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Development of an in house ELISA for human intact osteocalcin and its utility in diagnosis and management of osteoporosis

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ABSTRACT

Background: Serum Osteocalcin (OC) is a biomarker for evaluating bone turnover in humans. Commercial kits of OC in India are imported, hence the associated high cost prohibits their use in routine screening of osteoporosis. The present study describes the development, validation of human OC ELISA and establishes cut off values for its use in screening and management of women at risk for osteoporosis.

Methods: A sandwich OC ELISA was developed using immuno-reagents prepared indigenously and validated for analytical sensitivity, specificity, accuracy and compared with commercial kit using Bland-Altman method. The utility of OC assay was evaluated by ROC analysis.

Results: The new ELISA was sufficiently precise, accurate, matrix-free, sensitive and cost effective. The levels of OC were significantly different in women with osteopenia and osteoporosis (ANOVA, $p < .0001$) compared to women with normal BMD. ROC analysis demonstrated the cut off values of OC > 11.9 ng/mL for osteopenia and > 14.9 ng/mL for osteoporosis. The OC levels had maximum AUC of 0.831 in osteopenia and 0.932 in osteoporosis. Further, OC levels showed significant changes within 3 months in women monitored on therapy.

Conclusion: The developed OC ELISA has great potential to be used as a biomarker for routine screening and management of osteoporosis in Indian women.

1. Introduction

Osteoporosis is a common metabolic bone disorder characterized by a structural deterioration of bone tissue resulting in an increased risk of fragility fracture [1]. India is one of the leading countries affected by osteoporosis with estimates showing approximately 50 million people as osteoporotic or having low bone mass with one out of three women and one out of five men above the age of 50 years, at risk of osteoporosis [2,3]. Moreover, it is more prevalent in postmenopausal women and being a silent disease, it is a major concern in India as the prevalence rate is 35.7% in women over 50 years [4,5].

Currently, bone mineral density (BMD) measurement using dual-energy X-ray absorptiometry (DXA) scan is the gold standard for the diagnosis of osteoporosis [6–8]. However DXA scan has its own limitations being a static measure and expensive tool with limited availability in the many parts of India. This necessitates the need for an alternative diagnostic method for screening women at risk with a marker for management and treatment for osteoporosis. In recent times, cellular bone matrix components have been identified and categorised as either bone formation or resorption markers [9,10]. These biomarkers provide the useful information on assessment of osteoporosis at an earlier stage when the BMD measurement of DXA does not offer enough information to make the diagnosis [11]. Of late, these markers have been documented as tools in the clinical management of bone diseases. Therefore, a combination of BMD measurement by DXA and bone markers show the great potential to improve the early assessment of people with the high risk of osteoporosis.

Osteocalcin (OC) also known as Bone Gla Protein is a non-collagenous, 49 amino acid long single chain protein (MW 5.8 kDa), containing three γ-carboxyglutamic acids. It is secreted solely by osteoblasts and its biosynthesis is vitamin K dependent [12,13]. During bone formation, newly synthesized OC is incorporated into bone matrix, and a small fraction is secreted directly into the circulation, but its function remains elusive. Circulating OC concentrations have been broadly used for evaluating rate of bone turnover in metabolic bone diseases such as hyperparathyroidism, Paget's disease and renal osteodystrophy [14–17]. However its role in age related bone loss in women remains elusive.

Several immunoassays for OC have been reported which include Immuno-radiometric assay (IRMA) and enzyme- immunoassays (EIA

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and ELISA) and Enzyme Amplified Sensitivity Immunoassay (EASIA) using either monoclonal or polyclonal antibodies against bovine OC which shares 90% homology with human OC [18–20]. Despite sequence homology, there is dissimilarity observed at N-terminal region of the OC protein. Hence, antibody raised against bovine OC may react differently with human OC and may result in interpretation of false results. Of late, new developments in the assays include antibodies against human rather than bovine OC and sandwich assays with one or two monoclonal antibodies, so that the intact OC molecule is measured providing a valid indication of osteoblastic activity without any resorptive component. However, the commercial ELISAs available with the use of these antibodies need to be imported and relatively expensive.

In the present study, we report the development and validation of an in-house ELISA for the measurement of human intact OC and assessed the potential of the assay as an indicator of bone turnover in the clinical management of osteoporosis in women.

2. Materials and methods

2.1. Assay reagents

OC monoclonal antibody, Imject EDC mcKLH spin kit, Protein A IgG purification kit, HRP labelling kit were obtained from Thermo Scientific Co. USA. Intact OC human (1–49 aa), affinity purified goat anti-rabbit gamma globulin whole molecule (ARGG), Keyhole Limpet Haemocyanin (KLH), Freund's complete and incomplete adjuvant (FCA, FICA respectively) were purchased from Sigma Chemical Co. USA. Polyclonal antibodies to intact human OC were raised in two rabbits (New Zealand strain) at the Institute. The Institutional Animal ethics clearance was obtained to raise the antisera (No: 04/2012).

2.2. Solvents and solutions

All the buffer salts used were of analytical grade from Fischer Scientific Co.

a) Coating Buffer: 0.05 mmol carbonate – bicarbonate buffer, pH 9.6
b) Phosphate-Buffered Saline (100 mmol/L PBS)
c) Blocking Buffer: 2% Bovine serum Albumin (Sigma) in 0.1 M PBS, pH -7.2
d) Assay Buffer: 0.1% Bovine serum Albumin in 0.1 M PBS
e) Washing Solution: 0.15 M NaCl, 0.05% Teewn-20
f) 3,3,5,5′-tetramethylbenzidine (TMB, H_2O_2) (BioFx, Surmodics): liquid substrate system, ready to use solution
g) Stop Solution: 4 M H_2SO_4 in distilled water.

2.3. Study participants

The study was approved by Institutional ethics Committee for Clinical studies (172/2010). Blood samples were collected from normal healthy women participants (n = 138) aged between 25 and 65 years visiting the Institute's clinic after signing the informed consent form. Patients on oral contraceptives, having a chronic debilitating illness (cancer, AIDS), renal diseases, liver diseases, diabetes mellitus were excluded from this study. Since serum OC levels show diurnal variation, whole venous blood samples were drawn between 9 and 10 a.m. after an overnight fast in a plain vacutainer at room temperature and kept at 4 °C until separated. Serum was separated within an h by centrifuging the blood at 3000g for 10 min at 4 °C and stored as small aliquots for long term use at -80 °C till assayed for OC.

2.4. Methods

2.4.1. Preparation of immunogen

Human OC intact molecule (49 aa) was coupled to KLH using the Imject EDC-mcKLH spin kit according to the manufacturer's instructions. This enables effective one step conjugation of a hapten to a carrier protein using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as the cross-linker which reacts with the exposed carboxyl and amino groups on peptides and proteins to form stable bonds.

2.4.2. Immunization schedule for generation of antibody in rabbits

Two male New Zealand white rabbits 5–6 months old, weighing > 2 kg were selected for immunization. Approximately 5 mL of normal rabbit serum (NRS) was collected from non-immunised rabbits and stored in aliquots at −80 °C. This part of the study was approved by Institutional Animal Ethics Committee (NIRRH), Mumbai.

To immunogen OC-KLH (300 μg/600 μL), equal volume of 0.1 M PBS was added and emulsified with 1.2 mL of FCA at a ratio of 1:1. The emulsion 200 μg (1 mL) was administered to each rabbit (100 μg/mL) subcutaneously in small volumes (40 μL) at 20–25 sites along the back of each rabbit. Test bleed (2 mL) was collected after 4 weeks of primary immunization followed by 2 boosters every 2 weeks. Booster immunizations (50 μg) emulsified in FICA were given and rabbits were bled (10 mL) through ear lobes 10 days after the last booster immunization. Antisera were separated by centrifugation at 2500 rpm for 10 min and stored in aliquots at −80 °C until processed.

2.4.3. Checking of titers of antisera by an indirect ELISA

100 μL of the human OC (2.5 μg/mL) antigen (Sigma, USA) in coating buffer was dispensed in microtitre ELISA plate (Nunc-Immuno Modules, Maxisorp polystyrene strips) and incubated overnight at 4 °C. Following incubation, plate was blocked with 250 μL of blocking buffer for 1 h at room temperature. Different dilutions of anti–OC antiserum and NRS prepared in Assay buffer were dispensed to the antigen coated wells and incubated for 1 h at 37 °C. Further, plates were washed and probed with 100 μL of ARGG-HRP (1:3000 dilution). A difference of > 2.0 OD between the specific antibody binding well and zero binding (NRS) was selected as optimum working titer of raised antisera.

2.4.4. Purification of antisera

Initially, antiserum was purified using 33% ammonium sulphate precipitation technique as per laboratory protocol followed by Protein A-IgG purification kit procedure. The purified IgG fraction of the anti-OC antiserum was subjected to protein estimation using Lowry's method and aliquots of optimum concentration were prepared and stored at -80 °C for use in the development of sandwich ELISA.

2.4.5. Preparation of enzyme labelled (ARGG-HRP) antibody

Purified IgG fraction of ARGG was coupled to HRP using the HRP labeling kit (Bangalore Genei, India). In brief, 0.2 mL of water was added to the reaction tube containing the enzyme and mixed well for 1 h at room temperature to oxidize the enzyme. ARGG, 0.2–0.5 M equivalents was then added to the oxidized enzyme and mixed for 3–4 h at 4-8 °C. Later 10 μL of the reductant solution was added and tubes left for 15 min followed by 20 μL of Quench solution for 15 min at room temperature. The conjugate was passed onto the desalting column and the colored fractions (0.5 mL) containing the ARGG-HRP conjugate was collected. Approximately 1.5 mL of the enzyme conjugate was obtained. The conjugate was mixed with the stabilizer and stored at -20 °C for specific length of time. The optimum titer of this conjugate was determined in the sandwich ELISA by the checker board titration method and dilution of 1:3000 to specific target that was deemed acceptable.

2.4.6. Standardisation of sandwich ELISA for human OC

Mouse anti-human OC (monoclonal antibody), dilution of 1:3000 in coating buffer, pH 9.6 was coated onto the plate by incubating overnight at 4 °C. This was followed by washing and later blocking for 1 h. 100 μL of OC standard (0–80 ng/mL) or serum samples (1:3 diluted) prepared in assay buffer was added in duplicates. The plate was

incubated for 1 h at room temperature followed by addition of 100 µL/well of IgG purified polyclonal anti-OC antiserum (1:3000). Further, 100 µL of HRP labelled anti rabbit gamma globulin was added to the plate for 1 h. To measure enzyme activity of bound fraction, TMB, H_2O_2 was added to all the wells. After 20 min, reaction was stopped by adding 100 µL of stop solution and colour intensity was measured at 450 nm ELISA reader (MicroQuant, Bio-Tek Instrument Inc) using Gen 5.0 data analysis software.

2.4.7. Assay validation

The newly developed ELISA was validated for analytical sensitivity and specificity, accuracy and precision as per the WHO criteria. Analytical sensitivity was determined by calculating the concentration corresponding to the value obtained by adding 2SD to the mean OD of 8 replicates of blank readings in a single assay. The Analytical specificity of the assay was indirectly evaluated by comparing slopes of sample dilution curve (1:2, 1:4 and 1:8 of serum) with that of standard curve. Accuracy was measured by calculating the percentage of analyte recovered when known amounts of the sample were spiked to a serum having inherent low concentration of OC. The % recovery of spiked amount in 5 samples with inherent low concentration of OC was determined. The precision of the assay was calculated at low and high concentrations of OC. The coefficient of variation (CV%) for both intra assay ($n = 6$ consecutive replicates in a single assay) and inter assay ($N = 6$ assays carried out on 6 separate days) was determined. Intact OC levels were measured in duplicates for all the samples using the developed assay. Further, 35 samples were measured by a commercial kit (Human OC N-mid terminal ELISA kit from IDS, USA) and In-house developed assay. The measurements of the assays were compared using Deming regression analysis and Bland-Altman method.

2.4.8. Statistical analysis

All the statistical analysis were performed using Graph Pad prism version 7.0 (Graph Pad Software, Inc. USA). The baseline characteristics like age, BMI, BMD, T scores were compared using Student's *t*-test. Difference between measurements of OC by the two assays was tested using Deming linear regression and Bland - Altman plot. OC levels in different groups were compared by one-way non parametric ANOVA. Receiver operating Characteristic (ROC) curve analysis was performed to determine Area under Curve (AUC), diagnostic sensitivity and specificity of OC. Associations were considered statistically significant at $p < .05$ level.

3. Results

3.1. Development of sandwich ELISA for human intact OC

The Composite Standard curve represents the mean of six assays of intact OC wherein the increasing concentrations (0–80 ng/mL) of OC were plotted versus the binding of the respective standards (B/B0% represents the percent binding in presence of different standard concentration to that of zero binding well). The slope (b) and intercept (a) of the curve were calculated using logit-log transformation of the data (Fig. 1).

3.2. Validation of the intact OC ELISA

The developed sandwich ELISA for intact OC was validated for analytical sensitivity, analytical specificity, accuracy, precision and correlated with the commercial ELISA kit.

a) **Analytical Sensitivity:** the lower detection limit of the assay was 1.0 ng/mL, enabling proper measurements of intact OC concentrations in the study cohort.
b) **Analytical Specificity:** estimated using sera from different individuals at different dilution of serum. The standard plot was parallel to the sample dilution curve (Fig. 2) demonstrating the absence of matrix effect and indirectly supporting specificity of the assay.
c) **Accuracy**: Accuracy was determined by the developed OC assay by spiking varying concentrations of human OC in five serum samples. The average recovery of the spiked OC ranged between 92 and 105% (Table 1).
d) **Precision of the developed ELISA for OC:** estimated by incorporating two serum pools of low and high concentration within the single assay (intra-) and in six separate assays (inter-). Table 2 depicts inter assay and intra assay coefficient of variations which were < 10.0%
e) **Comparison of OC values by two assays:** Analytical method agreement (Deming's linear regression analysis) and possible systematic bias (Bland-Altman plot) were investigated in 35 samples with the commercial ELISA and the in-house developed ELISA for OC. The Deming linear regression analysis of the in-house developed

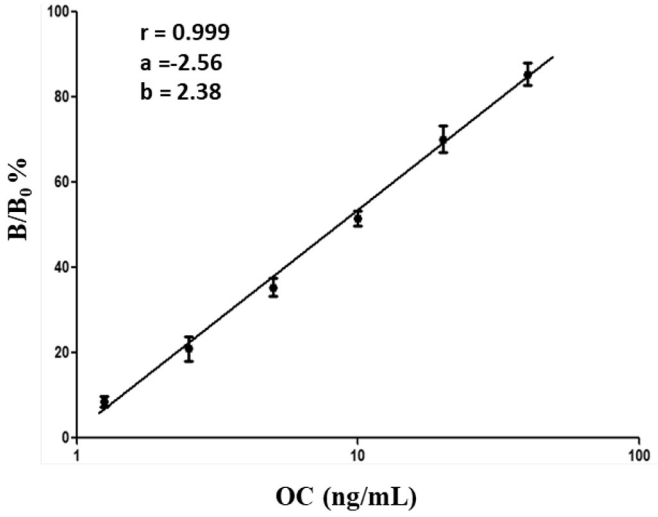

Fig 1. Composite standard curve of ELISA for intact Osteocalcin (n = 6). Composite standard curve of ELISA for intact Osteocalcin (n = 6). B/B0% is the ratio of the absorbance of percent binding in presence of different standard concentration to that of zero binding.
Osteocalcin (ng/mL), Slope = 2.38, Intercept = −2.56, $r = 0.999$.

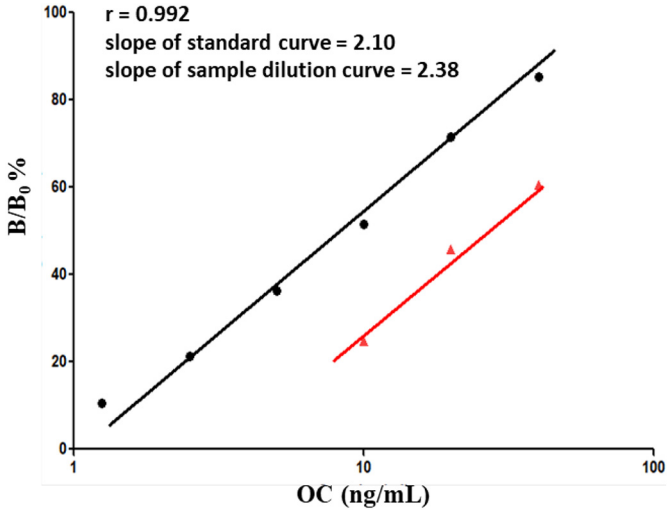

Fig. 2. Sample (patient serum) dilution plot showing specificity of the assay. Sample (patient serum) dilution plot showing specificity of the assay. B/B0% is the ratio of the absorbance of percent binding in presence of different standard concentration to that of zero binding. Osteocalcin (ng/mL), $r = 0.992$.

Table 1
Recovery of Osteocalcin from exogenously spiked serum samples.

Unspiked samples (n = 5) (ng/mL)	Added analyte (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
X = 12.6	X + 10	22.6	20.88	92.38
X = 16.3	X + 5	21.3	22.57	105.96
X = 10.2	X + 2.5	12.7	12.07	95.03
X = 19.8	X + 4	23.8	24.42	102.61
X = 26.0	X + 2	28.0	27.70	98.93

% Recovery was calculated using {100 - [(observed – expected /expected) x 100]}. Observed = Spiked sample value; Expected = Amount spiked into sample.

Table 2
Precision of human intact osteocalcin ELISA.

Osteocalcin pool	Concentration (ng/mL) (mean ± SD)	Coefficient of variation of assay (%CV)	
		Intra Assay (n = 6)	Inter Assay (N = 6)
Low	10.56 ± 1.11	9.3	10.5
High	47.3 ± 3.70	7.8	8.28

The coefficient of variation (% CV) was calculated for both intra assay (n = 6) consecutive measurements within one single assay and inter assay (N = 6) carried out between 6 different assay.

assay and commercial ELISA showed (n = 35) good agreement (R = 0.98), with an intercept (95% confidence interval, CI) of 0.909 (−3.652 to 3.449) and slope (95% CI) of 0.102 (0.620 to 1.198) (Fig. 3A). In Bland Altman analysis mean bias with limit of agreements was 1.353 (−1.562 to 4.268) (Fig. 3B).

3.3. Clinical parameters of the study participants

Several parameters such as age, BMI, menopausal status, serum calcium, phosphorous and BMD were estimated to study their association with OC levels. Since age is the confounding factor, the study participants were further classified into two groups as premenopausal women (21–40 years) and postmenopausal women (45–65 years). The serum Ca and Ph levels in the participants were within the normal range with no significant differences between the two groups. The anthropometric and BMD measurements of the study participants

Table 3
Baseline characteristics and BMD measurements of the study participants (n = 138).

Parameters		Premenopausal Mean ± SD (n = 70)	Postmenopausal Mean ± SD (n = 68)	p value
Age (Years)		30.47 ± 8.44	52.5 ± 6.52	–
BMI (Kg/m^2)		22.04 ± 4.14	25.10 ± 4.79	< **0.001**
Spine	BMD (g/cm^2)	1.087 ± 0.14	0.884 ± 0.17	< **0.001**
	T Score	−0.46 ± 1.33	−1.91 ± 1.61	< **0.0001**
Femur	BMD (g/cm^2)	0.936 ± 0.12	0.778 ± 0.11	< **0.001**
	T Score	−0.32 ± 1.06	−1.28 ± 1.17	**0.0002**
Calcium (mg/dL)		8.89 ± 1.23	8.21 ± 1.86	0.89
Phosphorous (mg/dL)		5.12 ± 1.89	5.89 ± 1.49	0.71
Osteocalcin (ng/mL)		10.83 ± 2.1	17.71 ± 2.44	< **0.0001**

p < .05 considered statistically different between groups. Significant values appear in boldface.

(n = 138) are shown in Table 3. The Body Mass Index (BMI) and Bone Mineral Density (BMD) measurements at spine and femur were significantly different between pre- and postmenopausal women.

The OC levels measured by the new ELISA were also significantly different among premenopausal (7.1–19.8 ng/mL) and postmenopausal women (12.8–28.6 ng/mL) (p < .0001) (Fig. 4a). The study cohort (n = 138) was further categorised as per WHO criteria as Normal (T score ≥ −1 at both the sites; Osteopenia (T score between −1 and − 2.5 at either of the two sites) and Osteoporosis (T score ≤ −2.5 at either of the two sites). The OC levels in women with osteopenia and osteoporosis were observed to be statistically different (p < .0001) when compared to the women with normal BMD as depicted in (Fig. 4b).

3.4. Monitoring hormone replacement therapy (HRT) by OC levels

In a small group of postmenopausal women (n = 20) OC levels were used for monitoring women on hormone replacement therapy. The levels were estimated at baseline and after therapy at 3, 6 and 12 months. Significant changes (p < .05) were observed in OC levels from the baseline within three months in women on HRT therapy suggesting OC assay can be utilised for monitoring treatment as well (Fig. 5)

3.5. Predictive ability of OC marker

On computing the odds ratio for estimating association of OC levels

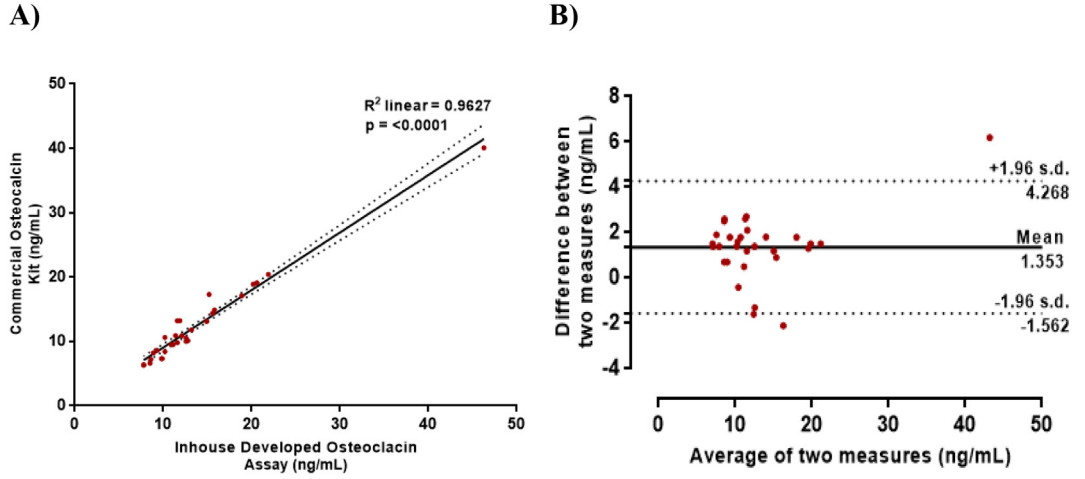

Fig. 3. Comparison of In-house Osteocalcin ELISA and commercial ELISA kit.
Deming Linear regression fit (A) and Bland-Altman plot (B) of the osteocalcin measurements (ng/mL) in human sera by commercial kit and In-house developed assay. p < .05 was considered statistically significant. s.d. Standard Deviation.

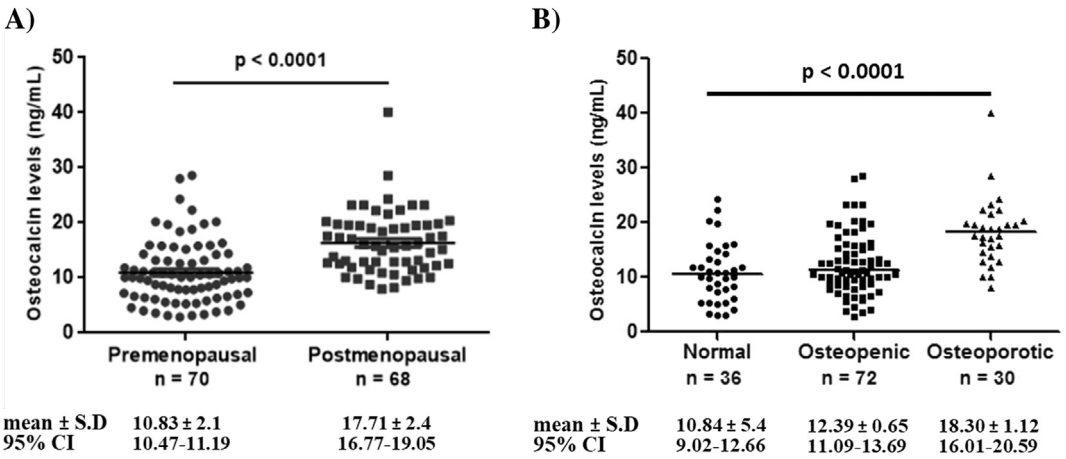

Fig. 4. Comparisons of Osteocalcin levels (ng/mL) in the study subjects. A) Scatter plot showing osteocalcin levels (ng/mL) in pre and postmenopausal women. Non parametric Mann-Whitney *U* test was used for comparison. B) Scatter plot showing osteocalcin levels (ng/mL) in women classified on the basis of BMD. One–way ANOVA test for analysis of variance with Kruskal Wallis correction for non-parametric test was carried out. p < .05 was considered statistically significant. Values are mean ± SD with 95% CI. CI = Confidence Interval.

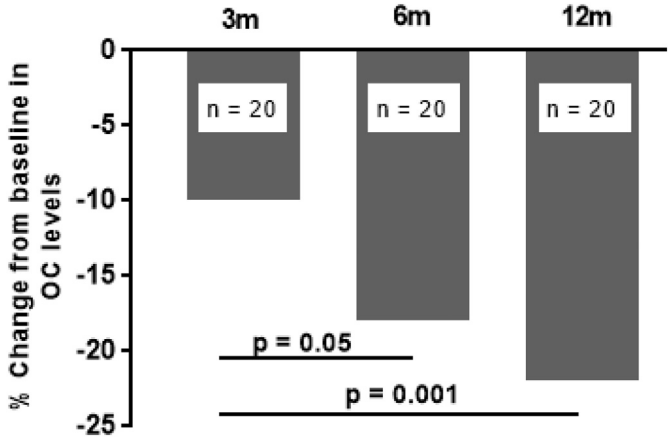

Fig. 5. Percentage change in osteocalcin levels from baseline at different time intervals to monitor women on therapy. One–way ANOVA test for analysis of variance with Kruskal Wallis correction for non-parametric test was carried out. p < .05 was considered statistically significant. m = Months. Percentage change in osteocalcin levels at different time intervals of 3 m, 6 m and 12 m after therapy as compared to the osteocalcin levels before therapy. m = Months.

with osteoporosis, it was observed that OC may be a significant predictor (*p* value < .0001) of low BMD. In total cohort, to distinguish between normal and low BMD, the odds ratio was 9.625 (95% CI = 3.115–29.74) and in pre and postmenopausal category, the odds ratio were 1.643 (95% CI = 0.4232–3.024) and 17.50 (95% CI = 1.700–18.01) respectively. However it acted as a better predictor in postmenopausal women (p value 0.0096).

3.6. Receiver operator curve (ROC) analysis and cut off values

ROC analysis performed on serum OC levels in premenopausal: Normal v/s women with osteopenia (Fig. 6A) and in postmenopausal: Normal and women with osteoporosis women (Fig. 6B) determined the cut off levels. Receiver Operating Curve (ROC) curve determined a cut off value of serum OC level as > 11.9 ng/mL between normal and women with osteopenia and > 14.90 ng/mL between normal and women with osteoporosis in postmenopausal women which were significantly different (*p* < .0001). The AUC generated for distinguishing osteopenia and osteoporosis was 0.831 and 0.932 respectively. Using the above cut off values, the diagnostic potential of OC can be assessed on a larger population.

4. Discussion

DXA (Dual Energy X-Ray Absorptiometry) scan, though considered as a gold standard and the most acceptable modality worldwide for the diagnosis of osteoporosis, is relatively expensive and not widely available in India thus making population screening of all high-risk women impossible. Although BMD is the best quantifiable predictor of osteoporotic fractures, the efficacy of the treatment cannot be judged immediately, since the structural recovery of the bone takes time. To overcome these difficulties there is a need to assess the bone turnover by biochemical markers in screening women with osteoporosis and to monitor the success of treatment [21]. Of late, serum OC has emerged as a promising bone turnover marker. Our study was designed with the aim to develop and validate an ELISA for measurement of serum OC and to assess its utility as a promising marker of bone turnover in the diagnosis of osteoporosis and follow-up of women on treatment [22].

In the current study a sensitive, specific and accurate ELISA was developed to measure serum OC levels which was precise (inter and intra assay coefficient of variation < 15%) and recovery of spiked OC was found within the expected range. Furthermore, for validation of the method the OC levels were measured by in-house ELISA which showed a good agreement higher than 0.9 by Deming linear regression with the commercial kit. Along the same lines, the result of the Bland-Altman plot showed acceptable limits of agreement for the OC.

Serum OC levels were significantly higher in postmenopausal women as compared to premenopausal women as reported in earlier studies [23–25].We also observed significant difference in OC levels in women when classified on the basis of BMD, with high in women with osteoporosis, intermediate in women with osteopenia and least in women with normal BMD [26]. This shows an inverse relationship of serum OC with BMD in present study. The elevated concentrations may be attributed to imbalance between bone resorption and bone formation due to estrogen deficiency in postmenopausal women with osteoporosis [27] which correlates with other similar study [28].

OC has high affinity for calcium and promotes absorption to hydroxyapatite in bone matrix, for mineralization. In osteoporotic women, deficiency of calcium and phosphorus may lead to decreased formation of hydroxyapatite crystals, thus making free OC available in the blood circulation. This explains the increased concentration of OC in circulation which may have prognostic significance for better management of postmenopausal osteoporosis. In our study cohort, the serum calcium levels were low in postmenopausal women but did not

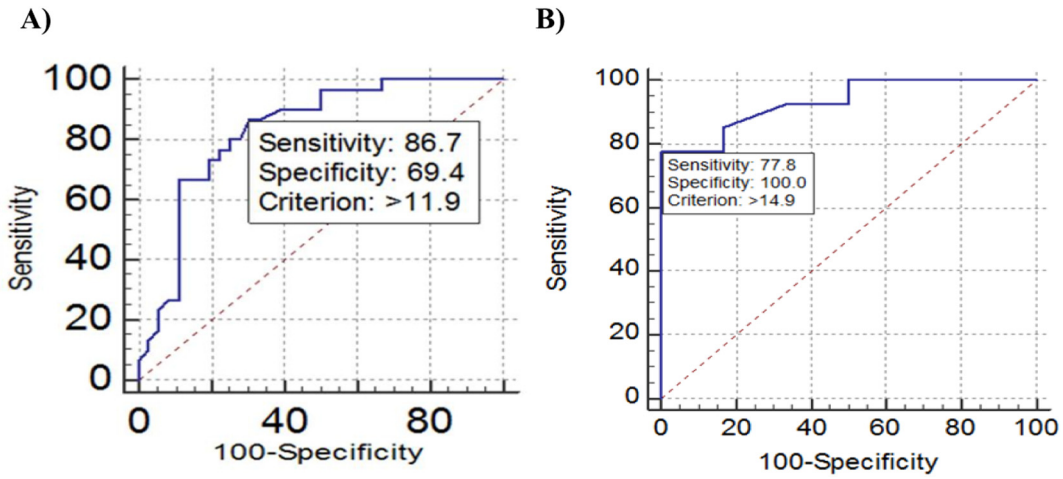

Fig. 6. ROC curve analysis to determine cut-off levels of serum Osteocalcin levels (ng/mL) in women with osteopenia and osteoporosis.

vary significantly. This may be due to the decreased estrogen concentration during menopause that leads to a lower intestinal absorption of calcium resulting in increased bone turnover in postmenopausal women.

Interestingly we observed that OC had a significant predictive ability for low BMD. In postmenopausal women, OC levels showed higher ability (odds ratio) to distinguish between low and high BMD as compared to premenopausal women. The ROC analysis was performed to assess the diagnostic sensitivity, specificity and to establish a cut off point for OC. Further, the cut-off value of > 11.9 ng/mL between premenopausal normal and women with osteopenia had sensitivity of 86.9% and specificity of 69.4%. Similarly the cut off value of > 14.9 ng/mL between postmenopausal women with normal BMD and postmenopausal women with osteoporosis showed sensitivity (77.78%) and higher specificity (100%).This suggests that serum OC level is able to distinguish women with low BMD from normal BMD. Notably, OC had higher AUC for osteoporotic women as compared to women with osteopenia though the difference was not significant. This could be due to the small number of participants studied. Thus AUC data suggests that the OC has a diagnostic ability to detect women with low BMD.

Further we assessed the efficacy of the treatment at specific time intervals by monitoring the OC levels in small group of women undergoing therapy by comparing it with its baseline level. Significant changes in OC levels were seen in women within 3 months of treatment unlike BMD measurements where changes could be detected only after a year. Thus, the assessment of osteoporotic risk fractures can be done effectively by a combination of BMD, which provides a static feature of the skeleton and the bone marker OC which provides a dynamic measure of the bone remodelling unit as reported by Vanitha et al. [29].

In summary, our result suggests that serum OC, a bone turnover marker may be useful for screening of elderly women with low bone mass (osteopenia and osteoporosis). The total cost of single OC estimation is approximately 6USD (₹400) with our indigenous assay as compared to the imported commercial kit, which is almost double the cost. Moreover the cost of BMD estimation is also relatively higher around 29USD (₹2000) in India. Hence we recommend the use of serum OC level for the routine screening of women at risk for osteoporosis who can later be confirmed using DXA scan if needed.

The limitation of our study is that the developed ELISA measures only intact OC (1-49aa) in serum samples, whereas N-terminal mid fragment (1-43aa) is reported to be more stable in circulation. Hence, an assay that measures both of these fragments has greater significance in clinical practice. Secondly, the analytical specificity of the new ELISA was not measured with closely related substances but demonstrated indirectly by the absence of matrix effect with parallelism study. Further, the cut off limits of OC levels derived in the study are based on the small sample size and gender specific. Hence, large multi-centric studies may be required for confirmation of the OC levels to be used for screening of low bone mass across India in both male and females.

5. Conclusions

In this study, we report a sufficiently precise, accurate, sensitive, matrix free and cost effective ELISA for measuring OC in serum samples. The derived cut off values with the developed assay can differentiate women with osteopenia, and osteoporosis from normal women independent of BMD measurements in Indian population. Thus OC, a bone turnover marker has great diagnostic potential for screening and management of postmenopausal Indian women at risk for osteoporosis.

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Conflicts of interest

All authors have no conflicts of interests to disclose.

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