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Biochemical markers in preclinical models of osteoporosis

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Abstract

Although several treatments for osteoporosis exist, further understanding of the mode of action of current treatments, as well as development of novel treatments, are of interest. Thus, preclinical models of osteoporosis are very useful, as they provide the possibility for gaining knowledge about the cellular mechanisms underlying the disease and for studying pharmaceutical prevention or intervention of the disease in simple and strictly controlled systems. In this review, we present a comprehensive collection of studies using biochemical markers of bone turnover for investigation of preclinical models of osteoporosis. These range from pure and simple *in vitro* systems, such as osteoclast cultures, to *ex vivo* models, such as cultures of embryonic murine tibiae and, finally, to *in vivo* models, such as ovariectomy and orchidectomy of rats. We discuss the relevance of the markers in the individual models, and compare their responses to those observed using 'golden standard' methods.

Keywords: *Biomarkers, preclinical models, osteoporosis*

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Introduction

Osteoporosis

Osteoporosis is a large and continuously growing problem worldwide. The number of patients suffering from osteoporosis and other bone diseases is rising due to the increasing lifetime of the elderly population in the industrialised countries (Riggs et al. 1998). Forty per cent of Caucasian women with an age older than 80 years have osteoporosis (Lazner et al. 1999, Rodan & Martin 2000). In men, osteoporosis occurs in approximately 10% of the population in western countries (Looker et al. 1997, Riggs et al. 1998, Kamel 2005).

Osteoporosis often occurs as a consequence of decreased production of gonadal steroids in both men and women resulting in an increase in bone turnover; however, with resorption exceeding formation this leads to pathological bone loss (Riggs et al. 1998). Decreased production of sex hormones is seen in women after menopause,

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whereas in men it occurs gradually as the production of sex hormones is reduced with age (Riggs et al. 1998).

Osteoporosis is a systemic skeletal disease characterised by low bone mass and microarchitectural reduction of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (Rodan & Martin 2000, Cooper 2003). Other causes of osteoporosis include glucocorticoid treatment, smoking and excessive alcohol intake (Scane et al. 1999, Hannan et al. 2000). A less clinically relevant explanation is space flight which, however, still provides useful information about bone loss after reduction of mechanical pressure (Collet et al. 1997, Lang et al. 2004, Iwamoto et al. 2005).

As detailed studies in humans are difficult, time consuming and expensive, preclinical models of osteoporosis are very useful, and they have been used both for studying the molecular and cellular basis of osteoporosis and for testing a high number of compounds for their ability to reduce disease progression. The models range from very simple *in vitro* testing systems for resorption and formation (Baron et al. 1985, Nefussi et al. 1985, Bellows et al. 1986, Nordstrom et al. 1995, Li et al. 1999, Massey & Flanagan 1999, Shalhoub et al. 2000, Karsdal et al. 2003, Rawadi et al. 2003, Ogasawara et al. 2004), to the more complex *ex vivo* systems (Dieudonne et al. 1991, Blavier & Delaisse 1995, Mundy et al. 1999, Engsig et al. 2000a, Chiusaroli et al. 2003, Garrett 2003, Henriksen et al. 2003), and finally to the *in vivo* models, such as the rat ovariectomised (OVX) model, which mimics human osteoporosis to some extent (Vanderschueren et al. 1992, Dempster et al. 1995, Cenci et al. 2000, Wu et al. 2003, Schaller et al. 2004, Karsdal et al. 2005).

Bone turnover and bone cells

The correct maintenance of a healthy skeleton is dependent on bone resorption and bone formation. Loss of regulation of this process leads to pathologies, such as osteoporosis. The main type of cell responsible for bone resorption is the osteoclast (Roodman 1999), whereas the main type of cell responsible for bone formation is the osteoblast (Manolagas 2000, Aubin 2001). The resorption level and the formation level are tightly coupled under normal circumstances, and resorption is always followed by the same amount of formation (Parfitt 1982).

The osteoclasts derive from haematopoietic stem cells, from which they are differentiated into mature, multinuclear bone-resorbing osteoclasts when stimulated with the cytokines, receptor activator of NFkappaB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (Lacey et al. 1998). The osteoclasts are characterised by the presence of multiple nuclei, a sealing zone and the formation of a ruffled border. Further characteristics of the osteoclasts include the expression of the calcitonin receptor, cathepsin K and tartrate-resistant acid phosphatase (TRACP) (Roodman 1999). The hallmark of osteoclast function is the ability to form a resorption lacuna, which allows the osteoclasts to resorb calcified bone matrix. After having finished bone resorption the osteoclast undergoes apoptosis, and bone formation is initiated (Roodman 1999).

The osteoblasts derive from mesenchymal stem cells, and they are recruited and differentiated into mature bone-forming osteoblasts in the presence of the right stimuli (Aubin 1998a, Aubin 1998b, Manolagas 2000, Aubin 2001). The characteristics of osteoblasts include their cuboidal shape, their ability to produce type I collagen and

the expression of alkaline phosphatase and osteocalcin (Midy & Plouet 1994, Shui & Scutt 2001, Rawadi et al. 2003, Kawazoe et al. 2004, Eghbali-Fatourechi et al. 2005). After bone formation, the osteoblasts either differentiate into osteocytes, become bone-lining cells or undergo apoptosis (Manolagas 2000, Aubin 2001).

Biochemical markers of bone turnover

The biomarkers for bone turnover can be divided into three categories: (1) markers that reflect bone resorption; (2) markers that reflect osteoclast number, and (3) markers that reflect bone formation. Bone resorption markers are different matrix-derived fragments that are generated during the osteoclastic resorption activity, such as the C-terminal cross-linked telopeptide of type I collagen (CTX-I) fragment and the N-terminal telopeptide of type I collagen (NTX) fragment (Hanson et al. 1992, Gertz et al. 1998, Rosenquist et al. 1998, Karsdal et al. 2003), which are both generated mainly by cathepsin K produced by the osteoclasts. Measurement of the deoxypyridinolines (DPYR/DPD), pyridinolines (PYR) and hydroxyprolines also reflects bone resorption; although DPYR and PYR are collagen type I fragments they are less sensitive markers of bone resorption than CTX-I and NTX (Okabe et al. 2004). Also, hydroxyprolines are less specific compared to CTX-I and NTX as they are released from all types of collagen (Chaki et al. 2000, Liesegang 2003). In addition, the matrix metalloproteinase (MMP)-generated C-terminal telopeptide of type I collagen (ICTP) fragment can be used to monitor bone resorption under certain circumstances, such as the resorption induced by bone metastasis, whereas it does not reflect increase in resorption seen in osteoporosis (Okabe et al. 2004, Leeming et al. 2006), probably because the MMPs do not play a major role in osteoclast-mediated resorption under osteoporotic conditions (Henriksen et al. 2006).

Markers that reflect osteoclast number are TRACP and, potentially, cathepsin K, which are enzymes produced and released into the circulation by the osteoclasts (Drake et al. 1996, Gelb et al. 1996, Halleen et al. 1998, Gowen et al. 1999, Halleen et al. 1999, 2000). The most well-characterised osteoclast marker is TRAP5b, which is a subset of the total TRACP only produced by the osteoclasts (Alatalo et al. 2003). Cathepsin K is still undergoing evaluation as an osteoclast marker (Meier et al. 2006) and further analysis will show whether it correlates to resorptive activity or osteoclast number.

The bone formation markers can be divided into two categories: (1) proteins, which reflect increased osteoblast differentiation, and thereby indicate increased bone formation, such as bone-specific alkaline phosphatase (BSAP) and osteocalcin (Brown et al. 1984, Delmas et al. 1985, Rosenquist et al. 1995, Broyles et al. 1998, Gundberg 2000, Peretz et al. 2003), or (2) fragments of pro-collagen, which are released during collagen incorporation into the newly formed bone matrix, and thereby directly reflect bone formation, such as the N-terminal and C-terminal pro-fragments of type I collagen, PINP and PICP, respectively, (Parfitt et al. 1987b, Suvanto-Luukkonen et al. 1997). Figure 1 and Table I summarise the origin of the different biochemical markers discussed in this review.

These markers often provide fast, sensitive and dynamic measurements of the different aspects of bone turnover. Furthermore, they often correlate to the 'golden standard' methods, such as various histomorphometrical indices (Visentin et al. 2000, Lark et al. 2002, Gopala et al. 2003, Schaller et al. 2004). In addition, CTX-I levels

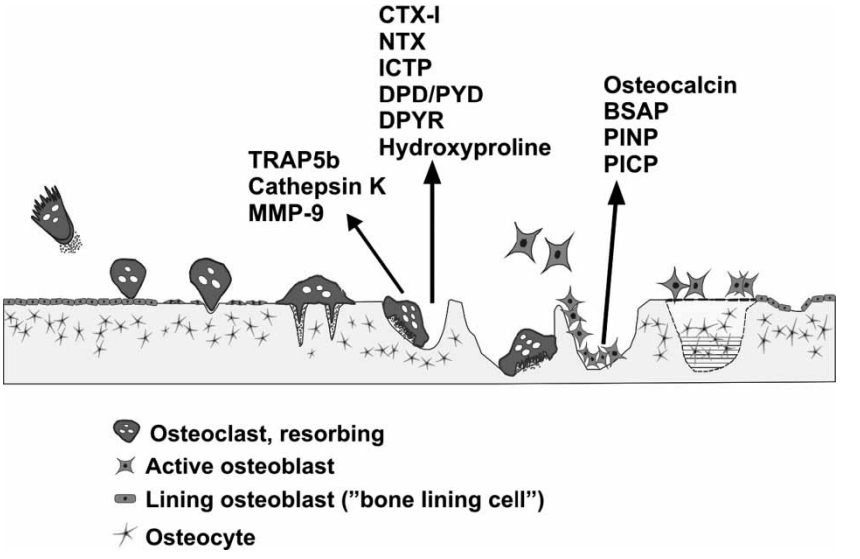

Figure 1. Schematic illustration of the origin of the markers. The figure illustrates that osteoclasts during resorption release CTX-I, NTX, DPYR/DPD/PYR, ICTP and hydroxyprolines from the bone matrix. cathepsin K and TRAP5b are released from the osteoclasts themselves. Osteocalcin and BSAP are secreted by bone forming osteoblasts and are indicative of bone formation. The pro-collagen fragments PINP and PICP are released during the incorporation of collagen into the bone matrix by the mature bone forming osteoblasts, and reflect bone formation.

have been shown to be an independent risk predictor for fractures, and thus the markers can also provide information about bone quality (Chapurlat et al. 2000, Garnero et al. 2000).

In this review, we will provide a description of various preclinical models including *in vitro*, *ex vivo* and *in vivo* models. We will focus on how biochemical markers can be used and to what extent they correlate to the validated measurements, such as histomorphometry, histology and strength tests.

In vitro models

Osteoclasts and bone resorption

The main function of osteoclasts is to resorb bone. Measurements of resorption can be done by two assays: (1) a direct evaluation of the pit area and volume of resorbed bone by stereology and/or scanning electron microscopy (Parikka et al. 2001, Henriksen et al. 2004, Schaller et al. 2004), or (2) measurement of the release of fragments of the organic matrix during the resorption, such as CTX-I, which is generated by the activity of cathepsin K (Apone et al. 1997, Parikka et al. 2001, Karsdal et al. 2003, Henriksen et al. 2004, Schaller et al. 2004, Henriksen et al. 2005, 2006). In addition measurement of hydroxyproline released into the cell culture supernatant can also be used to monitor resorptive activity (Yasuma et al. 1998, Furuyama & Fujisawa 2000). Both the measurement of biochemical markers and the evaluation of the pit area are commonly used to evaluate the resorptive activity of the osteoclasts. However, both assays have limitations as the first method evaluates the resorbed surface and the second the volume of collagen matrix resorbed, although in most cases these two

Table I. Origin of the biochemical markers.

Marker	Biochemical process	Description	References
CTX-I	Bone resorption	C-terminal cross-linked telopeptide of type I collagen. CTX-I is generated by cathepsin K and released during osteoclastic bone resorption.	(Rosenquist et al. 1998; Schaller et al. 2004; Henriksen et al. 2004)
NTX	Bone resorption	N-terminal telopeptide of type I collagen. NTX-I is generated by cathepsin K and released during osteoclastic bone resorption.	(Gertz et al. 1998; Hanson et al. 1992)
ICTP	Bone resorption in some cases	C-terminal telopeptide of type I collagen. ICTP is generated by MMPs and is released during bone resorption in special cases, such as bone metastases.	(Garnero et al. 2003; Leeming et al. 2006; Okabe et al. 2004)
DPD/DPYR/ PYD	Bone resorption	Deoxypyridinolines (DPD/DPYR) and pyridinolines (PYD) are found in mature collagen and are released during bone resorption.	(Seyedin et al. 1993; Robins et al. 1994)
Hydroxyproline	Bone resorption	Hydroxyprolines are found in the triple helices of collagen, and they are released during resorption.	(Liesegang 2003)
TRAP5b	Osteoclast number	Tartrate-resistant acid phosphatase (TRACP) is a family of enzymes, where TRAP5b is specifically produced by the osteoclasts	(Halleen et al. 1998; Halleen et al. 1999; Halleen et al. 2000; Alatalo et al. 2003)
Cathepsin K	Osteoclast number[a]	Cathepsin K is the essential protease for collagen type I degradation, and it is mainly produced by osteoclasts	(Meier et al. 2006)
Osteocalcin	Bone formation	Osteocalcin is a bone-specific protein produced by the osteoblast and inserted into the bone matrix.	(Delmas et al. 1985; Brown et al. 1984; Rosenquist et al. 1995)
BSAP	Osteoblast differentiation	Bone-specific alkaline phosphatase is an enzyme, which is an early marker of osteoclastogenesis.	(Peretz et al. 2003; Broyles et al. 1998; Gundberg 2000)
PINP/PICP	Bone formation	The C- and N-terminal propeptides of type I collagen. The propeptides are released from newly synthesised pre-procollagen prior to the incorporation of collagen molecules into the bone matrix.	(Parfitt et al. 1987b; Suvanto-Luukkonen et al. 1997)

[a]Under investigation.

measurements will correlate well (Henriksen et al. 2004, Schaller et al. 2004). On the other hand, the markers have two major advantages as they can provide dynamic data and the measurements are not as time consuming as the stereological scoring of resorption pits.

Measuring the TRACP activity or the level of TRAP5b in the supernatants reflects osteoclast numbers in cell cultures (Ylipahkala et al. 2005a). These measurements will

in most cases provide the osteoclast number, although TRACP activity is sensitive to some resorption inhibitors, probably due to processing of TRACP by cathepsin K (Ljusberg et al. 2005, Ylipahkala et al. 2005b). Finally, measurement of cathepsin K levels will probably predict osteoclast number, but may also indicate the activity. Published data demonstrate that the cathepsin K levels are increased in postmenopausal women, and the cathepsin K levels are reduced by bisphosphonate therapy (Holzer et al. 2005, Meier et al. 2006). However, as both these conditions are associated with both changes in osteoclast numbers and activity (Rodan & Martin 2000, Rodan 2003), there is no clear-cut answer to whether cathepsin K levels reflect osteoclast activity or number.

Osteoblasts and bone formation

The differentiation of mesenchymal stem cells into bone-forming osteoblasts can be done in the presence of β-glycerophosphate and ascorbic acid (Nefussi et al. 1985, Bellows et al. 1986). Studies of the factors involved in osteoblast differentiation (Rawadi et al. 2003, de Jong et al. 2004, Ogasawara et al. 2004) as well as enzymatic systems involved in osteoblast maturation, function and survival (Karsdal et al. 2001, 2002, 2004) can be performed using this method.

The most common assays for studying aspects of osteoblast biology are measurement of alkaline phosphatase activity as a marker for early stages of differentiation (Midy & Plouet 1994, Shui & Scutt 2001, Rawadi et al. 2003). Furthermore, the expression of the osteocalcin is also a characteristic of the development of bone-forming osteoblast (Eghbali-Fatourechi et al. 2005, Shaama 2005). During bone formation, pro-fragments type I collagen, such as PICP and PINP are released and can be measured in supernatants of cultured osteoblasts, thereby providing additional tools for studies of osteoblasts (Palosaari et al. 2001, Torricelli et al. 2002).

The 'golden standard' methods for *in vitro* measurement of osteoblast-mediated bone formation are measurement of alizarin red accumulation and Von Kossa staining, which, respectively, measure the amount of phosphate and calcium deposited in the newly formed bone (Nefussi et al. 1985, Bellows et al. 1986, Rawadi et al. 2003, Kawazoe et al. 2004). However, combining these static methods with dynamic measurement of the various biochemical markers will provide a more detailed picture of different aspects of osteoblast biology, such as differentiation.

***Ex vivo* models**

Resorption

In vivo osteoclasts are embedded in the matrix, which most likely will influence their biology. However, as *in vivo* models are expensive and time-consuming models for studying osteoclast activity, *ex vivo* models for bone culture systems have been developed. To study osteoclast development and function three *ex vivo* models are generally used. These are the murine metatarsal model, which is a development model, and the tibia and calvaria models, which are resorption models (Dieudonne et al. 1991, Corboz et al. 1992, Hofstetter et al. 1992, Henriksen et al. 2003, Leloup et al. 1994, Blavier & Delaisse 1995, Engsig et al. 2000a, Engsig et al. 2000b). Originally, all three models were based on radiolabelling, where pregnant mice were labelled with $^{45}Ca^{2+}$ and bones from the embryos were isolated. The resorption was

then followed by quantification of $^{45}Ca^{2+}$ release into the culture medium (Blavier & Delaisse 1995, Engsig et al. 2000a). Recently, it was demonstrated that measurement of the cathepsin K-generated CTX-I fragment could replace the use of radioactive $^{45}Ca^{2+}$ (Garnero et al. 2003). In addition, the ICTP fragment also reflected resorption to some extent (Garnero et al. 2003), as this fragment is generated by MMP activity and only reflects the resorption level under special circumstances (Nishi et al. 1999, Okabe et al. 2004, Henriksen et al. 2006, Leeming et al. 2006).

In the metatarsals model, the osteoclastic resorption is represented as the release of $^{45}Ca^{2+}$ into the culture medium. A characteristic of this model is that the release of $^{45}Ca^{2+}$ is dependent on differentiation and recruitment of osteoclasts into the developing site in the marrow (Dieudonne et al. 1991, Blavier & Delaisse 1995). Furthermore, the bone resorption and recruitment of the osteoclasts can in the metatarsals model be investigated histologically (Leloup et al. 1994, Blavier & Delaisse 1995, Engsig et al. 2000a,b). However, this model is based on resorption of the calcified cartilage present in the bone rudiments before marrow cavity formation, which contains both type I collagen and type II collagen (Blavier & Delaisse 1995). Thus, combining CTX-I and CTX-II will most likely reflect the actual resorption level (Hoegh-Andersen et al. 2004, Ishikawa et al. 2004, Sondergaard et al. 2006a,b).

The tibia and the calvaria models directly detect bone resorption (Dieudonne et al. 1991, Engsig et al. 2000a, Lacey et al. 2000), as they are based on the presence of mature resorbing osteoclasts. The difference between the two models lies in the bone type evaluated. The tibia model is a model for long bone, while the calvaria model is for flat bone (Dieudonne et al. 1991, Corboz et al. 1992, Hofstetter et al. 1992, Everts et al. 1999, Engsig et al. 2000a, Lacey et al. 2000, Chiusaroli et al. 2003, Garnero et al. 2003, Henriksen et al. 2003, Shorey et al. 2004). In these models the resorptive activity can be quantified by measuring the CTX-I or the ICTP level (Garnero et al. 2003).

In conclusion, the resorption markers can be used for studying resorption in the *ex vivo* models, which reflect osteoclast-mediated bone resorption, and are therefore very useful for studying both biological phenomena and pharmacological intervention in the *ex vivo* systems.

Bone formation

Bone formation can be studied *ex vivo* by using murine calvarias. Calvarias are isolated from 4-day-old mice and cultured for 3–7 days. Hereafter, the bone formation can be evaluated histologically (Traianedes et al. 1998, Mundy et al. 1999, Garrett et al. 2003, Garrett 2003). Furthermore, early stages of osteoblast differentiation can also be monitored using alkaline phosphatase (ALP) measurement in this assay (P. Hoegh-Andersen, Pharmos Bioscience, unpublished data). Finally, it would be of interest to be able to measure osteocalcin levels or type I collagen production, as a measurement of bone formation in this assay. However, whether these methods are applicable is currently not known, although it is likely as these markers can be measured in supernatants from *in vitro* cultures (Palosaari et al. 2001, Torricelli et al. 2002, Eghbali-Fatourechi et al. 2005, Shaama 2005).

A similar local bone formation assay can be performed *in vivo* using local injections of a desired compound into murine calvarias over a short period of time. This method is normally analysed using histomorphometry (Mundy et al. 1999, Garrett et al.

2003), and whether bone turnover markers will reflect the local changes in bone formation remains to be determined.

In conclusion, bone formation models *ex vivo* and *in vivo* are very useful methods and further development of the existing bone formation markers is likely to provide robust and rapid tools for studying these models.

In vivo models

Models of osteoporosis

Models for studying different forms of osteoporosis, such as glucocorticoid-induced osteoporosis, hypercalcaemia induced by retinoic acid treatment of thyroparathyroidectomized (TPTX) rats (Trechsel et al. 1987) and transgenic mouse models which also mimic osteoporosis, such as osteoprotegerin knockout mice, are numerous (Simonet et al. 1997, Bucay et al. 1998, Schilling et al. 2001, Priemel et al. 2002, Pogoda et al. 2005). However, two of the most commonly used models, are the models for studying osteoporosis reflecting the disease caused by loss of gonadal function. Ovariectomy (OVX) of female rats and mice is used to mimic postmenopausal osteoporosis in women (Dempster et al. 1995, Cenci et al. 2000, Roggia et al. 2001, Gao et al. 2004, Schaller et al. 2004, Karsdal et al. 2005). In male animals, orchidectomy (ORX) is used to mimic osteoporosis in men (Vanderschueren et al. 1992, Weinstein et al. 1997, Onoe et al. 2000, Erben 2001, Wu et al. 2003).

Both OVX and ORX lead to a decreased production of sex steroids, which subsequently leads to a rapid change in the bone turnover, characterised by both higher bone resorption and formation, although the increase in bone formation is lower than the increase in resorption (Wakley et al. 1991, Vanderschueren et al. 1992, Turner et al. 1993, Ke et al. 1998, Baldock et al. 1999, Rico et al. 2000, Bauss et al. 2002). These models are commonly used to study both the biological background of osteoporosis and potential treatments for the diseases (Nuttall et al. 1998, Visentin et al. 2000, Ke et al. 2001, Lark et al. 2002, Schaller et al. 2004, Karsdal et al. 2005).

Monitoring treatment effects is easily done using biomarkers of osteoblastogenesis and thereby bone formation, such as osteocalcin and BSAP (Gopala et al. 2003, Karsdal et al. 2005), whereas the pro-collagen fragments PINP and PICP cannot be used owing to species specificity. Furthermore, biomarkers of osteoclast number, such as TRACP (Karsdal et al. 2005, Rissanen et al. 2005b), and markers of osteoclast-mediated bone resorption such as DPD, PYR, NTX and CTX-I (Visentin et al. 2000, Schaller et al. 2004, Karsdal et al. 2005), can all be measured in either serum, urine or both (Karsdal et al. 2005).

Several studies have used biochemical markers of bone turnover to follow the response to treatment in different preclinical models of osteoporosis. The studies include oestradiol, various bisphosphonates, calcitonin and a group of novel drugs including inhibitors of the V-ATPase, ClC-7 and cathepsin K inhibitors and anti-RANKL therapy (see Table II).

The findings from study to study using the same drug, such as oestradiol, are generally consistent with the application of the marker, e.g. both CTX, PYR and TRACP levels are decreased when using oestradiol, reflecting the decreased resorption and decreased osteoclast number observed using this treatment (Frolik et al. 1996, Srivastava et al. 2000, 2002). In addition, the oestradiol-mediated

Table II. Bone markers used in preclinical osteoporosis models.

Target	Class of drug	Name of drug	Marker	References
Estrogen receptors	Estrogen agonists	Estradiol	TRACP	(Srivastava et al. 2002)
Estrogen receptors	Estrogen agonists	Estradiol	Osteocalcin CTX-I	(Srivastava et al. 2000)
Estrogen receptors	Estrogen agonists	Estradiol	Osteocalcin PYR	(Frolik et al. 1996)
Osteoclasts	Bisphosphonates	Zoledronate	Osteocalcin PYR	(Glatt et al. 2004)
Osteoclasts	Bisphosphonates	Incadronate	Osteocalcin PYR	(Teramura et al. 2002)
Osteoclasts	Bisphosphonates	Alendronate	TRACP	(Srivastava et al. 2002)
Osteoclasts	Bisphosphonates	Clodronate	Osteocalcin ALP DPD	(Kippo et al. 1998)
Calcitonin receptor	Calcitonin	Calcitonin	Hydroxyprolines	(Davey & Morris 2005)
RANKL	Anti-RANKL	OPG	BSAP TRACP	(Valenta et al. 2005)
V-ATPase	V-ATPase inhibitor	FR167356	DPYR	(Niikura et al. 2005)
V-ATPase	V-ATPase inhibitor	SB 242784	DPD PYR	(Visentin et al. 2000)
ClC-7	ClC-7 inhibitor	NS3736	CTX-I Osteocalcin	(Schaller et al. 2004)
ClC-7	ClC-7 inhibitor	NS3696	TRACP DPYR	(Karsdal et al. 2005)
Cathepsin K	Cathepsin K inhibitor	SB 331750	DPYR	(Lark et al. 2002)

impairment of bone formation seen as a consequence of the coupling, is reflected in the bone formation marker osteocalcin (Frolik et al. 1996, Srivastava et al. 2000).

A second example of the normally robust correlations between marker responses in different studies, is the study of ClC-7 inhibitors in the OVX rat model (Schaller et al. 2004, Karsdal et al. 2005). These studies show stable reductions in the resorption markers CTX-I and DPYR using two different inhibitors, and in addition both studies also observed that the osteocalcin levels were unaltered by the treatment (Schaller et al. 2004, Karsdal et al. 2005).

Thus, in general, the studies using the same class of inhibitors observe consistent results, and most of the differences are explained rather by differences in the individual experiment, such as age of the animals or dosing procedure, than by instability in the marker measurements.

The use of biomarkers is constantly being validated with reference to the more laborious histomorphometrical methods (Visentin et al. 2000, Schaller et al. 2004). In addition, in most cases, biochemical markers are used in combination with DXA, MR or CT scans, which provide both invasive and non-invasive measurements of bone structure and mass that the markers cannot supply (Bollerslev & Andersen Jr. 1989, Takahashi et al. 1999, Jiang et al. 2000, Binkley et al. 2003, Hornby et al. 2003, Iida-Klein et al. 2003, Munns et al. 2004). Furthermore, the labour-intensive histomorphometrical methods are often used to provide information about the dynamics of bone turnover in the different cases (Visentin et al. 2000, Stroup et al. 2001, Lark et al. 2002, Schaller et al. 2004). The methods for evaluating the strength of the bones, and thereby indicating the quality of the bones, are based on invasive techniques, such as three- and four-point bending tests and compression tests (Kang et al. 1998, Ke et al. 2000, 2001, Bauss et al. 2002, Schaller et al. 2004). Prediction of bone strength using non-invasive methods is difficult; however, measurement of bone mineral density (BMD) has been shown to be an individual and independent risk

predictor for fractures (Chapurlat et al. 2000, Garnero et al. 2000). Interestingly, measurement of CTX-I was also shown to be an individual and independent risk predictor for fractures and the outcome of treatment with respect to quality (Garnero et al. 1998). In addition, combination of BMD and CTX-I measurements provided a good prediction of fracture risk (Garnero et al. 1998, Chapurlat et al. 2000, Garnero et al. 2000). Furthermore, recent evidence has shown that bone turnover markers can reflect whether a given treatment will increase bone strength (Schaller et al. 2004).

For all the measurements described, such as biochemical markers, histomorphometry and DXA scanning, there are advantages and disadvantages. The markers have two main advantages: they are easily obtained from blood or urine and the measurements can be done dynamically. The major disadvantage with the markers is that they are not all fully validated, and, thus, a secondary measurement such as histomorphometry is required (Gopala et al. 2003, Schaller et al. 2004). In contrast, using dynamic histomorphometry is labour intensive, but the result is validated and the information is detailed, although it requires termination of the animals. However, it should be mentioned that in most cases, the biochemical markers correlate with the histomorphometrical data (Visentin et al. 2000, Gopala et al. 2003, Schaller et al. 2004).

The different scanning techniques provide good structural data, and some of them can be used dynamically to monitor changes in structure; however, the problem with the structural data, is that they do not always correlate very well to strength measurements (Bollerslev & Andersen Jr. 1989, Binkley et al. 2003, Hornby et al. 2003, Iida-Klein et al. 2003). The use of biomechanical testing is very useful, as a combination of these techniques will provide the strength of individual bone compartments, such as both the bending strength of the femur, and the compression strength of the vertebral body (Ke et al. 2000). The problem with these measurements is that they are not dynamic, and for obvious reasons they will never be applicable in clinical trials. Thus, all the different methods provide useful data; however, all have their drawbacks as well, and it still remains to be seen whether any non-invasive measurement can provide a robust indication of bone strength. A summary of the advantages and disadvantages of the different methods for analysis of *in vivo* models for osteoporosis is shown in Table III.

In conclusion, bone turnover markers are very useful for monitoring the *in vivo* osteoporosis models, providing dynamic and sensitive measurements of bone turnover and, in many cases, they reflect measurements of histomorphometric indices, and therefore can save time and money.

Conclusions

This review has focused on the use of bone turnover markers in preclinical models of osteoporosis ranging from very simple and easily interpretable *in vitro* models to the more complicated *ex vivo* models and, finally, the *in vivo* models used to study the treatments under development for osteoporosis. In the *in vitro* resorption models, measurement of CTX-I release, in most cases, reflects the total osteoclastic resorption activity, and correlates to the pit volume (Henriksen et al. 2004, Schaller et al. 2004). Measuring CTX-I release under either non-treated circumstances or using anti-resorptives, such as bisphosphonates or acidification inhibitors, will accurately reflect the resorptive activity of the osteoclasts (Henriksen et al. 2004, Schaller et al. 2004,

Table III. Advantages and disadvantages of biochemical markers compared to other assays for analysis of *in vivo* models.

	Method	Advantages	Disadvantages	References
Turnover	Markers	Blood/urine =easy, dynamic, correlate with histomorphometry	Not all are generally accepted	(Karsdal et al. 2005; Schaller et al. 2004; Gopala, V et al. 2003; Visentin et al. 2000; Lark et al. 2002)
	Histomorphometry	Validated, detailed information, dynamic	Labor-Internationalensive	(Parfitt et al. 1987a; Baddeley et al. 1986; Eriksen 1986; Gundersen et al. 1988; Vesterby et al. 1987; Hauge et al. 1999; Hauge et al. 2001)
Structure	DXA scanning	Non-invasive/dynamic	High density does not imply strength, does not always correlate to strength	(Iida-Klein et al. 2003; Binkley et al. 2003; Hornby et al. 2003)
	QCT/microCT scanning	Detailed scan, better for strength than DXA,	Termination of animals, not dynamic, does not always correlate to strength	(Barbier et al. 1999; Rhee et al. 2004; Schaller et al. 2004; Bollerslev & Andersen Jr. 1989; Tuukkanen et al. 2000; Munns et al. 2004; Moisio et al. 2003)
	MR scanning	High resolution, dynamic measurement	Expensive, does not always correlate to strength	(Jiang et al. 2000; Takahashi et al. 1999; Bollerslev & Andersen Jr. 1989; Tuukkanen et al. 2000; Munns et al. 2004)
Biomechanical	Compression	Strength, trabecular bone	Not dynamic	(Ke et al. 2000; Ke et al. 2001; Bauss et al. 2002)
	Bending	Strength	Not dynamic, mainly cortical bone	(Bauss et al. 2002)
	Indentation	Strength, trabecular bone	Not dynamic	(Kang et al. 1998; Schaller et al. 2004)

Karsdal et al. 2005, Ylipahkala et al. 2005a). Finally, the major advantage of the markers is that they can be measured dynamically during resorption experiments and therefore provide additional information, such as reversibility of an effect, loss of sensitivity to a treatment and fast responses to treatment, when compared to endpoint measurements of pit area, number and volume.

In vitro bone formation measurements are mainly confined to measuring ALP activity in the cell lysates and supernatants, although calcium deposition can also be measured (Midy & Plouet 1994, Shui & Scutt 2001, Rawadi et al. 2003). Studies using dynamic measurement of osteocalcin, PICP and PINP are still few (Palosaari et al. 2001, Torricelli et al. 2002, Shaama 2005, Eghbali-Fatourechi et al. 2005). However, we believe that use of the biomarkers will increase, as they supply easily accessible information.

In the *ex vivo* models, the resorption marker CTX-I reflects osteoclast activity quite well; however, as both cathepsin K and MMPs are involved in resorption of the developing bones in these models the marker ICTP, which reflects MMP-generated collagen fragments, can also be used (Garnero et al. 2003), and both of these correlate to analyses of $^{45}Ca^{2+}$ release (Garnero et al. 2003). The *ex vivo* formation model and the local *in vivo* formation model have not been studied in great detail with respect to bone formation markers, although ALP activity can be used for the *ex vivo* model (P. Hoegh-Andersen, Pharmos Bioscience, unpublished data). The results obtained in the models are normally obtained using histomorphometry and histology, which are time-consuming approaches, and therefore there is a need for formation markers which will allow dynamic and detailed quantification of responses to treatment in the models. It should be noted that whether the existing bone formation markers can measure the locally induced formation in the *in vivo* calvaria model is not known.

In vivo models of osteoporosis are routinely used for understanding the disease pathology and for testing potential new treatments. The classical analysis methods for the models are BMD, bone mineral content (BMC) measurements, strength tests and both static and dynamic histomorphometric indices as well as several other tests (Bollerslev & Andersen Jr. 1989, Kang et al. 1998, Barbier et al. 1999, Takahashi et al. 1999, Jiang et al. 2000, Binkley et al. 2003, Hornby et al. 2003, Iida-Klein et al. 2003, Munns et al. 2004). In recent years, the use of bone turnover markers has increased due to the increased numbers of tests available, but also due to the increased validation of the different markers (Schaller et al. 2005). The value of the markers has also increased as measurements of bone formation and bone resorption using histomorphometry can be reflected to a high extent by the measurement of the respective markers (Visentin et al. 2000, Stroup et al. 2001, Lark et al. 2002, Schaller et al. 2004), and thus these can save important time in the development of new drugs. In addition, measurement of osteoclast numbers using TRAP5b provides additional important information (Rissanen et al. 2005b), and the ratio between CTX-I and TRAP5b provides a resorption index for the individual osteoclast, and thereby provides very useful knowledge for investigators testing anti-resorptives for osteoporosis (Rissanen et al. 2005a, 2005b).

Furthermore, the bone formation markers also provide very useful information in the different studies; in particular, recent studies have indicated that the coupling between resorption and formation can be attenuated using certain anti-resorptive therapies, and thus the bone formation can be maintained (Schaller et al. 2004, Karsdal et al. 2005). These studies, and the studies focusing on characterising the

anabolic effects of parathyroid hormone (PTH) (Liu et al. 1991, Dempster et al. 1993, Shen et al. 2000), are using the bone formation markers extensively, and the markers provide useful information with respect to gaining knowledge on the further development of the different drugs.

In summary, biochemical markers of bone turnover are very useful for assessing response to treatment, onset and progression of disease pathology, specific cellular activities and especially dynamic information in preclinical models for osteoporosis, and thereby provide stable and easy access to important information in these models. However, there are still limitations with respect to their use in the different models and with respect to the validation of some of the markers, but these are areas undergoing intense investigation and these issues will likely be solved sooner rather than later.

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