

## Calcium, phosphate and hydroxyproline as bone turnover markers in adults of 45 to 60 years age group.

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### ABSTRACT

The present study is an attempt to monitor the role of bone biochemical markers (calcium, phosphorous and hydroxy proline) in males and females of 45 to 50 years age group and compare their levels with osteoporotic condition. Blood and urine samples was collected from the individuals and subjected to marker analysis. Significantly higher levels of calcium, phosphorous and hydroxy proline were observed in osteoporotic patients compared to normal patients. Female osteoporotic patients possess higher levels of calcium, phosphorous and hydroxy proline compared to male. Assessment of bone biochemical markers provides the valuable information about the risk of the individual towards bone diseases.

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### Introduction:

Bone is a living, growing tissue that turns over at a rate of about 10% a year. It is composed primarily of the inorganic minerals (calcium and phosphate) and an organic matrix (type I collagen). Collagen gives tensile strength and framework. Calcium phosphate mineralized complex that hardens the framework. This combination of collagen and calcium makes bone strong and yet flexible enough to bear weight and to withstand stress (Raisz and Rodan, 2003).

Bone metabolism is a dynamic and continuous process in order to maintain a balance between the resorption of old and injured bone initiated by osteoclasts and the formation of new bone under the control of osteoblasts (Simsek et al., 2004). In general, the process of bone formation and resorption are coupled processes and there is no net change in the bone mass. Through childhood and early adulthood, formation exceeds resorption so that bone density increases and then plateaus until the age of 30 to 40 years. After that, resorption exceeds formation and bone density decreases through the rest of life, which in turn may lead to osteoporosis (Vipla Puri., 2003).

The majority of the total body stores of calcium and phosphorus are located in bone in the form of hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ . Bone consists principally (90%) of highly organized cross-linked fibers of type I collagen; the remainder consists of proteoglycans, and “non-collagen” proteins. Osteoclasts are the bone resorbing cells and derive from circulating hematopoietic cells, and osteoblasts are the bone forming cells that derive from the marrow. Bone is a dynamic organ and remodels or turns over in response to hormones, cytokines, and changes in mechanical forces. At any one time, less than 15–20% of the bone surface is undergoing remodeling, controlled via the osteoprotegerin pathway by nearly every cytokine and hormone including PTH and calcitriol as well as inflammatory cytokines (Hofbauer et al., 2001). Thus, alterations in bone remodeling can affect calcium and phosphorus homeostasis.

Serum calcium levels are tightly controlled within a narrow range, usually 8.5–10.5 mg/dl (2.1–2.6 mmol/L). However, the serum calcium level is a poor reflection of overall total body calcium, as serum levels are only 0.1–0.2% of extracellular calcium, which in turn is only 1% of total body calcium. The remainder of total body calcium is stored in bone. Ionized calcium,

generally 40% of total serum calcium level is physiologically active, while the non-ionized calcium is bound to albumin or anions such as citrate, bicarbonate and phosphorus. In the presence of hypoalbuminemia, there is a relative increase in the ionized calcium relative to the total calcium, thus total serum calcium may underestimate the physiologically active (ionized) serum calcium.

Total adult body store of phosphorus is approximately 700 g, of which 85% is contained in bone in the form of hydroxyapatite. Of the remaining, 14% is intracellular, and only 1% is extracellular. Of this extracellular phosphorus, 70% is organic and contained within phospholipids, and 30% is inorganic, 15% is protein bound, and the remaining 85% is either complexed with sodium, magnesium, or calcium or circulates as the free mono hydrogen or dihydrogen forms. It is this latter 0.15% of total body phosphorus (15% of extracellular phosphorus) that is freely circulating and measured. At pH of 7.4, it is in a ratio of about 4:1  $\text{HPO}_4^{-2}$  to  $\text{H}_2\text{PO}_4^{-1}$ . For that reason, phosphorus is usually expressed in mmol rather than meq/L. Thus, similar to calcium, serum measurements only reflect a minor fraction of total body phosphorus, and therefore do not consistently reflect total body stores.

Age-related bone loss affects both women and men (Raisz, 2005). Recent studies indicate that significant trabecular bone loss begins as early as the twenties in men and women- long before any major hormonal changes (Khosla *et al.*, 2005). In women, however, bone loss accelerates for 5 to 10 years after menopause due to the rapid decline in estrogen levels; after this phase, bone loss continues at approximately the same rate as in elderly males. These observations indicate that there is an element of the aging process in bone, other than an age-associated failure of other organs or tissues that is a common cause of bone loss in both aging women and men.

Osteoporosis is a major health and economic problem and it is characterized by low bone mass and deterioration of bone architect, with a consequent increase in bone fragility and susceptibility to fracture. This metabolic bone disease is more predominant in India, and osteoporotic fractures are a common cause of morbidity and mortality in adult Indian men and women (Gupta, 1996). The prevalence of osteoporosis increases with age for all sites. Two principle factors determines the adult bone health (a) Maximum attainment of peak bone mass in young adulthood, and (b) the rate of bone loss with advancing age.

As being the major components of bone metabolism calcium and phosphorous levels are strictly maintained in the serum, hence any disturbance in their levels should manifest about the disturbance in their regulation so an ailment can be predicted. Hydroxyproline is an amino acid present in appreciable quantities in collagen and excreted in the urine after

collagen breakdown. Because urinary Hydroxyproline is derived almost entirely from collagen, it reflects the rate of collagen catabolism. Biochemical markers of bone turnover have been shown to provide valuable information for the diagnosis and monitoring of metabolic bone diseases (Woitge *et al.*, 1998). Bone turnover markers reflect the whole body rates of bone metabolism; 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 bone mineral density at specific skeletal sites (Delmas, 2000).

In the present study we aimed at identifying the role of calcium, phosphorous and Hydroxyproline as markers in the preliminary diagnosis of osteoporotic patients of 45 to 50 years male and female. The aim of the work is to measure the levels of these markers in males and females of 45 to 50 years age group and correlate their levels with osteoporotic condition.

### Materials and methods

The study was conducted with the assistance of individuals suffering osteoporosis belonging to 45-50 age groups. In each test and control group there were 20 members. All the participants were non smokers, non alcoholic and ambulatory. The test groups were requested not to take non-vegetarian diets for 48 hrs before the test sample collected.

On the day of sample collection 5 ml of venous blood was collected aseptically from antecubital vein and serum was separated immediately by centrifuging at 3000 rpm for 10 min to separate the serum.

For the purpose of hydroxyproline estimation urine sample was collected two hours after passing first urine.

In case of urine, hydrolyzed urine samples with equal volumes of concentrated HCl (100  $\mu\text{l}$  Urine + 100  $\mu\text{l}$  HCl) in a pressure tight, teflon capped vial. Hydrolyzed at 120°C for 3 hrs. Vortexed and centrifuged at 10000 x g for 3min. to remove precipitate. Transferred 10  $\mu\text{l}$  of each hydrolyzed sample to a 96 well plate and evaporated to dryness under vacuum.

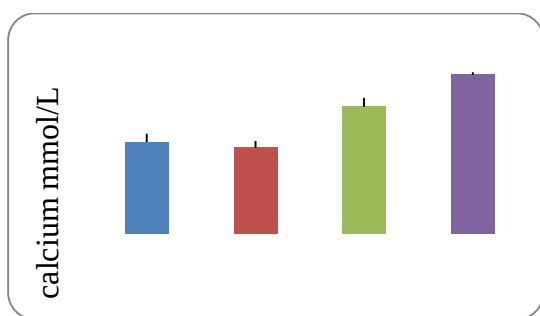
Calcium estimation was carried out by OCPC Method (Connerty and Briggs, 1966). Calcium in an alkaline medium combines with O-Cresolphthalein complexone to form a purple coloured complex. Intensity of colour formed is directly proportional to the amount of calcium present in the sample. Phosphorus estimation was carried out by Fiske and Subbarow method (Fiske and Subbarow, 1925). Phosphorous is converted to phosphomolybdate by the addition of ammonium molybdate. Phosphomolybdate on reduction with aminonaphthosulphonic acid (ANSA) gives a blue colour which is measured at 650 nm in a photoelectric colorimeter.

Urinary hydroxyproline was estimated by the modified method of Neuman and Logan (Mitoma *et al.*,

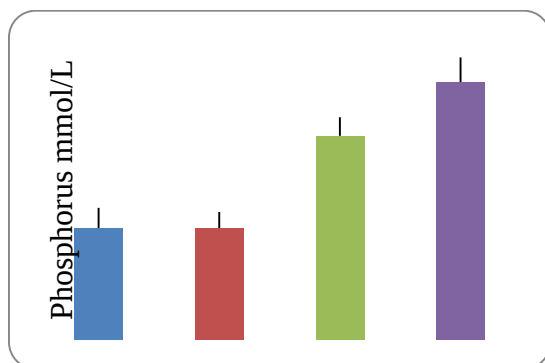
1959). Hydroxyproline is treated with  $\text{CuSO}_4$  and  $\text{H}_2\text{O}_2$  in an alkaline solution results in the formation of pyrroline-4-carboxylic acid, which upon acidification is converted to pyrrole-2-carboxylic acid. The latter condenses with p-dimethylaminobenzaldehyde to give the coloured complex which is measured at 540nm.

### Results:

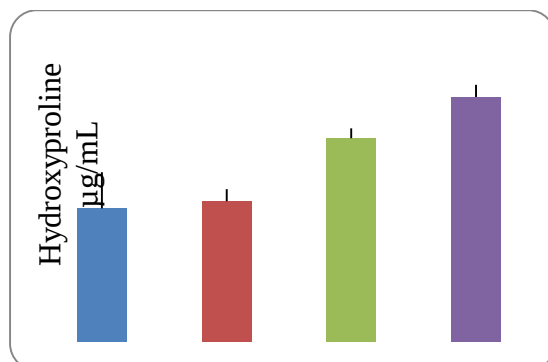
The present study was mainly focused on analysis of total calcium, phosphorous and hydroxyproline levels in normal and osteoporotic patients (both male and female). All the three markers were found to be higher in osteoporotic patients compared to the normal patients. A further comparison between male and female osteoporotic patients manifests that the three markers are higher in case of females than in males.



**Fig. 1:** Free serum calcium levels expressed in mmol/L in normal and osteoporotic male and female patients.



**Fig. 2:** Free phosphorus levels expressed in mmol/L in normal and osteoporotic male and female patients.



**Fig. 3:** Urinary Hydroxy proline levels expressed in µg/mL in normal and osteoporotic male and female patients.

### Discussion:

Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and of degradation products produced during bone resorption. Various biochemical markers are now available that allow a specific and sensitive assessment of the rate of bone formation and bone resorption of the skeleton. Rate of bone resorption was monitored by changes in urinary excretion of calcium, hydroxyl proline, lysylpyridinoline and hydroxy lysylpyridinoline (Eastell *et al.*, 2001). During bone resorption, highly active osteoclasts may secrete factors into the space between the cell and bone surface such as acids, matrix metalloproteinases and cathepsin K. These factors can degrade collagen type I into hydroxyproline which intum excreted through urine.

In the present study increased calcium and phosphorous content was observed in osteoporotic males and females compared with healthy individuals of relevant gender. This is due to the increased resorption of bone releasing free calcium and phosphorous. The results were agreed with the findings of Khatak *et al.* (2013) and Sadaf *et al.* (2014). Changes in the calcium metabolism related to metabolic syndromes of hypertension, impaired glucose tolerance and hyperlipidemia (Rajiv and Deborah, 1997). However higher levels of phosphates in the blood reduce formation of vitamin D in the kidneys there by decreasing the blood calcium. Similar results were reported by Jowsey *et al.* (1974).

Hydroxyproline is found mainly in collagen, imparts stability to the molecule and represents about 13% of the amino acid content collagen (Udenfriend, 1966). Hydroxyproline is derived from proline by a post translational hydroxylation occurring within the peptide chain. As free hydroxyproline released during degradation of collagen cannot be reutilized in collagen synthesis, most of the endogenous hydroxyproline present in biological fluids is derived from various forms of collagen (Prockop and Kivirikko., 1967). Urinary hydroxyproline is thus considered as an index of bone resorption and a major determinant of bone status (George, 2003). Urinary hydroxyproline is expressed as mg of hydroxyproline per gram of creatinine (Justesen *et al.*, 2006; George, 2003).

In the present study there was a rise in urinary hydroxyproline level in osteoporotic males and females compared to healthy individuals. This might be due to the degradation of collagen of bone there by increasing the urinary excretion of hydroxyproline. The results of the study were agreed with the findings of Sachdeva *et al.*, (2005) and Lofman *et al.*, (2005). Deficiency of estrogen at menopause, receptors on the osteoclasts do not function properly, which leads to the significant variation in the bone formation and bone resorption markers. Thus simple, direct urinary assay to measure bone resorption have clinical applications as part of

screening programs to assess the risk of osteoporotic fractures. Monitoring bone status through urinary excretion of hydroxyproline could serve as a surveillance measure in early intervention against excessive bone loss (Simsek *et al.*, 2004).

### Conclusions:

The results from the present study indicate that calcium, phosphorous and hydroxyproline would be worthy markers in indexing the bone turnover. Their raise specially reports the increased bone resorption; hence these markers may help to identify women at greater risk. However detailed analysis should need to be the outcome of further studies to make use of these markers.

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