Measurement of osteocalcin

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INTRODUCTION

Bone turnover may be assessed by the measurement of enzymes or matrix proteins produced by osteoblasts (which form bone) or osteoclasts (which resorb bone).[1] The introduction of reliable, specific tests for the biochemical markers of bone metabolism would aid in the clinical management of metabolic bone diseases, including osteoporosis.

Osteocalcin, also known as bone Gla protein, is a marker of bone formation. It is a vitamin K- and vitamin D-dependent protein produced by osteoblasts and is the most abundant and most widely studied of the non-collagenous proteins in bone.[2–4]

STRUCTURE OF OSTEOCALCIN

Osteocalcin is a 49-residue (5·8 kDa) polypeptide which is highly conserved between species. In humans the osteocalcin gene is located on chromosome 1 (1q25-q31) and is regulated at the transcriptional level by 1,25-dihydroxy-vitamin D_3.[5]

Osteocalcin is synthesized as an 11 kDa preproosteocalcin of 98 residues. This molecule consists of three parts, a 23-residue signal peptide that is cleaved during translation, a 26-residue propeptide that targets the protein for γ carboxylation and the 49-residue mature protein.[6–7] The mature osteocalcin peptide consists of two anti-parallel α-helical domains (residues 16–25 and 30–41) connected by a β turn (residues 26–29). There are two further β turns and a β-sheet structure at the C-terminal end. The structure is stabilized by a Cys_{23}–Cys_{29} disulphide bond (*see* Fig. 1).[8,9]

γ-CARBOXYLATION OF OSTEOCALCIN

Osteocalcin is one of three known vitamin K-dependent proteins produced by osteoblasts, the other proteins being matrix Gla protein[10] and protein S.[11]

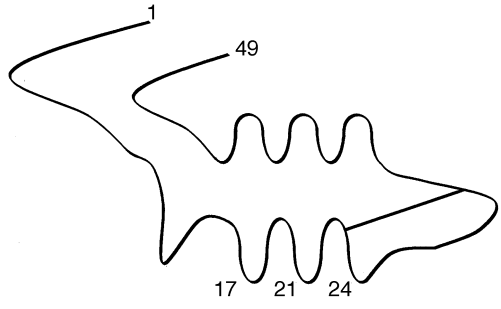

FIGURE 1. *Diagram of the secondary structure of osteocalcin. Gla residues are present at positions 17, 21 and 24 and a disulphide bridge is present between residues 23 and 29.*

Vitamin K_1 or phylloquinone (a lipid-soluble vitamin better known for its association with the coagulation cascade) is an essential co-factor for the post-translational γ-carboxylation of osteocalcin. During carboxylation, a second carboxyl group is added to specific glutamyl residues (Glu) at positions 17, 21 and 24 forming γ-carboxyglutamyl residues (Gla) (*see* Fig. 2). This modification leads to a conformational change, stabilizing the α-helical portion of the protein and conferring a greater affinity for calcium and hydroxyapatite.[9]

There is no homology between the mature osteocalcin protein and the vitamin K-dependent

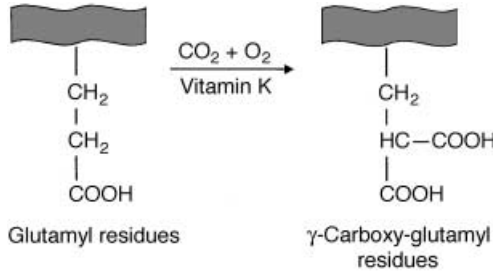

FIGURE 2. *The γ-carboxylation of glutamyl residues. This step is catalysed by the vitamin K-dependent carboxylase with vitamin K as a cofactor.*

This article was prepared at the invitation of the Analytical Investigations Standing Committee of the Association of Clinical Biochemists.

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coagulation factors, but there is a region of homology in the propeptides of these proteins.[12]

SYNTHESIS AND CATABOLISM OF OSTEOCALCIN

Osteocalcin has long been accepted as an osteoblast-specific product.[3,13,14] Recently, however, there have been reports of megakaryocyte and adipocyte expression of osteocalcin mRNA and protein.[15,16] It should be noted that this expression has been reported *in vitro*, in immortalized cell lines, and not *in vivo*.

The actual function of osteocalcin is not known, but the 'osteocalcin knockout' mouse reported by Ducy *et al*.[17] showed no abnormal phenotype until 6 months of age, at which time there was a marked increase in bone formation. This suggests that osteocalcin may have a role in regulation of osteoblast function.

The majority of osteocalcin secreted by the osteoblast is deposited in extracellular bone matrix; serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite.

HETEROGENEITY OF CIRCULATING OSTEOCALCIN

The heterogeneity of circulating osteocalcin was first reported in 1985.[18] Serum osteocalcin has a short half-life and is hydrolysed in the kidney and liver.[19] The C-terminal fragment is easily cleaved and the N-terminal mid-fragment shows greater apparent stability. Residues 19–20 and 43–44 are susceptible to tryptic hydrolysis.[20] It has been proposed that the arginyl–arginyl residues at positions 19–20 are protected from proteolysis by their incorporation in the Gla helix[9] or that this protection is derived from the highly negatively charged γ-carboxyglutamyl residues at positions 17, 21 and 24.[21] The residues at positions 43 and 44, which are highly conserved between species, are probably more labile due to their incorporation in the C-terminal β-sheet.[22] Garnero *et al*.[23] used monoclonal antibodies to identify circulating fragments in healthy subjects and patients with metabolic bone disease (*see* Fig. 3). The intact molecule and the N-terminal mid-fragment were the most abundant immunoreactive forms in normal and osteoporotic subjects. Additionally, they found that there was an increase in the N-terminal mid-fragment in patients with Paget's disease, and concentrations of both intact and fragmented osteocalcin were increased in patients with chronic renal failure. Different metabolic bone diseases may have different characteristic patterns of immunoreactive forms of osteocalcin[24,25] highlighting the possibility of measuring different forms in the clinical investigation of bone pathologies. The detection of intact osteocalcin or fragments is an important issue in the choice of a commercial assay for the clinical laboratory. Assays detecting only intact osteocalcin will be particularly sensitive to *in vitro* degradation, whilst assays detecting fragments may, depending on the fragments recognized, overestimate the concentration of intact osteocalcin.[20] Another difficulty arises from recent studies which suggest that the specificity of commercial assays may differ from that claimed by the manufacturers (*see* Table 1).[26,27]

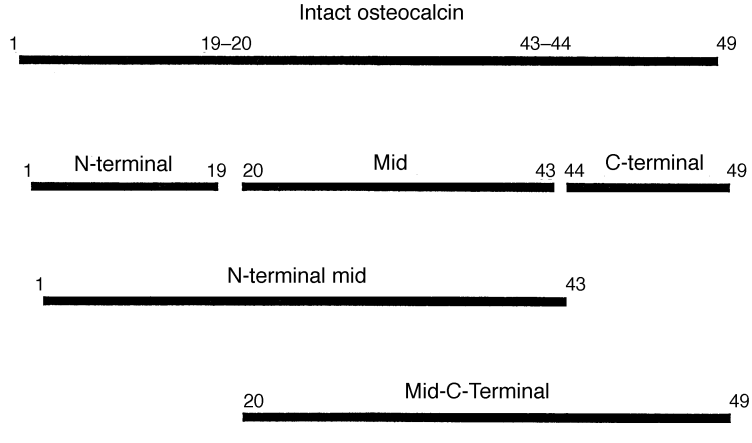

FIGURE 3. *The intact osteocalcin molecule and potential fragments. Reproduced with permission.*[23]

TABLE 1. *Expected tracer specificities compared with actual specificities for four commercially available assays*

	Commercial assays (expected tracer specificities)			
specificity	Incstar (1–49)	CIS ELSA-Osteo-Nat (1–49)	CIS ELSA-Osteo (1–43)	Nichols (1–49, 1–43)
1–49	✓	✓	✓	✓
1–43		✓	✓	✓
1–16		✓	✓	✓
7–19				✓
30–49	✓			✓
37–49	✓			✓

See Appendix for manufacturers' details. Adapted with permission.[26]

CLINICAL RELEVANCE OF SERUM OSTEOCALCIN

Bone markers are classified according to whether they reflect bone formation or resorption. As stated earlier, osteocalcin is a marker of bone formation and in some situations, such as corticosteroid therapy, is considered to be a more sensitive marker than serum alkaline phosphatase activity.[28]

Serum osteocalcin reflects the 10–40% of osteocalcin produced that is not incorporated into the bone matrix.[24] It is postulated that newly synthesized osteocalcin is released into the circulation as the intact (1–49) molecule.[29] Osteocalcin fragments may be derived from bone resorption and catabolism of the molecule *in vivo* before clearance by metalloproteases in the kidneys and the liver.[30] Quantitative bone histomorphometry and combined calcium balance/calcium kinetics studies have validated the use of osteocalcin as a marker of bone formation.[31–34]

In postmenopausal women serum osteocalcin levels correlate significantly with both the bone formation rate and the kinetically determined calcium accretion rate, but not with bone resorption.[31,33]

Postmenopausal osteoporosis

It has been suggested that there are discrete groups of postmenopausal osteoporotic women, with normal, high or low bone formation.[31] In support of this, osteocalcin concentrations have been reported as similar, higher or lower than normal age-matched controls.[31,35]

An elevation of 10% has been reported in the mean osteocalcin concentration in postmenopausal osteoporosis, in contrast to the greater elevation reported in bone resorption markers, such as deoxypyridinoline, which is increased by 50%.[33] Serum osteocalcin concentrations correlate with the rate of bone loss from the distal forearm.[36] However, the overlap of results is too great for the diagnosis of osteoporosis in individuals and the scatter around the regression line is too great to identify individuals with accelerated bone loss.[37] Monitoring osteocalcin concentration may be useful in determining the response to treatment for metabolic bone diseases or the prediction of bone loss in postmenopausal women.[31,38–40]

Osteomalacia

In osteomalacia, serum osteocalcin correlates with parameters of osteoid formation and is significantly elevated. This is due to secondary hyperparathyroidism and to the defect in mineralization in osteomalacia, which prevents the incorporation of *de novo* osteocalcin into the bone and results in raised serum concentration.[41]

Hyper- and hypoparathyroidism

Circulating osteocalcin is increased in hyperparathyroidism and decreased in hypoparathyroidism.[42] Osteocalcin concentration correlates with parathyroid hormone concentration better than does serum alkaline phosphatase activity and urinary hydroxyproline:creatinine ratio.[43]

Paget's disease of bone

In Paget's disease, serum osteocalcin concentrations are significantly raised. However, the magnitude of this is smaller than for other markers of bone formation such as bone alkaline phosphatase. The reason for this discrepancy is not known, but it could relate to the greater affinity of osteocalcin for the woven bone of Paget's disease.[39,41,44–46]

TABLE 2. *Effect of medical conditions on circulating osteocalcin concentration*

Condition	Effect on circulating osteocalcin	References	Other information
Multiple myeloma	Decrease	47	Correlates with disease stage
Bone metastases	No change	48, 49, 50	
Hypercalcaemia of malignancy	Decrease	48, 50	
Liver cirrhosis	Decrease	51, 52	Not a consistent finding
Hyperthyroidism	Increase	53, 54	Decreases with treatment
Fracture	Increase	55, 56, 57	For up to 1 year

Other conditions

The effects of other pathological conditions on osteocalcin are listed in Table 2.

Therapeutic agents

Some therapeutic agents affect circulating concentrations of osteocalcin. 1,25-dihydroxyvitamin D_3 stimulates osteocalcin production.[35] A calcitriol stimulation test has been proposed by Duda *et al.*,[58] in which 2 μg calcitriol per day is given orally and the osteocalcin response at 7 days is used to assess osteoblast responsiveness. Glucocorticoids, on the other hand, decrease serum osteocalcin within a day of starting therapy and the effect is dose-dependent.[59–64] Low osteocalcin concentration has been used to identify patients with adrenal insufficiency given excessive replacement doses.[65] It has been suggested that the increase in osteocalcin concentrations seen in anticonvulsant therapy may reflect an increase in bone turnover, which may contribute to osteopoenia, a complication of anticonvulsant therapy in children.[66]

Circulating osteocalcin is decreased in patients receiving heparin[67] and some, but not all, studies of warfarin-treated patients also show a decrease in osteocalcin.[68,69] Coumarins such as warfarin are considered further in the discussion of undercarboxylated osteocalcin below.

UNDERCARBOXYLATED OSTEOCALCIN

Low concentrations of serum vitamin K_1 (phylloquinone) and vitamin K_2 (the menaquinones) have been associated with bone fragility and increased risk of fracture.[70–73] Osteocalcin is a vitamin K-dependent protein and the percentage of osteocalcin that has not undergone modification by the vitamin K-dependent carboxylase has recently been the focus of much attention.

Concentration of undercarboxylated osteocalcin is significantly increased with age,[74] but can be normalized by vitamin K supplementation.[75,76] In addition, undercarboxylated osteocalcin correlates with bone mineral density at the femoral neck.[77] Furthermore, the fraction of undercarboxylated osteocalcin has been suggested to be a predictor of hip fracture in elderly women.[78,79] Early studies of undercarboxylated osteocalcin were criticized because the populations studied were predominantly institutionalized women, who may have had inadequate nutrition, a recognized factor in osteoporosis in the elderly.[80,81] This issue has recently been addressed and a recent report confirms undercarboxylated osteocalcin as an independent predictor of hip fracture in a population of ambulatory elderly women, 90% of whom were free-living.[82] Total osteocalcin concentration is not predictive of hip fracture, suggesting that the association between undercarboxylated osteocalcin and hip fracture is not due to increased bone formation. This may indicate that undercarboxylated osteocalcin is an indicator of poor bone quality rather than an indicator of bone turnover. Another suggestion that requires further investigation is that undercarboxylated osteocalcin may inhibit osteoblast function to a greater degree than the carboxylated form of the protein.[83]

Warfarin and other coumarin derivatives are used clinically as anticoagulants. These drugs inhibit the vitamin K-dependent carboxylase, thus preventing the post-translational modification of the vitamin K-dependent clotting factors produced by the liver. The carboxylation of osteocalcin is impaired in warfarin-treated patients,[84] i.e. the percentage of undercarboxylated osteocalcin is higher in these subjects, and this may have implications for their bone health.

HISTORICAL PERSPECTIVE OF OSTEOCALCIN ASSAYS

Many immunoassays have been developed to detect human osteocalcin. Bovine osteocalcin is more abundant and more stable than human osteocalcin. As osteocalcin is highly conserved

between species, many assays are directed against bovine osteocalcin.

In 1980 Price *et al.* described the first assay for osteocalcin.[85] This assay employed a rabbit antibody directed against calf osteocalcin to detect human osteocalcin in plasma, with a sensitivity of 0·1 ng.

By 1985 there were several methods for the determination of osteocalcin. Gundberg *et al.* compared two commercial kits with an in-house assay and showed a good correlation between methods when the assay standard and normal ranges were used.[18] This was the first report to draw attention to differences between methods and to suggest that one assay cannot be substituted for another. In 1986 Tanaka *et al.* reported the first enzyme immunoassay (EIA) developed for osteocalcin.[86] This assay was based on competition of 'sample' osteocalcin and β-galactosidase-labelled bovine osteocalcin for a rabbit polyclonal anti-osteocalcin antibody. It gave a good recovery and correlated well with radioimmunoassay (RIA).

There are now several commercial immunoassays available in RIA, immunoradiometric assay (IRMA) and enzyme immunoassay (ELISA) formats for the detection of total (fully and under-carboxylated) osteocalcin. Some of these assays are described in more detail below.

The carboxylation of osteocalcin confers a greater affinity for hydroxyapatite, and Price *et al.* demonstrated that undercarboxylated osteocalcin can be measured by an indirect method based on this property.[87] Typically, total osteocalcin is measured by immunoassay followed by incubation with hydroxyapatite and centrifugation. The supernatant, which contains osteocalcin that has not adsorbed to hydroxyapatite (i.e. undercarboxylated osteocalcin) is then measured using the same immunoassay. The results of this procedure can be expressed either as absolute concentrations or as a ratio of undercarboxylated to carboxylated osteocalcin. The method is still susceptible to problems associated with osteocalcin assays and non-specific binding to hydroxyapatite may occur,[82] but it has been utilized in many studies and is well characterized. Barium sulphate has been proposed as an alternative to hydroxyapatite for this procedure.[88]

Recently, monoclonal antibodies have been developed which are claimed to be specific for undercarboxylated osteocalcin.[82,89] Vergnaud *et al.* reported a significant correlation ($r = 0{\cdot}82$) between results obtained with an ELISA based on these antibodies and the hydroxyapatite method and a low (5%) rate of cross-reactivity of the undercarboxylated osteocalcin specific antibodies with carboxylated osteocalcin.[82,89] A commercial immunoassay based on monoclonal antibodies, for the measurement of undercarboxylated osteocalcin, is now available (*see* Table 4).

These are exciting developments that will allow the measurement of undercarboxylated osteocalcin without use of the cumbersome and time-consuming hydroxyapatite method. However, these assays have not been fully characterized and further work will be required before they are accepted for widespread use.

There is no definitive or reference method for the measurement of either total or undercarboxylated osteocalcin in serum or plasma.

COMMERCIAL ASSAYS FOR OSTEOCALCIN

There are many commercial assays available for the measurement of osteocalcin. A selection, together with their performance characteristics, is shown in Tables 3 and 4.

STANDARDIZATION OF METHODS

Several studies have compared commercial assays; all have found a marked difference between methods irrespective of whether they were commercial kits or in-house assays.

In 1985 Gundberg *et al.* demonstrated that three different assays gave widely differing results for identical samples; nevertheless the results were consistent when the reference ranges for the different assays were taken into account.[18] In 1990 Delmas and co-workers[28] highlighted the poor comparability of osteocalcin assays in a series of blinded lyophilized serum samples and a lyophilized reference standard analysed by eight in-house assays and two well-characterized commercial assays (*see* Fig. 4). Different assays were found to have different absolute concentrations of osteocalcin, sometimes differing by up to 50%. However, all of the assays gave acceptable results when compared by assay-to-assay correlations and when the results were expressed as a percentage of the mean of two controls.[28] A similar analysis of commercially available assays, which used Z scores (Z score = sample osteocalcin − assay mean osteocalcin/SD) to compare the different kits, found that this procedure reduced the differences between results.[90] The divergent results from different assays arise from several

TABLE 3. *Radiolabelled commercial assays available for the detection of osteocalcin*

Assay	Supplier	Method	Fragments detected*	Volume	Sample	Approximate assay time	Notes
CIS ELSA-Osteo-Nat	CIS Bio Int	IRMA	N-terminal mid (1–43)	50 μL	P, S	4 h plus counting time	Not citrated plasma
CIS ELSA-Osteo	CIS Bio Int	IRMA	Intact (1–49)	50 μL	P, S	4 h plus counting time	Not citrated plasma
CIS Ostk-PR	CIS Bio Int	RIA	Not specified	50 μL	P, S	2 days	Not citrated/heparin plasma
N-tact® Osteo SP	Diasorin	IRMA	Intact (1–49)	20 μL	S	2 h plus counting time	
Osteocalcin ^{125}I RIA Kit	Diasorin	RIA	Intact (1–49) N-terminal mid (1–43)	50 μL	P, S	2 days	Not EDTA plasma
BTI Human Osteocalcin RIA Kit	IDS Ltd	RIA	Not specified	50 μL	P, S	4 h plus counting time	Not citrated/EDTA plasma
Medgenix h-OST IRMA	Lifescreen Ltd	IRMA	Intact (1–49)	50 μL	P, S	4 h plus counting time	Not heparinzed/ citrated plasma
Human Osteocalcin Kit	Nichols Institute	IRMA	Intact (1–49) N-terminal mid (1–43)	10 μL	P, S	5 h plus counting time	Not citrated plasma

(b)

Assay	Supplier	Intra-assay precision (%CV)	Inter-assay precision (%CV)	Recovery (%)	Limit of detection (μg/L)
CIS ELSA-Osteo-Nat	CIS Bio Int	3·5–3·9	3·7–4·2	90–110	0·3
CIS ELSA-Osteo	CIS Bio Int	3·8–3·9	4·5–5·2	95–105	0·4
CIS Ostk-PR	CIS Bio Int	3·0–3·7	5·5–6·6	95–105	0·5
N-tact Osteo SP	Diasorin	7·1–9·0	4·5–6·3	78–101	0·2
Osteocalcin^{125}I RIA Kit	Diasorin	Not given	Not given	Not given	0·2
BTI Human Osteocalcin RIA Kit	IDS Ltd	Not given	Not given	Not given	1·0
Medgenix h-OST IRMA	Lifescreen Ltd	2·9–4·7	5·3–6·3	86·1–95·5	0·15
Human Osteocalcin Kit (IRMA)	Nichols Institute	3·9–5·2	5·5–6·7	91–108	0·05

See Appendix for manufacturers' details. *Manufacturers' claims for detection of fragments of osteocalcin; *see* Table 1.
P = plasma; S = serum; IRMA = immunoradiometric assay; RIA = radioimmunoassay; %CV = % coefficient of variation; not given = information unavailable from manufacturer.

TABLE 4. *Enzyme-labelled commercial assays available for the detection of osteocalcin*

(a)

Assay	Supplier	Method	Fragments detected*	Volume	Sample	Approximate assay time	Notes
BTI Intact Human Osteocalcin EIA	IDS Ltd	ELISA	Intact (1–49)	25 μL	S, P	5 h	
BTI Mid-Tact Human Osteocalcin EIA	IDS Ltd	ELISA	Intact (1–49) N-terminal mid (1–43)	25 μL	S, P	5 h	
Metra Novocalcin	Metra Biosystems	ELISA	Intact (1–49)	25 μL	S, P	5 h	Not EDTA plasma
Human Osteocalcin Kit (CLA)	Nichols Institute	CLA	Intact (1–49) N-terminal mid (1–43)	10 μL	S, P	4 h plus reading time	Not citrated plasma
Undercarboxylated Osteocalcin (Glu-OC) EIA Kit	Takara Biomedicals	ELISA	Undercarboxylated osteocalcin	100 μL	S, U	4 h	Not EDTA plasma

(b)

Assay	Supplier	Intra-assay precision (%CV)	Inter-assay precision (%CV)	Recovery (%)	Limit of detection (μg/L)
BTI Intact Human Osteocalcin EIA	IDS Ltd	7·0	10·5	Not given	0·5
BTI Mid-Tact Human Osteocalcin EIA	IDS Ltd	7·0	10	Not given	0·5
Metra Novocalcin	Meta Biosystems	4·8–10	4·8–9·8	Not given	0·45
Human Osteocalcin Kit (CLA)	Nichols Institute	3·9–4·5	5·5–5·9	96–108	0·04
Undercarboxylated Osteocalcin (Glu-OC) EIA Kit	Takara Biomedicals	4·58–6·66	5·67–9·87	71–124	Not quoted

See Appendix for manufacturers' details. *Manufacturer's claims for detection of fragments of osteocalcin; *see* Table 1.
EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; S = serum; P = plasma; CLA = chemiluminescence assay; U = urine; not given = information unavailable from manufactuer.

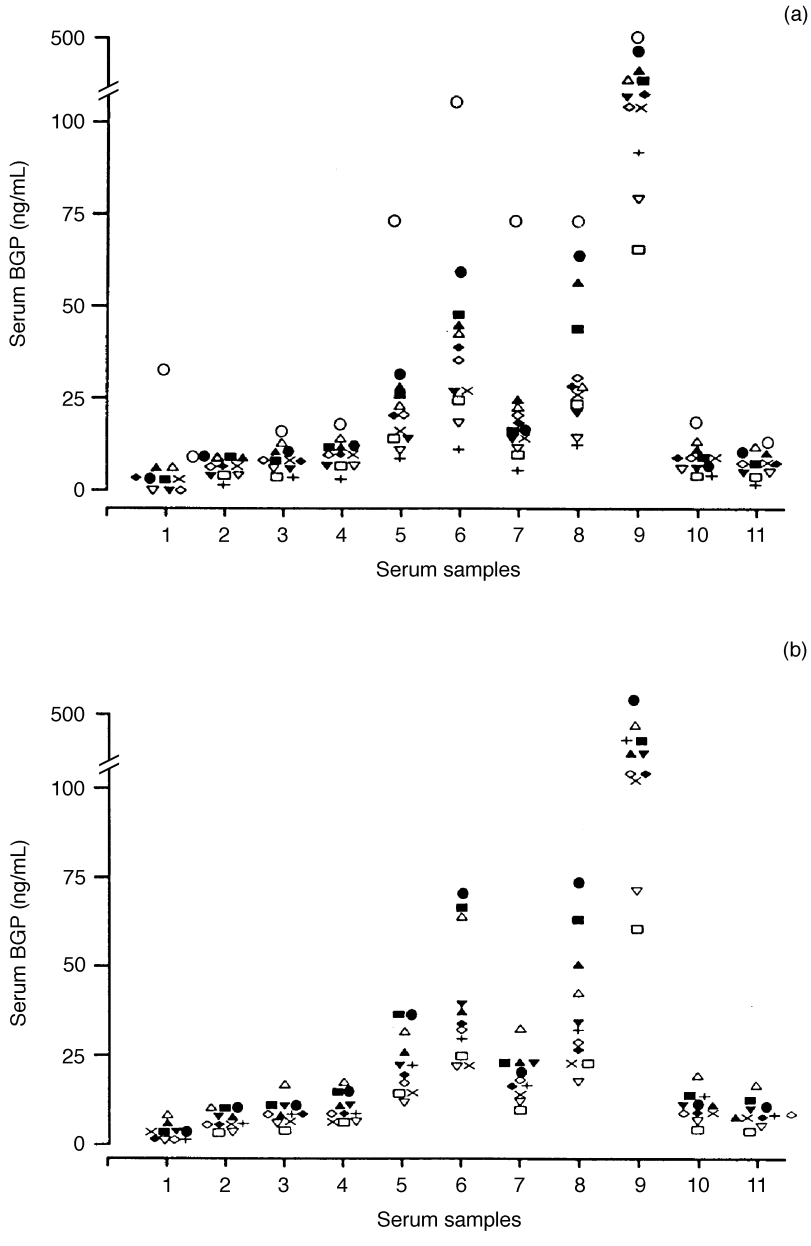

FIGURE 4. *Raw values of serum osteocalcin (bone Gla protein) concentration in 11 serum samples measured with differing assays (represented by different symbols). Symbols represent different assays. (a) Osteocalcin values obtained with the in-house osteocalcin standard curve. (b) Osteocalcin values obtained with the osteocalcin reference standard curve. Reproduced with permission.*[28]

factors, including cross-reactivity of the antibodies to other molecules, especially at lower concentrations of the analyte, differences in immunorecognition of fragments and differences in standards.[20,24]

SAMPLE STABILITY AND HANDLING

Osteocalcin is markedly unstable *in vitro*,[23,91] rapidly degrading in samples at room temperature and at 4°C.[20,92] Garnero *et al.* reported an *in vitro* degradation of 17% of intact osteocalcin

over 2 h incubation at room temperature.[23] This group also described at 4% *in vitro* degradation of intact and N-terminal mid-fragment after 2-h incubation at room temperature. This instability may occur in samples, standards or tracer and it has been suggested that there may even be degradation of these during the assay procedure itself.[24]

Protease inhibitors may reduce deterioration of osteocalcin in samples,[23,93] but it is recommended that samples are kept at 4°C immediately upon venepuncture, serum or plasma separated as soon as possible, and samples frozen until assay. For short-term storage e.g. 1 month, freezing at −20°C will suffice,[24] but for long-term storage it is advisable to store samples at −70°C (*see* Fig. 5).

Osteocalcin is sensitive to freeze–thaw cycles and haemolysis. Repeated freeze–thaw cycles have been shown to reduce immunoreactivity by up to 40%.[18,20,94] Haemolysis decreases apparent osteocalcin concentrations in both in-house and commercial assays, possibly owing to proteolysis by enzymes released from lysed red blood cells.[95]

Most commercial assays measure osteocalcin in either serum or plasma, but the anticoagulant used in plasma samples may affect the results obtained. In particular, potassium oxalate/sodium fluoride should be avoided.[94] Furthermore, some commercial assays may also be sensitive to calcium ions. Unfortunately, the manufacturers do not make available information on the calcium content of their buffers.[90] This has clear implications for samples from patients with differing clinical conditions and samples collected using EDTA or other calcium chelators.

In this laboratory, samples for osteocalcin measurement are separated within 1 h of venepuncture and immediately frozen at −20°C. For long-term storage they are then transferred to a freezer at −70°C.

QUALITY CONTROL

The National Supraregional Assay and Advisory Service in England measures biochemical markers of bone metabolism. An External Quality Assessment Programme (EQAS) has been initiated to monitor the performance of these and other laboratories that offer assays of biochemical markers of bone metabolism. Osteocalcin will be included in the scheme in 1999.[96]

The lack of international standards for osteocalcin has hindered progress in the standardization of commercial assays and at present there is no consensus among laboratories concerning the fragment(s) of osteocalcin that are of greatest clinical use.

BIOLOGICAL VARIABILITY

Serum osteocalcin has been shown to have a large diurnal variation (peaking in the early morning at approximately 0400 h).[35,95,97] Samples for osteocalcin determination should be collected at the same time of day if comparisons between serial samples are required. There is also a seasonal variation, with concentrations declining from January to July and then increasing to a peak in winter.[98,99] During the menstrual cycle osteocalcin reaches its highest levels during the luteal phase.[40]

Serum osteocalcin concentrations are higher in children than in adults, rising during puberty and then falling to adult levels.[100–102] Most studies show a higher concentration of osteocalcin in adult males than in adult females and show an age-related increase after the fifth decade of life in women.[42,103,104] The postmenopausal rise in serum osteocalcin can be reversed by hormone replacement therapy.[35,105]

REFERENCE RANGES

The majority of commercial kits supply reference ranges for serum osteocalcin. However, many of these data do not reflect the populations in which osteocalcin is most likely to be measured, such as postmenopausal women or children, and have been derived from a small sample of subjects. It is recommended that, once a laboratory has decided on which method to use, a local reference range is determined in the clinical population for which the assay is likely to be requested.

PROBLEMS WITH ASSAYS AND DIFFERENCES BETWEEN METHODS

In considering which method to employ there are certain factors that need to be considered. Firstly, a decision is needed regarding the use of radiolabelled or enzyme-labelled assays. For the majority of clinical chemistry laboratories, systems will be in place for safe working practices with radiation, but this incurs extra training costs, etc.

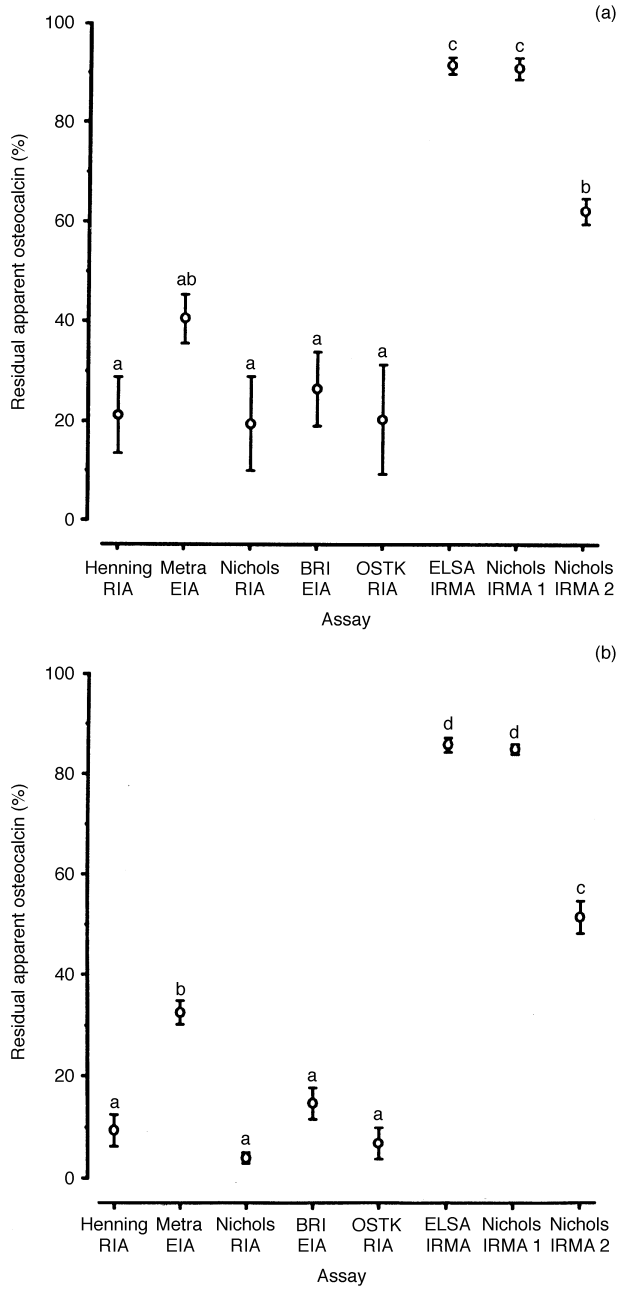

FIGURE 5. *Effect of sample storage at 4°C for 2 weeks (a) and at 4°C for 4 weeks (b) on the concentration of osteocalcin measured by eight different assays. Results are expressed as mean + standard error of the mean, relative to control samples stored at −75°C. Symbols without a common letter are significantly different (*$P < 0{\cdot}05$*, Scheffe test). Overall significance of the differences between methods by analysis of variance was* $\leqslant 0{\cdot}0001$ *for both storage conditions. RIA = radioimmunoassay; EIA = enzyme immunoassay; IRMA = immunoradiometric assay; Metra EIA = Metra Novocalcin; Nichols RIA = Nichols osteocalcin RIA; BRI RIA = BRI Oc Assay; OSTK RIA = CIS Ostk-PR; ELSA IRMA = CIS ELSA-Osteo; Nichols IRMA 1 = Nichols Institute OC IRMA Version 1; Nichols IRMA 2 = Nichols OC IRMA Version 2. See Appendix for manufacturers' details. Copyright © 1995 The American Association for Clinical Chemistry, Inc. Reproduced with permission.*[24]

The clinical interpretation of osteocalcin measurements relies, to a certain extent, upon methodological differences, but there is no consensus regarding the most clinically informative fragment(s) of osteocalcin to measure. This may depend on the patient groups for which the assay is requested. As stated earlier, there is no standardization of methods at present. It is strongly recommended that when a laboratory has decided on the assay most suited to its needs, a local reference range should be determined.

CONCLUSION

There is increasing demand for clinically relevant biochemical markers of bone health. Osteocalcin is one of the best characterized of the markers of bone formation. However, as this review indicates, it is unclear which assay system provides results with the greatest clinical utility.

The application of osteocalcin assays to routine clinical practice has been hampered by poor comparability between different methods and the instability and heterogeneity of circulating osteocalcin.[20,28]

Osteocalcin assays are available in many formats using both polyclonal and monoclonal antibodies and different standards. Given this, it is not surprising that osteocalcin values determined by different assays give discordant results. The natural outcome of the research reported in the sections of this review on sample stability and biological variability is an absolute need for continuity in the methodology for sample collection. This includes the avoidance of haemolysis and freeze–thaw cycles.

The poor comparability of methods and problems with sample handling are compounded by a lack of consensus regarding the most clinically informative fragment(s) of osteocalcin. We suggest that this will depend on the patient group for which the assay is likely to be requested.

The establishment of a Quality Assurance scheme will aid considerably the standardization of methodologies. However, until there is consensus on which fragment(s) of osteocalcin to measure and the introduction of national or international standards, it is recommended that laboratories determine a local reference range with the system of their choice and establish standard operating procedures for continuity in the measurement of osteocalcin.

APPENDIX

Manufacturers' details

BTI Human Osteocalcin RIA Kit, BTI Intact Human Osteocalcin EIA, BTI Mid-Tact Human Osteocalcin EIA: IDS Ltd, Tyne and Wear, UK

BRI Oc Assay: BRI-Diagnostics, Dublin, Ireland

CIS ELSA-Osteo-Nat, CIS ELSA-Osteo, CIS Ostk-PR: CIS Bio International, Gif-sur-Yvette, France

Medgenix h-OST IRMA: Lifescreen Ltd, Hertfordshire, UK

Metra Novocalcin: Metra Biosystems, Mountain View CA, USA

Human Osteocalcin Kit, Human Osteocalcin Kit (CLA), Human Osteocalcin Kit (IRMA), Nichols Institute OC IRMA Versions 1 and 2, Nichols Osteocalcin RIA: Nichols Institute, San Juan Capistrano, California, USA

N-tact® Osteo SP, Osteocalcin ^{125}I RIA Kit, Incstar: Diasorin, Berkshire, UK

OSCAtest: Henning, Berlin, Germany

Undercarboxylated Osteocalcin (Glu-OC) EIA Kit: Takara Biomedicals, Takara Shuzo, Japan

REFERENCES

1 Eastell R, Blumsohn A. The value of biochemical markers of bone turnover in osteoporosis. *J Rheumatol* 1997; **24**: 1215–7
2 Price PA, Nishimoto SK. Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. *Proc Natl Acad Sci USA* 1980; **77**: 2234–8
3 Price PA, Parthemore JG, Deftos LJ. New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. *J Clin Invest* 1980; **66**: 878–83
4 Price PA, Baukol SA. 1,25-dihydroxyvitamin D increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. *J Biol Chem* 1980; **255**: 11660–3
5 Puchacz E, Lian JB, Stein GS, Wozney J, Huebner K, Croce C. Chromosomal localization of the human osteocalcin gene. *Endocrinology* 1989; **124**: 2648–50

6 Gundberg CM, Clough ME. The osteocalcin propeptide is not secreted in vivo or in vitro. *J Bone Miner Res* 1992; **7**: 73–80
7 Houben R, Soute BA, Knapen MH, Vermeer C. Strategies for developing human osteocalcin standards: a critical evaluation. *Scand J Clin Lab Invest Suppl* 1997; **227**: 100–4
8 Hauschka PV. Osteocalcin: the vitamin K-dependent Ca^{2+}-binding protein of bone matrix. *Haemostasis* 1986; **16**: 258–72
9 Hauschka PV. Calcium-dependent alpha-helical structure in osteocalcin. *Biochemistry* 1982; **21**: 2538–47
10 Price PA, Urist MR, Otawara Y. Matrix Gla protein, a new gamma carboxyglutamic acid containing protein which is associated with the organic matrix of bone. *Biochem Biophys Res Commun* 1983; **117**: 765–71
11 Maillard C, Berruyer M, Serre CM, Dechavanne M, Delmas PD. Protein-S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. *Endocrinology* 1992; **130**: 1599–604
12 Pan LC, Price PA. The propeptide of rat bone gamma carboxyglutamic acid protein shares homology with other vitamin K-dependent protein precursors. *Proc Natl Acad Sci USA* 1985; **82**: 6109–13
13 Price PA, Poser JW, Raman N. Primary structure of the gamma-carboxyglutamic acid-containing protein from bovine bone. *Proc Natl Acad Sci USA* 1976; **73**: 3374–5
14 Owen TA, Aronow M, Shalhoub V, Barone LE, Wilming L, Tassinari MS, *et al*. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the extracellular matrix. *J Cell Physiol* 1990; **143**: 1241–5
15 Thiede MA, Smock SL, Petersen DN, Grasser WA, Thompson DD, Nishimoto SK. Presence of messenger ribonucleic acid encoding osteocalcin, a marker of bone turnover, in bone marrow megakaryocytes and peripheral blood platelets. *Endocrinology* 1994; **135**: 929–37
16 Benyahu D, Shamay A, Wientroub S. Osteocalcin (BGP) gene expression, and protein production by marrow stromal adipocytes. *Biochem Biophys Res Commun* 1997; **231**: 442–6
17 Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, *et al*. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; **382**: 448–52
18 Gundberg CM, Wilson PS, Gallop PM, Parfitt AM. Determination of osteocalcin in human serum: results with two kits compared with those by a well-characterized assay. *Clin Chem* 1985; **31**: 1720–3
19 Farrugia W, Melick RA. Metabolism of osteocalcin. *Calc Tiss Internat* 1986; **39**: 234–8
20 Diaz DE, Guerrero R, de la Piedra C. Six osteocalcin assays compared. *Clin Chem* 1994; **40**: 2071–7
21 Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum by ELISA with two monoclonal antibodies. *Clin Chem* 1995; **41**: 1439–45
22 Hauschka PV, Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. *Physiol Rev* 1989; **69**: 990–1047
23 Garnero P, Grimaux M, Seguin P, Delmas PD. Characterization of immunoreactive forms of human osteocalcin generated in vivo and in vitro. *J Bone Miner Res* 1994; **9**: 255–64
24 Blumsohn A, Hannon RA, Eastell R. Apparent instability of osteocalcin in serum as measured with different commercially available immunoassays. *Clin Chem* 1995; **41**: 318–9
25 Chen JT, Hosoda K, Hasumi K, Ogata E, Shiraki M. Serum N-terminal osteocalcin is a good indicator for estimating responders to hormone replacement therapy in postmenopausal women. *J Bone Miner Res* 1996; **11**: 1784–92
26 Colford J, Sailer D, Langman C. Five osteocalcin assays compared: tracer specificity, fragment interference and calibration. *Clin Chem* 1997; **43**: 1240–1
27 Souberbielle JC, Marque D, Bonnet P, Herviaux P, Sachs C. Simple method to evaluate specificity of osteocalcin immunoassays. *Clin Chem* 1997; **43**: 1663–4
28 Delmas PD, Christiansen C, Mann KG, Price PA. Bone Gla protein (osteocalcin) assay standardization report. *J Bone Miner Res* 1990; **5**: 5–11
29 Diaz DE, Nacher M, Rapado A, Serrano S, Bosch J, Aubia J, *et al*. Immunoreactive osteocalcin forms in conditioned media from human osteoblast culture and in sera from healthy adult control subjects and patients with bone pathologies. *Eur J Clin Invest* 1998; **28**: 48–58
30 Baumgrass R, Williamson MK, Price PA. Identification of peptide fragments generated by digestion of bovine and human osteocalcin with the lysosomal proteinases cathepsin B, D, L, H, and S. *J Bone Miner Res* 1997; **12**: 447–55
31 Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ. Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. *Lancet* 1984; **i**: 1091–4
32 Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL. Serum bone gla-protein compared to bone histomorphometry in endocrine diseases. *Bone* 1985; **6**: 339–41
33 Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL. Bone formation rate in older normal women: concurrent assessment with bone histomorphometry, calcium kinetics, and biochemical markers. *J Clin Endocrinol Metab* 1988; **67**: 741–8
34 Charles P, Poser JW, Mosekilde L, Jensen FT. Estimation of bone turnover evaluated by 47 Ca-Kinetics. Efficiency of serum bone gamma-carboxygluatmic acid-containing protein, serum alkaline phosphatase, and urinary hydroxyproline excretion. *J Clin Invest* 1985; **76**: 2254–8
35 Markowitz ME, Gundberg CM, Rosen JF. The circadian rhythm of serum osteocalcin concentrations: effects of 1,25 dihydroxyvitamin D administration. *Calcif Tissue Int* 1987; **40**: 179–83

36 Christiansen P, Steiniche T, Brixen K, Hessov I, Melsen F, Charles P, *et al*. Primary hyperparathyroidism: biochemical markers and bone mineral density at multiple skeletal sites in Danish patients. *Bone* 1997; **21**: 93–9
37 Blumsohn A, Eastell R. Prediction of bone loss in postmenopausal women. *Eur J Clin Invest* 1992; **22**: 764–6
38 Eastell R, Robins SP, Colwell T, Assiri AM, Riggs BL, Russell RG. Evaluation of bone turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption. *Osteoporos Intl* 1993; **3**: 255–60
39 Deftos LL, Parthemore JG, Price PA. Changes in plasma bone gla protein during treatment of bone disease. *Calcif Tissue Int* 1982; **34**: 121–4
40 Nielsen HK, Brixen K, Bouillon R, Mosekilde L. Changes in biochemical markers of osteoblastic activity during the menstrual cycle. *J Clin Endocrinol Metab* 1990; **70**: 1431–7
41 Hannon R, Eastell R. Biochemical markers of bone turnover. In: Tovey FI, Stamp TCB, eds. *The Measurement of Metabolic Bone Disease*. London: Parthenon Publishing Group, 1995: 21–47
42 Duda RJ Jr, O'Brien JF, Katzmann JA, Peterson JM, Mann KG, Riggs BL. Concurrent assays of circulating bone Gla-protein and bone alkaline phosphatase: effects of sex, age, and metabolic bone disease. *J Clin Endocrinol Metab* 1988; **66**: 951–7
43 Minisola S, Scanecchia L, Carnevale V, Bigi F, Romagnoli E, Pacitti MT, *et al*. Clinical value of the measurement of bone remodelling markers in primary hyperparathyroidism. *J Endocrinol Invest* 1989; **12**: 537–42
44 Delmas PD, Demiaux B, Malaval L, Chapuy MC, Meunier PJ. Serum bone gla-protein is not a sensitive marker of bone turnover in Paget's disease of bone. *Calcif Tissue Int* 1986; **38**: 60–1
45 Wilkinson MR, Wagstaffe C, Delbridge L, Wiseman J, Posen S. Serum osteocalcin concentrations in Paget's disease of bone. *Arch Int Med* 1986; **146**: 268–71
46 Ingram RT, Collazo-Clavell M, Tiegs R, Fitzpatrick LA. Paget's disease is associated with changes in the immunohistochemical distribution of non-collagenous matrix proteins in bone. *J Clin Endocrinol Metab* 1996; **81**: 1810–20
47 Carlson K, Ljunghall S, Simonsson B, Smedmyr B. Serum osteocalcin concentrations in patients with multiple myeloma – correlation with disease stage and survival. *J Int Med* 1992; **231**: 133–7
48 Delmas PD, Demiaux B, Malaval L, Chapuy MC, Edouard C, Meunier PJ. Serum bone carboxyglutamic acid-containing protein in primary hyperparathyroidism and in malignant hypercalcaemia. *J Clin Invest* 1986; **77**: 985–91
49 Vinholes J, Guo CY, Purohit OP, Eastell R, Coleman RE. Metabolic effects of pamidronate in patients with metastatic bone disease. *Br J Cancer* 1996; **73**: 1089–95
50 Vinholes JJ, Guo CY, Purohit OP, Eastell R, Coleman RE. Evaluation of new bone resorption markers in a randomized comparison of pamidronate or clodronate for hypercalcemia of malignancy. *J Clin Oncol* 1997; **15**: 131–8
51 Pietschmann P, Resch H, Muller CH, Woloszczuk W, Wilsvonseder R. Decreased serum osteocalcin levels in patients with liver cirrhosis. *Bone Miner* 1990; **8**: 103–8
52 Hodgson SF, Dickson ER, Eastell R, Eriksen EF, Bryant SC, Riggs BL. Rates of cancellous bone remodeling and turnover in osteopenia associated with primary biliary cirrhosis. *Bone* 1993; **14**: 819–27
53 Lukert BP, Higgins JC, Stoskopf MM. Serum osteocalcin is increased in patients with hyperthyroidism and decreased in patients receiving glucocorticoids. *J Clin Endocrinol Metab* 1986; **62**: 1056–8
54 Guo CY, Weetman AP, Eastell R. Longitudinal changes of bone mineral density and bone turnover in postmenopausal women on thyroxine. *Clin Endocrinol* 1997; **46**: 301–7
55 Ingle BM, Hay SM, Bottjer HM, Eastell R. Changes in bone mass and bone turnover following distal forearm fracture. *Osteoporosis Int* 1999; **10**: 399–407
56 Ingle BM, Hay SM, Bottjer HM, Eastell R. Changes in bone mass and bone turnover following ankle fracture. *Osteoporosis Int* 1999; **10**: 408–415
57 Obrant K, Merle B, Bejui J, Delmas PD. Serum bone-gla protein after fracture. *Clin Orthop* 1990; **258**: 300–3
58 Duda RJJ, Kumar R, Nelson KI, Zinsmeister AR, Mann KG, Riggs BL. 1,25-Dihydroxyvitamin D stimulation test for osteoblast function in normal and osteoporotic postmenopausal women. *J Clin Invest* 1987; **79**: 1249–53
59 Nielsen HK, Charles P, Mosekilde L. The effect of single oral doses of prednisone on the circadian rhythm of serum osteocalcin in normal subjects. *J Clin Endocrinol Metab* 1988; **67**: 1025–9
60 Nielsen HK, Brixen K, Kassem M, Mosekilde L. Acute effect of 1,25-dihydroxyvitamin D3, prednisone, and 1,25-dihydroxyvitamin D3 plus prednisone on serum osteocalcin in normal individuals. *J Bone Miner Res* 1991; **6**: 435–41
61 Struijs A, Mulder H. The effects of inhaled glucocorticoids on bone mass and biochemical markers of bone homeostasis: a 1-year study of beclomethasone versus budesonide. *Neth J Med* 1997; **50**: 233–7
62 Lane SJ, Vaja S, Swaminathan R, Lee TH. Effects of prednisolone on bone turnover in patients with corticosteroid resistant asthma. *Clin Exp Allergy* 1996; **26**: 1197–201
63 Godschalk MF, Downs RW. Effect of short-term glucocorticoids on serum osteocalcin in healthy young men. *J Bone Miner Res* 1988; **3**: 113–5
64 Kotowicz MA, Hall S, Hunder GG, Cedel SL, Mann KG, Riggs BL. Relationship of glucocorticoid dosage to serum bone Gla-protein concentration in patients with rheumatologic disorders. *Arthritis Rheum* 1990; **33**: 1487–92
65 Peacey SR, Guo CY, Robinson AM, Price A, Giles MA, Eastell R, *et al*. Glucocorticoid replacement therapy: are patients over treated and does it matter? *Clin Endocrinol (Oxf)* 1997; **46**: 255–61

66 Takeshita N, Seino Y, Ishida H, Tanaka H, Tsutsumi C, Ogata K, *et al*. Increased circulating levels of gamma-carboxyglutamic acid-containing protein and decreased bone mass in children on anticonvulsant therapy. *Calcif Tissue Int* 1989; **44**: 80–5
67 Cantini F, Niccoli L, Bellandi F, Di Munno O. Effects of short-term, high dose, heparin therapy on biochemical markers of bone metabolism. *Clin Rheumatol* 1995; **14**: 663–6
68 van Haarlem LJ, Knapen MH, Hamulyak K, Vermeer C. Circulating osteocalcin during oral anticoagulant therapy. *Thromb Haemost* 1988; **60**: 79–82
69 Jamal SA, Browner WS, Bauer DC, Cummings SR. Warfarin use and risk for osteoporosis in elderly women. Study of Osteoporotic Fractures Research Group. *Ann Intern Med* 1998; **128**: 829–32
70 Hart JP, Shearer MJ, Kenerman L, Catterall A, Reeve J, Sambrook PN, *et al*. Electrochemical detection of depressed circulating levels of vitamin K1 in osteoporosis. *J Clin Endocrinol Metab* 1985; **60**: 1268–9
71 Hodges SJ, Pilkington MJ, Stamp TCB, Catterall A, Shearer MJ, Bitensky L, *et al*. Depressed levels of circulating menaquinones in patients with osteoporotic fractures of the spine and femoral neck. *Bone* 1991; **12**: 387–9
72 Hodges SJ, Pilkington MJ, Shearer MJ, Bitensky L, Chayen J. Age-related changes in the circulating levels of congeners of vitamin K_2, menaquinone-7 and menaquinone-8. *Clin Sci* 1990; **78**: 63–6
73 Hodges SJ, Akesson K, Vergnaud P, Obrant K, Delmas PD. Circulating levels of vitamins K_1 and K_2 decreased in elderly women with hip fracture. *J Bone Miner Res* 1993; **8**: 1241–5
74 Plantalech L, Guillaumont M, Vergnaud P, Leclercq M, Delmas PD. Impairment of gamma carboxylation of circulating osteocalcin (bone gla protein) in elderly women. *J Bone Miner Res* 1991; **6**: 1211–6
75 Knapen MHJ, Hamulyak K, Vermeer C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla protein) and urinary calcium excretion. *Ann Intern Med* 1989; **111**: 1001–5
76 Knapen MH, Jie KS, Hamulyak K, Vermeer C. Vitamin K-induced changes in markers for osteoblast activity and urinary calcium loss. *Calcif Tissue Int* 1993; **53**: 81–5
77 Szulc P, Arlot M, Chapuy MC, Duboeuf F, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J Bone Miner Res* 1994; **9**: 1591–5
78 Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest* 1993; **91**: 1769–74
79 Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. *Bone* 1996; **18**: 487–8
80 Older MWJ, Delyth E, Dickerson JWT. A nutrient survey in elderly women with femoral neck fracture. *Br J Surg* 1980; **67**: 884–6
81 Delmi M, Rapin CH, Bengoa JM, Delmas PD, Vasey H, Bonjour JP. Dietary supplementation in elderly patients with fractured neck of the femur. *Lancet* 1990; **335**: 1016
82 Vergnaud P, Garnero P, Meunier PJ, Breart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: the EPIDOS Study. *J Clin Endocrinol Metab* 1997; **82**: 719–24
83 Seibel MJ. Editorial: Serum undercarboxylated osteocalcin and the risk of hip fracture. *J Clin Endocrinol Metab* 1999; **82**: 717–8
84 Menon RK, Gill DS, Thomas M, Kernoff PBA, Dandona P. Impaired carboxylation of osteocalcin in warfarin-treated patients. *J Clin Endocrinol Metab* 1987; **64**: 59–61
85 Price PA, Nishimoto SK. Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. *Proc Natl Acad Sci USA* 1980; **77**: 2234–8
86 Tanaka H, Kuwada M, Shiraki M, Katayama Y. An enzyme immunoassay for osteocalcin. *J Immunol Methods* 1986; **94**: 19–24
87 Price PA, Williamson MK, Lothringer JW. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J Biol Chem* 1981; **256**: 12760–6
88 Sokoll LJ, O'Brien ME, Camilo ME, Sadowski JA. Undercarboxylated osteocalcin and development of a method to determine vitamin K status. *Clin Chem* 1995; **41**: 1121–8
89 Koyama N, Ohara K, Yokota H, Kurome T, Katayama M, Hino F, *et al*. A one step sandwich enzyme immunoassay for γ-carboxylated osteocalcin using monoclonal antibodies. *J Immunol Methods* 1991; **139**: 17–23
90 Masters PW, Jones RG, Purves DA, Cooper EH, Cooney JM. Commercial assays for serum osteocalcin give clinically discordant results. *Clin Chem* 1994; **40**: 358–63
91 Banfi G, Daverio R. In vitro stability of osteocalcin. *Clin Chem* 1994; **40**: 833–4
92 Power MJ, O'Dwyer B, Breen E, Fottrell PF. Osteocalcin concentrations in plasma prepared with different anticoagulants. *Clin Chem* 1991; **37**: 281–4
93 Noonan K, Kalu ME, Holownia P, Burrin JM. Effect of different storage temperatures, sample collection procedures and immunoassay methods on osteocalcin measurement. *Eur J Clin Chem Clin Biochem* 1996; **34**: 841–4
94 Power MJ, O'Dwyer B, Breen E, Fottrell PF. Osteocalcin concentrations in plasma prepared with different anticoagulants. *Clin Chem* 1991; **37**: 281–4
95 Tracy RP, Andrianorivo A, Riggs BL, Mann KG. Comparison of monoclonal and polyclonal antibody-based immunoassays for osteocalcin: a study of sources of variation in assay results. *J Bone Miner Res* 1990; **5**: 451–61

96 Supraregional Assay and Advisory Service. *Biochemical Markers of Bone Turnover*. Sheffield, UK: PRU Publications, 1998
97 Gundberg CM, Markowitz ME, Mizruchi M, Rosen JF. Osteocalcin in human serum: a circadian rhythm. *J Clin Endocrinol Metab* 1985; **60**: 736–9
98 Thomsen K, Eriksen EF, Jorgensen JC, Charles P, Mosekilde L. Seasonal variation of serum bone GLA protein. *Scand J Clin Lab Invest* 1989; **49**: 605–11
99 Nielsen HK, Brixen K, Mosekilde L. Diurnal rhythm and 24-hour integrated concentrations of serum osteocalcin in normals: influence of age, sex, season and smoking habits. *Calcif Tissue Int* 1990; **47**: 284–90
100 Gundberg CM, Lian JB, Gallop PM. Measurements of gamma-carboxyglutamate and circulating osteocalcin in normal children and adults. *Clin Chim Acta* 1983; **128**: 1–8
101 Cioffi M, Molinari AM, Gazzerro P, Di Finizio B, Fratta M, Deufemia A, *et al*. Serum osteocalcin in 1634 healthy children. *Clin Chem* 1997; **43**: 543–5
102 Blumsohn A, Hannon RA, Wrate R, Barton J, al-Dehaimi AW, Colwell A, *et al*. Biochemical markers of bone turnover in girls during puberty. *Clin Endocrinol (Oxf)* 1994; **40**: 663–70
103 Epstein S, Poser J, McClintock R, Johnston CCJ, Bryce G, Hui S. Differences in serum bone GLA protein with age and sex. *Lancet* 1984; **i**: 307–10
104 Delmas PD, Stenner D, Wahner HW, Mann KG, Riggs BL. Increase in serum bone gamma-carboxyglutamic acid protein with aging in women. Implications for the mechanism of age-related bone loss. *J Clin Invest* 1983; **71**: 1316–21
105 Hannon R, Blumsohn A, Naylor K, Eastell R. Response of biochemical markers of bone turnover to hormone replacement therapy: impact of biological variability. *J Bone Miner Res* 1998; **13**: 1124–33

Accepted for publication 31 January 2000