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## **STUDY OF SERUM ALKALINE PHOSPHATASE, CALCIUM AND URINARY HYDROXYPROLINE AS BONE BIOMARKERS IN POSTMENOPAUSAL WOMEN**

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### **ABSTRACT**

Osteoporosis is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Postmenopausal women due to the various hormonal factors are at increased risk. Bone mineral density (BMD) measurements are gold standard in calculating bone mass, the changes are usually late and the damage is irreversible. This study was done to evaluate the rate of bone loss in postmenopausal women by measuring Serum Total Alkaline Phosphatase (ALP), Total Calcium, Urinary hydroxyproline and to evaluate the correlation of Age, Body Mass Index (BMI), and above biomarkers as indicators of increased bone turnover in postmenopausal women. Bone formation markers, serum total ALP were significantly increased ( $p < 0.001$ ) and total calcium was significantly decreased ( $p < 0.001$ ) in cases compared to controls. Bone resorption marker, urinary hydroxyproline was significantly increased ( $p < 0.001$ ) in cases. This study concluded that, Serum total ALP, total calcium and Urinary hydroxyproline combined together provided fairly useful index of bone resorption in postmenopausal women and these common biochemical parameters can be used to categorize postmenopausal women into rapid and slow bone losers. Preventive measures like calcium supplementation or Hormone replacement therapy can be initiated early in those who are rapid bone losers and prevent the osteoporotic fractures

**Keywords:** *Alkaline Phosphatase, Calcium, Hydroxyproline*

### **INTRODUCTION**

Osteoporosis is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration, with a consequent increase in bone fragility and susceptibility to fracture (Brown and Josse, 2002). According to World Health Organization (WHO), Osteoporosis is second only to cardiovascular disease as a global healthcare problem and medical studies show that a 50 year old woman has a similar lifetime risk of dying from hip fracture as from breast cancer (Anonymous, 2003). The number of osteoporotic patients in India is approximately 26 million (2003 figures), with the numbers projected to increase to 36 million by 2013. In most western countries, while peak incidence of osteoporosis occurs at about 70-80 years of age, in India it may affect those 10-20 years younger at age 50-60 years (Anonymous, 2003). It is imperative for health care providers to diagnose not only osteoporotic patients but also to identify risk at asymptomatic subject early in premenopausal age group (Acharya *et al.*, 2010). According to national osteoporosis foundation and U.S preventive services task force recommendations, women 65 years of age and older should be screened for osteoporosis (Kern *et al.*, 2005). Measurement of bone mineral content may identify women likely to develop osteoporosis. Tools like Dual Emission X-ray Absorptiometry (DEXA) for estimating Bone Mineral Density (BMD) are not yet easily available in India. Very few centers are equipped with these facilities (Gandhi and Shukla, 2005) and also changes in BMD are late and relatively irreversible. Hence it is important to have a means of identifying high risk individuals and to monitor their treatment before fracture occurs (Masse *et al.*, 2005).

Biochemical markers of bone turnover may be of value for prediction of individual bone loss and can also be used to provide information about future bone loss of that individual (Lofman *et al.*, 2005). Elevated bone turnover, assessed by biochemical parameters are negatively correlated with bone mineral density.

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Since biochemical markers are able to estimate the rate of bone formation and resorption and also provide unique information about rapid bone loss. Combined use of biochemical markers and BMD screening may provide a better prediction of osteoporosis than BMD measurements alone (Melton *et al.*, 1997). Bone markers are usually classified as markers of bone formation –serum total alkaline phosphatase (ALP), osteocalcin, bone - specific alkaline phosphatase (B- ALP) and procollagen type-1 extension peptides and markers of bone resorption are urinary hydroxylysine glycosides, hydroxyproline, plasma tartrate resistant acid phosphatase (TRAP) and collagen pyridinium cross-links (Eastell, 2009).

By diagnosing osteoporosis before the disease is florid, the incidence of post-menopausal osteoporosis and fractures can be reduced with evidence-based measures like maintaining a healthy lifestyle, maintaining a balanced diet to achieve adequate calcium and vitamin D intake, avoid smoking and high intakes of alcohol and to take regular, weight-bearing exercise (Gandhi and Shukla, 2005). Hence this study was taken up to measure the levels of serum alkaline phosphatase, calcium and urinary hydroxyproline to creatinine ratio in post-menopausal women and to compare these parameters with the pre-menopausal women.

## **MATERIALS AND METHODS**

The study was conducted from April 2009 to April 2010, at Bapuji Hospital and Chigateri General Hospital, Davangere. The ethical clearance was obtained from institutional ethical committee. Informed consent was given by all participants. Fifty healthy women who had ceased menstruation for 3 to 5 years and not started taking hormone replacement therapy, non-obese (BMI < 30 kg/m<sup>2</sup>) and without regular use of calcium supplementations or any other medications known to affect bone metabolism were considered as cases and 50 healthy women of reproductive age group (30-40 years) with regular menstruation who were non-pregnant and not taking oral contraceptive pills were considered as controls. Women with history of known osteoporotic fractures, diabetes mellitus, renal failure, major liver diseases or those on hormone replacement therapy and oral corticosteroids for more than six months were excluded from the study. A detailed history was taken regarding age, parity, duration since menopause, socioeconomic status, past history, family history and personal history in a proforma. General examination was conducted in all the cases. Routine investigations were done in all the cases.

Five milliliter of blood sample was collected aseptically; serum was separated immediately by centrifuging at 3,000 rpm for 10 minutes and kept at 4°C until analysis was carried out. A random urine sample was collected at the same time in a clean plastic container.

The concentration of hydroxyproline and creatinine in urine samples were estimated within 24 hours of collection of urine. Serum total calcium (Moorehead and Briggs, 1974; Tietz, 1986), ALP (Tietz, 1976; Wilkinson and Winsten, 1969) and albumin (Doumas *et al.*, 1972; Tietz, 1986) were analyzed by using diagnostic kits from Erba Company on Microlab Semiautoanalyzer (ERBA). Urinary hydroxyproline was estimated by spectrophotometric method using the Modified Neuman and Logan method at 540 nm (Sachdeva *et al.*, 2005; Mitoma *et al.*, 1959; Garnero *et al.*, 1996). Urine creatinine concentration was determined by Jaffe's reaction at 520 nm (Rock *et al.*, 1987; Bartels and Bohmer, 1971).

All the reagents used in the estimation were of analytical grade.

## **RESULTS**

Table 1 shows comparative analysis of BMI, serum albumin, total ALP, total calcium and urinary hydroxyproline-creatinine ratio. The statistical analysis by unpaired t -test shows that BMI and levels of serum total ALP, and urinary hydroxyproline corrected to creatinine is increased in post - menopausal women and are statistically highly significant (p < 0.001). The serum albumin though increased in postmenopausal women was not statistically significant. Serum calcium levels are significantly (p < 0.05) decreased in postmenopausal cases compared to premenopausal women.

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**Table 1: BMI, serum levels of Albumin, Total ALP, Calcium and urinary Hydroxyproline-Creatinine ratio between premenopausal women and postmenopausal women**

	Premenopausal women	Postmenopausal women	't' value	'p' value
<b>BMI (Kg/mt<sup>2</sup>)</b>	23.38±1.88	27.23±2.07	9.75	< 0.001
<b>Albumin (g/dl)</b>	4.32±1.02	4.36±1.09	0.16	<0.88
<b>ALP (mkat/ml)</b>	2.09±0.40	8.64±0.78	12.45	< 0.001
<b>Calcium (mmol/L)</b>	2.10 ± 0.54	1.94 ± 0.12	2.004	< 0.05
<b>OHP: Cre</b>	16.26±5.23	31.37±7.14	12.07	< 0.001

All the values are expressed in mean ±SD

'p' value less than 0.001 is considered as statistically significant

BMI: Body mass index

ALP: Alkaline phosphatase

OHP:Cre: Hydroxyproline-creatinine ratio

The mean age of healthy postmenopausal women was 60.4±9.6 years and premenopausal controls were 30.1±6.3 years.

**DISCUSSION**

The results of the present study show that the increase in serum ALP levels and urinary hydroxyproline levels corrected to creatinine in postmenopausal women were highly significant with p value <0.001 when compared with premenopausal controls and also the serum calcium levels were decreased in postmenopausal women than in premenopausal controls and were statistically significant (p < 0.05) these findings were in accordance with several studies (Sachdeva *et al.*, 2005; Mitoma *et al.*, 1959; Garnero *et al.*, 1996; Rock *et al.*, 1987; Bartels and Bohmer, 1971; Indumati *et al.*, 2007; Agrawal *et al.*, 2009; Brazier *et al.*, 1995; Civitelli *et al.*, 1988; Riggs *et al.*, 1969; Reid *et al.*, 2006; Suresh and Naidu, 2006).

The mean age at menopause was observed to be 49.66 years (Agrawal *et al.*, 2009). The prevalence of osteoporosis increases with age for all sites, and by WHO definition upto 70 % of women over the age of 89 years have osteoporosis (Indumati *et al.*, 2007). A high mortality is well recognized in osteoporotic patients with hip fracture compared with the general population (Adami and Kanis, 1995). The fracture burden is expected to increase in India from the current 26 million to 36 million by 2013 (Indumati *et al.*, 2007). Bone mass decreases with aging, and it is now well established that a low bone mass is the major determinant of all osteoporotic fractures. The dramatic increase in the bone turnover rate with an imbalance between bone formation and bone resorption in the first year after the cessation of ovarian function is responsible for the accelerated rate of postmenopausal bone loss. High bone turnover rate seems to play an increasing role as a determinant of bone mass with increasing postmenopausal age (Iuaska *et al.*, 2007).

Measurement of Bone Mineral Density (BMD) with other risk factors can potentially improve the identification of osteopenic women with high risk of developing osteoporosis. One risk factor is increased rate of bone remodeling, which has been shown to be associated with bone loss and fractures independently of BMD. Bone metabolism can be assessed by measuring bone turnover markers (BTMs) in serum or urine (Iuaska *et al.*, 2007). The main aim of the study was to estimate Serum Albumin, Total Alkaline phosphatase (ALP), Total calcium and urinary Hydroxyproline/ Creatinine ratio in postmenopausal women and premenopausal controls and to find out whether the measurement of the above parameters could be useful in assessing the increased bone turnover in postmenopausal women.

A total number of 100 subjects were studied comprising of 50 premenopausal women as controls and 50 postmenopausal women (20 early postmenopausal women and 30 late postmenopausal women) as cases. ALP is a ubiquitous enzyme that plays an important role in osteoid formation and mineralization (Indumati *et al.*, 2007).

Our results show that the increase in serum ALP levels were highly significant (p < 0.001) in both early postmenopausal women and late postmenopausal women when compared to premenopausal controls.

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These findings are in accordance with several studies (Sachdeva *et al.*, 2005; Indumati *et al.*, 2007; Agrawal *et al.*, 2009; Brazier *et al.*, 1995; Civitelli *et al.*, 1988; Riggs *et al.*, 1969; Reid *et al.*, 2006; Suresh and Naidu, 2006).

Serum alkaline phosphatase activity is the most commonly used marker of bone formation. A moderate increase of serum alkaline phosphatase is ambiguous; since it may reflect a mineralization defect in elderly patients (Delmas, 1993). Elevated blood levels of serum alkaline phosphatase activity indicate increased activity of the osteoblasts (Kaveh *et al.*, 2010).

At menopause, the rate of bone remodeling increases precipitously. This fact may be explained by evidence, derived primarily from studies in mice, that loss of sex steroids up-regulates the formation of osteoclasts and osteoblasts in the marrow by up regulating the production and action of cytokines that are responsible for osteoclastogenesis and osteoblastogenesis (Manolagas, 2000). It is suggested that in most clinical situations, measurement of serum total ALP provides sufficient diagnostic information at a good cost-benefit ratio (Melton *et al.*, 1997; Fountia).

#### **Hydroxyproline:**

Fibrillar collagens are rich in the amino acid hydroxyproline, which is excreted in the urine after collagen degradation and are considered to be markers of bone resorption. The urinary excretion of hydroxyproline is increased in states of physiologically high turnover, such as somatic growth, during menopause and high turnover osteopathies (Auth *et al.*, 2008)

This increased excretion is due to increase in bone loss which was a characteristic feature of the postmenopausal period (Sachdeva *et al.*, 2005).

Our results show that urinary hydroxyproline corrected to creatinine was increased in postmenopausal women compared to premenopausal controls and also urinary hydroxyproline levels were increased more in late postmenopausal women than in early postmenopausal women. These findings were in accordance with other studies (Masse *et al.*, 2005; Agrawal *et al.*, 2009; Brazier *et al.*, 1995; Riggs *et al.*, 1969; Reid *et al.*, 2006). Estrogen deficiencies at the menopause increase the rate of bone remodeling, which results in high turnover bone loss. There are recognized receptors on the osteoblast which do not function optimally due to lack of hormones. This is reflected by a significant increase in the mean value of markers of resorption and formation from premenopause to postmenopause. Thus simple, direct urinary assay of hydroxyproline to measure bone resorption have clinical applications as part of screening programs to assess the risk of osteoporotic fractures (Eastell, 2009).

#### **Serum total calcium:**

Approximately 99% of body's calcium is contained in bone (Murray *et al.*, 2006). In elderly women, bone resorption is markedly increased in part because of reduced calcium intake. Bone formation is also inhibited, in part to preserve serum calcium (Storm *et al.*, 1998).

Our results showed that the level of serum total calcium was decreased in postmenopausal women compared to premenopausal controls and was statistically significant ( $p < 0.05$ ). These findings were in accordance with the studies of other investigators (Indumati *et al.*, 2007; Riggs *et al.*, 1969). But there was no significant decrease in serum total calcium in postmenopausal cases compared to premenopausal controls according to Sachdeva (2005).

Our results also show that the correlation between hydroxyproline / creatinine ratio (OHP/Cre) with age was significant but not with the other biochemical parameters. The correlation between the various biochemical parameters with BMI was not significant.

These results demonstrate that biochemical parameters can give an idea as to the rates of bone formation and resorption. It also suggests that simple, easy, common biochemical markers such as urinary hydroxyproline, total serum ALP, total serum calcium could be used as indicators of increased bone turnover, to enable early intervention so as to minimize fractures due to osteoporosis. Combined biochemical and BMD screening may provide better prediction of future fracture risk than BMD alone. If preventive measures are to be initiated prior to the onset of excessive bone loss, measurement of bone turnover through urinary hydroxyproline could form a tool available to assist health care professionals to predict fracture risk (Indumati *et al.*, 2007).

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Only fifty healthy premenopausal women were included as controls and fifty healthy postmenopausal women were included as cases in this study because of the strict inclusion and exclusion criteria. Given that the participants were both from the general population and patients who had visited the hospital, they may not be representative of Indian postmenopausal women. The small sample size and the use of hospital based participants may have influenced our findings. The purpose of the study was to estimate the biochemical markers of bone formation (Serum Total ALP and Total Calcium) and bone resorption (Urinary hydroxyproline) in postmenopausal women. Further studies that include a larger population of participants are necessary to confirm our findings. Another limitation is that the serum total ALP used as a marker of bone formation is not bone-specific but as the other causes of rise in total ALP have been ruled out in the participants during the study, the rise in total ALP is mainly due to the bone isoenzyme. However, we suggest that the bone specific alkaline phosphatase is a more specific marker of bone formation than total ALP.

However, there is a need to establish normal acceptable ranges for the above biochemical markers in various communities beyond which individuals will be at risk of excessive bone loss and consequently be predisposed to fractures.

### **Conclusion**

Early detection of bone loss by measurement of Bone Mineral Density (BMD) helps to confirm the diagnosis of osteoporosis and assesses the future risk of osteoporotic fractures so that timely therapy can be instituted. Dual X-ray Absorptiometry (DXA) is the gold standard to assess BMD but is not always available to the general population.

The changes in BMD are late and relatively irreversible; therefore it is important to have a means of identifying high risk individuals and to monitor their treatment before fractures occur. Bone turnover assessed by histomorphometric analysis of bone biopsies is the most reliable method available at present. However, bone biopsy is an invasive procedure and yields information only on a restricted area of bone, which may not represent the entire skeleton. Biochemical markers of bone turnover have been shown to provide valuable information for the diagnosis and monitoring of metabolic bone disease like postmenopausal osteoporosis. They reflect the whole body rates of bone resorption (Resorption markers) and bone formation (Formation markers). Therefore they may provide a more representative index of the overall skeletal bone loss than would be obtained by measuring the rates of change in BMD at specific skeletal sites.

The results of the current study support the concept that the common biochemical parameters of bone turnover can identify postmenopausal women who are at an increased risk of developing osteoporotic fractures.

The high turnover bone loss which occurs in postmenopausal women is due to the estrogen deficiency at the menopause which increases the rate of bone remodeling.

The receptors on the osteoblasts do not function optimally due to the lack of hormones. Monitoring bone status using biochemical parameters could serve as a screening measure in early intervention against excessive bone loss. However, studies are needed to establish normal acceptable ranges for various bone biomarkers in various communities beyond which individuals will be at risk of excessive bone loss and consequently be predisposed to fractures.

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