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Title Page

Bone turnover markers and Bone Mineral Density in healthy Mother-daughter pairs from South India.

Running Title: BTMs and BMDs in mother-daughter pairs

1. Sahana Shetty ¹	Assistant Professor
2. Nitin Kapoor ¹	Assistant Professor
3. Joseph Dian Bondu ²	Lecturer
4. Belavendra Antonisamy ³	Professor
5. Nihal Thomas ¹	Professor
6. Thomas Vizhalil Paul ¹	Professor

¹ Department of Endocrinology, Diabetes & Metabolism,

² Department of Clinical Biochemistry,

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3 Department of Biostatistics

Christian Medical College, Vellore, South India – 632 004.

Corresponding Author (proof to be sent to the following address)

Dr. Thomas V Paul, MD, DNB(Endo), PhD(Endo),
Professor,
Department of Endocrinology, Diabetes & Metabolism,
Christian Medical College, Vellore – 632 004.
Mobile: +91-9566920379
E-mail: thomasvpaul@yahoo.com
Fax: +91-416-4203570

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Conflict of Interest:

The authors have no conflicts of interest to disclose.

INTRODUCTION:

Osteoporosis is an emerging public health problem worldwide and is expected to increase with rising life expectancy. India has a high prevalence of osteoporosis; with one out of two women and one out of five men above the age of 50 years at risk.^{1,2,3} Complications of osteoporosis in the form of fractures, the most dreaded being hip fractures, contribute to an increased morbidity and mortality in the elderly population.⁴ The current gold standard test for the diagnosis of osteoporosis is bone mineral density (BMD) assessment using Dual energy X-ray Absorptiometry (DXA). However, about 50% of women who sustain a fragility fracture may not have BMD in the osteoporotic range.⁵ Hence there is a need for assessment of other risk factors for osteoporosis using tools such as FRAX, and also for the use of bone turnover markers (BTMs) which reflect the underlying bone turnover process. Microarchitectural alterations affecting bone quality and predisposing to fractures can be assessed by BTMs and may thus serve as a complementary tool to BMD in the assessment of fracture risk.⁶

BTMs provide insights into the dynamics of bone remodelling. In recent years, there has been an increase in utilisation of BTMs, especially in clinical trials in regard to the monitoring of treatment with new agents. The International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry & Laboratory Medicine (IFCCCLM) have proposed that serum CTX-1 and serum PINP can be used as reference markers of bone resorption and formation, respectively, in clinical settings for assessment of fracture risk and monitoring therapeutic response to osteoporosis treatment.⁷

Reliable, rapid and cost effective assays to estimate BTMs are available with improved sensitivity and specificity. However, there is limited information available with regards to BTMs and their relation to BMD and population-based normative data of BTMs from India. The manufacturer-provided reference range may not be the same for various ethnicities and may also depend upon pre-analytical variables such as age, gender, race, diurnal and seasonal variation.

Therefore, this study was undertaken to derive the reference range for various BTMs in healthy premenopausal and postmenopausal women, to look at the correlation between BTMs

and BMD at different sites, and also to study the diagnostic performance of BTMs in the diagnosis of osteoporosis. We also compared the BMD and BTMs among the daughters of mothers with osteoporosis at the spine and the femoral neck with those women whose mothers did not have osteoporosis.

MATERIALS AND METHODS:

This was a cross sectional study conducted over a period of 3 months. Institutional review board (IRB Min.No.8343 **OBSERVE** dated 10.06.2014) approval was obtained. Healthy premenopausal women and their mothers who had reached the menopause were recruited from an urban community from the NorthArcot district in the Southern part of India. There is significant homogeneity in the ethnicity of the population in this region. Daughters and their mothers were enrolled, as they would be expected to have similar environmental and genetic background. Recruitment was undertaken by random cluster sampling. The houses in the study sample recruitment area were numbered and divided into clusters of ten houses in continuity. Computer generated numbers were taken for random sampling of the clusters. Among the cluster samples, women who met the inclusion criteria were recruited after obtaining written informed consent. Only the eldest daughter who resided in the study area was recruited which enabled us to get equal numbers of pre- and postmenopausal women. Thus, study subjects comprised of 2 groups which included healthy premenopausal women between 25 and 45 years of age with regular menses (occurring every 25-35 days within the past year) and mothers of the pre-menopausal women aged above 50 years of age who had attained menopause (cessation of menstruation for a minimum period of 12months). Subjects with systemic illnesses, hyperthyroidism, hyperparathyroidism, Cushing's syndrome, history of fracture within 6 months, immobilisation and who were on medications that interfere with bone mineral metabolism were excluded from the study.

Details were obtained regarding their age, menstrual cycles, parity, menopausal age, socioeconomic status, physical activity, any systemic illness and medication use. Physical activity was assessed using an internationally validated standardised questionnaire: the International Physical Activity Questionnaire (IPAQscoring).⁸ Dietary calcium intake was assessed by using a semi-quantitative food frequency questionnaire,⁹ which included a typical daily meal plan recall for a duration of a week. Published data on individual nutrient composition of Indian food was utilised for estimating the dietary calcium intake.¹⁰ Data were also collected with regard to the duration of sunlight exposure (number of minutes per

day) in addition to the usual type of clothing to determine the extent of the body area that had been exposed to sunlight.¹¹

Subjects were examined on the day of BMD assessment. Weight was measured using the same weighing scale throughout the study, and height was measured using a Harpenden stadiometer (UK). The body weighing machine and stadiometer were calibrated on a weekly basis. Blood samples were collected for assessing the following biochemical parameters after an overnight fast: albumin-corrected calcium (N: 2.07- 2.59 mmol/L), phosphorus (N: 2.5 - 4.6 mmol/L), alkaline phosphatase (N: 40 - 125 u/l), creatinine (N: 61.9- 123.7 μ mol/L), parathyroid hormone (PTH) (N: 0.85 – 5.3 pmol/L), 25OH vitamin-D (N:75-175nmol/L). Biochemical parameters were measured in a fully automated computerised microanalyser (Hitachi model 911; Boehringer Mannheim, Mannheim, Germany). Intact PTH and 25(OH) vitamin D were measured by a chemiluminescence immunoassay using an Immulite analyser 2000. Vitamin D deficiency was defined as a serum 25(OH) vitamin D level of less than 50 nmol/L and severe vitamin D deficiency as less than 25nmol/L.

Fasting blood and second void urine samples were obtained for the measurement of serum C-Terminal Telopeptide Type I Collagen (CTX), Serum Procollagen Type 1N-terminal propeptide (PINP), serum osteocalcin (OC) and urine deoxypyridinoline (DPD), respectively. All blood and urine samples were collected between 8 am and 10 am after an overnight fast. Samples of venous blood were collected from study subjects into serum separator tubes. The blood was allowed to clot at room temperature for 30 minutes then centrifuged at 2500 rpm for 10 minutes at 4°C. The samples were stored at minus 70°C until the assay was performed. Urine samples were collected and stored at minus 20°C until the assay was performed. Serum CTX was measured using electro-chemiluminescence immunoassay (ECLIA) on a Roche elecsys Modular E170 analyser. The measuring range as described in the insert kit was 10–6000 pg/ml with an analytical sensitivity of 10 pg/ml and intra- and inter-assay CV of 5.5% and 7.6%, respectively. The manufacturer's reference range was 299 $\pm$ 137 pg/ml (mean $\pm$ 2SD) for premenopausal women and 556 $\pm$ 226 pg/ml (mean $\pm$ 2SD) for postmenopausal women.

Serum PINP was measured by electro-chemiluminescence immunoassay (ECLIA) on a Roche elecsys Modular E170 analyser. The measuring range as described in the insert kit was 5-1200 μ g/L with an analytical sensitivity of 5 μ g/L (ng/mL), and intra- and inter-assay CV of 2.9 % and 3.8 %, respectively. The manufacturer's reference range was 10 $\pm$ 10.86 ng/mL

(mean $\pm$ 2SD) for premenopausal women and 40.44 $\pm$ 14.40 ng/mL (mean $\pm$ 2SD) for postmenopausal women.

Serum OCN was also measured by electro-chemiluminescence immunoassay (ECLIA) on a Roche elecsys Modular E170 analyser. The measuring range as described in the insert kit was 0.5-300ng/ml with an analytical sensitivity of 0.5ng/ml and intra- and inter-assay CV of 3.3 % and 3.8 %, respectively. The manufacturer's reference range was 21.5 $\pm$ 6.30 ng/mL (mean $\pm$ 2SD) for premenopausal women and 28.99 $\pm$ 10.02 ng/mL (mean $\pm$ 2SD) for postmenopausal women.

Urine DPD was measured using a chemiluminescence immunoassay (CLIA) on an Immulite 2000 analyser. The measuring range as described in the insert kit was 7- 300 mmol/L with an analytical sensitivity of 7 mmol/L and intra- and inter-assay CV of 5.9 % and 7.1 %, respectively. The manufacturer's reference range was 5.2 $\pm$ 1.1 mmol/L (mean $\pm$ 2SD) for premenopausal women and 8.2 $\pm$ 2.7 mmol/L (mean $\pm$ 2SD) for postmenopausal women.

Bone mineral density was assessed using the Hologic DXA Discovery QDR 4500 at the lumbar spine (LS) and femoral neck (FN) by the same technician. The WHO classification of osteoporosis (T score $\leq$ minus 2.5) was used for categorisation. For premenopausal women, a low bone mass was defined as a Z-score of less than minus 2 at either site.

Sample size calculation:

A sample size of 272 (136 premenopausal and 136 postmenopausal women) was required to estimate a 95% CI ($Z_{1-\alpha/2}=1.96$) for the 95% serum CTX reference limit ($z_p=0.845$), with a relative margin of error of 10%, when compared with the 95% reference range ($z_{1-\beta/2}=1.96$).

Statistical analysis:

Data were expressed as mean and 95% confidence interval. Categorical variables were reported when appropriate. For each BTM, a premenopausal and postmenopausal reference range was established by calculating the mid 95% confidence interval. The reference range derived in our study comprised the 2.5 - 97.5 percentile which represented the mid 95% reference range of the study population thus removing the 5% values on the extremes. The reference intervals were calculated using parametric methodology as the data was normally distributed. A similar methodology has been used in deriving reference range for BTMs in other studies.^{12,13}

The independent T-test was used to compare the means of two continuous variables which were normally distributed and nonparametric tests were used if their distribution was not

normal. A difference between 2 groups was considered significant when $P \leq 0.05$. A correlation between 2 continuous variables was performed using Pearson and Spearman correlation based on the distribution of the variables. Receiver Operating Characteristics (ROC) curves were constructed using different cut-offs of BTMs and T-scores of the lumbar spine and femoral neck BMD in a cohort of postmenopausal women, to derive the cut-offs for each BTM which could best predict the risk of osteoporosis (T-score ≤ -2.5). Statistical analysis was undertaken using the SPSS 16.0 software package.

RESULTS:

Three hundred and fifty subjects, comprising 175 postmenopausal women and their premenopausal daughters ($n=175$), were recruited by random cluster sampling. Of these, 50 subjects (25 pairs) were excluded based on exclusion criteria for either the postmenopausal women or their daughters (Figure-1).

Finally, three hundred subjects which included 150 postmenopausal women and their daughters ($n=150$) who met the inclusion criteria were recruited from the community after obtaining written informed consent.

Demography, biochemistry and BMD of study subjects are shown in table 1. The mean age (SD) of the premenopausal and postmenopausal group was 35.6 ± 5.4 years and 59.0 ± 5.4 years, respectively. The prevalence of vitamin D deficiency ($< 50 \text{ nmol/L}$) was 131/300 (43.6%) overall with 69 (46%) premenopausal women and 63 (42%) postmenopausal women having vitamin D deficiency. The mean BMD at the LS and femoral neck was significantly lower in the postmenopausal group as compared to the premenopausal group ($P < 0.001$), in accordance with their advancing age and menopausal status. Premenopausal women were more physically active as assessed by IPAQ questionnaire. 65.3% premenopausal women were categorised as having high physical activity (>3000 met equivalents) as compared to 43% in the postmenopausal group.

In the postmenopausal group, 45% ($n=66$) had osteoporosis at the spine and 25.3% ($n=38$) had osteoporosis at the femoral neck. In the premenopausal women, low bone mass was seen in 5.3% ($n=8$) at the spine and 0.7% ($n=1$) at the femoral neck.

The distribution of BTMs was analysed for premenopausal and postmenopausal women. Both bone resorption markers (serum CTX & Urine DPD) and bone formation markers (serum OC & serum P1NP) were significantly higher in postmenopausal women

than premenopausal women. The reference range for all BTMs for premenopausal and postmenopausal women was derived based on the mid 95% confidence interval. The mean and reference range for each marker is shown in table 2. The reference range calculated in this study includes subjects with vitamin D deficiency as a large proportion of the healthy Indian population is vitamin D deficient, and further analysis excluding subjects with severe vitamin D deficiency did not reveal a significant difference.

■ Serum CTX, P1NP, OC and urine DPD had a significant negative correlation with BMD at the lumbar spine ($P < 0.05$). At the femoral neck, a statistically significant negative correlation of serum CTX, P1NP, OC and BMD was seen. Although there was a negative correlation between urine DPD & FN BMD, it did not reach statistical significance (Figure-2). Serum CTX (pg/ml) and P1NP ($\mu\text{g/ml}$) were significantly higher in women with osteoporosis ($N=67$) at the spine when compared to the women who did not have osteoporosis ($N=83$) (674.01 ± 246.56 Vs 524.46 ± 277.10 , $P=0.01$ and 72.40 ± 36.73 Vs 61.41 ± 25.12 , $P=0.039$ respectively). Serum OC and urine DPD were also higher in postmenopausal women, but this did not attain statistical significance.

The analytical performance of each BTM for the diagnosis of osteoporosis at the lumbar spine and femoral neck was studied by using Receiver Operating Curve (ROC) analysis in the postmenopausal women (Figure-3). All the BTMs showed a statistically significant area under the curve (AUCs) except urine DPD. These values did not show statistically significant difference after excluding subjects with severe vitamin D deficiency. The cut-off values of BTMs for prediction of osteoporosis at the spine were 413.40 pg/ml for CTX (sensitivity-91%, specificity-41%), 42.2 $\mu\text{g/ml}$ for P1NP (sensitivity-91%, specificity-33%), 16.15 ng/ml for OC (sensitivity-91%, specificity-34%) and 9.12 nmol/l for urine DPD (sensitivity-77%, specificity-30%). The cut-off values of BTMs for prediction of osteoporosis at the femoral neck were 405.40 pg/ml for CTX (sensitivity-86%, specificity-30%), 49.85 $\mu\text{g/ml}$ for P1NP (sensitivity-84%, specificity-38%), 18.65 ng/ml for OC (sensitivity-81%, specificity-30%), and 9.28 nmol/L for urine DPD (sensitivity-73%, specificity-30%).

BMD of the daughters with osteoporotic mothers was compared with the daughters of non-osteoporotic mothers. Daughters born to mothers with osteoporosis at the lumbar spine had a significantly lower BMD at the spine compared to those whose mothers did not have osteoporosis. However, there were no significant differences at the femoral neck. Similarly daughters of mothers with osteoporosis at the femoral neck had a lower BMD at the femoral

neck with no statistically significant difference at the spine (Table-3). There was no significant difference between serum CTX, P1NP, OC and urine DPD of daughters born to mothers with osteoporosis at the LS versus those whose mothers did not have osteoporosis (P=NS). **DISCUSSION:**

This community based study was undertaken in a unique cohort of healthy mothers and their daughters, where we studied the reference range of BTMs, their relationship with BMD and also compared the BMD and BTMs of daughters of the women who had osteoporosis with those whose mothers did not have osteoporosis. Nearly 45% of these postmenopausal women had osteoporosis at the spine, which is similar to that reported in previously published studies from India.^{7,12} A quarter had osteoporosis at the femoral neck, which constitutes an important finding in view of the high risk of hip fractures and its associated morbidity and mortality. The high prevalence of osteoporosis seen in our study subjects is in contrast to an approximate 30% prevalence reported in the Caucasian population.¹⁴ This is not explained by the database used, as in a recent study the Indian reference database underperformed in the diagnosis of osteoporosis compared to a Caucasian database.¹⁶ When considering the number of postmenopausal women in India (around 100 million), the magnitude of the problem is huge. Among the premenopausal women, around 5% had a low bone mass at the spine which is akin to a study by Harinarayan *et al* in this age group.¹⁷ Vitamin D deficiency was seen in 43% of the subjects, unlike previously published Indian studies which have shown a much higher prevalence of around 50-80%.^{7, 18} This is probably due to an increase in awareness and supplementation provided by the various educational programmes conducted by the local community health services.

Postmenopausal women had higher levels of both bone formation and resorption markers when compared to premenopausal women in our study. The reference range derived in the postmenopausal women showed a steep increment from premenopausal to the postmenopausal age group, which has also been shown in recent studies from Saudi Arabia and Japan.^{12,19} This emphasises the need for different reference ranges for premenopausal and postmenopausal women.

The reference range for each of the BTMs in our study, which is representative of the South Indian urban population, was higher when compared to the reference values derived in a Spanish study, with the exception of sP1NP.²⁰ The variation in the reference ranges found across different populations could be secondary to differences in the assays used in individual

studies, pre-analytical variations of the BTMs, ethnicity-specific differences, lifestyle, epigenetic and genetic influences on the rate of bone remodelling.^{13,21} This necessitates the use of an ethnicity-specific reference range in clinical practice. To reduce the pre-analytical variability in our study, samples for BTMs were collected in the morning after an overnight fast during the same season of the year. BTMs were measured by an electrochemiluminescence immunoassay which is a robust assay when compared to other immunoassays.

BMD at the spine and the femoral neck showed a significant correlation with all the BTMs except for urinary DPD at the femoral neck. Similar correlations between BTMs and BMD have been shown in studies by Ardawi *et al* and Botella *et al*.^{12,20} Urinary DPD did not show a good correlation, probably due to its known analytical variability. In addition, although corrected for creatinine DPD was collected as a spot sample which may not have reflected the true dynamics of bone turnover. A significant inverse correlation observed in our study between BTMs and BMD may have to be studied longitudinally in an Indian context, where the interplay of various factors such as vitamin D deficiency and poor nutrition may further influence bone remodelling, especially in this age group. A significantly higher CTX and PINP in those with osteoporosis at the spine when compared to those without osteoporosis in our study was also seen in a Spanish study which showed a higher level of CTX, PINP, OC and sclerostin in osteoporotic subjects.²² Women with elevated BTMs have been found to have a three- to five- times higher rate of bone loss.²³ In addition to being cost-effective, the change in BTMs following initiation of treatment is rapid and can be seen as early as 3 months.

In our study, a trade-off between sensitivity and specificity of BTM assays for the diagnosis of osteoporosis was seen, with a poor specificity and a moderate area under curve. Similar analytical performance has also been shown in recent studies.^{20,21}

Daughters of mothers with osteoporosis at the spine and the femoral neck had a significantly lower BMD at the spine and the femoral neck, respectively, when compared to those whose mothers did not have osteoporosis at these sites. However, there was no significant difference in BTMs between daughters of mothers with osteoporosis when compared to those whose mothers did not have osteoporosis. The mother-daughter concordance in this study not only reflects the influence of genetic factors on BMD but also the shared environmental factors in view of the same locality and similar lifestyles in terms of nutrition, socioeconomic status,

sunlight exposure and physical activity. Differences in BMD observed between 2 groups implicate the role of genetic, epigenetic as well as similar environmental factors in the attainment of peak bone mass for both trabecular and cortical bone. Similar findings were seen in a study from China, where daughters of mothers with osteoporosis had a lower peak bone mass as compared to daughters whose mothers did not have osteoporosis.²⁴ Previously published paired studies have also shown that the inheritance of bone mass in women is determined by the peak bone mass attained, and thereafter a decline in the bone mass secondary to bone loss that occurs especially after the menopause, which runs in families between mothers and daughters.²⁵ Daughters of mothers with low BMD and osteoporotic fractures have been shown to have significantly lower BMD (5.0–8.0%, $P < 0.017$) as compared to daughters of mothers with normal BMD.²⁶ The differences observed in BMD but not in BTMs between the daughters of osteoporotic and non-osteoporotic mothers may be attributable to a difference in peak bone mass and may not reflect the variations seen in bone remodelling between the two groups. Genetic influence plays an important role in the acquisition of peak bone mass, in the rate of bone loss and the risk of an osteoporotic fracture in an individual. Parental hip fracture has been shown to be one of the major risk factors for fragility fracture.²⁷ This familial predisposition to fracture risk could be secondary to both genetic susceptibility and environmental risk factors shared by the family members. The genetic influence on osteoporosis is complex, with multiple candidate genes implicated to have small-to-moderate effects in the attainment of peak bone mass and bone loss.²⁸

LIMITATIONS OF THE STUDY

This study was undertaken in an urban area and may not represent a rural community. In premenopausal women, the collection of blood or urine samples for BTMs was performed regardless of the particular phase of the menstrual cycle.

CONCLUSION

This is the first community study from India to establish normative data of BTM and to derive a reference range in a cohort of premenopausal women and their mothers recruited by a cluster sampling technique. [A significant inverse correlation between BTMs \(except for urine DPD\) and BMD at all sites was seen in our study.](#) Daughters born to mothers with osteoporosis at the spine and femoral neck had a significantly lower BMD at the spine and femoral neck, respectively, when compared to those whose mothers did not have osteoporosis

at these sites, suggesting the probable interplay of genetic, epigenetic and similar environmental factors in the attainment of peak bone mass.

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Table 1: Demography, Biochemistry and BMD of study subjects

Variables	Postmenopausal	Premenopausal	P value
	Women	Women	
	(N=150) Mean $\pm$ SD	(N=150) Mean $\pm$ SD	
Age (years)	58.9 $\pm$ 5.4	35.1 $\pm$ 5.2	< 0.001
BMI (kg/m ²)	25.2 $\pm$ 4.9	26 $\pm$ 4.9	NS
Parity	3.6 $\pm$ 1.7	2.4 $\pm$ 1.2	< 0.001
Dietary calcium intake (mg/24 hrs)	501 $\pm$ 124	511 $\pm$ 120	NS
Sunlight exposure (min/day)	116 $\pm$ 92	122 $\pm$ 106	NS
Menopausal age (yrs)	45.7 $\pm$ 4.7	-	-
Years since menopause	13.5 $\pm$ 7.4	-	-
Corrected Calcium (mmol/L)	2.25 $\pm$ 0.12	2.27 $\pm$ 0.12	NS
Albumin (g/L)	44 $\pm$ 5	44 $\pm$ 07	NS
Phosphorus(mmol/L)	4.0 $\pm$ 0.5	3.6 $\pm$ 0.5	< 0.001
25(OH)Vitamin D (nmol/L)	56.0 $\pm$ 26.7	50.0 $\pm$ 21.5	0.049
Alkaline phosphatase (U/L)	93.3 $\pm$ 24	72.9 $\pm$ 25	< 0.001
Creatinine (μ mol/L)	62.8 $\pm$ 8.8	62.78 $\pm$ 8.1	NS
PTH (pmol/L)	5.6 $\pm$ 3.6	5.2 $\pm$ 2.2	NS
Lumbar spine BMD (g/cm ²)	0.801 $\pm$ 0.142	0.993 $\pm$ 0.112	<0.001
Femoral Neck BMD (g/cm ²)	0.658 $\pm$ 0.111	0.812 $\pm$ 0.113	<0.001

Table-2: Mean and 95% reference range of BTMs in pre and postmenopausal women

Parameters	Postmenopausal women (N=150)		Premenopausal women (N=150)		P value
	Mean $\pm$ 2SD	Reference range (2.5-97.5 percentile)	Mean $\pm$ 2SD	Reference range (2.5-97.5 percentile)	
SerumCTX (pg/ml)	592 $\pm$ 274	134.2 – 1352.9	373 $\pm$ 200	120.3 – 915.9	<0.001
SerumPINP (μ g/ml)	66.3 $\pm$ 31.2	27.11 – 148.41	52.5 $\pm$ 34.6	24.53 - 99.48	<0.001
SerumOC (ng/ml)	26.2 $\pm$ 13.5	9.39 - 61.56	17.8 $\pm$ 7.1	8.41 – 33.45	<0.001
UrineDPD (n.mol/m.mol creatinine)	11.2 $\pm$ 3.3	5.93- 21.12	9.8 $\pm$ 2.9	5.10 – 15.33	<0.001

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ROC curve of BTMs in relation to LS BMD T-score

ROC curve of BTMs in relation to FN BMD T-score

Variable	AUC	P value	95% CI
Serum CTX	0.68	< 0.001	0.59-0.76
Serum P1NP	0.59	0.05	0.50-0.68
Serum OC	0.66	0.001	0.57-0.75
Urine DPD	0.51	0.89	0.41-0.60

Variable	AUC	P value	95% CI
Serum CTX	0.61	0.038	0.50-0.72
Serum P1NP	0.65	0.008	0.54-0.77
Serum OC	0.62	0.026	0.51-0.73
Urine DPD	0.52	0.717	0.40-0.63

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Author/s:

Shetty, S;Kapoor, N;Dian Bondu, J;Antonisamy, B;Thomas, N;Paul, TV

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