



Screening and Assessment of Bone Health in Indian Women Using an Indigenous ELISA of Human Osteocalcin a Bone Turnover Marker

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Abstract Osteoporosis a major public health problem of the elderly, is associated with substantial morbidity and socio economic burden. The aim of the study was to screen women with low bone mass using the indigenously developed Osteocalcin (OC) ELISA kit and compare it with commercial ELISA kit and evaluate. The diagnostic potential of the assay was assessed in 359 samples from neighboring tertiary care hospitals over a period of 2 years. OC levels were estimated by the developed indigenous assay in samples, correlated with the Bone Mineral Density (BMD) measurements and compared by a commercial ELISA kit. On the basis of T-scores the women were stratified into Normal and case groups as Osteopenia and Osteoporosis. The serum biochemical parameters calcium and phosphorus were estimated on an auto-analyzer. To compare two different assays Bland–Altman plot and Deming linear regression analysis was performed. The prevalence of Osteopenia was high (56%) and Osteoporosis (13%) in the healthy Indian women aged 21–65 years with significant differences in OC levels in normal and women with low bone mass. Good correlation ($p < 0.0001$) in the OC levels by the two assays was observed. Cut off limits established earlier with indigenous assay (11.9 ng/mL and 14.9 ng/mL) for Osteopenia and Osteoporosis were similar to those with the commercial kit (13.2 ng/mL and 16.8 ng/

mL) respectively. The diagnostic sensitivity, specificity and accuracy of the OC prototype was $> 85\%$. The cost effective OC prototype can be used in screening and management of Indian women with low bone mass.

Keywords Osteocalcin ELISA · Correlation · Method comparison · Bland–Altman · Agreement analysis

Introduction

Currently India has more than 36 million population affected by osteoporosis. Due to an increase in longevity of life, osteoporotic fractures are a major cause of morbidity and mortality. Approximately 10% of the Indian population is older than 50 years and these figures are likely to go up by 34% till 2050 [1–3]. Bone mineral density measurements (BMD) by dual energy X-ray absorptiometry (DXA) is considered as the gold standard for the diagnosis of osteoporosis. Estimates suggest that ~ 50 million people in India over the age of 50 have low bone mass (T-scores of < -3), with 20% women and 10–15% of men as osteoporotic [4, 5]. Access to BMD measurements is limited and expensive as very few government hospitals have the facility of DXA machines [6]. Hence identification of high risk groups, early detection in terms of diagnosis, prevention and timely treatment is important in Indian context. Further there is a need to generate ethnic-specific reference data to be used in the diagnosis of osteoporosis. [7, 8]. Serum Osteocalcin (OC) levels may be used to assess bone turnover associated with decreased bone mass in women. Serum OC levels are also used extensively to evaluate bone remodeling and metabolic bone disease conditions like osteoporosis [9–11]. We had developed an ELISA for OC a sensitive, specific and accurate bone turnover marker and documented its utility in osteoporosis [12].

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Objectives

The study aims to evaluate the potential of OC assay in screening, diagnosis and management of osteoporosis by correlating with BMD the gold standard and comparing the levels with a commercial ELISA.

Material

Study Design

The cross sectional study was carried out for a period of 2 years (May 2016 to May 2018) in the Molecular Immunodiagnostics Department at the Institute with 359 samples from participants attending the various clinics. “The Institutional Ethics Committee for Clinical Research approved the study and informed consent was obtained from all individual participants included in the study”. The premenopausal women were with regular cycles for last 6 months while post-menopausal women with more than 1 year of menopause, apparently healthy, physically active, having normal blood pressure, non-obese, not consuming steroids and bisphosphonates and willing to participate in this study were included. Women on Hormone Replacement Therapy (HRT), abnormality of liver and kidney functions, endocrine disorders and acute infections and menopause due to pre ovarian failure or surgical removal were excluded from the study. A total of 359 women were included in this study and their clinical history was recorded at the respective centers. The BMD measurements by DXA scans were available for all the women ($n = 359$). Study subjects were classified into two groups as premenopausal and postmenopausal women and further as Normal, Osteopenic and Osteoporotic on the basis of their T-score (World Health Organization criteria). Normal was defined as T-score of above -1 SD, Osteopenia was defined as T-score between -1 and -2.5 SD and Osteoporosis was defined as T-score equal to or less than -2.5 SD. The serum OC levels were estimated with indigenous developed Sandwich ELISA and correlated with BMD measurements ($n = 359$) as well compared with commercial ELISA kit ($n = 257$).

Methods

Aliquots of the coded serum samples received from the validation centers stored at -20°C till use were tested for OC levels by the indigenous developed ELISA and by a commercial ELISA kit from Ray Biotech USA. Serum calcium and phosphorus were estimated by an auto-analyser using the ERBA reagents.

Indigenous Sandwich ELISA for OC

In brief 100 μl of the standard and diluted serum sample was added to the respective wells of a microtiter ELISA plate pre-coated with a monoclonal antibody to intact OC and incubated at room temperature (RT) for 1 h (hr) allowing the OC antigen to bind to the antibody. This was followed by three washes with 250 μl 1X wash buffer using microplate washer. Later a purified polyclonal antibody (IgG) 100 μl in an appropriate dilution was added to the plate and incubated for 1 h at RT washed and probed with secondary antibody Anti Rabbit Gamma Globulin coupled to Horse Radish Peroxidase (ARGG-HRP) for 1 h at RT. The plate was washed, substrate added and incubated in dark for 30 min. The reaction was stopped with 50 μl of stop solution and was read immediately at 450 nm (BioTek, μ Quant plate reader). A four-parameter logistic curve using Gen5 Data Analysis software was generated.

Commercial Sandwich ELISA Kit for OC

In contrast, the commercial OC ELISA uses biotinylated anti-human OC antibody. The standards and samples are sandwiched between the pre-coated OC specific antibody and biotinylated anti-human OC antibody further detected by HRP-conjugated streptavidin followed by TMB substrate addition for color development. The intensity of the color developed is directly proportional to the amount of OC, which is measured at 450 nm in an ELISA reader.

Statistical Analysis

All the statistical analysis was performed using Graph Pad prism version 7.0 (Graph Pad Software, Inc. USA). OC levels in the three different groups were compared by one-way non parametric ANOVA. Both the ELISA methods were compared using Bland–Altman plots and Deming linear regression analysis [13–15]. To assess the diagnostic sensitivity and specificity of OC, Receiver operating Characteristic (ROC) curve analysis was performed to determine Area under Curve (AUC). All associations were considered to be statistically significant at $p < 0.05$ level.

Results

A defined validation protocol was used for the analysis of the data of the subjects. BMD reports were available for all the 359 participants. In all the samples OC was estimated with Indigenous Sandwich ELISA and by Commercial ELISA kit. On the basis of the T-score the study cohort was classified into Normal, Osteopenia and Osteoporosis. In the present study 56% women had Osteopenia, 13% had

Osteoporosis while only 31% of the women were Normal (See supplemental data Figure S1).

Characteristics of the study population (N = 359 women) classified as premenopausal and postmenopausal group is given in Table 1.

The BMI of the postmenopausal women though high was not significantly different from the premenopausal women). Serum calcium and phosphorus levels of the study cohort were normal. The mean OC level by Indigenous ELISA in the pre and postmenopausal women was (12.01 ± 6.32 ng/mL) (15.55 ± 6.92 ng/mL), while with the Commercial ELISA (13.11 ± 6.86 ng/mL) (16.91 ± 6.25 ng/mL) as depicted in the Fig. 1 (See supplemental data table S2).

A significantly increased OC level ($p < 0.0001$) indicated an increased bone turnover with a decrease in the bone mass and the T-score of the women. The OC levels in the study cohort were also significantly different in the three groups of women with varying bone mass Fig. 2 (See supplemental data table S3), indicating that the OC levels by indigenous ELISA and the commercial ELISA were similar.

An inverse correlation was also observed with the BMD and OC level, thereby showing increased bone turnover in postmenopausal women.

Precision of the Immunoassays of Indigenous ELISA and Commercial ELISA

Both the ELISAs showed an intra assay (within-run) and inter assay (between-run) precision of < 10 and $\leq 15\%$ at each concentration level (Low and High) as shown in Table 2.

The OC levels estimated by the Indigenous Sandwich ELISA were correlated and compared with a commercial ELISA kit (N = 359). A good correlation was obtained in the OC values by the two methods ($r = 0.499$ and $p < 0.0001$) (See supplemental data Figure S4).

Comparison of OC Values by Two Methods

Analytical method agreement and possible systematic bias in the levels were assessed in 359 samples by both methods. The Bland–Altman plot is illustrated in Fig. 3.

Compared to the commercial ELISA kit the Indigenous assay demonstrated mean relative bias of -0.377 ng/mL with limits of agreement 14.85 – 15.60 (95% confidence interval). The Deming linear regression analysis between the two methods showed line of equation $Y = 0.469X + 7.462$ with the intercept (95% confidence interval) 7.462 ± 0.896 as depicted in Fig. 4 (See supplemental data table S5).

A linearly increasing difference in set of values with a range up to 40 ng/mL was observed by both methods. The probable reason is both the ELISAs have different assay formats and also differ in assay characteristics. The cut off limits observed for Osteopenia (> 11.9 ng/mL) and Osteoporosis (> 14.9 ng/mL) with the commercial kit and those established with the indigenous assay were more or less similar. The overall diagnostic sensitivity, specificity and accuracy of the OC prototype in assessing the bone health of Indian women was $> 85\%$.

Discussion

Currently the prevalence rate of osteoporosis in India is not known. However, the prevalence of Osteopenia and Osteoporosis in Indian women is quite high and many women are unaware about osteoporosis. It is also important to create awareness about osteoporosis. [16]. Benu et al., have reported 61 million Indians have low bone mass; of which 80% patients are females and 62.2% and 10.9% Osteopenic and Osteoporotic respectively [16]. In the present study 56% of the women were observed to be Osteopenic while 13% of the women were Osteoporotic also the prevalence of low bone mass was observed to be 70% in the age group of 40 years and above, which corroborates well with the earlier study by Babu et al [17] and

Table 1 Characteristics of premenopausal and postmenopausal women (N = 359)

Parameters		Premenopausal (N = 232)	Postmenopausal (N = 127)	P value
Age (years)		36.04 ± 9.40	53.43 ± 6.18	< 0.0001
BMI (kg/m^2)		23.96 ± 4.78	25.59 ± 4.50	0.0026
Osteocalcin (ng/mL)		12.01 ± 6.32	15.55 ± 6.91	0.0009
Calcium (mg/dL)		8.64 ± 0.83	8.67 ± 0.71	ns
Phosphorus (mg/dL)		5.15 ± 1.56	5.07 ± 1.09	ns
Spine	T-score	-0.91 ± 1.02	-2.13 ± 1.04	< 0.0001
	BMD (g/cm^2)	1.09 ± 0.13	0.91 ± 0.14	< 0.0001
Femur	T-score	-0.90 ± 0.80	-1.58 ± 0.80	< 0.0001
	BMD (g/cm^2)	0.91 ± 0.11	0.81 ± 0.11	< 0.0001

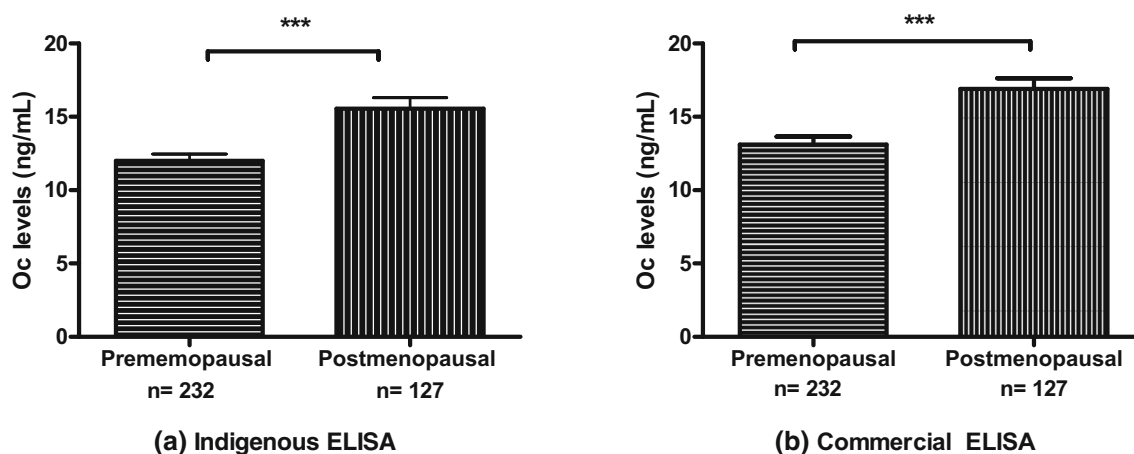


Fig. 1 Osteocalcin levels in premenopausal and post-menopausal women by Indigenous (a) and commercial (b) ELISA (N = 359)

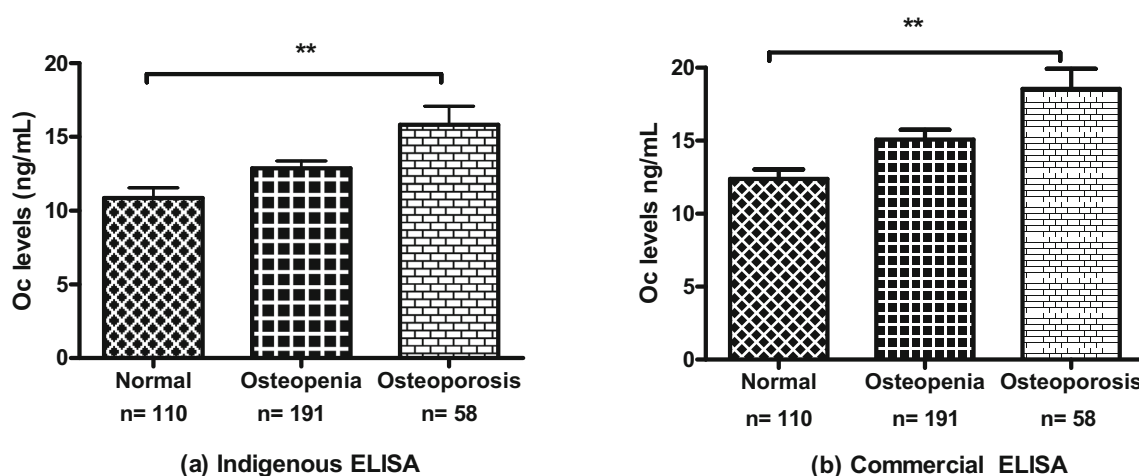


Fig. 2 Osteocalcin levels in Normal, Osteopenic and Osteoprotic women by Indigenous (a) and commercial ELISA (b) (N = 359)

Table 2 Precision of indigenous ELISA and commercial ELISA

ELISA assays		Indigenous ELISA (ng/mL)		Commercial ELISA (ng/mL)	
		Low	High	Low	High
Intra assay	Mean \pm SD	10.3 \pm 0.8	32.3 \pm 1.7	11.5 \pm 0.8	35.6 \pm 2.1
	CV (%)	7.4	6.9	8.7	5.8
Inter assay	Mean \pm SD	10.7 \pm 0.9	38.8 \pm 4.8	10.5 \pm 1.1	39.5 \pm 5.5
	CV (%)	8.8	12.3	10.4	13.9

significant correlation was observed with the BMD and bone marker levels. Bone turnover markers are not used routinely in clinical practice for diagnosis though they can aid in therapeutic diagnosis, due to the rapid changes seen within 3 months with the medications [18, 19]. Osteoporosis is more common in post-menopausal women, due to estrogen deficiency and OC a sensitive bone marker, is significantly elevated during menopause due to the high bone turnover [9].

Correlation and regression analysis have reported in this study for comparison of two quantitative immunoassays. However, correlation studies evaluate the relationship between two quantitative variables but do not compare them. Though a good correlation was observed between the OC levels by the two ELISAs, it only quantified the degree to which two variables are related. The alternative method Bland–Altman analysis plots showed the difference between two paired measurements against their mean which in turn indicated the agreement between the values

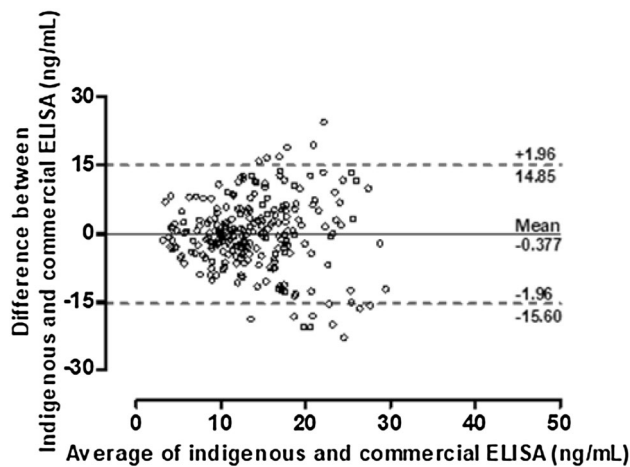


Fig. 3 Bland–Altman Plot

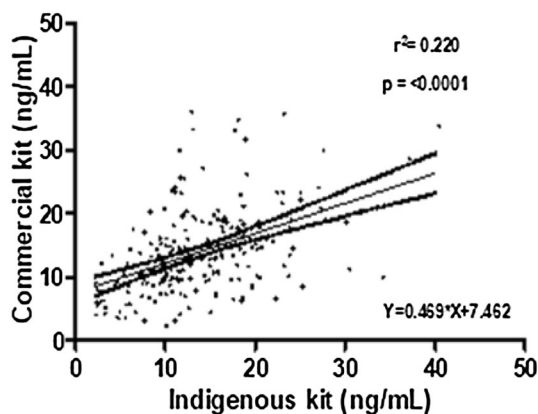


Fig. 4 Deming linear regression analysis

with a low bias. The assay performance was evaluated with a large matched samples ($N = 359$) through a series of validation tests thus meeting the recommendation of the Clinical and Laboratory Standards Institute and also compared with the gold standard BMD [20]. The difference in the cut off values may be due to the variability in the two ELISAs. Though both the assays are Sandwich ELISAs the commercial ELISA is based on the amplification system wherein one of the antibodies is biotinylated and then probed with streptavidin- HRP label [21]. However diagnostic potential of the indigenous OC ELISA evaluated for sensitivity, specificity, accuracy was $> 85\%$ thus proving its ability in screening and evaluation of women at risk for osteoporosis [22]. The kit is prepared indigenously and its performance is similar to the commercially available imported kit, as observed by the good correlation which is the major advantage. Moreover, the polyclonal detection antibody and other key reagents are indigenously raised at the Institute thereby making it cost effective. The assay is also sensitive enough to detect lower levels of OC (1 ng/mL) in serum and can be completed in 3 and half hours,

which is less time consuming compared to commercial ELISA kit. However the limitation is that, the monoclonal capture antibody is from a commercial source. Also osteocalcin is not stable at room temperature/higher temperature and degrades easily due to its limited half-life, thus necessitating that the serum samples require proper storage conditions. Also serum OC shows diurnal variation in circulating level, so the blood samples need to be collected after overnight fast between 9 and 10 a.m. [9]. The developed assay is for intact osteocalcin though N-terminal mid fragment (1-43aa) of osteocalcin is reported to be more stable in circulation. Strength of the study lies in the kit being validated using large number of samples from Indian women. The key reagents are prepared indigenously thereby reducing the cost per sample as compared to the commercial ELISA kit. OC is a promising bone turnover marker as it reflects both formation and resorption [9]. Though BMD is a better predictor of fracture risk it gives static measurement at a particular site and if used in conjunction with OC levels it can aid in evaluating low bone mass and monitoring therapy [10]. The developed kit can be thus serve as a diagnostic tool for screening and identifying women at risk for osteoporosis who can be subsequently put on therapy and simultaneously the changes in OC levels can be monitored by the assay which is accessible and inexpensive [11].

This study documents that the Indigenous developed OC assay appears to be a potential screening tool in identifying Osteopenia and Osteoporosis in substantial women who otherwise would have remain undiagnosed and may face the consequences associated with low bone mass. At present validation of OC kit is ongoing at neighboring Government Medical and teaching hospitals and also at the Bone health clinic. After successful completion of validation studies at users hands, our efforts will be directed towards finding a suitable entrepreneur to make the kit available in India.

Conclusion

In conclusion, indigenous OC prototype kit evaluated in the study is useful for estimating OC levels in women with low bone mass. The developed indigenous OC ELISA can be used as a tool for screening women at risk for osteoporosis. Thus early detection of osteoporosis with the developed cost effective OC kit and timely treatment may improve the quality of life of Indian women providing a replacement for the expensive commercial ELISA kit.

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Compliance with Ethical Standards

Conflict of interest Sonam Hatkar, Seema Kadam, M Ikram Khatkhatay and Meena Desai declare that they have no conflict of interest.

Human and Animal Rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee, NIRRH ethics committee for clinical studies (292/2016) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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