was shown to colocalize with bone degradation products at the transcytotic route in resorbing osteoclasts (Halleen *et al.,* 1999b) and with internalised *S. aureus* in alveolar macrophages (Räisänen *et al.,* 2001). The transcytotic route of osteoclasts is analogous to the antigen presentation route of activated macrophages, as both are a transport route from a late endosomal/lysosomal compartment to a cell membrane. This data indicates that TRACP may be localized into some new still unidentified vesicular compartment both in osteoclasts and in macrophages.

Researchers are in agreement that TRACP is secreted from osteoclasts during bone resorption (Minkin 1982; Chambers *et al.,* 1987; Zaidi *et al.,* 1989; Moonga *et al.,* 1990) and active enzyme can be detected in the serum of mammals as a complex with α_2-macroglobulin (Brehme *et al.,* 1999; Ylipahkala *et al.,* 2003). Because the biological role of TRACP in osteoclasts and other cells is still unknown, several secretory pathways related to distinct intracellular and extracellular functions may exist.

2.5.5 Protein structure

Purple acid phosphatase (PAP) from the red kidney bean (Protein Data Bank code, 1KBP) was the first member of the TRACP family for which the crystal structure became available at 2.9 Å resolution in 1995 (Sträter *et al.,* 1995). In 1999, the three-dimensional structures for three mammalian TRACP enzymes were published almost simultaneously: for pig TRACP (1UTE) at 1.55 Å resolution (Guddat *et al.,* 1999), for rat TRACP from bone (1QHW) at 2.2 Å resolution (Lindqvist *et al.,* 1999), and for recombinant rat TRACP (1QFC) at 2.7 Å resolution (Uppenberg *et al.,* 1999). Despite of less than 15% nucleotide sequence homology between distinct mammalian TRACP enzymes, the amino acid sequences exhibit over 80% identity (Ketcham *et al.,* 1985; Hunt *et al.,* 1987) and the protein folds are very similar. The structure of mammalian TRACP enzymes is approximately spherical with dimensions of 45 × 45 × 40 Å. The enzymes are formed of two sandwiched β-sheets flanked by α-helical segments facing the solvent. The enzymes show internal symmetry, with the metal ions bound at the interface between the two halves, comprising the active site (Fig 6). Mammalian enzymes are approximately 35 kD monomeric proteins, whereas plant enzymes are typically dimers of 55 kD subunits (Schenk *et al.,* 2000).

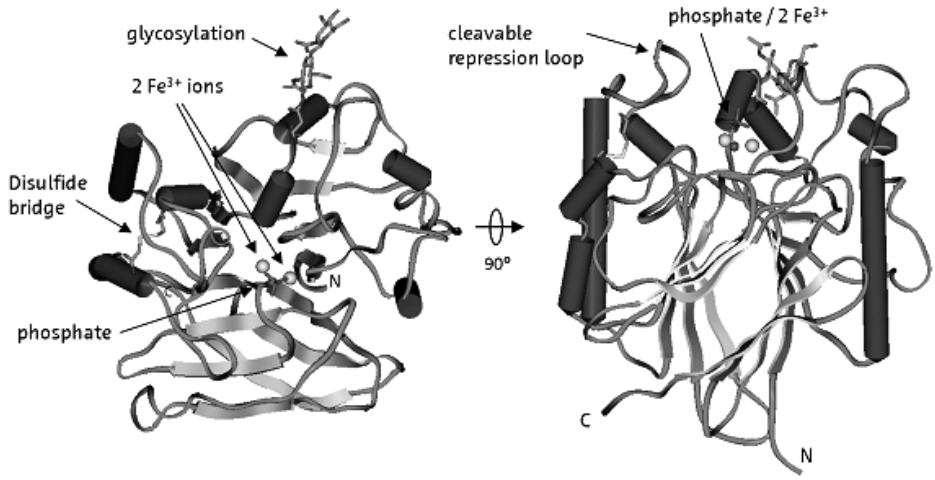

Figure 6. The three-dimensional structure of porcine TRACP, uteroferrin (PDB code 1UTE). The core is formed from two seven-stranded β-sheets, which are surrounded by twelve α-helices. The active site with two iron atoms and a phosphate ligand is located at the interface between the β-sandwich. The disulphide bridge is between cysteine residues Cys142 and Cys200, and the high-mannose type glycosylation site is in asparagine residue Asn97. The protease sensitive loop is located close to the active site, as shown in the right image.

Mammalian TRACPs contain two iron atoms in their active site, while plant enzymes have one iron atom and either zinc or manganese (Davis *et al.,* 1981; Sträter *et al.,* 1995; Schenk *et al.,* 1999). The anti-ferromagnetically spin-coupled binuclear iron center of the mammalian TRACP enzymes exists in two stable interconvertible states: pink, reduced and enzymatically active, with a mixed-valent Fe^{3+}-Fe^{2+} cluster; and purple, oxidized and catalytically inactive, with a binuclear pair as Fe^{3+}-Fe^{3+} (Davis and Averill 1982). Another of the iron atoms in the active site of mammalian TRACPs is stabilized into the ferric (Fe^{3+}) form by a tyrosine residue (Tyr55; numbering according to human sequence), accounting for the characteristic purple colour of TRACPs. A histidine (His223) and an aspartate (Asp14) residue coordinate the ferric iron among tyrosine residue. A μ-hydroxo bridge and an aspartate carboxylate (Asp52) connect the ferric iron to the redox active iron ($Fe^{2+/3+}$), which is in turn coordinated by two histidines (His186, His221) and an asparagine (Asn91) residue (Sträter *et al.,* 1995). The residues coordinating the binuclear active site of TRACPs are essentially identical to those of plant TRACPs and the regulatory Ser/Thr protein phosphatases (PPs) (Vincent and Averill 1990b; Egloff *et al.,* 1995; Goldberg *et al.,* 1995; Griffith *et al.,* 1995; Klabunde *et al.,* 1995; Schenk *et al.,* 2000). However, both plant TRACPs and PPs have a ferric iron together with a zinc or manganese in their active site instead of two iron atoms. Additionally, PPs differ essentially from the TRACP family by having a water molecule instead of the tyrosine residue coordinating the ferric iron, accounting for the loss of purple color (Egloff *et al.,* 1995; Sträter *et al.,* 1995).

Near the active site pocket is an exposed, protease sensitive loop (residues 142-159 in uteroferrin, corresponding to residues 145-160 in human TRACP) and a covalent disulphide bond linking cysteine residues Cys142 and Cys200 both in uteroferrin and in human TRACP (Fig. 6) (Guddat *et al.,* 1999; Lindqvist *et al.,* 1999). The loop structure of mammalian enzymes can be cleaved by trypsin or cathepsins, yielding two fragments connected by a disulphide bridge. Proteolytic cleavage shifts the pH optimum approximately from 5 to 6 and activates the acid phosphatase activity of TRACP by 3-8 fold, also causing changes in the EPR spectrum (Orlando *et al.,* 1993; Marshall *et al.,* 1997; Kaija *et al.,* 1999; Ljusberg *et al.,* 1999; Funhoff *et al.,* 2001a). Enzymatic activation was shown to be not due to the removal of steric hindrance or conformational changes, but rather due to changes in the molecular interaction between residue Asp146 of the loop region and residue Asn91 coordinating the redox active iron in the active site (Funhoff *et al.,* 2001a; Funhoff *et al.,* 2001b).

From the two putative N-linked glycosylation sites Asn97 and Asn128, the previous one was shown to be glycosylated (Guddat *et al.,* 1999; Lindqvist *et al.,* 1999; Uppenberg *et al.,* 1999). In uteroferrin, the carbohydrate side chain consists of phosphorylated, high-mannose-type oligosaccharide composed of six mannose residues and two N-acetylglucosamine residues (Baumbach *et al.,* 1984; Saunders *et al.,* 1985). Similar to uteroferrin, human bone TRACP also contains only N-linked high-mannose carbohydrate (Halleen *et al.,* 1996).

2.5.6 Isoforms 5a and 5b

Soon after the discovery of TRACP, Lam and co-workers were able to demonstrate using native electrophoresis and CM-Sepharose chromatography that TRACP exists in two distinct isoforms in human serum (Lam *et al.,* 1978a). Two tartrate-resistant bands with slightly different molecular weights were observed and named according to their electrophoretic mobility as TRACP 5a and TRACP 5b. The difference in the size was shown to be due to additional sialic acid in the carbohydrate side chain of TRACP 5a. After sialidase treatment TRACP 5a was converted to TRACP 5b (Lam *et al.,* 1981). TRACP 5a has a pH optimum of 5.2 and relatively low specific activity, whereas TRACP 5b has a pH optimum of 5.8 with substantially higher specific activity. In addition to a different pH optimum and carbohydrate content, TRACP 5a and 5b are also distinguishable by antigenic properties (Table 5) (Lam *et al.,* 1978a). Recently, Janckila and colleagues were able to demonstrate that TRACP 5b is identical to a two-chain form generated by protease cleavage, whereas TRACP 5a is the non-cleaved form (Janckila *et al.,* 2002b).

Based on current knowledge, osteoclasts contain and secrete only TRACP 5b isoform, while activated macrophages express both TRACP 5a and 5b, but secrete only TRACP 5a into the circulation (Janckila *et al.,* 2002a; Janckila *et al.,* 2002b). The osteoclastic origin of TRACP 5b makes it a specific marker for osteoclasts and bone resorption (Halleen *et al.,* 2001; Halleen *et al.,* 2002).

Table 5: Properties of TRACP 5a and 5b

	TRACP 5a	**TRACP 5b**
Sialic acid	Present	Not present
pH optimum for AcP	4.9-5.1	5.7-5.9
Specific activity of AcP	Low	High
Polypeptide chain	Intact ~35 kD	Cleaved 16 kD + 20-25 kD
Origin	Macrophages and dendritic cells	Osteoclasts

2.5.7 TRACP gene

The mammalian TRACP genes have been cloned from mouse (Cassady *et al.,* 1993; Reddy *et al.,* 1993), human (Lord *et al.,* 1990; Cassady *et al.,* 1993; Fleckenstein *et al.,* 1996), rat (Ek-Rylander *et al.,* 1991) and pig (Vallet and Fahrenkrug 2000). The exon – intron structure of the TRACP gene is highly conserved, consisting of five exons with the translation initial signal ($A^{+1}TG$) at the beginning of exon 2 (Fig. 7) (Cassady *et al.,* 1993; Fleckenstein and Drexler 1997; Vallet and Fahrenkrug 2000). Uteroferrin was originally shown to have only three exons (Cassady *et al.,* 1993), but later Vallet and Fahrenkrug showed that a similar five exon structure also existed in the uteroferrin gene (Vallet and Fahrenkrug 2000). In contrast to most plants studied, mammalians have only a single TRACP gene in their genome (Lord *et al.,* 1990;

Cassady *et al.,* 1993; Ling and Roberts 1993). The mouse TRACP gene is located to chromosome 9 and the human TRACP gene to chromosome 19p13.2 - 13.3 (Grimes *et al.,* 1993; Leach *et al.,* 1994). The TRACP gene in mammalians is 975 – 1020 bp long, and it encodes approximately 1.5 kb mRNA. Translation will encode a protein with 323 - 325 amino acids, including a signal peptide of 19 residues and two potential sites for N-glycosylation (Lord *et al.,* 1990).

Very recently multiple tissue-specific promoters were identified (Walsh *et al.,* 2003), solving the problem with contradictory results with several transcription start points (*tsp*) (Cassady *et al.,* 1993; Reddy *et al.,* 1993; Alcantara *et al.,* 1994). Walsh and co-workers were able to identify three distinct mRNAs with differential 5'UTRs, but similar 3' ends of the mRNA from the first base of exon 2 (Walsh *et al.,* 2003). The novel 5'-UTRs represent alternative first exons located upstream of the known 5'-UTR (Reddy *et al.,* 1993). This genomic structure is conserved at least between mouse and human (Fig. 7). Expression of the most distal 5'-UTR (exon 1A) in mouse is restricted to adult bone and spleen tissue, while exon 1B is expressed primarily in tissues containing TRACP-positive non-haematopoietic cells. The known 5'-UTR (exon 1C) is expressed in cells originating from a myeloid lineage, in osteoclasts and macrophages (Walsh *et al.,* 2003). In mouse genome, exon 1C comprises two distinct transcription start regions, with an osteoclast-specific *tsp* at –445 bp and a macrophage-specific *tsp* upstream of osteoclast-specific *tsp* at –495 bp (Walsh *et al.,* 2003). Although the genomic structure of the 5'-flanking region of the human and mouse TRACP genes is conserved, the sequence identity is limited.

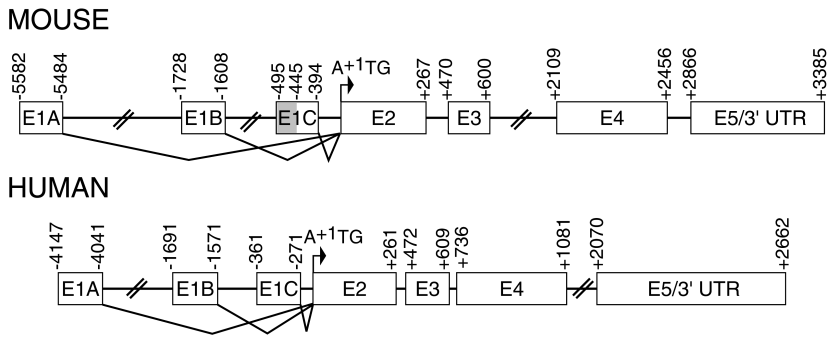

Figure 7: The exon – intron structures of mouse and human TRACP genes are highly conserved. The alternative first exons are marked as E1A, E1B and E1C. In mouse genome, exon 1C comprises of two distinct transcription start regions, with an osteoclast-specific *tsp* at –445 bp and a macrophage-specific *tsp* upstream of osteoclast-specific *tsp* at –495 bp. The translation initial signal $A^{+1}TG$ is at the beginning of exon 2.

2.5.8 Regulation of transcription

Studies on the 5'-flanking region of the TRACP gene have indicated a complex transcriptional regulation. The most conserved region of the TRACP 5'-flanking region lies within the 2 kb segment from the *tsp* $A^{+1}TG$, and this area has been under

extensive study. The possibility that TRACP might be involved in iron transport or storage has led several groups to examine the regulatory influence of intracellular iron on TRACP expression. Simmen *et al.* found a putative iron response element in the 5'-flanking region of the uteroferrin gene at 1989 (Simmen *et al.,* 1989), and a few years later Alcantara and colleagues were able to show that expression of TRACP was regulated by iron and the iron responsive element was located between base pairs –1846 and –1240 in the mouse promoter (Alcantara *et al.,* 1994). This iron regulation is very complex and occurs at the transcriptional level (Reddy *et al.,* 1995). Two iron-delivering agents, hemin (ferric protoporphyrin IX) and transferrin have opposite effects on TRACP expression. TRACP expression is decreased by both hemin and protoporphyrin IX (Reddy *et al.,* 1996) and increased by transferrin (Alcantara *et al.,* 1994; Reddy *et al.,* 1995). Two hemin-responsive elements (HRE) were found within the human 5'-flanking region, representing binding sites for an HRE binding protein (HRE-BP) (Fleckenstein *et al.,* 1996), and both are needed for the hemin-induced regulation of the human TRACP gene expression (Fleckenstein *et al.,* 2000).

The promoter area of TRACP contains numerous candidate transcription factor (TF) binding sequences, including those for Sp1, AP1, PU.1, GT-1 and H-APF-1 (Reddy *et al.,* 1993). PU.1 is a monocyte-associated TF regulating the initial commitment in the differentiation pathway of macrophages and osteoclasts (Tondravi *et al.,* 1997; Fisher and Scott 1998). PU.1 acts synergistically with osteoclast commitment factor MiTF (microphthalmia transcription factor) regulating the TRACP expression during terminal differentiation of osteoclasts (Luchin *et al.,* 2000; Luchin *et al.,* 2001; Cassady *et al.,* 2003). IL-6 activates the H-APF-1 site, and stimulates osteoclast formation and activity (Kurihara *et al.,* 1991). TRACP expression is also stimulated by RANKL, and at least upstream stimulatory factors (USF) 1 and 2, and TF called YY1 are involved in the activation (Liu *et al.,* 2003; Shi *et al.,* 2003). However, it is likely that other DNA binding proteins are involved in the RANKL-mediated upregulation of TRACP expression, but they are still unknown (Selski *et al.,* 2003).

2.6 Functions of TRACP

TRACP has been shown to be a bi-functional protein *in vitro* with an acid phosphatase and ROS generating activity. However, the biological function(s) as well as natural substrate(s) of TRACP are still unknown despite intensive studies within last three decades. The highly conserved structure of the TRACP enzymes, and especially the active site structure throughout the animal kingdom from microorganisms to mammalians provides strong evidence for an important biological role for the enzyme. Location in several tissues and cells suggests that TRACP may function as a multifunctional protein in different cells and cell compartments.

2.6.1 Acid phosphatase (AcP)

TRACP can catalyze the hydrolysis of a wide range of phosphate monoesters such as β-umbelliferyl phosphate, *p*-nitrophenyl phosphate, α-naphthyl phosphate and

phosphotyrosine (Schlosnagle *et al.,* 1974; Janckila *et al.,* 1992; Marshall *et al.,* 1997). Also phosphoanhydrides such as pyrophosphate and nucleoside tri- and diphosphates are hydrolyzed by TRACP (Anderson and Toverud 1986; Andersson and Marks 1989), but aliphatic phosphoesters such as monophosphates, β-glycerophosphate, mannose-6-phosphate, phosphoserine and phosphothreonine are not hydrolyzed (Antanaitis and Aisen 1983; Lau *et al.,* 1987a). However, TRACP can catalyze the release of phosphate from certain phosphoproteins that carry phosphoserine residue such as osteopontin and osteonectin (Roberts and Bazer 1976; Ek-Rylander *et al.,* 1994). Nevertheless, the AcP activity of TRACP prefers phosphotyrosine containing proteins over other phosphoproteins, suggesting a specific protein tyrosine phosphatase (PTPase) activity for TRACP (Lau *et al.,* 1987a; Janckila *et al.,* 1992; Nash *et al.,* 1993; Nuthmann *et al.,* 1993; Halleen *et al.,* 1998b). Phosphorylation of specific tyrosine residues by kinases plays an important role in various cell events such as signal transduction, activation, proliferation and differentiation. As a PTPase, TRACP could regulate protein-tyrosine kinases and thereby influence various cellular events (Janckila *et al.,* 1992; Nuthmann *et al.,* 1993).

The AcP activity of TRACP is competitively inhibited by inorganic phosphate and its analogs e.g. vanadate, arsenate and molybdate. Fluoride, tungstate, copper and zinc cause non-competitive inhibition of AcP activity (Schlosnagle *et al.,* 1974; Davis *et al.,* 1981; Lau *et al.,* 1987a; Janckila *et al.,* 1992). Reducing agents such as β-mercaptoethanol, dithiothreitol, ascorbic acid, cysteine and glutathione activate the AcP activity of TRACP by reducing the ferric iron into the ferrous form and simultaneously changing the purple colour to pink (Schlosnagle *et al.,* 1976; Campbell *et al.,* 1978; Davis *et al.,* 1981). The reverse reaction from pink to purple ($Fe^{2+} \rightarrow Fe^{3+}$) causes inactivation and is achieved by oxidizing agents such as hydrogen peroxide and ferricyanide (Antanaitis and Aisen 1983). Dithionite inactivates TRACP irreversibly by removing the metal ions from the active site, thereby bleaching its colour (Schlosnagle *et al.,* 1974; Keough *et al.,* 1980; Halleen *et al.,* 1998b). In contrast, cyanide, azide, tartrate, and p-nitrophenol show no inhibition of the AcP activity of TRACP (Schlosnagle *et al.,* 1976; Davis *et al.,* 1981).

Rat, mouse and human TRACP, but not uteroferrin, contain a consensus motif for PTPase; $Cys(X)_5Arg$ (Zhang *et al.,* 1994). The conserved cysteine residue in the motif for PTPases forms a covalent bond with phosphate during catalysis and is essential for enzyme activity [reviewed by (Tonks 2003)]. However, in TRACPs the conserved cysteine forms a disulphide bridge, being unable to participate in the catalysis. Additionally, the cysteine is located far away from the binuclear iron center, suggesting that it has no role in the enzymatic activity of TRACP. Combined structural and mechanical studies provide a model for the catalytic mechanism of TRACP. In the first step, the phosphate group of the substrate is coordinated to the divalent metal ion. Formation of the enzyme-substrate complex is followed by a nucleophilic attack on the phosphorus, leading to the release of the product alcohol. Three possible candidates have been proposed for the attacking group: 1) a terminal Fe^{3+}-bound hydroxide (Klabunde *et al.,* 1996); 2) a bridging hydroxide (Wang *et al.,* 1996); or 3) a hydroxide residing in the second coordination sphere of Fe^{3+} (Merkx *et al.,* 1999). In the final step of catalysis, the metal-bound phosphate group is released.

TRACP may participate in bone resorption by dephosphorylating bone matrix phosphoproteins osteopontin, osteonectin and bone sialoprotein (Ek-Rylander *et al.,* 1994; Andersson and Ek-Rylander 1995). Osteopontin has been shown to bind to osteoclast cell surface integrins via the RGD motif, thereby mediating substrate adhesion, at least *in vitro* (Flores *et al.,* 1992). After dephosphorylation osteopontin is no longer able to support osteoclast binding to the substrate, suggesting that TRACP regulates osteoclast attachment (Ek-Rylander *et al.,* 1994). This detachment would allow migration across the bone surface to new resorption sites. Dephosphorylation of bone matrix phosphoproteins suggests a catabolic function for TRACP in bone resorption. This is similar to the function proposed for TRACP in the degradation of red cell membrane and cytoskeletal phosphoproteins during erythrophagocytosis by macrophages (Schindelmeiser *et al.,* 1987).

2.6.2 Generator of reactive oxygen species (ROS)

Three individual research groups have been able to demonstrate by different methods that TRACP is capable of generating ROS via Fenton's reaction (Sibille *et al.,* 1987; Hayman and Cox 1994; Halleen *et al.,* 1999b). In an enzymatically active reduced state, TRACP has a mixed-valent Fe^{3+}-Fe^{2+} active site, in which the ferrous iron (Fe^{2+}) is able to react with hydrogen peroxide and produce ferric iron (Fe^{3+}) and hydroxyl radical (•OH) (Equation 1). The newly formed ferric iron is still able to react with hydrogen peroxide to form superoxide anion ($\bullet O_2^-$) and a ferrous iron (Equation 2).

Hydroxyl radical formation:

$$\text{TRACP-}Fe^{2+} + H_2O_2 \rightarrow \text{TRACP-}Fe^{3+} + \bullet OH + OH^- \quad (1)$$

TRACP regeneration and superoxide formation:

$$\text{TRACP-}Fe^{3+} + H_2O_2 \rightarrow \text{TRACP-}Fe^{2+} + \bullet O_2^- + 2H^+ \quad (2)$$

The generation of these destructive oxygen radicals can continue as long as H_2O_2 is available. When hydroxyl radicals alone attack proteins, they cause protein aggregation (Davies and Delsignore 1987; Berlett and Stadtman 1997), but in combination with superoxide anions they cause protein fragmentation without formation of aggregates (Davies and Delsignore 1987). Halleen and co-workers were able to demonstrate *in vitro* that TRACP is able to destroy the major component of the bone matrix, type I collagen, without disruption of the enzyme itself (Halleen *et al.,* 1999b). They also showed that TRACP is localized in transcytotic vesicles of osteoclasts together with bone degradation products, hypothesizing that TRACP would participate in the final degradation of bone matrix molecules intracellularly in the transcytotic vesicles (Halleen *et al.,* 1999b). In general, ROS have been shown to stimulate osteoclastic bone resorption both *in vivo* and *in vitro*, and they also enhance the recruitment of osteoclasts (Garrett *et al.,* 1990; Key *et al.,* 1994). Based on studies with TRACP deficient or TRACP over-expressing macrophages, TRACP may also

have a role in antigen processing by generating destructive ROS (Bune *et al.,* 2001; Räisänen *et al.,* 2001).

Both the ROS generation and the AcP activity of TRACP use the redox active iron for the catalysis. However, the acid phosphatase activity has an acidic pH-optimum, whereas ROS are generated in a neutral pH (Kaija *et al.,* 2002). Also, single amino acid TRACP mutants that are completely inactive as phosphatase are still able to produce ROS, suggesting that the two activities are functionally independent (Kaija *et al.,* 2002). Beck and co-workers were able to demonstrate that AcP activity of TRACP is inhibited by the substrate of ROS generating activity, H_2O_2 (Beck *et al.,* 1999), and vice versa, inorganic phosphate blocks H_2O_2 inhibition, confirming the use of the same active site (Beck *et al.,* 1999).

2.6.3 Participation in iron metabolism and homeostasis

Studies with uteroferrin suggest that TRACP may function as an iron transporter from mother to fetus (Buhi *et al.,* 1982; Ducsay *et al.,* 1984; Roberts *et al.,* 1986). Uteroferrin is produced and secreted into the uterus up to 2g/day during midpregnancy and transported across the chorioallantoic membrane to the blood circulation of the fetus (Renegar *et al.,* 1982). Further evidence for participation in iron metabolism is the direct regulation of TRACP expression by iron (Alcantara *et al.,* 1994; Reddy *et al.,* 1995).

2.7 Mouse models for TRACP

2.7.1 TRACP deficient mice

The role of TRACP in bone metabolism and also in other tissues has been elucidated by the TRACP knockout mouse model (Hayman *et al.,* 1996). Mice with targeted disruption of the TRACP gene totally lack TRACP activity, but they are nevertheless viable under laboratory conditions. However, they suffer from progressive foreshortening and deformity of the long bones and axial skeleton. Tooth eruption and skull plate development is normal in TRACP deficient mice, indicating a role for TRACP in endochondral ossification. The height of the epiphyseal growth plate is increased with disorganized columns of chondrocytes, in particular hypertrophic chondrocytes (Hollberg *et al.,* 2002). Adult TRACP deficient mice have higher mineralization density of the bone in all regions, reflecting a mild osteopetrosis resulting from defective bone resorption (Hayman *et al.,* 1996). The osteopetrotic phenotype is apparent as early as 4 weeks of age.

The formation of osteoclasts is normal in TRACP deficient mice, suggesting that TRACP is not essential during osteoclastogenesis (Hollberg *et al.,* 2002). However, osteoclasts isolated from TRACP deficient mice demonstrate defective bone resorption also *in vitro* determined by a conventional resorption pit assay (Hayman *et al.,* 1996). Ultrastructural examination of young TRACP null mice revealed that osteoclasts exhibit an increased relative area of ruffled border and accumulation of

cytoplasmic vesicles (Hollberg *et al.,* 2002) containing filamentous material (Suter *et al.,* 2001). The accumulation of intracellular vesicles is probably not due to the impaired secretion, since the location of cathepsin K is normal. Some explanations have been offered for the accumulated vesicles: 1) TRACP deficient osteoclasts are incapable of totally digesting the internalised bone degradation products, and vacuoles containing filamentous material accumulate into the cytoplasm instead of normal transcytosis and secretion through the FSD (Halleen *et al.,* 1999b); 2) TRACP may have a function in modulating intracellular vesicular transport by dephosphorylating phosphoproteins (Hollberg *et al.,* 2002).

A possible role for TRACP in the immune system was detected in TRACP deficient mice (Bune *et al.,* 2001). TRACP null macrophages have disordered inflammatory responses and delayed clearance of *Staphylococcus aureus in vivo*. TRACP-deficient macrophages exhibit enhanced superoxide and nitrite production, and additionally increased secretion of proinflammatory cytokines such as TNFα, IL-1β and IL-12. The activity of tartrate-sensitive LAP was increased in TRACP deficient macrophages, indicating a compensatory activation of acid phosphatase activity. Whether the reduced clearance of bacteria was due to the defective recruitment of macrophages or a direct defect in bactericidal activity remains unclear, because *in vitro* phagocytosis and bacterial killing were normal in TRACP null macrophages (Bune *et al.,* 2001).

The biological roles and functional relationship between two acid phosphatases, TRACP and LAP, have remained unknown. Single TRACP (Hayman *et al.,* 1996) or LAP (Saftig *et al.,* 1997) knockouts have distinct, but rather mild phenotypes, suggesting that the phosphatases may in part substitute each other. The double knockout mouse model of both TRACP and LAP demonstrates a more severe phenotype than the sum of the single knockouts, thus strengthening the hypothesis (Suter *et al.,* 2001). Mice deficient for both phosphatases showed massive lysosomal storage, for example in Kupffer cells, in bone marrow macrophages and in osteoclasts. Also the bone phenotype, which was more evident in TRACP than in LAP deficient mice, was even more severe in double deficient mice. The dephosphorylation of osteopontin was severely affected in double knockout mice, and the authors suggest that TRACP represents the major enzyme implicated in this process (Suter *et al.,* 2001). Nevertheless, no data is available from dephosphorylation of osteopontin in single knockout mice to verify this hypothesis. Osteopontin, which is identical to the T-cell cytokine η-1, may be the substrate for TRACP both in bone resorption and in inflammatory responses (Hayman and Cox 2003). Interestingly, the authors do not address any interest to the ROS generation activity of TRACP, which is not possible to overcome by LAP.

2.7.2 Over-expression of TRACP in mice

Angel and colleagues chose a complementary approach to examine the biological function of TRACP (Angel *et al.,* 2000). They introduced additional copies of the TRACP gene with an SV40 enhancer element into mouse genome. A transgene gave a copy number-dependent increase in TRACP mRNA levels and TRACP activity.

Transgenic mice exhibited changes in bone structure consistent with changes in both osteoclasts and osteoblasts. Over-expression of TRACP caused an increase in bone resorption that was accompanied by increased bone formation, yielding a net increase in bone turnover. The mechanism by which TRACP increases bone resorption remains unclear, but nevertheless the TRACP transgenic mice exhibit decreased trabecular bone consistent with mild osteoporosis (Angel *et al.,* 2000).

TRACP over-expressing macrophages were used to study the free radical profile and bacterial killing *in vitro* (Räisänen *et al.,* 2001; Räisänen 2004). The overall ROS level was increased in activated macrophages over-expressing TRACP (Räisänen *et al.,* 2001), which was shown to be as a result of elevated superoxide production after phorbol 12-myristate 13-acetate (PMA) stimulus (Räisänen 2004). In contrast, both lipopolysaccharide (LPS) and PMA induced nitric oxide production was not significantly different in TRACP over-expressing macrophages compared with wild type macrophages. Macrophages over-expressing TRACP showed a tendency to higher capacity for bacterial killing but the statistical difference was not reached between the wild type and over-expressing cells, possibly due to the insensitivity of the method used. However, the direct bactericidal activity of recombinant TRACP was clearly shown for the first time, and the effect is perhaps due to the radical forming activity of TRACP (Räisänen 2004).

2.8 TRACP as a clinical tool

2.8.1 TRACP in hairy cell leukemia (HCL) and Gaucher's disease

In the early 1970s, the identification of leukemic cells of HCL was difficult and it was important to develop more specific cytochemical methods for accurate identification. Yam and co-workers noted that leukemic cells had strong AcP activity in the presence of tartrate and naphtol-ASBI phosphate as substrate, whereas most normal cells remained negative (Yam *et al.,* 1971). Therefore, the histochemical staining method for TRACP using naphtol-ASBI phosphate as a substrate to identify hairy cells of HCL has been the first diagnostic use of TRACP activity (Janckila *et al.,* 1978; Yam *et al.,* 1987). Another disease characterized with elevated TRACP activity is Gaucher's disease (Robinson and Glew 1980b). Gaucher's disease results from mutations in β-glucocerebrosidase, leading to accumulation of the lipid glucocerebroside in the lysosomes of monocytes and macrophages (Frenkel 1993). These cells also stain strongly for TRACP (Troy *et al.,* 1985), and elevated TRACP levels can be detected in serum and also in the spleen of patients with Gaucher's disease (Lam *et al.,* 1981; Chamberlain *et al.,* 1995). The main isoform in Gaucher's serum and hairy cells of HCL is TRACP 5b, whereas the isoform found in a Gaucher's spleen is normally TRACP 5a (Lam *et al.,* 1981).

2.8.2 TRACP as a marker for osteoclasts

TRACP was considered as a specific histochemical marker for osteoclasts more than 20 years ago (Minkin 1982). Since then TRACP has been widely accepted as a histological marker of osteoclasts both in tissue sections and *in vitro* cell cultures. Without knowing the exact biological role of TRACP in bone metabolism it has become one of the best-known markers for bone-resorbing osteoclasts and their precursors. At present there are up to 1000 references in the database of National Library of Medicine with keywords "TRACP and osteoclast".

2.8.3 Detection of TRACP from serum or plasma

In the past, the best ways to assess TRACP from serum or plasma were electrophoresis (Lam *et al.,* 1978a; Lam *et al.,* 1978b), CM-Sepharose or heparin column chromatography (Lam *et al.,* 1978a; Igarashi *et al.,* 2001) and isoelectric focusing. These methods are normally not used for analytical purposes, but mainly in the purification and characterization of the TRACP enzymes.

The first assays for analytical purpose were kinetic assays to measure the total TRACP activity in serum or plasma (Table 6) (Lam *et al.,* 1978b; Schiele *et al.,* 1988). The main problem with these assays was the lack of specificity for TRACP, since they also detected tartrate-resistant AcPs from erythrocytes and platelets, which are not related to type-5 TRACP (Fenton and Richardson 1967; Sorenson 1972). Kinetic assays were improved by inhibiting non-TRACP AcPs by using fluoride (Nakanishi *et al.,* 1998) or incubating the samples at 37°C for an hour before measurement (Lau *et al.,* 1987b). However, these assays still measured total TRACP activity, and lacked the specificity for osteoclast-derived TRACP 5b. An important finding was the substrate specificity of distinct AcPs and also TRACP 5a and 5b. The most common substrate used to detect AcP activity is a 4-nitrophenylphosphate (4-NPP), which is hydrolysed well by non-type 5 AcPs, as well as TRACP 5a and 5b. Instead, α-naphthyl phosphate and naphthol-ASBI phosphate have been shown to be more selective substrates and are hydrolysed effectively by TRACP 5b, but only poorly by TRACP 5a and not at all by other non-type 5 AcPs (Seiler *et al.,* 1983; Janckila *et al.,* 2001a). Thus, by using α-naphthyl phosphate or naphthol-ASBI phosphate as a substrate, kinetic assays are more specific to osteoclastic TRACP 5b (Schiele *et al.,* 1988; Rico *et al.,* 1990). Another approach for TRACP 5b specific kinetic assay was developed using fluoride to inhibit non-type 5 AcPs and heparin to inhibit TRACP 5a activity during measurement (Nakanishi *et al.,* 2000).

Table 6: Kinetic assays detecting TRACP from serum or plasma

REFERENCE	MEASURES	SUBSTRATE	OTHERS USING THE SAME ASSAY
(Lam *et al.,* 1978b)	Total TRACP activity including AcPs from erythrocytes and platelets	4-NPP, pH 5.5	(Lam *et al.,* 1978a; Stepan *et al.,* 1983)
(Lau *et al.,* 1987b)	Total TRACP activity	4-NPP, pH 5.5	(Jalovaara *et al.,* 1990; Scarnecchia *et al.,* 1991; Wada *et al.,* 1999; Willig and Jalovaara 2000; Janckila *et al.,* 2001a, b; Minisola *et al.,* 2002)
(Schiele *et al.,* 1988)	Prefers TRACP 5b activity	α-naphthyl phosphate	(Plebani *et al.,* 1996)
(Rico *et al.,* 1990)	Prefers TRACP 5b activity	α-naphthyl phosphate	(Rico and Villa 1993; Revilla *et al.,* 1998; Rico *et al.,* 2002)
(Nakanishi *et al.,* 1998)	Fluoride-sensitive TRACP	2,6-dichloro-4-acetylphenyl phosphate, pH 6.2	(Nakanishi *et al.,* 1999)
(Nakanishi *et al.,* 2000)	Fluoride-sensitive, heparin-resistant TRACP 5b	2,6-dichloro-4-acetylphenyl phosphate, pH 6.6	

The development of TRACP immunoassays started after the production of antibodies against TRACP. Immunoassays detect TRACP activity or TRACP protein concentration from serum or plasma (Table 7). Most probably these assays are not selective for TRACP 5a or TRACP 5b, since the TRACP isoforms show very high immunological identity (Lam *et al.,* 1980). Until this millennium, the lack of absolute specificity for bone-specific TRACP 5b has held back progress on the use of biochemical assay of TRACP 5b activity or protein concentration as a marker of bone resorption.

Table 7: Immunoassays detecting TRACP from serum or plasma

REFERENCE	TECHNIQUE	ANTIGEN	SUBSTRATE	OTHERS USING THE SAME ASSAY
(Lam *et al.,* 1982)	Antibody-bound total TRACP activity	TRACP from HCL spleen	P-NPP, pH 5.5	
(Echetebu *et al.,* 1987)	Antibody-bound total TRACP activity	Uteroferrin	α-naphthyl phosphate, pH 5.6; 4-NPP, pH 5.0	(Whitaker *et al.,* 1989)
(Kraenzlin *et al.,* 1990)	Competitive ELISA for total TRACP mass	TRACP from HCL spleen	-	
(Cheung *et al.,* 1995)	Competitive ELISA for total TRACP mass	TRACP from human cord plasma	-	
(Chamberlain *et al.,* 1995)	Two-site immunoassay for total TRACP mass	Recombinant human TRACP	-	
(Halleen *et al.,* 1996)	Competitive ELISA for total TRACP mass	TRACP from human bone	-	
(Halleen *et al.,* 1999a)	Two-site immunoassay for total TRACP mass	Recombinant or human bone TRACP	-	
(Nakasato *et al.,* 1999)	Antibody-bound total TRACP activity and two-site immunoassay for total TRACP mass	TRACP from HCL spleen	4-NPP, pH 5.5	(Janckila *et al.,* 2001b; Janckila *et al.,* 2002a)
(Miyazaki *et al.,* 2003)	Antibody-bound total TRACP activity and two-site immunoassay for total TRACP mass	Recombinant human TRACP	4-NPP, pH 5.5	

2.8.3.1 Total serum TRACP

Both kinetic assays and immunoassays for TRACP showed that individuals with normally or pathologically high rates of bone turnover have increased levels of serum total TRACP activity or protein concentration. Children with physiologically active bone growth and postmenopausal women were shown to have significantly elevated serum TRACP levels compared to healthy premenopausal adults (Lam *et al.,* 1978a; Chen *et al.,* 1979; Scarnecchia *et al.,* 1991; Chamberlain *et al.,* 1995; Cheung *et al.,* 1995; Halleen *et al.,* 1996; Nakanishi *et al.,* 1998; Halleen *et al.,* 1999a). Marker levels were also elevated in cases where bone resorption is known to be increased, such as osteoporosis, Paget's disease, HHM, MM, osteomalacia, immobilization and chronic renal failure (Stepan *et al.,* 1983; Lam *et al.,* 1984; Lau *et al.,* 1987b; de la Piedra *et al.,* 1989; Kraenzlin *et al.,* 1990; Scarnecchia *et al.,* 1991; Torres *et al.,* 1991; Cheung *et al.,* 1995; Minisola *et al.,* 2002). An early finding of elevated serum TRACP levels in patients with metastatic bone diseases (Li *et al.,* 1973) was later confirmed by several research groups (Tavassoli *et al.,* 1980; Lam *et al.,* 1984; Lau *et al.,* 1987b; Scarnecchia *et al.,* 1991; Wada *et al.,* 1999; Miyazaki *et al.,* 2003). Also, patients with primary hyperparathyroidism had markedly elevated TRACP levels, which after removal of parathyroid adenoma decreased to the normal level within two weeks (Stepan *et al.,* 1983; Lau *et al.,* 1987b; Stepan *et al.,* 1987; Kraenzlin *et al.,* 1990; Scarnecchia *et al.,* 1991; Cheung *et al.,* 1995; Minisola *et al.,* 2002; Miyazaki *et al.,* 2003). In contrast, individuals with hypoparathyroidism who were expected to have decreased rates of bone resorption, had appropriately lesser TRACP in serum than healthy controls (Lau *et al.,* 1987b; Minisola *et al.,* 2002).

The association between serum TRACP and bone resorption was also confirmed by investigating bone density simultaneously with serum TRACP. A significant inverse correlation was observed between serum TRACP and BMC or BMD in the distal radius or lumbar spine (de la Piedra *et al.,* 1989; Scarnecchia *et al.,* 1991). Similarly, bone density assessed by QUS from calcaneus showed a significant negative association with serum TRACP (Nakanishi *et al.,* 1999). Additionally, therapies known to decrease bone resorption, such as HRT, also decreased serum TRACP, confirming the close relation of the enzyme to bone degradation (Halleen *et al.,* 1996). Similar to other bone metabolic markers, levels of serum TRACP are also strongly influenced by age, therefore age-matched reference material is essential for proper experimentation (Nakanishi *et al.,* 1998; Nakanishi *et al.,* 2000; Willig and Jalovaara 2000).

Despite the encouraging results observed by several groups, assays for total TRACP were not proven to be specific and accurate enough in clinical use for assessing the rate of bone resorption. The non-type 5 TRACP enzymes from erythrocytes and platelets, as well as bilirubin interfered with the kinetic measurement of total TRACP (Alvarez *et al.,* 1999). Furthermore, serum contained an inhibitory factor for TRACP activity, which was not overcome by multiple freezing and thawing cycles or by preincubation at 37°C (Lau *et al.,* 1987b). Dilution of serum samples improved the situation, but did not totally resolve it (Lau *et al.,* 1987b). Also, the instability of TRACP activity was a problem, since enzyme activity was markedly decreased when stored at -20°C (Lau *et al.,* 1987b). In healthy individuals, the activity

of total serum TRACP measured in the pH optimum 5.5 is composed approximately equally from isoforms TRACP 5a and 5b (Janckila *et al.,* 2001b). However, total serum TRACP protein measured by two-site immunoassays contains almost 90% of non-osteoclastic, low-activity TRACP 5a isoform, the remaining 10% being osteoclastic, highly active TRACP 5b (Janckila *et al.,* 2001b). Thus, neither total TRACP activity nor total TRACP protein provides a sensitive and specific tool for assessing the rate of bone resorption.

2.8.3.2 Serum TRACP 5b as a bone resorption marker

TRACP 5b expression is strictly limited to bone-resorbing osteoclasts, activated macrophages and dendritic cells, which in principle could all be the sources of circulating active TRACP 5b (Efstratiadis and Moss 1985b; Andersson and Marks 1989; Yaziji *et al.,* 1995). However, it has been shown in *in vitro* studies that TRACP 5b is retained almost entirely intracellular in macrophages and dendritic cells, which secrete exclusively TRACP 5a (Janckila *et al.,* 2002a; Janckila *et al.,* 2002b). Thus, serum TRACP 5b is exclusively secreted by bone-resorbing osteoclasts. Indeed, the existing data demonstrates that osteoclastic TRACP has identical biochemical and physical properties as serum TRACP 5b (Chen *et al.,* 1979; Lam *et al.,* 1980; Minkin 1982; Halleen *et al.,* 1996). Therefore, serum TRACP 5b should provide an excellent tool for assessing the rate of bone resorption, which has been noted by several researchers who have developed assays for serum TRACP activity or mass (Lam *et al.,* 1978a; Echetebu *et al.,* 1987; Lau *et al.,* 1987b; Kraenzlin *et al.,* 1990). Despite realizing the connection between bone resorption and serum TRACP 5b, it has been very difficult to develop assays having absolute specificity for 5b isoform only.

So far no-one has been able to develop TRACP 5b specific antibodies, and better specificity of kinetic assays for osteoclastic TRACP 5b has been achieved by using α-naphthyl phosphate as a substrate (Schiele *et al.,* 1988; Rico *et al.,* 1990), or by using heparin to inhibit TRACP 5a (Nakanishi *et al.,* 2000). Results obtained using α-naphthyl phosphate are normally interpreted as total TRACP, even if they may reflect more specifically osteoclastic TRACP 5b (Seiler *et al.,* 1983). Patients with Paget's disease or metastatic bone diseases were shown to have significantly elevated levels of serum TRACP, probably isoform 5b (Rico and Villa 1993; Plebani *et al.,* 1996; Revilla *et al.,* 1998). Furthermore, a recent study showed that the baseline measurement of serum TRACP, again probably isoform 5b, can predict bone loss after two years in postmenopausal women (Rico *et al.,* 2002).

2.8.3.2 Clinical significance of serum TRACP 5a

Based on the recent data, it seems that serum TRACP 5a has its own specific biological significance in some specific diseases such as rheumatoid arthritis (RA) (Nakasato *et al.,* 1999). Serum total TRACP concentration, but not activity, was shown to be significantly elevated in patients with RA (Nakasato *et al.,* 1999; Takahashi *et al.,* 2000). The elevated levels of TRACP concentration did not correlate with bone metabolic markers bone ALP or NTX, suggesting that the TRACP elevation

was not osteoclastic in origin and may not be related to bone turnover (Takahashi *et al.,* 2000). Instead, the levels of TRACP concentration in RA were significantly correlated with C-reactive protein, an acute-phase protein marker of inflammation and indicator of disease activity in RA (Janckila *et al.,* 2002a). Since as much as 90% of the total serum TRACP protein in RA is TRACP 5a, the total TRACP concentration gives a good estimate of TRACP 5a concentration (Janckila *et al.,* 2001b). Based on these results, the authors conclude that increased TRACP protein is a low activity TRACP 5a isoform from a source other than osteoclasts, probably inflammatory macrophages and dendritic cells abundant in the synovial tissues of affected joints (Janckila *et al.,* 2002a). Further investigations are needed to confirm the clinical significance of serum TRACP 5a concentrations in RA and other chronic inflammatory conditions.

3. AIMS OF THE PRESENT STUDY

This study focused on the use of TRACP 5b as a clinical and diagnostic marker for osteoclasts and bone diseases with increased rate of bone resorption. Bone-resorbing osteoclasts secrete active TRACP 5b into the circulation, from where it can be detected kinetically or immunologically. Assays measuring serum total TRACP activity or mass have shown the correlation between serum TRACP and the rate of bone resorption. However, these assays lack both the specificity and sensitivity for osteoclastic TRACP 5b. The specific aims of this study were as follows:

1. To characterize anti-TRACP antibodies.
2. To use the antibodies for the development of TRACP 5b specific immunoassays.
3. To study human serum TRACP 5b as a marker of bone resorption in healthy and diseased individuals.
4. To investigate TRACP 5b as a marker of bone resorption in animal models and *in vitro* osteoclast cultures.

4. MATERIALS AND METHODS

4.1 Monoclonal and polyclonal antibodies against TRACP (I, II, IV)

TRACP was purified from human bone as described (Halleen *et al.,* 1996) and used as an antigen to produce monoclonal antibodies (MAbs) O1A and J1B in Diabor Ltd (Oulu, Finland) and polyclonal antiserum in rabbits. MAb DB-130Z1 against recombinant rat TRACP (Kaija *et al.,* 1999) was also produced at Diabor Ltd. MAb 4E6 was produced against recombinant human TRACP at SmithKline Beecham Pharmaceuticals (Harlow, UK) and was a generous gift from Dr. Stephen D. Holmes.

The ability of MAb O1A to bind to type-5 TRACP, erythrocytic AcP, and platelet-derived AcP, the three completely different tartrate-resistant AcPs that can be found in human serum, was tested by PhD Anthony J. Janckila. Briefly, pure TRACP (0.3 U enzyme activity/ml), crude platelet lysate (0.18 U/ml), and crude erythrocyte lysate (0.3 U/ml) were mixed with 10 µg of O1A antibody and 10 µl rabbit anti-mouse IgG-agarose (Sigma, St. Louis, MO, USA) at 4°C for 6 hours. The same amount of control antibody not binding the studied enzymes was used as a negative control in place of O1A.

4.2 Sialidase treatment (I)

MAbs 4E6 and O1A (400 ng/well) were incubated separately in anti-mouse IgG-coated microtiter wells (PerkinElmer Life Sciences - Wallac Oy, Turku, Finland) for 1 h. After washing (6×) serum samples were incubated in the wells for 1 h, followed by further washing. Different amounts of sialidase enzyme (Sigma) were added to the wells in 0.1 M sodium acetate buffer, pH 5.9, for 1 hour. After reaction, the buffer containing the sialidase and detached TRACP was removed from the wells, and the wells were washed again. Both the remaining and detached TRACP activities were measured. The amount of TRACP activity remaining in the microtiter wells was measured by incubating 200 µl of reaction mixture (0.1 M sodium acetate, pH 5.9, 8 mM 4-NPP and 40 mM sodium L(+)-tartrate) in the wells for 1 hour. The reactions were stopped by adding 16 µl of 0.5 M sodium hydroxide. Absorbance at wavelength 405 nm was measured with an ELISA plate reader (Labsystems Multiscan MCC, Labsystems Inc., Marlboro, MA, USA). The amount of TRACP activity detached from the wells was measured from the sialidase solution by adding 8 mM 4-NPP and 40 mM sodium L(+)-tartrate. After 1 hour, the reaction was terminated by adding 0.8 ml of 40 mM sodium hydroxide, and the absorbance at 405 nm was determined. For each amount of sialidase used, a zero control was measured containing the same amount of sialidase. The zero controls were incubated in empty anti-mouse IgG-containing wells for 1 hour, removed, and processed exactly as the corresponding samples. Results of the zero controls were subtracted from the results of the samples to eliminate non-specific acid phosphatase activity in the sialidase preparation. One unit of TRACP activity hydrolyzes one µmol of 4-NPP per minute at 37°C.

4.3 Western analysis (I, II)

Western analysis was performed on human bone homogenate and human serum that were fractionated into two fractions using CM-Sepharose cation-exchange chromatography equilibrated with 10 mM of Tris-HCl, pH 8.2. Bone homogenate and human serum were separately applied through the column. The flow-through fractions (containing most of the proteins) were collected and concentrated, and bound proteins (including TRACP) were eluted using 0.5 M sodium chloride in an equilibration buffer. Fractions with eluted proteins were combined and concentrated. Proteins from the obtained two fractions (flow-through fraction and fraction with eluted proteins) were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Schleicher & Schull, Dassel, Germany). Non-specific binding was blocked by incubation in phosphate buffered saline containing 1% bovine serum albumin. TRACP was detected using Mab O1A (10 μg/ml) as a primary antibody. Biotinylated anti-mouse IgG and horseradish peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) were used as secondary reagents to enhance the signal. Bands were visualized with the peroxidase substrate diaminobenzidine (DAB, Sigma).

4.4 Native acidic polyacrylamide gel electrophoresis and pH optimum (IV)

TRACP 5a and 5b activity were demonstrated in 3 individual rat and human serum samples using native acidic polyacrylamide gel electrophoresis as described (Lam *et al.,* 1978b). The pH-optimum of serum TRACP was determined from the rat and human serum samples by measuring acid phosphatase activity using 8 mM 4-NPP as substrate in a 0.1 M sodium acetate buffer pH 4.5, 5.0, 5.5, 6.0 and 6.5 in the presence of 40 mM L(+)-tartrate.

4.5 TRACP 5b specific immunoassays

The basic principle for each TRACP 5b immunoassay is the same: anti-TRACP antibody is captured on the microtiter plates (PerkinElmer Life Sciences - Wallac Oy), which specifically binds total TRACP from the sample. Based on the pH curves of TRACP 5a and 5b within the pH range from 4.1 to 6.5, the pH 6.1 offers the best discrimination between the two forms. Therefore, the specificity for TRACP 5b is achieved by determining the TRACP 5b activity at the pH 6.1, where TRACP 5a activity is less than 10% of the detected TRACP activity (Lam *et al.,* 1978a).

4.5.1 Human serum TRACP 5b assay (I)

Mab O1A (400 ng/well) was incubated for 1 h in anti-mouse IgG-coated microtiter wells (PerkinElmer Life Sciences - Wallac Oy). After washing, recombinant human TRACP as a calibrator or serum samples both as duplicates were incubated in the wells for 1 hour. Bound TRACP 5b activity was detected using 8 mM 4-NPP as a

substrate in a 0.1 M sodium acetate buffer, pH 6.1, for 1 hour at 37°C. The reactions were determined by the addition of 25 µl of 0.32 M sodium hydroxide into the wells, and the absorbance at 405 nm (A_{405}) was determined using Victor II equipment (PerkinElmer Life Sciences - Wallac Oy).

4.5.2 Medium TRACP 5b assay (II)

The polyclonal rabbit anti-TRACP antiserum was diluted 1:1000 and incubated on anti-rabbit IgG-coated microtiter plates (PerkinElmer Life Sciences - Wallac Oy) for 1 hour. After washing, recombinant human TRACP as a calibrator or culture medium samples (200 µl) were incubated as a duplicates in the wells for 1 hour. Detection of bound TRACP 5b activity was determined as described in the human TRACP 5b assay with 2 hours incubation with 4-NPP as a substrate.

4.5.3 Rat serum TRACP 5b assay (IV)

Mab DB-130Z1 (400 ng/well) was incubated for 1 h in anti-mouse IgG-coated microtiter wells (PerkinElmer Life Sciences - Wallac Oy). Recombinant rat TRACP was used as a calibrator and rat serum samples, diluted 1:4 with 0.9% NaCl, were incubated as duplicates in the wells for 1 hour. Bound TRACP 5b activity was determined using 4-NPP as a substrate in a 0.1 M sodium acetate buffer, pH 5.8, for 1 hour at 37°C. Reactions were stopped and measured as described in the human TRACP 5b assay.

4.5.4 Characterization of immunoassays (I, II, IV)

The sensitivity of the assay was defined as 2 standard deviations above the mean value of 16 blank replicates. The stability of human serum TRACP 5b activity was studied using serum samples from 5 individuals. Used serum samples were stored at -80°C for several months before the testing. After thawing, aliquots of the samples were stored for up to 7 days at 25°C and at 4°C. Human serum stability during storage was studied by storing aliquots of the samples frozen at -20°C and at -80°C. The stability of TRACP activity in mouse bone marrow cell culture medium was tested by storing samples for up to 7 days at 25°C and at 4°C.

Recovery was determined by adding various amounts of purified human osteoclastic TRACP or recombinant human/rat TRACP into the serum or culture medium samples containing a known amount of TRACP 5b activity, and calculating the amount detected. Dilution linearity was determined for rat serum TRACP 5b from three serum samples diluted 1:2, 1:4, 1:6, and 1:8 with 0.9% NaCl.

Intra-assay coefficient variations (CVs) for serum assays were determined as the mean value obtained by measuring 8-12 replicates of 4 serum samples of different levels simultaneously. Intra-assay CV for medium TRACP 5b assay was determined as the mean value obtained from 5 replicates of three pooled medium samples simultaneously. Inter-assay CVs were determined as the mean of the same samples

measured in 5 to 6 consecutive days. Intraindividual variability for human serum TRACP 5b was calculated from 6 samples drawn on different days from 5 individuals.

4.6 Sampling and storing of the samples

Blood samples were taken in the morning unless otherwise stated, and serum was separated after coagulation by centrifugation at 2000 rpm at room temperature (RT) for 10 minutes. Serum samples were quick-frozen immediately after separation and stored throughout the storing period at -70°C to -80°C. Human urine samples were collected in the morning and stored similarly as serum samples.

4.7 Other bone markers (I, V)

4.7.1 Total serum TRACP mass or activity

Total serum TRACP mass was measured using a two-site assay with MAbs O1A and J1B as described previously (Halleen *et al.,* 1998a). In an HRT study, total serum TRACP activity was measured using the same protocol as described in section 4.5.1, but at pH 5.5. In an ADO2 study, total serum TRACP activity was measured at the Indiana University General Clinical Research Center laboratory as previously described (Lau *et al.,* 1987b).

4.7.2 Urinary deoxypyridinoline (DPD)

Urinary DPD was measured with the Pyrilinks-D enzyme immunoassay (Metra Biosystems Inc., Mountain View, CA, USA). The values were corrected with a creatinine concentration measured by a commercial method (Boehringer Mannheim, Mannheim, Germany). Thus, the corrected values are given as nM DPD/mM creatinine (DPD/Cr).

4.7.3 Total serum osteocalcin

Measurement of total serum osteocalcin was done as described earlier (Käkönen *et al.,* 2000). Briefly, MAbs detecting human osteocalcin were either biotinylated or europium-labeled as described by Hellman *et al.* (Hellman *et al.,* 1996). Commercial osteocalcin peptide was used as a calibrator. Samples and calibrators (10 µl of each) were added as duplicates into wells of streptavidin-coated microtiter plates (Perkin-Elmer – Wallac Oy), followed by a mixture of biotinylated and europium-labeled MAbs (200 mg/well of each) in 50 µl of Delfia® Assay Buffer (PerkinElmer Life Sciences - Wallac Oy) containing 5 mmol/L EDTA. After shaking at 35°C for 1h, the plates were washed followed by addition of 200 µl Delfia® Enhancement Solution (PerkinElmer Life Sciences - Wallac Oy). After shaking for 5 minutes at RT, the

fluorescence was measured by model 2 Victor equipment (PerkinElmer Life Sciences - Wallac Oy).

4.8 Subjects for human TRACP 5b studies (I, III, V)

All human studies were approved by the local ethics committees and performed in accordance with the Declaration of Helsinki. All individuals participating in HRT study as well as individuals with various bone diseases and nonskeletal diseases gave written informed consent before participating the study. The research protocol for the ADO2 study was approved by the Indiana University Institutional Review Board. All subjects participating in the ADO2 study or their parents (in the case of children under 18 years) gave written informed consent before participating in the study.

4.8.1 Osteoporotic women treated with hormone replacement therapy (HRT) or placebo (I)

Forty healthy white women aged 50 – 57 years who were within 0.5 – 5 years of the onset of menopause were chosen for the study from more than 1000 randomly drawn women from the city of Jyväskylä (Cheng *et al.,* 1999). In this double-blinded study, 20 women were treated with placebo and 20 with HRT (2 mg estradiol, 1 mg norethisterone [Kliogest; Novo Nordisk, Davis, CA, USA]) daily for 1 year with the guidance of a doctor. Of the subjects, 15 in the HRT group and 16 in the placebo group completed the study. Serum and urine samples were obtained at the baseline and after 6 months.

4.8.2 Patients with metabolic bone diseases (III)

For studying the clinical specificity and clinical sensitivity of serum TRACP 5b assay we obtained serum samples of 303 individuals with various bone diseases and nonskeletal diseases after written informed consent. At the time of sampling, none of these subjects was receiving antiosteoporotic treatment. Healthy pre- and postmenopausal women (n=60 and n=40, respectively), and breast cancer (BC) patients without evidence of bone metastases (BC-; n=18) were included as control populations. Patients with bone diseases included subjects with primary vertebral osteoporosis (n=16), osteopenia (n=29), active Paget's disease of bone (n=14), and BC patients with overt bone metastases (BC+; n=20). All patients with osteoporosis had at least one vertebral compression fracture. Patients with chronic hepatic dysfunction (HF; n=17), chronic renal failure (RF; n=49), and rheumatoid arthritis (RA; n=40) were included as reference populations. All patients with RF had endstage renal disease, and they had been on chronic hemodialysis for several years (mean duration, 5 years) at the time of sampling.

4.8.3 Patients with type II autosomal dominant osteopetrosis (ADO2) (V)

Ten families with ADO2 secondary to heterozygous ClCN7 gene mutations (G215R, R286W, L213F, R762L, R767W), as described previously by Waguespack and co-workers (Waguespack *et al.,* 2002; Waguespack *et al.,* 2003), participated in this study. Serum samples, clinical histories and available radiographs were obtained from all subjects during 1998 - 2001. Serum samples were stored in –70°C throughout the study. Two hundred and thirty three subjects were classified into three groups: clinically affected, unaffected gene carriers, and healthy controls. All clinically affected individuals and unaffected gene carriers had a ClCN7 gene mutation, whereas control subjects did not have a mutation in the ClCN7 gene. The diagnosis of ADO2 (and subsequent classification as an affected individual) was made if there was diffuse osteosclerosis and/or the pathognomonic findings of endobones in any portion of the visualized skeleton and if the subject had diagnostic levels of total TRACP and CK-BB, as previously described (Waguespack *et al.,* 2002). We defined a gene carrier as any individual with a ClCN7 mutation who had normal radiographs and normal serum biochemistries, again using the diagnostic cutoffs defined by the previous report (Waguespack *et al.,* 2002). Radiographs were not available in 13 individuals having a ClCN7 gene mutation. Because our previous data clearly demonstrated that levels of total TRACP and CK-BB effectively differentiate unaffected gene carriers from clinically affected subjects, we classified these individuals into unaffected gene carriers (n=12) and clinically affected subjects (n=1) based on biochemistries alone. Additionally, there was one carrier who had slightly elevated serum biochemistries but no clear radiographic evidence of osteopetrosis. Because radiographs were considered the gold standard, this subject was classified as a carrier. Finally, control subjects were family members without a ClCN7 gene mutation or family members related by marriage. The study participants were further stratified into children, age < 18 years, and adults, age > 18 years. Results from clinically affected individuals and unaffected gene carriers were compared with controls in the same age group.

The subjects were classified into three clinical severity score classes, 0, 1 or 2, based on their fracture history. Subjects with less than three fractures, excluding hip and femur fractures, were classified with score 0. Severity score 1 was given to subjects with a history of 3 – 10 fractures or with one hip or femur fracture. The most severely affected subjects were classified as a severity score 2, and they had either more than 10 fractures or more than one hip or femur fracture. Finger and toe fractures were considered as minor fractures and therefore left out of the calculations. Fracture history was unknown for four of the healthy controls, and they were excluded from the fracture analysis.

4.9 Mouse bone marrow cell culture (II)

Bone marrow cells from the femora and tibia of 8-week old NMRI mice were cultured for 3 to 7 days on plastic in the presence of $1,25(OH)_2D_3$ at 10 nmol/L concentration to induce osteoclast formation (Choi *et al.,* 1998; Hentunen *et al.,* 1998). Clodronate-containing liposomes were prepared as described (Mönkkönen *et al.,* 1995), and the effect of 10^{-5} to 10^{-8} mol/L clodronate was studied by adding the liposomes into the

culture medium (Selander *et al.,* 1996). After culturing, cells were fixed with 3% (w:v) paraformaldehyde and stained for TRACP using a histochemical kit according to manufacturer's instructions (Sigma). TRACP positive cells containing three or more nuclei were counted using a light microscope (Leica Aristoplan, Wetzlar, Germany).

4.10 Orchidectomy (ORC) for rats (IV)

Ten-week-old Spraque-Dawley male rats were divided into two groups, sham-operated (n=10) and orchidectomized (n=11) for the six-month experiment. The operations were performed under anesthesia induced by intramuscular injections of fentanyl-fluanisole (Hypnorm, 1 mg/kg; Pharmaceutical Ltd, Grove, Oxford, UK). An incision was made in the scrotum, and the testicles together with the epididymides were extruded and excised after ligating the internal spermatic vessels, deferential vessels, and ductus deferens. After the surgery, animals were housed 3 rats in one cage at 20°C under a 12 h/12 h light/dark cycle and maintained on tap water *ad libitum* and commercial laboratory food. The rats were killed 6 months after operation by CO_2 suffocation followed by decapitation. The animal experiments were carried out in the Central Animal Laboratory of Turku University that is managed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the Statute 1076/85 §3 and 1360/90 of The Animal Protection Law in Finland and EU Directive 86/609.

4.10.1 Peripheral quantitative computed tomography (pQCT) and serum sampling

Tomographic measurements were performed with pQCT (XCT540 Stratec, Norland Medical System, Germany) before the operation and at 5, 11, 17, 24, 40, 70, 110, 150 and 180 days after the operation under Hypnorm anesthesia. At each time point, the left proximal tibia was scanned from two distinct levels. The first reference line was placed at the lowest edge of the high dense area of the proximal tibia, and trabecular bone density was measured 2 mm below the reference line. Another reference line was placed at the tibiofibular junction, and cortical bone density was measured 6 mm above this reference line. A standardized analysis (peel mode 2, cort 1, contour mode 1, threshold 0.25 g/cm^3 for trabecular bone and 0.71 g/cm^3 for cortical bone) was applied to the images. Blood (200 µl) was drawn from the tail at the same time points and serum was collected after coagulation by centrifugation. Serum samples were stored at -70°C before analysis.

4.10.2 Histomorphometry

The right femur was cut off at the middle and lower third of the shaft, and the distal part of the bone was fixed in ethanol for embedding into methylmetacrylate. Longitudinal undecalcified sections (5 µm) of distal femur were prepared and stained by the Masson-Goldner-trichrome method. The histomorphometric data were

determined from distal femur, approximately 1 mm above the growth plate. The total tissue area (T.Ar) measured was 4.86 mm^2, consisting of 6 fields. Trabecular bone volume (BV/TV), trabecular bone perimeter (B.Pm), and the number of osteoclasts (N.Oc) were determined from the same area. For methodological details see (Peng 1996). The parameters were derived with the OsteoMeasure™ V. 2.31-program (OsteoMetrics, Inc., Atlanta, USA). The histomorphometric nomenclature, symbols, and units are described according to the Report of the American Society for Bone and Mineral Research (ASBMR) histomorphometry nomenclature committee (Parfitt *et al.,* 1987).

4.11 Statistical analysis (I – V)

Statistical analysis was performed using one-way analysis of variance (ANOVA). In the HRT study, the least significant change (LSC) was calculated for the treated individuals with the equation described earlier (Hannon *et al.,* 1998).

In the ADO2 study, the results are presented as a median with lower and upper quartiles because of the non-gaussian distributions. Comparison among the groups were made using non-parametric Wilcoxon's test with Bonferroni. Non-parametric Spearman correlation was used for correlation analysis. A *P*-value of less than 0.05 was considered statistically significant in each experiment.

5. RESULTS

5.1 Antigenic properties and pH-optimum of serum TRACP 5a and 5b (I)

Mab 4E6 recognized serum TRACP, which had a pH-optimum of 4.9-5.1, suggesting that it represents isoform 5a. To test this hypothesis further, we separated 5a and 5b from human serum by the protocol of Lam and co-workers (Lam *et al.,* 1978a). Analysis of these forms revealed that 4E6 did indeed bind to 5a, and did not bind to 5b. The pH-optimum of both the purified 5a and the serum TRACP bound by 4E6 was 4.9-5.1. Instead, the pH-optimum of purified 5b was 5.7-5.9, the same as that of purified human osteoclastic TRACP. MAbs O1A and J1B recognized both forms, TRACP 5a and 5b.

5.2 Sialidase treatment (I)

Next we studied the effect of sialidase treatment on the binding of serum TRACP by 4E6. After binding serum TRACP to 4E6 in anti-mouse IgG-coated microtiter wells, various sialidase amounts from 1 to 100 mU were added to the wells, and both the amount of bound and unbound TRACP activity after the sialidase reaction was determined. The bound TRACP activity decreased, and the unbound TRACP activity increased when the amount of sialidase was increased. Similar sialidase treatment did not have any effect on the ability of Mab O1A to bind serum TRACP. These results suggest that the epitope of TRACP recognized by 4E6 contains or is influenced by sialic acid, and the removal of the sialic acid prevents 4E6 from binding to serum TRACP. These results together confirm that 4E6 binds specifically to the sialic acid-containing TRACP 5a with a pH-optimum of 4.9-5.1. The results show that the antigenic properties and pH-optimum of osteoclastic TRACP are identical to those of serum TRACP 5b, and completely different from those of serum TRACP 5a, suggesting that serum TRACP 5b is derived from osteoclasts and 5a from some another, yet unidentified source.

5.3 Specificity of Mab O1A (I)

The specificity of Mab O1A was tested using type 5 TRACP and two other, completely different tartrate-resistant acid phosphatase enzymes, derived from platelets and erythrocytes. Crude platelet and erythrocyte lysates were used to demonstrate that neither the platelet-derived nor the erythrocyte-derived enzymes were bound to O1A, confirming that the only tartrate-resistant acid phosphatase bound by O1A from human serum is type 5 TRACP.

5.4 Western analysis (I, II)

We reported earlier that MAb O1A was not useful in Western analysis, as no bands were observed from human bone homogenate or from human serum (Halleen *et al.,* 1999a). However, when we partially purified TRACP from bone homogenate and serum using cation exchange chromatography and then concentrated the proteins bound to the column, we were able to show a specific TRACP band in both the bone and serum derived fractions. No other bands were detected in the concentrated fractions of bound proteins and unbound proteins, showing that O1A is specific for TRACP in both human bone and serum.

The polyclonal anti-TRACP antiserum bound a maximal amount of TRACP when diluted up to 1:1000. In Western analysis, the antiserum detected purified human osteoclastic TRACP, and it was specific for TRACP in mouse osteoclast culture medium, and in human bone homogenate.

5.5 Native acidic polyacrylamide gel electrophoresis and pH optimum (IV)

Native acidic polyacrylamide gel electrophoresis was performed for both rat and human serum samples. TRACP 5a was observed in human serum only, while TRACP 5b was present in both rat and human serum. The pH-optimum of human serum TRACP was 5.5 as a result of the presence of both TRACP 5a (pH optimum 5.2) and TRACP 5b (pH-optimum 5.8). The absence of TRACP 5a activity in rat serum was supported by the finding that the pH-optimum of rat serum TRACP was 6.0, reflecting TRACP 5b activity detected in native acidic polyacrylamide gel electrophoresis.

5.6 Development and characterization of novel TRACP 5b immunoassays (I, II, IV)

Based on our results with the differences of TRACP 5a and 5b, we developed a novel TRACP immunoassay specific for the osteoclast-derived form 5b utilizing the difference of the pH optima of the two forms. MAb O1A was used as a capture antibody to bind total TRACP (both 5a and 5b) to microtiter wells, and then bound enzyme activity was measured using 4-NPP as a substrate. Based on the pH curves of TRACP 5a and 5b, the pH 6.1 offers the best discrimination between the two forms. As shown in Table 8, the activity of TRACP 5a is relatively low at pH 6.1 (~18% of the maximal TRACP 5a activity observed at pH 4.9), whereas TRACP 5b is still almost as active as in the pH optimum 5.9 (~95% of the maximal TRACP 5b activity). Furthermore, since the highest enzyme activity of TRACP 5a is approximately 2 times lower than that of TRACP 5b, the final degree of cross reactivity of the 5a form in the assay is less than 10% at pH 6.1. Traditionally, serum TRACP activity was detected using pH 5.5, because this is the optimum pH value observed when measuring total serum TRACP activity (which is due to the fact that the combined activity of 5a and 5b is highest at pH 5.5).

Table 8: Activity of serum TRACP 5a and 5b within pH range from 4.1 to 6.5. Activities are shown as absorbance values (A405nm), and the percentage of TRACP 5a activity from the total TRACP activity is calculated from the equation [5a activity/(5a activity + 5b activity)] × 100.

pH	TRACP 5a activity	TRACP 5b activity	% of TRACP 5a from total activity
4.1	0	0	
4.3	0,032	0,023	58%
4.5	0,187	0,045	80%
4.7	0,256	0,098	72%
4.9	0,317	0,188	62%
5.1	0,266	0,477	35%
5.3	0,243	0,504	32%
5.5	0,211	0,549	27%
5.7	0,158	0,570	21%
5.9	0,103	0,583	15%
6.1	0,057	0,552	9,3%
6.3	0,033	0,308	9,6%
6.5	0,016	0,143	10%

The analytical sensitivity of human TRACP 5b assay was 0.06 U/L. The recovery of various amounts of purified human osteoclastic TRACP from serum samples was 96.6 ± 2.7%. In measurements with osteoporotic women with HRT or placebo treatment, the intra-assay CV was 3.2%, and inter-assay CV 6.9%; and with patients with metabolic bone diseases 1.8% and 2.2%, respectively. Intraindividual variability was 8.9%, and LSC 26.2%.

In medium TRACP 5b assay, the recovery of various amounts of purified human osteoclastic TRACP from mouse osteoclast cell culture medium (collected after a 6-day culture) was 104,6 ± 3.1%. Intra-assay CV was 1.6%, and inter-assay CV 5.1%.

The analytical sensitivity of rat TRACP 5b immunoassay was 0.1 U/L, and the recovery of various amounts of recombinant rat TRACP from serum samples was 99.1 ± 5.8%. Intra-assay CV was 4.5%, inter-assay CV 3.8%, and dilution linearity 104.6 ± 7.6%. Because the antibody used in the immunoassay was developed using recombinant TRACP as an antigen, the immunoassay seems to detect both TRACP 5a and TRACP 5b. However, because of the absence of TRACP 5a in rat serum, only TRACP 5b is detected from rat serum with the immunoassay.

5.7 Stability of serum or medium TRACP 5b (I, II, IV)

Human serum TRACP 5b activity was relatively stable. After 8 hours incubation of serum samples at 25°C, the immunoassay detected more than 90% of the amount that

was detected before starting the incubation period. After one-day incubation at 25°C, the assay detected 84%, and after 7 days incubation, 42% of the amount detected before starting the incubation period. The immunoassay detected approximately 90% of TRACP 5b activity after 3 days incubation, and approximately 80% after 7 days incubation of serum samples at 4°C. There was no change in the amount of 5b activity detected after 6 months storage at -80°C. Instead, only 63% of 5b activity was detected after 6 months storage at –20°C. Thus, it is essential to store serum samples at -80°C for prolonged times. Repeated freezing/thawing cycles up to 6 times had no effect on the amount of TRACP 5b activity detected (data not shown).

TRACP activity was stable in the culture medium (no statistically significant change from baseline) for 8 hours at 25°C, and for 3 days at 4°C. After 7 days incubation at 4°C and 25°C, 76% and 30% of the initial TRACP activity was detected, respectively.

5.8 Results from human studies

5.8.1 Osteoporotic women treated with HRT or placebo (I)

Serum TRACP 5b activity, total TRACP activity, total TRACP mass, and urinary DPD/Cr amounts were measured at baseline and after 6 months of HRT or placebo treatment. The baseline results of the placebo group were not statistically different from the baseline results of the HRT group in all markers. Changes in the marker levels were calculated during the 6-month period in the placebo and HRT groups, and the changes were compared between the placebo and HRT groups. The decrease of serum TRACP 5b activity in the HRT group was statistically significant ($p<0.0001$ vs. the change in the placebo group), as was also the decrease of another resorption marker urinary DPD/Cr ($p<0.001$). Interestingly, no significant differences were observed between the changes in the placebo and HRT groups in total serum TRACP mass measured using our previously published two-site assay, or in total serum TRACP activity measured similarly to TRACP 5b activity, but at pH 5.5 instead of 6.1.

In all of the 15 individuals in the HRT group, TRACP 5b activity decreased during 6 months HRT. This decrease was more than LSC in 13 of the 15 individuals, which gives an 87% clinical sensitivity to the assay in following HRT. Instead, only one of the 16 individuals in the placebo-group showed a decrease of more than LSC after 6 months compared with the baseline value, giving a 94% clinical specificity to the assay in following HRT. The amount of DPD/Cr decreased more than LSC in 45%, the amount of total serum TRACP mass in 0%, and the amount of total serum TRACP activity in 27% of the individuals in the HRT group.

5.8.2 Patients with metabolic bone diseases (III)

Serum TRACP 5b activity was measured from the serum samples of premenopausal and postmenopausal women, from patients with osteopenia or osteoporosis, and from

patients with Paget's disease or with BC with or without bone metastases. The reference interval was determined as a mean of ± 2 SD of healthy premenopausal women of TRACP 5b, and with these samples it was 0.50 – 3.80 U/L. Compared with healthy premenopausal women, serum TRACP 5b activity was significantly higher ($p<0.001$) in healthy postmenopausal women and in all groups of patients with bone diseases. The clinical specificity of TRACP 5b (percentage of healthy premenopausal women with TRACP 5b activity within reference interval) was 95%, and the clinical sensitivity (percentage of individuals in a disease group with TRACP 5b activity above reference value 3.8 U/L) was 48.3% in osteopenia, 81.3% in osteoporosis, 71.4% in Paget's disease, and 80% in BC patients with bone metastases.

Clinical sensitivity in the group of BC patients with bone metastases was substantially higher for TRACP 5b than for other serum markers of bone resorption, including NTX, CTX and BSP. Concentrations of all markers in the group of BC patients without bone metastases were within reference values, obtained from measurements of healthy premenopausal women. Clinical sensitivity was 80% for TRACP 5b, 35.3% for NTX, 18.8% for CTX, and 35.7% for BSP.

Accumulation of bone resorption markers into the blood circulation has been noticed with patients having chronic hepatic dysfunction (HF) or chronic renal failure (RF) (Woitge *et al.,* 1999). Mean serum TRACP 5b activity was not increased in patients with HF or RF, whereas NTX was significantly increased in both diseases ($p<0.001$) and were above the upper limit of normal in 58.8% of patients with HF and 93.2% of patients with RF. However, total TRACP mass was also significantly increased in RF ($p<0.001$), and 51.0% of the RF subjects had total TRACP mass values above the upper limit of normal. These results suggest that the function of the liver and the kidneys has no effect on circulating TRACP 5b activity, although NTX and total TRACP mass are being affected.

5.8.3 Patients with ADO2 (V)

Similar to total TRACP levels in the earlier study (Waguespack *et al.,* 2002), TRACP 5b levels were significantly elevated in clinically affected individuals compared with age-matched controls in both age groups. Carriers with the ClCN7 gene mutation but without classic clinical findings were indistinguishable from age-matched controls by TRACP 5b. One adult subject who was classified as an unaffected gene carrier had elevated serum levels of total TRACP and CK-BB (Waguespack *et al.,* 2002), as well as TRACP 5b. This individual did not have the classic radiographic findings of osteopetrosis, but there was some mild osteosclerosis noted in an x-ray of the upper humerus. Therefore, because the subject did not clearly fulfill the criteria to be called clinically affected, his data were analyzed as part of the carrier group. The correlation between total TRACP and TRACP 5b was significant in each study subgroup, and the overall correlation between total TRACP and TRACP 5b was also highly significant ($r=0.833$, $p<0.001$). Serum total osteocalcin levels were slightly yet significantly decreased in clinically affected children compared with healthy controls, not changed in unaffected adult gene carriers, and significantly elevated in adult affected individuals compared with adult controls. Osteocalcin was compared to both total

TRACP and TRACP 5b in each study subgroup, and a stronger correlation was observed for TRACP 5b than for total TRACP. The removal of the 13 individuals classified using serum biochemistries only did not change the outcome of the statistical analysis (data not shown).

5.8.3.1 Clinical specificity and sensitivity of serum TRACP

The usefulness of any diagnostic test lies in its ability to distinguish clinically affected subjects from healthy individuals. We investigated the diagnostic sensitivity and specificity for total TRACP and TRACP 5b in our study population, which is at risk for having a ClCN7 gene mutation and ADO2. Unaffected gene carriers were included in this analysis by combining them with healthy controls. Based on these data, empiric cut-off values were determined to maximize the separation between clinically affected subjects and others. In the pediatric group, both total TRACP and TRACP 5b are 100% sensitive and specific for the diagnosis of ADO2, if the diagnostic cut-offs of 35 U/L and 60 U/L are used, respectively. In adults, there was some overlap between unaffected gene carriers and clinically affected individuals, which may be due to the differential phenotypic expression of ADO2 and also due to the classification criteria used in this study. In adults, using a diagnostic cut-off of 17 U/L, the diagnostic sensitivity for total TRACP is 100% and specificity is 98%, even when carriers are included in the analysis. For TRACP 5b, the diagnostic sensitivity in the adult group is 97%, when a diagnostic cut-off is set to 13 U/L. The diagnostic specificity for TRACP 5b is 99%, and without the one enigmatic individual in the carrier group having elevated serum biochemistries, the specificity would be 100%. One individual with ADO2 had a TRACP 5b value below the cut-off, and this subject has had only one fracture during his life. It is postulated that this subject's false negative result may in part be secondary to a mild expression of ADO2. However, this same individual had high total TRACP activity, suggesting that this individual might have an altered serum TRACP 5a level.

5.8.3.2 Fracture prevalence and relationship to TRACP 5b in ADO2 patients

Individuals with ADO2 had more fractures compared to unaffected gene carriers and healthy controls, both in children ($p<0.05$) and in adults ($p<0.001$). All four pediatric subjects having a severity score of 1 or 2 had ADO2. In the adult group, all twelve subjects with a severity score of 2 were clinically affected. Twenty-three adult subjects had a severity score of 1, and were distributed equally among all populations: 43.5% (10/23) clinically affected, 13.0% (3/23) unaffected gene carriers, and 43.5% (10/23) controls. Adult healthy controls and unaffected gene carriers did not differ in their fracture prevalence: 8.2% (10/122) of healthy subjects and 10.7% (3/28) of unaffected gene carriers had a severity score of 1. In the pediatric group, all controls and unaffected gene carriers had a severity score of 0. Interestingly, a high number of clinically affected individuals did not have severe or multiple fractures with 40.5% (15/37) of affected adults and 75% (12/16) of affected children classified as a severity score of 0.

We further analyzed whether serum markers are correlated to the fracture severity score in clinically affected individuals only, because unaffected gene carriers did not differ from age-matched healthy controls in their fracture incidences. Both total TRACP and TRACP 5b showed a strong positive association with fracture severity scores both in the pediatric (n=16) and in the adult (n=37) affected subjects. In the pediatric group, one individual with severity score 1 and both individuals with severity score 2 had markedly elevated serum levels of both total TRACP and TRACP 5b. However, statistical analysis was not performed because of the small number of individuals in the pediatric group. In the adult group both total TRACP and TRACP 5b were significantly elevated in severity scores of 1 and 2 compared to a severity score of 0 ($p<0.01$ and $p<0.05$ respectively). A statistically significant difference was not observed between severity scores 1 and 2, but TRACP 5b levels were slightly more elevated in the latter group. Total osteocalcin had no association with fracture severity scores.

5.9 Medium TRACP 5b as an index of osteoclast number (III)

Both the number of differentiated osteoclasts and the amount of TRACP 5b activity released into the culture medium were significantly increased along the increasing culture time in mouse bone marrow cell cultures. Clodronate decreased the number of osteoclasts and TRACP 5b activity released into the culture medium dose-dependently. The number of formed osteoclasts correlated significantly with the amount of TRACP 5b released into the culture medium ($r=0.94$; $p<0.0001$; $n=120$).

5.10 Trabecular and cortical BMD, histomorphometric data and serum TRACP 5b activity in orchidectomized rats (IV)

Trabecular BMD of the proximal tibia was decreased throughout the study in both the ORC and Sham-operated rats. Trabecular BMD was significantly lower ($p<0.05$) in the ORC group compared with the Sham group as early as day 11, and at all later time points. Cortical BMD was the same in both the ORC and Sham-operated rats at all time points measured. Serum TRACP 5b activity was significantly elevated at 5 days after ORC, returned to the Sham level at 17 days, and was not increased above the Sham level at any of the later time points. When serum TRACP 5b activities are shown as relative activities normalized to the Sham level at each time point it demonstrates more clearly the differences between the Sham and ORC groups.

Histomorphometric data from the distal femur showed significant reduction of trabecular bone in ORC rats compared with Sham operated rats at the end of the study. Trabecular bone volume/tissue volume (BV/TV) was approximately 80% lower in the ORC group than in the Sham group, whereas the number of osteoclasts per trabecular bone perimeter (N.Oc/B.Pm) was slightly higher in ORC group. However, the absolute number of osteoclasts per total tissue area (N.Oc/T.Ar) was significantly lower in the ORC group than in the Sham group.

6. DISCUSSION

6.1 Characterization of TRACP specific antibodies

The first task on the way to developing TRACP 5b specific immunoassays was to produce and characterize specific antibodies against osteoclastic TRACP. MAbs O1A and J1B were produced against purified human osteoclastic TRACP (Halleen *et al.,* 1996; Halleen *et al.,* 1998a), MAb 4E6 against recombinant human TRACP (Chamberlain *et al.,* 1995), and MAb DB-130Z1 against recombinant rat TRACP (Kaija *et al.,* 1999). All these antibodies were species specific and did not cross-react with other TRACP enzymes of distinct species (unpublished observations). We developed a polyclonal antiserum against purified human osteoclastic TRACP in rabbits, which recognized TRACP from human, rat and mouse, but not from chicken (unpublished observations). Characterization of these antibodies revealed that O1A and J1B bound equally well both TRACP 5a and 5b isoforms separated from human serum, whereas 4E6 only bound to sialylated TRACP 5a. MAb O1A was further characterized and shown to specifically bind to TRACP, but not non-type 5 AcPs from other sources, such as erythrocytes or platelets. In Western analysis, O1A and polyclonal antiserum specifically detected only the intact 32 kD type 5 TRACP from human bone, human serum or mouse bone marrow cell culture medium, confirming specificity against TRACP.

6.2 Immunoassays for osteoclast-derived TRACP 5b

The potential of serum TRACP 5b as a bone resorption marker has been noted by several researchers for several decades (Lam *et al.,* 1978a; Chen *et al.,* 1979; Minkin 1982; Miller 1985; Echetebu *et al.,* 1987; Lau *et al.,* 1987b; Kraenzlin *et al.,* 1990). However, the lack of specific antibodies against osteoclastic TRACP 5b has hindered the development of specific and sensitive immunoassays for osteoclast-derived TRACP 5b. We have been able to develop highly specific antibodies against purified human osteoclastic TRACP and recombinant rat TRACP, which are suitable for use in immunoassay approaches. However, these antibodies also recognize non-osteoclastic TRACP 5a, which interferes with the measurement of osteoclastic TRACP 5b. Based on the difference in the pH optimum of TRACP 5a and 5b (Lam *et al.,* 1978a), we were able to select the pH 6.1 for the detection of bound TRACP 5b activity. At pH 6.1, TRACP 5a activity is less than 20% of the maximal TRACP 5a activity, whereas TRACP 5b activity is almost as high as in the pH optimum 5.9 (~95%). Furthermore, since the highest enzyme activity of TRACP 5a is approximately 2 times lower than that of TRACP 5b, the final degree of cross reactivity of the 5a form in the assay is less than 10% at pH 6.1. As TRACP 5a levels in circulation are rather constant and the specific activity of the 5a isoform is almost 6 times lower than that of the 5b isoform (Janckila *et al.,* 2001b), this cross-reactivity does not have a major impact on the specificity of the TRACP 5b activity assay. However, we investigated the TRACP 5b activity of serum samples after preadsorption with TRACP 5a specific antibody 4E6,

but the results were not further improved (unpublished data). It remains to be seen, if in the future it will be possible to raise isoform specific antibodies against TRACP 5b to further improve the analytical and clinical performance of the TRACP 5b assay. The basic principle of the novel solid-phase immunofixed TRACP 5b enzyme activity assay is shown in Fig. 8. Interesting observation was made using native acidic PAGE, that in contrast to human serum, rat serum does not contain TRACP 5a activity. This was confirmed by the finding that the pH optimum of rat serum total TRACP was higher than that of human serum TRACP. Thus, the rat serum immunoassay using MAb DB-130Z1 was performed at pH 5.8, which is the pH optimum of human TRACP 5b.

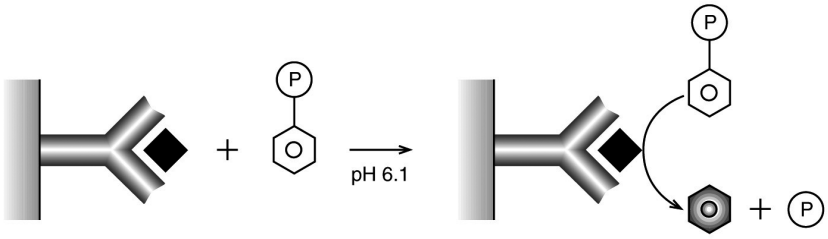

Figure 8: The principle of the solid-phase immunofixed TRACP 5b enzyme activity assay. Anti-TRACP antibody is captured in the microtiter plate. Both non-osteoclastic TRACP 5a and osteoclastic TRACP 5b are bound by the antibody from the sample, but only osteoclast-derived TRACP 5b activity is detected using 4-NPP as substrate at pH 6.1. The end-product para-nitrophenol (4-NP) is detected at wavelength 405 nm, demonstrating TRACP 5b activity in the sample.

TRACP is secreted from osteoclasts as an active enzyme into the circulation, where it is inactivated, loosing its iron content (Halleen *et al.,* 1998b). The completely inactivated enzyme is effectively hydrolysed by proteases, resulting in formation of fragments that are most probably metabolised through the liver or secreted into the urine (Buhi *et al.,* 1982). Based on competitive and two-site immunoassay studies, it has been estimated that approximately 90% of the serum TRACP circulates as fragments, and only 10% as active or inactive enzyme molecules (Halleen *et al.,* 1996; Halleen *et al.,* 1998a). Thus, by measuring enzymatically active TRACP 5b, we detect only the intact enzyme molecules that have been freshly liberated from osteoclasts into the circulation. Based on earlier studies, all known serum markers of bone resorption accumulate into the circulation in chronic renal or hepatic failure, creating "false-positives" although bone resorption is normal (Woitge *et al.,* 1999). Using patients with chronic hepatic dysfunction and chronic renal failure, we were able to demonstrate that, contrary to the other resorption markers, serum TRACP 5b activity does not accumulate into the circulation in renal or hepatic failure.

We were able to show that our TRACP 5b immunoassays have high recovery, reproducibility and sensitivity, in addition to their high specificity for osteoclastic

TRACP 5b. Also, the instability of TRACP activity observed during storage at -20°C was overcome by storing the serum sample at -70°C. Furthermore, Hannon and co-workers recently demonstrated that TRACP 5b has very low diurnal variation, which is a clear advantage when compared to other bone resorption markers, such as CTX (Qvist *et al.,* 2002; Hannon *et al.,* 2004). Since heparin inhibits the activity of TRACP 5a (Nakanishi *et al.,* 2000), we also tested heparin in our assay and found no effect on TRACP activity bound by our antibody O1A, suggesting that our assay is TRACP 5b specific even without addition of heparin (Halleen *et al.,* 2002). Thus, we have developed specific assays for human and rat serum TRACP 5b, as well as for *in vitro* osteoclast cultures, which are likely to be useful both in basic biology and clinical applications.

6.3 Clinical use of human TRACP 5b immunoassay

The investigation of biochemical markers of bone metabolism has provided new approaches in managing patients with bone disease. Bone metabolic markers assess the dynamics of the whole skeleton and provide useful tools for the prediction of bone loss and fracture risk in postmenopausal women, as well as for monitoring the efficacy of antiresorptive treatment. These markers may also have diagnostic value in diseases with high bone turnover, such as Paget's disease and hyperparathyroidism. We studied our TRACP 5b immunoassay in healthy individuals, patients with metabolic bone diseases, postmenopausal women with antiresorptive therapy and patients with ADO2.

Initially we made the comparison between conventional total TRACP activity and mass assays with both TRACP 5a and TRACP 5b activities. In every experimentation, serum TRACP 5b activity showed the best correlation with total TRACP activity (Halleen *et al.,* 2002), which would have been expected, since approximately half of the TRACP activity in serum is 5b activity (Janckila *et al.,* 2001b). TRACP 5b activity showed some association with total TRACP mass, but this association was significantly lower than the association with total TRACP activity, which is also expected because only approximately 10% of TRACP protein in serum is of form 5b. Conversely, TRACP 5a activity (pH 5.0) did not show any correlation with TRACP 5b activity (pH 6.1), or with any other bone metabolic markers determined (DPD, PINP, PICP) (Halleen *et al.,* 2002). In addition, TRACP 5b activity as well as total TRACP activity showed a significant negative association with BMD measured from the tibia, whereas TRACP 5a activity and total TRACP mass failed to show such an association (Halleen *et al.,* 2002). Finally, TRACP 5b activity showed a significant correlation with other bone metabolic markers, DPD, PINP and PICP. These results clearly indicate that serum TRACP 5b activity reflects bone resorption more specifically than total TRACP mass or activity that contains non-osteoclastic TRACP 5a.

Elevated levels of serum TRACP 5b were observed in conditions with increased bone resorption, such as healthy postmenopausal women and individuals with osteopenia, osteoporosis or Paget's disease. Others using the same assay have recently observed similar results (Xiang *et al.,* 2003). These results are in line with previous studies made with total TRACP assays (Kraenzlin *et al.,* 1990; Scarnecchia *et al.,*

1991; Chamberlain *et al.,* 1995; Halleen *et al.,* 1998a). Also children had significantly elevated levels of serum TRACP 5b, which were even further elevated in individuals with vitamin D deficiency (Cheng *et al.,* 2003). In contrast, antiresorptive treatment such as HRT and alendronate treatment decreased serum TRACP 5b levels in postmenopausal women, as expected (Alatalo *et al.,* 2002; Hannon *et al.,* 2004). The only disease studied in which serum TRACP 5b did not correlate with the rate of bone resorption was ADO2, a rate autosomal dominant osteopetrosis with increased BMD simultaneously with an increased rate of fractures. ADO2 is caused by heterozygous mutations in the ClCN7 gene, resulting in ineffective osteoclast-mediated bone resorption (Benichou *et al.,* 2001; Cleiren *et al.,* 2001; Kornak *et al.,* 2001; Waguespack *et al.,* 2003). Previously, it has been demonstrated that patients with ADO2 have several orders of magnitude higher serum total TRACP activity levels than healthy controls (Waguespack *et al.,* 2002), and we were able to show that this elevation was due to elevated levels of osteoclastic TRACP 5b. Histological evaluation of individuals with ADO2 has shown that these patients have highly increased numbers of dysfunctional osteoclasts (Bollerslev *et al.,* 1993; Teti *et al.,* 1999), suggesting that the elevation in serum TRACP 5b would reflect the increased number of inactive osteoclasts. Serum TRACP 5b was shown to have almost 100% specificity and sensitivity in diagnosing the clinically affected and unaffected ClCN7 gene mutation carriers for diseased and healthy subjects.

Cancers, especially breast and prostate, show a strong propensity to metastasize to bone. Bone metastases usually indicate progressive disease and are associated with severe pain, pathological fractures, and profound morbidity (Mundy *et al.,* 2002). The clinical symptoms of bone metastases are often late, since small lesions are difficult to identify with conventional X-ray imaging. Therefore, reliable bone metabolic markers would offer an appropriate tool for frequent and quantitative evaluations of developing lesions and monitoring of treatment (Mose *et al.,* 2003). Our results showing that breast cancer patients with bone metastases have significantly elevated serum TRACP 5b levels have also been confirmed by others (Capeller *et al.,* 2003; Koizumi *et al.,* 2003). Serum TRACP 5b levels were closely associated with the metastatic burden classified as extent of disease scores I (<6 lesions) to IV (generalized osseous metastases) (Koizumi *et al.,* 2003). Similar associations were also observed with other bone metabolic markers NTX, ICTP, PICP and PINP, but TRACP 5b showed the best ROC area under curve implicating the best sensitivity to detect osseous metastases (Koizumi *et al.,* 2003). Bisphosphonates are normally used in pain relief and improvement of symptoms of breast cancer patients with bone lesions. Two independent studies revealed that serum TRACP 5b levels specifically reflect the efficacy of bisphosphonate treatment (Martinetti *et al.,* 2002; Capeller *et al.,* 2003). Preliminary data has also been revealed from serum TRACP 5b in prostate cancer. Patients with a primary prostate tumour had similar TRACP 5b values as healthy individuals, while patients with bone metastases had significantly elevated marker levels (Alatalo *et al.,* 2003a; Maddison *et al.,* 2003). Similar to breast cancer, TRACP 5b was proven to be associated with the severity of bone disease in MM, and specifically monitors the efficacy of antimyeloma treatment (Terpos *et al.,* 2003a; Terpos *et al.,* 2003b). The progression of the disease was also reflected by serum

TRACP 5b, suggesting that TRACP 5b could be used as a predictive tool for bone disease in MM (Terpos *et al.,* 2003a).

The value of markers for bone metabolism is not only to detect the rate of high bone turnover, but also to predict future bone loss or fractures. Recent data with kinetic TRACP 5b assay using α-naphthyl phosphate as a substrate demonstrated that the baseline measurement of serum TRACP 5b can predict bone loss after 2 years in postmenopausal women (Rico *et al.,* 2002). Another large prospective study with over 1000 postmenopausal women showed that serum TRACP 5b, assessed by our immunoassay, and also urinary OC can predict fracture and, in particular, fractures that engage the trabecular bone, such as vertebral fractures (Gerdhem *et al.,* 2004). Both TRACP 5b and urinary OC showed better prediction for fractures than conventional bone resorption markers CTX and DPD (Gerdhem *et al.,* 2004). The predictive value of TRACP 5b was also noted in patients with ADO2. The clinically affected individuals having highest serum TRACP 5b levels were also the most severely fractured.

6.4 TRACP 5b in animal models

Animal models provide insight into *in vivo* skeletal events, and also improve understanding of the pathogenesis, prevention, and treatment of osteoporosis and other bone diseases. The most commonly used rat models for osteoporosis are ovariectomy and orchidectomy, recommended by the USFDA (USFDA 1994). Within the last few years, serum markers of bone metabolism have provided a convenient and reproducible alternative to conventional histochemical and biomechanical techniques commonly used to assess the effect of pharmacological agents and the pathogenesis of bone diseases in rat models.

In animal models, serum TRACP values have been traditionally assessed using conventional kinetic total TRACP activity assays, or more recently with a novel immunoassay for total TRACP protein at the endpoint of the experiment (Srivastava *et al.,* 2002a). The results have shown that total TRACP is elevated after OVX and decreased with the administration of alendronate, clodronate or estradiol, or with the supplementation of dietary calcium and exercise (Diaz Diego and de la Piedra 1993; Gala *et al.,* 2001; Srivastava *et al.,* 2002a). We used our novel rat serum TRACP 5b immunoassay to assess the serum TRACP 5b levels in male rats before the ORC and sequentially after the operation for up to six months. The results were compared with the pQCT measurements at the same time points. We observed that the levels of serum TRACP 5b were increased as early as 5 days after orchidectomy, whereas the significant bone loss, quantified by pQCT, was observed 11 days after operation. This suggests that elevated serum TRACP 5b levels would predict future bone loss. Interestingly, after the rapid elevation of serum TRACP 5b, it started to decline and returned to the control level within 17 days after ORC. Similar results were obtained earlier with the OVX model showing an even faster elevation after operation and reduction to the control level within the first weeks (Surve *et al.,* 2001). The mRNA levels confirm that TRACP expression is highly enhanced within a week after OVX, and also decreased rapidly within 18 hours after administration of estradiol (Zheng *et*

al., 1995). However, we did not treat rats after ORC, but we made the histomorphometric analysis at the endpoint of the experiment, revealing that almost 80% of the trabecular bone was lost within the six months. The number of osteoclasts per trabecular bone was still slightly elevated, but the *absolute* number of osteoclasts per bone tissue was significantly decreased (~70%). We concluded that the novel serum TRACP 5b assay reflects the absolute number of osteoclasts and the absolute rate of bone resorption throughout the study.

The development of mice specifically lacking estrogen receptor α (ERKO), estrogen receptor β (BERKO) or both receptors (DERKO) (Couse *et al.,* 1999) has provided new insight into the regulation of estrogen in bone metabolism. As might have been expected, female and male mice lacking estrogen receptors have distinct skeletal phenotypes due to the differential participation of estrogen receptors α and β. Female ERKO mice exhibit clear bone phenotype with shorter bones, but increased trabecular BMD (Lindberg *et al.,* 2001a). Surprisingly, serum OC levels were decreased and serum CTX levels increased in female ERKO mice, while serum TRACP 5b levels were marginally decreased supporting the low bone turnover phenotype of ERKO mice (Lindberg *et al.,* 2001a). In ERKO and DERKO male mice, the high OPG/RANKL ratio suggested that the ERα might be involved in the regulation of bone turnover in males. As a result of a high OPG/RANKL ratio, serum levels of IL-6 and TRACP 5b were significantly decreased in ERKO and DERKO male mice (Lindberg *et al.,* 2001b). The complicated regulation of estrogen through estrogen receptors α and β needs further investigations in animal models. Serum TRACP 5b, as well as other bone metabolic markers, might provide a useful tool in assessing the changes in osteoclasts and the bone microenvironment.

6.6 TRACP 5b as a marker of osteoclast number

TRACP 5b has been generally accepted as a marker for osteoclasts, but there is still an open question whether secreted TRACP 5b reflects the activity or the number of osteoclasts. Our *in vitro* and *in vivo* data clearly demonstrate that secreted TRACP 5b activity is strongly associated with the number of existing multinucleated osteoclasts. In mouse bone marrow cell cultures the secreted TRACP 5b activity in the culture medium was strongly associated with the number of formed multinucleated non-resorbing osteoclasts ($r=0.94$; $p<0.0001$). In contrast, we were not able to demonstrate such an association with secreted TRACP 5b activity and the amount of bone resorption using primary rat osteoclasts on bovine bone slices (unpublished observations). Nevertheless, other researchers have been able to demonstrate this connection using other types of *in vitro* assays (Zaidi *et al.,* 1989; Moonga *et al.,* 1990; Scheven *et al.,* 1997). Clearly, the secretion of TRACP is not only restricted to resorbing osteoclasts, since also non-resorbing osteoclasts grown on glass or plastic surfaces secrete equal or even higher amounts of the enzyme (Moonga *et al.,* 1990).

Results obtained from *in vivo* rat ORC experiments showed that serum TRACP 5b peaked rapidly after the operation, but returned to the control level as early as two to three weeks after ORC. This data is contradictory with the presented data from other bone resorption markers that increase after ORC or OVX, and remain

elevated after prolonged periods (Ederveen *et al.,* 2001; Srivastava *et al.,* 2002a). However, TRACP 5b is a product of bone-resorbing osteoclasts, whereas the other resorption markers are released from degraded bone matrix. Therefore, TRACP 5b could reflect the number of existing osteoclasts, which is increased shortly after operation (Ikeda *et al.,* 1996), but probably reaches a plateau and decreases later on. Nevertheless, overall bone resorption may still be elevated after a prolonged period when compared with controls. More direct evidence with the association of the number of osteoclasts and serum TRACP 5b has been observed from naturally occurring osteopetrotic rats. Three distinct rat strains, toothless (tl/tl), osteopetrotic (op/op) and incisors absent (ia/ia) rats all have osteopetrosis but totally different phenotypes. Toothless rats have a severely reduced number of osteoclasts; osteopetrotic rats have a slightly reduced number of deficient osteoclasts, and incisors absent rats have a markedly increased number of inactive osteoclasts. In parallel to the osteoclast numbers, serum TRACP 5b is significantly decreased in toothless rats, unchanged in osteopetrotic rats, and significantly increased in incisors absent rats (Alatalo *et al.,* 2003b).

Also patients with ADO2 have a clear association between serum TRACP 5b and the number of osteoclasts. ADO2 is characterized by a significantly increased amount of non-resorbing multinucleated osteoclasts that are highly TRACP positive (Bollerslev *et al.,* 1993; Waguespack *et al.,* 2002). Clearly serum TRACP 5b does not reflect the resorptive activity of osteoclasts, but more likely the number of multinucleated osteoclasts in ADO2. Data supporting these results was observed from uremic patients on maintenance hemodialysis, in which serum TRACP 5b levels correlated more strongly with the histological parameters of osteoclasts (both numbers and osteoclast surface) than those of erosion (Chu *et al.,* 2003). However, at the same time serum TRACP 5b correlated about equally to that of intact PTH and ICTP with histological parameters of erosion, suggesting that serum TRACP 5b is the most specific marker for assessing the number of osteoclasts and, therefore, also osteoclastic activity in these individuals (Chu *et al.,* 2003).

Finally, in normal physiological conditions, and in most of pathological conditions, the number of resorbing osteoclasts is tightly coupled with the resorptive activity of those cells. Thus, by measuring the level of serum TRACP 5b, it is possible to assess the amount of currently present osteoclasts, and the rate of ongoing bone resorption.

6.7 Comparison of TRACP 5b with other bone resorption markers

Biochemical markers of bone resorption are mainly products of degraded bone matrix, such as CTX, NTX, DPD, PYD and BSP. TRACP 5b is unique, since it is a product of bone-resorbing osteoclasts. Therefore, these different markers may reflect different phases in bone degradation, providing new aspects in the field of bone markers. Theoretically, TRACP 5b may be produced and secreted prior to other resorption markers, already during osteoclastogenesis, whereas other markers are released during the active resorption phase.

TRACP 5b has an advantage over other resorption markers, since TRACP 5b is exclusively secreted from osteoclasts, whereas degradation products of type I collagen

may also be derived from other tissues than bone and from diet. Additionally, TRACP 5b has lower within-subject and diurnal variation compared to other resorption markers e.g. CTX (Hannon *et al.,* 2004). Furthermore, the accumulation of collagen fragments, but not the active form of TRACP 5b, is obvious in renal or hepatic failure causing "false-positives" in the case of normal bone resorption. The lower stability of serum TRACP 5b compared with collagen fragments is clearly the main disadvantage of TRACP 5b in clinical use. When type I degradation products are very stable at -20°C, TRACP 5b activity is decreased significantly after some months. However, this problem is easy to eliminate by storing samples at -70°C for longer periods. The rare occasions where a low bone resorption rate is associated with elevated serum TRACP 5b, such as ADO2, may cause "false-positives". However, these cases are extremely rare and easy to recognize by observing the symptoms and phenotype of each individual. Furthermore, the elevations of serum TRACP 5b in ADO2 are several orders of magnitude higher than in any high bone turnover disease such as osteoporosis, making the interpretation of the results very simple.

The percentage of response to antiresorptive treatment has been reported to be much higher for collagen fragments than for TRACP 5b. This observation is easy to understand when thinking of the mechanism of action of most antiresorptive drugs. Bisphosphonates, such as alendronate and clodronate inhibit the action of osteoclasts, thereby decreasing bone resorption, but not necessarily the number of inactivated osteoclasts. This is reflected by a remarkable decrease in the secretion of bone matrix degradation products, with simultaneously smaller changes in secretion of TRACP 5b. However, the changes in serum TRACP 5b are significant due to smaller biological and analytical variability than other resorption markers.

In summary, markers of bone resorption reflect different phases of bone resorption, and they therefore offer tools to investigate more precisely specific events during bone degradation and antiresorptive treatment. At present, there is no one superior bone marker and the best results in patient management may be obtained by combining a panel of bone metabolic markers with BMD measurements.

7. CONCLUSIONS

Novel and conventional biochemical markers of bone metabolism will provide adjunctive tools to assess patients with bone diseases. We have developed immunoassays for serum TRACP 5b, an enzyme released specifically from osteoclasts during bone resorption. Serum TRACP 5b has low diurnal variation and does not accumulate into the circulation in renal or hepatic failure. Our serum TRACP 5b immunoassay can be used to detect increased rates of bone resorption in patients with high bone turnover diseases, such as osteoporosis, malignancies metastatic to bone, Paget's disease and hyperparathyroidism. It is also useful for monitoring the efficacy of antiresorptive treatments, such as HRT and bisphosphonate treatment. Preliminary data suggests that serum TRACP 5b can predict future bone loss and fractures, in particular fractures engaging in the trabecular bone. Our present data suggests that circulating TRACP 5b reflects the number of multinucleated osteoclasts and therefore indirectly assesses the rate of bone resorption. In most cases, the number and activity of osteoclasts are tightly coupled, suggesting that circulating TRACP 5b is a useful marker of bone resorption. The novel TRACP 5b immunoassays may prove to be valuable either as independent or combinatory tools with other bone metabolic markers and BMD measurements in clinical practice and bone research.

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Sari Alatalo

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