Serum levels of phosphorylated heat shock protein 27 (pHSP27) are associated with bone mineral density in pre- & postmenopausal women: A pilot study

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Background & objectives: Phosphorylated heat shock protein 27 (pHSP27) has been implicated in the pathogenesis of osteoporosis. Oxidative stress and proinflammatory cytokines, which are known to be involved in aetiology of osteoporosis, can trigger HSP27 phosphorylation. Since pHSP27 is present in circulation, it was hypothesized that serum pHSP27 would be elevated in low bone mineral density (BMD) condition and might serve as an indicator of osteoporosis/osteopenia. Hence, the aim of this study was to examine serum levels of pHSP27 in relation with BMD in pre- and postmenopausal women.

Methods: Premenopausal (30 to 40 yr) and postmenopausal (50 to 60 yr) women having either low BMD (osteopenia/osteoporosis) or high BMD were selected (n=80) from a prospective cohort (n=200). Serum levels of pHSP27; along with levels of oestradiol, malondialdehyde, total antioxidant capacity, interleukin (IL)-1, IL-6, tumour necrosis factor - alpha, (TNF-α), c-telopeptide fragments of collagen type I (CTX-1) and osteocalcin were estimated.

Results: The serum levels of pHSP27 were significantly elevated in low BMD groups in premenopausal and postmenopausal categories ($P<0.05$). It also exhibited a significant odds ratio (OR) to differentiate between low and high BMD in both premenopausal (OR=1.734, P=0.013) and postmenopausal (OR=1.463, P=0.042) categories. Additionally, area under the curve to predict low BMD was non-significantly higher for pHSP27 than CTX-1 in premenopausal and postmenopausal categories.

Interpretation & conclusions: This study highlights a novel relation between serum pHSP27 and BMD in Indian women however, these findings need to be confirmed in larger studies.

Key words Bone mineral density (BMD) - osteoporosis - phosphorylated HSP27 - postmenopause - premenopause

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and increased susceptibility for fragility fracture. Recently it has been reported that 50 million people have osteoporosis or low bone mass in India[1]. It is more prevalent in females than in males, and more so, in postmenopausal women due to decline in levels of estradiol. Besides its direct protective actions on

the skeleton, estradiol also has antioxidant and anti-inflammatory properties[2,3]. Hence, menopause is usually accompanied with a rise in oxidative stress and proinflammatory cytokines[4,5]. This leads to the enhanced formation and activity of osteoclasts (bone resorbing cells) compared with that of osteoblasts (bone forming cells), leading to a net decrease in BMD[6]. It is well recognized that oxidative stress and proinflammatory cytokines exert adverse effects on skeletal health. Even so, the mechanisms underlying the complex aetiology of postmenopausal osteoporosis are not completely understood.

Oxidative stress and proinflammatory cytokines can directly induce phosphorylation of heat shock protein 27 (HSP27)[7-10]. HSP27 is a molecular chaperone belonging to the small heat shock protein group. Phosphorylated HSP27 (pHSP27) level has been found to be significantly elevated in serum of patients with advanced lung cancer compared with early stages[11]. In our recent proteomics study, we found pHSP27 to be upregulated in osteoclast precursors in low BMD condition and exogenous pHSP27 could increase the migration of osteoclast precursors towards RANKL representing the bone milieu[12]. In addition, it has been previously shown that number of osteoclasts formed is inversely related to BMD[13]. Thus, it can be deduced that elevated pHSP27 in osteoclast precursors may lead to their increased migration towards the bone milieu and thereby increased osteoclastogenesis which can contribute to the pathogenesis of osteoporosis. As oxidative stress and proinflammatory cytokines can drive phosphorylation of HSP27, it would be imperative to know whether elevation of pHSP27 in low BMD condition can also be seen in serum and used as an indicator of bone health.

Hence, to examine the association of serum pHSP27 with BMD, in unison with its inducers, we sought to determine serum levels of pHSP27 in pre- and postmenopausal Indian women with low (osteopenia/osteoporosis) versus high BMD, along with oxidative stress indicators (malondialdehyde and total antioxidant capacity), proinflammatory cytokines [interleukin (IL)-1, IL-6 and tumour necrosis factor-α) (TNFα)] and also oestradiol, in a nested case control study. We also compared the predictive ability of pHSP27 with bone turnover markers, osteocalcin (bone formation marker) and C-telopeptide fragments of collagen type I or CTX-1 (bone resorption marker).

Material & Methods

This study was carried out at the National Institute for Research in Reproductive Health (NIRRH), Mumbai, India, from March 2011 to April 2013. The protocol was approved by the Institutional Ethics Committee for clinical studies. Premenopausal women (n=100) aged 30 to 40 yr with regular menstrual cycles and postmenopausal women (n=100) aged 50 to 60 yr having achieved menopause at least one year prior attending the health clinic of NIRRH, were consecutively enrolled. All these women gave their written informed consents to participate in the study. All the participants were apparently healthy, physically active, and none had nutritional deficiencies as per their dietary recall. Exclusion criteria comprised smoking habits, alcohol consumption, obesity, diabetes, hypertension, chronic disorders or malignancies, arthritis or any other bone disease, regular intake of any drugs or medication. Dual energy X-ray absorptiometry (DXA, *QRR* 1000, Hologic Inc., USA) was used for measurement of BMD (g/cm^2) at hip (femoral neck or FN) and at spine (lumbar spine 1-4 or LS) for all 200 enrolled participants. Participants were categorized as osteoporotic (T score ≤ - 2.5 at either of the two sites); osteopenic (T score between -1 and -2.5 at either of the two sites) and normal (T score ≥ -1 at both the sites) in accordance with World Health Organization criteria[14].

The classification of low and high BMD subjects was essentially based on centile distribution of BMD values only. Accordingly, BMD values of the women belonging to pre- and postmenopausal categories were arranged according to centile, of whom, women with either low BMD (osteoporosis/osteopenia) *i.e.* the women having the lowest BMD (≤20th centile) or high BMD *i.e.* those having the highest BMD (≥ 80th centile) were selected. The selected women (total n = 80) included 20 women in each of the four categories *(i)* premenopause low BMD, *(ii)* premenopause high BMD, *(iii)* postmenopause low BMD, and *(iv)* postmenopause high BMD.

Fasting blood was collected from the selected participants (n = 80) within a period of three months from DXA measurement and serum was separated immediately and stored at -80°C. Blood was collected from 0900 to 1000 h. For premenopausal women blood collection was done on menstrual day 5 to day 8 and for postmenopausal women on any convenient day.

HSP27 phosphorylation occurs at serine 15, 78 and 82, however, serine 78 and 82 are the major sites of phosphorylation[15]. Hence, serum levels of HSP27 phosphorylated at serine 78/82 and also total HSP27 were estimated using commercial sandwich ELISA kits (R & D systems, USA). Phosphorylated HSP27 was expressed as percentage of phosphorylation calculated by estimating ratio of phosphorylated fraction to total HSP27 multiplied by 100 for each individual sample. This was done to exclude the possibility that the rise in its phosphorylated form was simply due to rise in total expression, hence, to assess an actual change in phosphorylation status. Further, 17 - β estradiol was measured in serum using a competitive ELISA kit (ImmunoDiagnostic Systems, Canada). The thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical, USA) was used for estimation of malondialdehyde (MDA), a byproduct of lipid peroxidation which is used as an indicator of oxidative stress. Total Antioxidant Capacity kit (Cayman Chemical, USA) was used for estimating antioxidant status. That proinflammatory cytokines IL - 1β, IL-6 and TNF-α were measured using sandwich ELISA kits (BD Biosciences, USA). Intact human osteocalcin was measured using a commercially available solid phase Enzyme Amplified Sensitivity Immunoassay (DIASource Immunoassays, Belgium) and C-telopeptide fragments of collagen type I or CTX-I was measured using Serum CrossLaps® ELISA (Nordic Bioscience Diagnostics, Denmark). The intra-

and inter-assay coefficients of variations were less than 10 and 15 per cent, respectively for all assays.

Statistical analysis: Data were represented as median and interquartile range (IQR). Graphpad Prism version 6 (GraphPad Software, Inc., USA) was used to compare parameters between two independent groups using Mann Whitney U tests and also for Spearman rank correlation. Multivariate logistic regression was performed using Statistical Package for Social Sciences (SPSS) version 16 for windows (SPSS, IBM, USA) Receiver operating characteristic (ROC) curve was computed to calculate area under the curve (AUC) using MedCalc software version 13 (Med Calc Software, Ostend, Belgium). For correlation, data of all samples were considered (n=80). DeLong test was applied to compare AUCs using MedCalc software version 13. For logistic regression and ROC curve, data were categorized as *(i)* premenopause low BMD (n=20) and premenopause high BMD (n=20), and *(ii)* postmenopause low BMD (n=20) and postmenopause high BMD (n=20).

Results

Baseline characteristics of the selected participants are shown in Table I. Age and body mass index (BMI) were not significantly different, whereas, BMD (z and T scores) at FN and at LS were significantly different (*P*<0.0001) in cases and controls. All women in the low BMD group were previously undiagnosed and untreated cases of osteopenia/osteoporosis at FN and/

Parameters	Premenopausal women (30 to 40 yr)			Postmenopausal women (50 to 60 yr)		
	Low BMD	High BMD	*P*	Low BMD	High BMD	*P*
No. of samples	20	20	-	20	20	-
Age (yr)	36.5 (32.5 - 39)	36 (31.5 - 39)	0.419	55 (52 - 58.8)	53.5 (52 - 55)	0.250
BMI (kg/m²)	24.5 (22.1 - 25.6)	26.3 (24.6 - 7.6)	0.055	25.1 (23.8 - 27.3)	26.2 (24.5 - 29)	0.141
FN BMD (g/cm²)	0.82 (0.79 - 0.84)	1.07 (1.03 - 1.1)	<0.001	0.726 (0.6 - 0.75)	1.02 (0.98 - 1.1)	<0.001
T score FN	-1.4 (-1.6 - 1.2)	0.75 (0.43 - 1.05)	<0.001	-2.3 [(-2.8) - (-1.9)]	0.3 (0 - 0.75)	<0.001
Z score FN	-1.2 [(-1.5) - (-0.83)]	0.9 (0.7 - 1.08)	<0.001	-1.1 (-1.6 - -0.83)	1.1 (0.5 - 1.4)	<0.001
LS BMD (g/cm²)	0.99 (0.95 - 1.1)	1.22 (1.17 - 1.27)	<0.001	0.79 (0.75 - 0.89)	1.2 (1.09 - 1.2)	<0.001
T score LS	-1.6 [(-1.9) - (-0.7)]	0.4 [(-0.1) - (0.8)]	<0.001	-3.3 [(-3.5) - (-2.4)]	-0.1 [(-0.7) - 0.5]	<0.001
Z score LS	-1.1 (-1.5 - -0.28)	-0.4 (-0.45 - 0.78)	0.005	-2.1 [(-2.4) - (-1.3)]	0.4 [(-0.18) - (1.03)]	<0.001
OPP/OP/N	OP (20/20)	N (20/20)	-	OPP (17/20), OP (3/20)	N (20/20)	-

Table I. Baseline characteristics and bone mineral density (BMD) measurements of the study participants (n=80)

Values expressed as median (interquartile range). BMI, body mass index; FN, femoral neck; LS, lumbar spine; OPP, osteoporosis; OP, osteopenia (at one or both sites); N, normal BMD at both sites

Table II. Serum estimations in premenopausal and postmenopausal women with low versus high bone mineral density (BMD)

Serum parameters	Premenopausal women (30 - 40 yr)		Postmenopausal women (50 - 60 yr)	
	Low BMD	High BMD)	Low BMD	High BMD
pHSP27 (%)	5.25 (4.01 - 6.71)	3.24 (1.86 - 4.97)**	4.6 (3.5 - 8.1)	3.63 (3- 4.2)*
E2 (pg/ml)	84.3 (49.7 - 101)	86.8 (53.9 - 122)	28.4 (17.6 - 50.8)† ††	39 (26.6 - 59.8)δδ
MDA (μM)	2.85 (2.5 - 3.6)	2.57 (2.5 - 2.9)	3.77 (3.4 - 3.9)†	3.5 (2.7 - 3.7)δδδ
TAC (mM)	0.86 (0.7 - 1.2)	0.96 (0.7 - 1.5)	0.56 (0.5 - 0.9)†	0.72 (0.6 - 0.9)δδ
IL-1 (pg/ml)	6.5 (4.8 - 9.6)	6 (5.1 - 9.3)	15.3 (11.5 - 17.6)†††	11.5 (8.9 - 15.2)*δδδ
IL-6 (pg/ml)	8.2 (4.7 - 10.7)	5.6 (7.8 - 12)	15.4 (13.7 - 20.6)†††	10.8 (9.5 - 14.6)*δ
TNF-α (pg/ml)	8.33 (6.8 - 10.5)	8.08 (6.5 - 9.7)	14.6 (10.7 - 17.7)†††	10.6 (9.5 - 12.3)*δδ
CTX-1 (pg/ml)	402 (364 - 434)	324 (284 - 425)*	619 (501 - 697)†††	475 (456 - 577)*δδδ
OC (ng/ml)	6.32 (5.72 - 7.7)	5.76 (5.06 - 6.87)	7.9 (6.77 - 8.65)†	7.02 (6 - 7.92)δ

Values expressed as median (interquartile range). BMD, bone mineral density; HSP27, Heat shock protein 27; p, percentage of phosphorylated protein; E_2, estradiol; MDA, malondialdehyde; TAC, total antioxidant capacity; IL, interleukin; TNF, tumour necrosis factor; CTX-1, C-telopeptide fragments of collagen type I; OC, osteocalcin
P*<0.05 **<0.01 compared to low BMD in the respective group
P†<0.05 †††<0.001 compared to low BMD premenopausal group
Pδ<0.05 δδ<0.01 δδδ<0.001 compared to high BMD premenopausal group

or LS; while participants in the high BMD group were normal at both FN and LS.

Comparison of serum parameters based on BMD and menopause: Table II shows the comparison of serum parameters based on BMD and menopause. The pHSP27 was significantly elevated in low BMD groups in both pre- and postmenopausal categories. Also, C-telopeptide fragments of collagen type I (CTX-1) was significantly higher in low BMD in both categories. Median levels of oestradiol (E_2), total antioxidant capacity (TAC) were marginally raised in high BMD condition, whereas, MDA and OC were marginally raised in low BMD condition, in both pre- and postmenopausal categories, however, not significantly different. Further, IL-1, IL-6 and TNF-α were significantly higher in low BMD condition in post- but not in premenopausal category.

On comparing premenopausal versus postmenopausal women within similar BMD conditions, E_2 and TAC were significantly higher in pre, while, MDA, IL-1, IL-6, TNF-α, CTX-1 and OC were significantly higher in post menopausal category. Besides, pHSP27 was not significantly different in pre versus post categories within similar BMD conditions.

Relation of serum parameters with BMD: pHSP27 showed a significant inverse correlation with BMD at FN (r = -0.320, P = 0.004) and at LS (r = -0.315, P

= 0.004) (Fig.1). Further, E_2 positively correlated with BMD at FN (r = 0.268, P= 0.016) and at LS (r = 0.321, P= 0.004). Also, TAC positively correlated with BMD at FN (r = 0.358, P = 0.001) and at LS (r = 0.354, P = 0.001). Conversely, MDA inversely correlated with BMD at FN (r = -0.276, P = 0.01) and at LS (r = -0.367, P = 0.001). IL-1, IL-6 and TNF-α exhibited inverse correlation with BMD at FN (IL-1 r = -0.321, P = 0.004; IL-6 r = -0.275, P = 0.01; TNF -α r = -0.304, P = 0.006) and at LS (IL-1 r = -0.368, P = 0.001; IL-6 r = -0.228, P = 0.04; TNF-α r = -0.327, P = 0.003). Besides, OC was inversely correlated with BMD at FN (r = -0.248, P = 0.027) and at LS (r = -0.221, P = 0.049). Also, CTX-1 was inversely correlated with BMD at FN (r = -0.404, P<0.0001) and at LS (r = -0.330, P= 0.003).

Relation of serum parameters with pHSP27: pHSP27 showed significant positive correlation with MDA (r = 0.456, P< 0.0001), IL-1 (r = 0.318, P = 0.004) and TNF-α (r = 0.369, P = 0.0008) and an inverse correlation with TAC (r = -0.363, P = 0.0009) (Fig. 2). However, pHSP27 did not correlate with IL-6 and E_2, and there was no correlation between pHSP27 and OC or between pHSP27 and CTX-1.

Predictive ability of pHSP27: Using multivariate logistic regression, it was found that pHSP27 and CTX-1 exhibited significant odds ratios to predict low BMD condition (Table III). The odds ratio exhibited
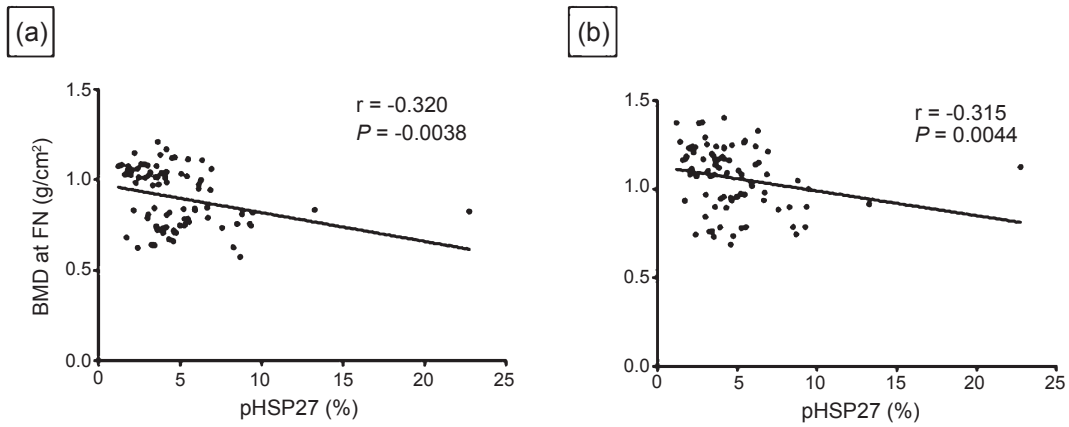
Fig. 1. Correlation of phosphorylated HSP27 with BMD at **(a)** Femur neck (FN) and **(b)** Lumbar spine (LS) in women (n=80).
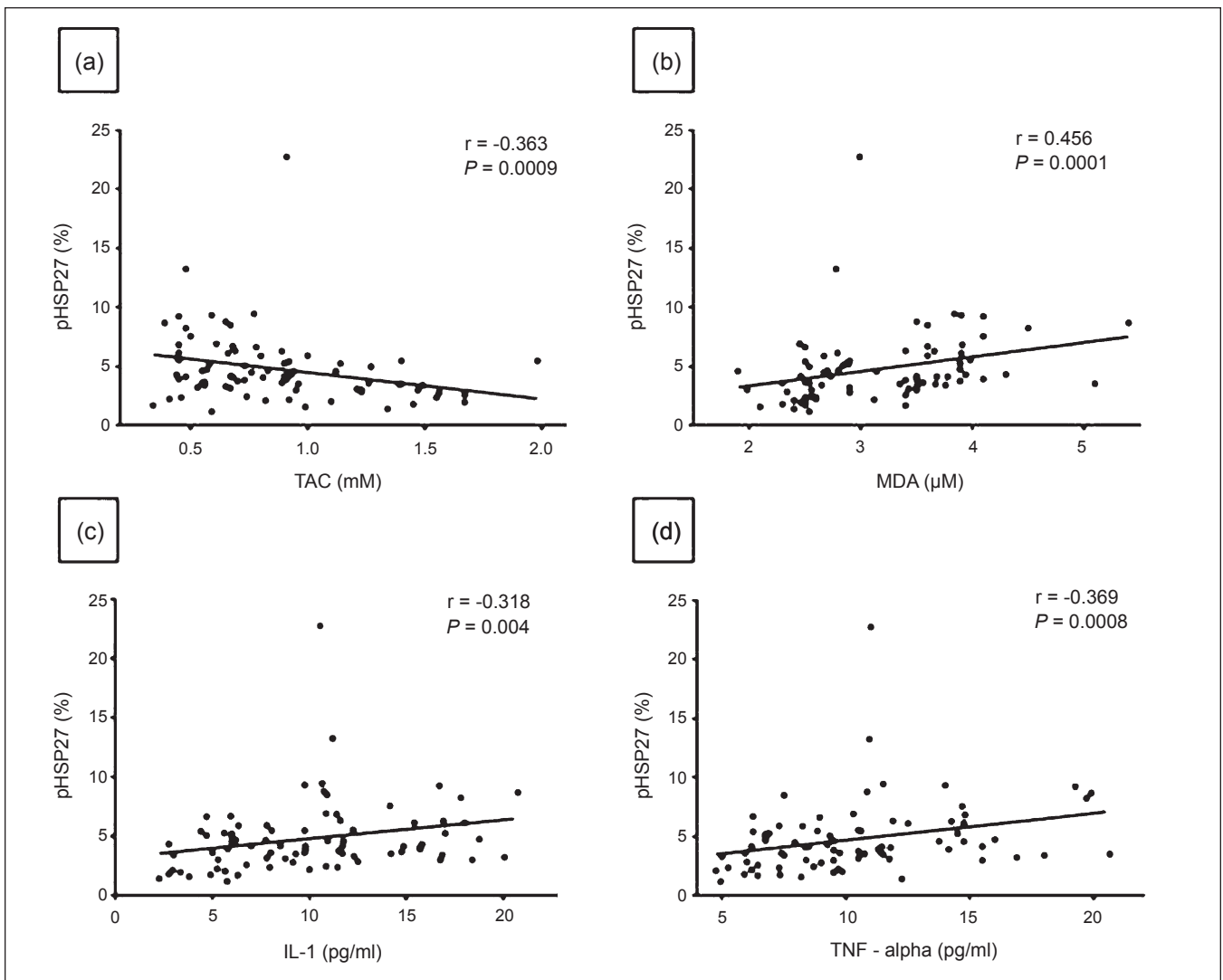

Fig. 2. Correlation between phosphorylated HSP27 (pHSP27) and **(a)** Total antioxidant capacity (TAC), **(b)** Malondialdehyde (MDA), **(c)** Interleukin -1 (IL-1) and **(d)** Tumour necrosis factor - alpha (TNF-α), in Indian women (n=80).

Table III. Multivariate logistic regression analysis with odds ratios of serum parameters for predicting low BMD in premenopausal (n=40) and postmenopausal (n=40) women

| Serum parameters	Premenopausal women			Postmenopausal women		
	OR	95% CI	*P*	OR	95% CI	*P*
pHSP27	1.734	1.123 - 2.676	0.013	1.463	1.013 -2.112	0.042
CTX-I	1.591	1.153 - 1.907	0.032	1.303	0.1 - 1.553	0.044

OR, odds ratio; CI, confidence interval; pHSP27, phosphorylated heat shock protein 27; CTX-1, C-telopeptide fragments of collagen type I

by pHSP27 was higher than CTX-1 for both pre and post categories. Further, since pHSP27 and CTX-1 showed significant odds ratio in both categories, ROC curves were computed to assess their predictive ability. The pHSP27 showed significant AUC for predicting low BMD in both pre (AUC = 0.765, *P* = 0.0004) and post (AUC = 0.688, *P* = 0.0308) categories (Fig. 3). Likewise, CTX-1 showed significant AUC for predicting low BMD in pre (AUC = 0.695, *P* = 0.0271) and post (AUC = 0.686, *P*= 0.0001) categories. In both categories, the AUC exhibited by pHSP27 was higher

than CTX-1, although the difference in AUCs was not significant.

Discussion

In this study, pHSP27 was found to be significantly elevated in low BMD condition compared with high BMD condition. Oxidative stress and proinflammatory cytokines can trigger phosphorylation of HSP27[7-10]. In agreement with this, the MDA, TAC, IL-1 and TNF - α showed direct correlation with pHSP27 in our study. Although IL-6 is considered a proinflammatory
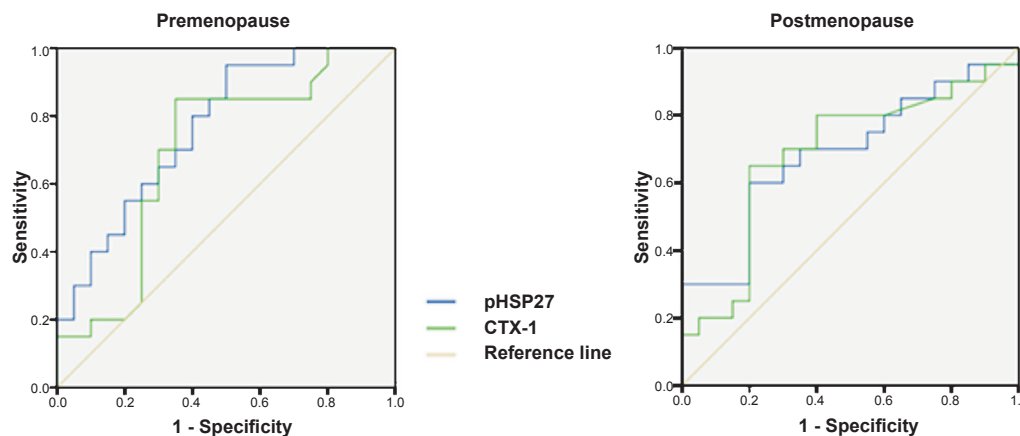
Parameter	AUC (95% CI)	*P* value	Sensitivity (%)	Specificity (%)	Associate criterion
		Premenopause			
pHSP27	0.765 (0.604 - 0.884)	0.0004	80	60	>3.63
CTX-1	0.695 (0.529 to 0.830)	0.0271	85	65	>0.337
		Postmenopause			
pHSP27	0.686 (0.522 - 0.824)	0.0308	60	80	>4.18
CTX-1	0.686 (0.520 - 0.8323)	0.0365	65	80	>0.585

ACU, area under the curve computed by receiver operating characteristic curve; CI, confidence interval; HSP27, heat shock protein 27; p, stands for percentage of phosphoted protein; CTX-1, c-telepeptide fragments of collagen type I

Fig. 3. Receiver operating characteristic (ROC) curves showing area under the curve (AUC) exhibited by phosphorylated HSP27 and CTX-1 to distinguish between low BMD (n=20) and high BMD (n=20) in pre- and postmenopausal Indian women.

cytokine, it also has anti-inflammatory properties, which may be a possible reason for lack of association with pHSP27[16]. Bone turnover markers (CTX-1 and OC) showed no correlation with pHSP27 but serum levels of pHSP27 were associated with oxidative stress and proinflammatory cytokines.

It is well known that oxidative stress and proinflammatory cytokines have adverse effects on bone density. Concordantly, we observed that MDA, IL-1, IL-6 and TNF - α were inversely correlated with BMD, while, TAC was positively correlated with BMD. Yet, median levels of MDA and TAC were not significantly higher and lower in low BMD condition, respectively, for both pre- and postmenopausal categories. This implies a marginally altered oxidative balance in low BMD and is consistent with a previous study addressing the link between oxidative stress and BMD, although, significant alterations have also been reported in another study[17,18]. In our study, IL-1, IL-6, TNF- α were significantly raised in women with low BMD compared to women with high BMD in post menopausal women. However, this was not the case in pre category, probably since low BMD group in precategory comprised women with osteopenia (20/20), whereas, low BMD group in post category comprised women mostly with osteoporosis (17/20). Studies in the past have also shown a significant rise in proinflammatory cytokines in postmenopausal osteoporosis and have postulated a synergistic mechanism resulting in their simultaneous elevation[19-21].

The median levels of E_2 marginally declined in low BMD condition, in both pre and post categories. This is plausible since significant decline in E_2 may not be the only basis for postmenopausal osteoporosis[22]. E_2 and BMD showed a direct correlation with each other, consistent with another study, though a few studies have refuted an association[23-25].

Bone turnover markers, OC (bone formation marker) and CTX-1 (bone resorption marker) had an inverse correlation with BMD as shown in earlier studies[26,27]. In both pre- and postmenopausal categories, CTX-1 was significantly higher in low BMD condition, which was concordant with earlier reports[27,28].

On comparing pre- versus postmenopausal categories within similar BMD conditions, E_2 and TAC were significantly elevated in pre, whereas, MDA, IL-1, IL-6, TNF- α, CTX-1 and OC were significantly elevated in postmenopausal group. These results were expected as menopause results in decline in levels of E_2 with a concomitant rise in oxidative stress, proinflammatory cytokines and bone turnover markers[4,5,29,30]. However, pHSP27 was not significantly different in pre versus post categories within similar BMD conditions. This may be attributed to the fact that E_2 can induce HSP27 expression and secretion, and in turn, HSP27 can modulate E_2 signaling[31,32]. Hence, E_2 being significantly elevated in pre, can induce HSP27 expression and secretion, whereas, oxidative stress and proinflammatory cytokines, being significantly elevated in post category, can trigger HSP27 phosphorylation, perhaps leading to a net uniformity in serum levels of pHSP27. Therefore, pHSP27 may serve as an early indicator of bone health, irrespective of menopausal status.

In our study, serum pHSP27 was able to distinguish between low and high BMD in both pre and post categories. Apart from pHSP27, CTX-1 also showed significant ability to predict low BMD in both pre- and post categories, although with a lower odds ratio than pHSP27. Further, ROC curves revealed that both pHSP27 and CTX-1 exhibited a significant area under the curve to predict low BMD in pre and post categories. Taken together, pHSP27 may be a candidate serum marker of low BMD and may potentially aid in the diagnosis of osteoporosis. A study on non-small cell lung cancer reported significantly raised pHSP27 in serum of patients with advanced stage of cancer compared with early stage[11].

Our preliminary data show that pHSP27 may be a potential marker for diagnosis of osteoporosis/osteopenia in premenopausal as well as postmenopausal women; however, its usefulness needs to be addressed through larger studies. Although BMD measurement is gold standard for diagnosis of osteoporosis/osteopenia, the translational utility of pHSP27 may be important especially in conditions wherein DXA may not be a suitable option like pregnancy, *etc*. The case control nature of this study limited our ability to study a linear trend between pHSP27 and BMD; hence, prospective studies with large sample size including co-morbid diseases are needed.

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