A COMPARATIVE STUDY OF BONE RELATED BIOCHEMICAL MARKERS IN POSTMENOPAUSAL WOMEN WITH AND WITHOUT DIABETES MELLITUS

BY

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Dissertation Submitted to

Sri Devaraj Urs Academy of Higher Education and Research, Kolar.

In Partial fulfillment of the Requirements for the Degree of

M.D in BIOCHEMISTRY

Under the guidance of

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MAY 2014.

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"A COMPARATIVE STUDY OF BONE RELATED BIOCHEMICAL MARKERS IN POSTMENOPAUSAL WOMEN WITH AND WITHOUT DIABETES MELLITUS"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. K.N SHASHIDHAR M.D, Professor and HOD, Dept of Biochemistry,** Sri Devaraj Urs Medical College, Kolar.

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LIST OF ABBREVIATIONS

AGE- Advanced glycation end products

ALP – Alkaline phosphatase

BAP- Bone isoform of ALP

BMD – Bone mineral density.

Ca^{2+}- Calcium

CTX- C-Telopeptide of collagen cross-links

DEXA- Dual-energy X-ray Absorptiometry

GHb- Glycated Hemoglobin

HbA_{1c}- Glycated Hemoglobin

ICTP- Cross-linked C-telopeptide of type I collagen

IDDM- Insulin-Dependent Diabetes Mellitus

IGF 1- Insulin like growth factor-1

NIDDM-Non-Insulin Dependent Diabetes Mellitus

NTX -N-Telopeptide of collagen cross-links

OC - Osteocalcin

P- Phosphorus

PICP - Procollagen I Carboxy-terminal extension peptide

PINP- Procollagen I Amino-terminal extension peptide

PPARγ - Peroxisome proliferator -activated receptor gamma

PTH- Parathyroid hormone

QCT- Quantitative computed tomography

RANKL- Receptor activator of nuclear factor- k B ligand

RBS – Random blood sugar

SD – Standard deviation

TRAP –Tartarate resistant acid phosphatase.

WHO – World Health Organization

BACKGROUND:

Postmenopausal osteoporosis is characterized by abnormal reduction in bone density and thus increased risk of fractures. The impact of diabetes on bone is still incompletely understood. Bone turnover is regulated by local cytokines cell matrix interactions and systemic hormones and hyperglycemia may affect any of these micro-environments that regulate bone turnover. Some studies have shown postmenopausal diabetic women to have reduced bone marrow density (BMD). On the contrary several cross sectional studies have found diabetics to have elevated bone mass. Due to these conflicting results we took up the study to know the effects of Diabetes on bone markers.

OBJECTIVES OF THE STUDY:

1. To study and compare various bone related biochemical markers in normal postmenopausal women and type 2 diabetic postmenopausal women in a predominantly rural population.
2. To compare the values between the two groups.
3. To study the effect of duration and severity of diabetes on bone turnover.

MATERIALS AND METHODS:

A hospital based study with 30 postmenopausal diabetic women and 30 postmenopausal women without diabetes was done beginning from February 2012 at R.L.Jalappa Hospital, Kolar. Bone related biomarkers such as calcium, phosphorus; alkaline phosphatase, 24 hour urinary hydroxyproline and glycemic markers random blood sugar and glycated hemoglobin were assessed. These parameters were compared between the two groups.

RESULTS:

In our study the postmenopausal women with and without Diabetes mellitus had significant differences in the RBS and glycated hemoglobin values, with a p value <0.001. Mean levels of 24 hr urinary excretion of hydroxyproline in diabetic women was 37.12±6.5 mg /day and in non diabetic was 33.55±8.2 mg/day ie, within the reference range for the age group. However there was no significant difference observed in 24 hour urinary hydroxyproline levels with p value of 0.068. Calcium, Phosphorus and alkaline phosphatase didn't show significant difference. Calcium was found to be on the lower side of reference range in both groups.

CONCLUSION

- Urinary hydroxyproline, a bone resorption marker in diabetic postmenopausal women was higher when compared to non diabetic group but in reference range. Its value correlated positively with serum calcium levels, showing that as bone resorption occurred, the calcium from bone got released, thereby increasing serum calcium levels. Hydroxyproline being a collagen degradation product can be used as an inexpensive and reliable tool for assessing bone loss.
- Serum calcium was found to be towards the lower side of reference range in both groups indicating a need for supplementation of calcium in this age group.
- The study was inconclusive to comment on effect of diabetes on bone, as the rest of the biomarkers didn't show any significant difference between the groups and further studies need to be conducted to know exactly how diabetes effects bone.

Key words: Diabetes, calcium, hydroxyproline, postmenopausal women.

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Osteoporosis is a common dreaded complication of menopause in old women. Around 60 million women in India are above the age of 55 years and most would spend almost one third of their life in the post menopausal stage.[1]

Osteoporosis is a systemic skeletal disease characterized by a decreased bone mineral density resulting in increase in bone fragility and susceptibility to fracture.[2] According to World Health Organization (WHO), Osteoporosis ranks second as a non-communicable global healthcare problem compared to cardiovascular disease and has been operationally defined on the basis of bone mineral density (BMD) assessment. The WHO criteria defines osteoporosis as a BMD that lies 2.5 standard deviations or more below the average value for young healthy women (a T-score of <-2.5 SD).[3]

Osteoporosis is a common complication of menopause occurring due to lack of estrogen hormone that causes increased bone turnover. This causes increased bone resorption rate compared to bone formation rate resulting in negative bone balance.[4] Studies conducted by Handa R et al have extrapolated that in India the number of osteoporotic patients will increase to 36 million by 2013.[5]

Thirty percent of post menopausal women are osteoporotic at the hip, lumbar spine or distal forearm. This is comparable with the risk of fracture for a 50 year old woman at one of these three sites. By the age of 80 years, 70% of women are osteoporotic at the hip, lumbar spine or distal forearm.[6] There is a considerable amount of morbidity and mortality resulting from these osteoporotic fractures.

Majority of the bone diseases arise due to abnormalities in the bone remodeling. Osteoporosis too occurs due to this bone remodeling imbalance, where the bone

resorption rate exceeds the bone formation rate, thereby causing a net loss in bone mass. Genetic and environmental factors are the determinants of peak bone mass and these with the lack of estrogen at menopause causes increased bone turnover and an imbalance between bone resorption and bone formation.[7]

Diabetes is a chronic multi-systemic disease which can be regarded as a pandemic affecting around 150 million people worldwide. Its prevalence in India is 2-4% in rural areas and 4-11.6% in urban areas.[8] Diabetes through multiple pathways can affect the bone and hence can be referred to as an unconventional risk factor for fractures in older women. [9]

Chronic hyperglycemia of diabetes results in non-enzymatic glycation of various proteins and the same holds true for bone proteins and collagen, especially type I collagen. The principle source of energy for osteoclasts is glucose.[10] These factors along with effect of hyperglycemia on the local microenvironment and cytokines could impair the bone quality.

Studies have shown a reduction in bone mass in type 1 diabetes which is associated with increased fracture risk.[9, 11] With regard to effect of hyperglycemia on bone marrow density in type 2 diabetes there have been conflicting results. Some studies have shown postmenopausal diabetic women to have reduced bone mineral density (BMD).[12] However, other studies have found diabetics to have elevated bone mass.[11,13,14] In view of these conflicting results it is imperative for health care providers to detect the osteoporotic changes in diabetic individuals and monitor the disease progression.[15]

Hence, it seems worthwhile to study the pattern of bone loss in postmenopausal women with diabetes mellitus.

Markers of bone turnover are proteins that originate from osteoclastic and osteoblastic activity or fragments released during the formation or degradation of type I collagen. Some of these peptides are small enough to get filtered into urine, while the larger fragments may be detected in blood. These markers provide an assessment of the rate of bone turnover. These bone turnover markers are released during normal bone turnover. The concentrations may rise in metabolic bone diseases and during physiological processes such as fracture healing and growth spurts.[16]

Bone turnover markers are not disease specific. These are used by bone specialists mainly to monitor treatment response and disease progression in several metabolic bone diseases including postmenopausal osteoporosis, corticosteroid-induced osteoporosis and Paget's disease.[16]

Bone markers can be broadly classified into two groups:

a) **Markers of bone formation**- Alkaline Phosphatase (ALP), Osteocalcin, Amino and carboxy terminal peptides of type 1 procollagen
b) **Markers of bone resorption**- N Terminal and C Terminal telopeptide, Pyridinoline crosslinks, Deoxypyridinoline, Urinary Hydroxyproline, Hydroxylysine glycosides, Plasma Tartrate Resistant Acid Phosphatase (TRAP).

Multiple biomarker measurements are better compared to single biochemical marker in assessment of bone loss in postmenopausal women with or without diabetes mellitus.[17]

Assessment of increased bone turnover by biochemical markers negatively correlates with bone mineral density and provide an estimate of the rate of bone formation and resorption. Combined use of biochemical markers with BMD screening may provide information about rapid bone loss and a better prediction of osteoporosis than BMD measurements alone.[18]

The onus is on the early detection of bone loss in type 2 diabetic postmenopausal women so that evidence based therapy can be instituted such as good glycemic control, maintaining a balanced diet to achieve optimum calcium and vitamin D intake, and weight-bearing exercise.[15]

In view of these observations, it would be useful to evaluate the bone turnover markers serum total alkaline phosphatase (ALP), total calcium, phosphorus and urinary hydroxyproline in postmenopausal diabetic women and non diabetic postmenopausal women.

1. To study and compare various bone related biochemical markers in normal postmenopausal women and type 2 diabetic postmenopausal women in a predominantly rural population.

2. To compare the values between the two groups.

3. To study the effects of duration and severity of Diabetes on bone turnover markers.

OSTEOPOROSIS

Definition: The definition of osteoporosis is based on the bone mineral density assessment. WHO defines Osteoporosis as a BMD that lies 2.5 standard deviations (SD) or more below the average value for young healthy women (a T-score of < -2.5 SD).[3]

In other words osteoporosis is "a disease characterized by low bone mass and micro- architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk".[2]

HISTORY OF OSTEOPOROSIS

Osteoporosis has affected women since the beginning of mankind. The tell tale sign of osteoporosis "Dowager's lump" has been found even in the Egyptian mummies. In the early 1820s French pathologist Jean Georges Chretien Frederic Martin Lobstein, noticed that some patients' bones had larger than normal holes. Thus he coined the term "osteoporosis" which described a pathological state of a bone riddled with larger than usual holes.[19]In the early 19th century, a distinguished English surgeon, Sir Astley Cooper, noted that the lightness and softness of bones favored the occurrence of fractures. He noticed that older individuals were at an increased risk of fracture due to lower bone density. [20]

In 1940s, Fuller Albright noted that most of his patients with osteoporosis were postmenopausal women. He was the first to propose the role of estrogen hormone on the bone. He stated that the buildup of calcium reserves in bone was triggered mainly by estrogen. The drastic fall in estrogen that occurs due to menopause causes the boss loss in

postmenopausal women.[21] In 1970s, it was reported that osteoclasts were produced by bone marrow and the activity of bone marrow was influenced by various cytokines. Hence, the bone turnover markers serum calcium, alkaline phosphatase and phosphorus would be altered if there was an imbalance in the bone formation and bone resorption.

Collagen degradation product hydroxyproline levels can be altered in osteoporosis. Urinary hydroxyproline and other bone markers reveal changes in bone metabolism much earlier than radiographic methods. Hydroxyproline is considered as an index of resorption of bone. Hydroxyproline, thus could be used as a tool to asses osteoporotic fractures.[22]

While there is an enormous amount of understanding about osteoporosis and the treatment options, it still affects a lot of people especially the postmenopausal women. Till date research continues not only for the cure and prevention of osteoporosis but also for earlier diagnosis and monitoring of disease progression.

EPIDEMIOLOGY OF OSTEOPOROSIS

Osteoporosis is one of the most dreaded complications of menopause responsible for osteoporotic vertebral fractures in nearly 1/3rd of women older than 65 years and their lifetime risk of hip fractures is 15%.[23] By extreme old age, one of every three women and one of every six men will have a hip fracture.[24]

Osteoporosis is a global health problem, responsible for at least 1.2 million fractures in the United States each year.[24] Among Asian postmenopausal women one in two are likely to have an osteoporotic fracture at some time during their lifetime. [25]

The number of osteoporosis patients in India was approximately 26 million in the year 2003 as per the report of Osteoporosis Society of India and was been projected to increase upto 36 million by 2013. The prevalence of osteoporosis increases with age for all sites, and by World Health Organization (WHO) definition up to 70% of women over the age of 80 years have osteoporosis.[26]

According to recent reports, India is one of the largest affected countries in the world in which one out of every eight males and one out of every three females suffer from osteoporosis. [27] In India the peak incidence of osteoporosis is observed at around 50 -60 years whereas in western countries it is around 70 -80 years.[28]

BONE TISSUE

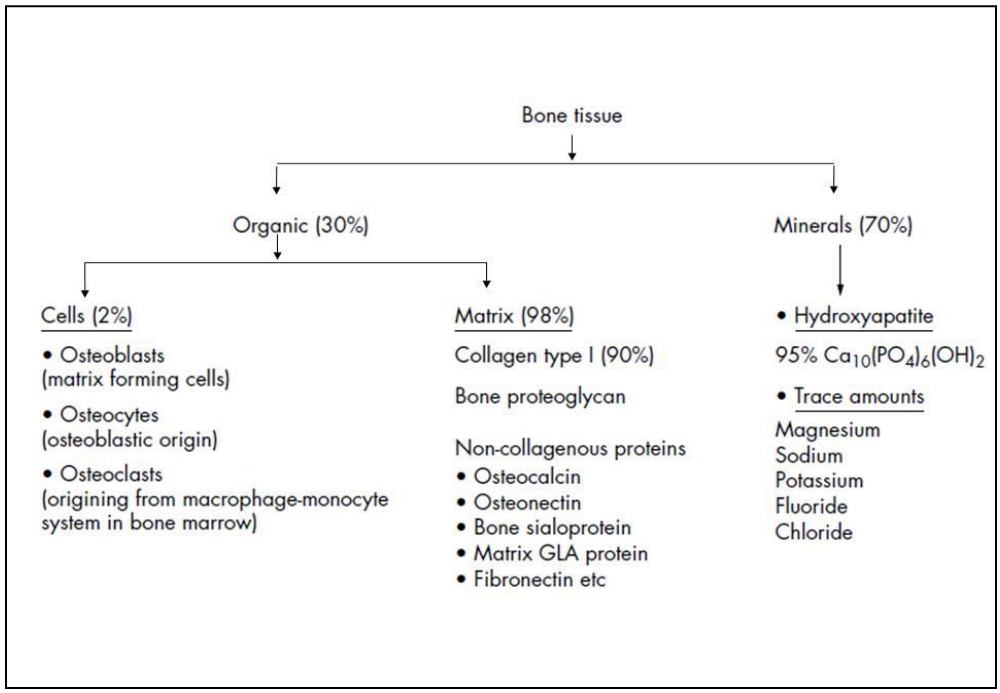
Seventy per cent of bone tissue constitutes the inorganic component, mainly minerals and the rest thirty percent is organic in origin. Organic bone tissue is mainly made up of a protein matrix (98%) and cells (2%).The cell types mainly being osteocytes, osteoclasts and osteoblasts. Bone matrix principally consists of type I collagen (90%). Other matrix proteins being proteoglycans and other non-collagenous proteins, namely osteocalcin, bone gamma-carboxyglutamic acid (GLA) protein, matrix GLA protein, osteonectin, and cellular binding proteins like fibronectin, bone sialoproteins. [29]

Bone tissue is formed by the deposition of hydroxyapatite crystals on an organic protein matrix. As shown in Table 2, almost ninety nine per cent of total body calcium

and phosphorus are found in bone alone, where they are stored on the extracellular matrix, in the form of hydroxyapatite crystals. These crystals precipitate on the collagen matrix. Glycoproteins and proteoglycans also participate in the binding of these crystals to the matrix.

The bone mainly constitutes cortical (compact) and trabecular bone. Cortical bone constitutes approximately 90% of the skeleton. It is found mainly in the diaphysis of long bones and on the surface of flat bones. Cortical bone is composed of a concentric compact bone mass located around a central canal (haversian canal). The haversian canal mainly contains the blood vessels, lymphatics, nerves, and connective tissue. Trabecular bone is found in the distal ends of long bones and in the inner surface of flat bones, and it involves bone marrow. Cortical bone is mainly for the mechanical support, whereas the trabecular bone is for the metabolic support.[29]

TABLE (1) CONSTITUENTS OF BONE TISSUE[29]



BONE REMODELLING

The bone remodeling process is often referred to as being "coupled". Coupling means that bone formation is linked to bone resorption. Bone being a dynamic tissue, is always in a state of constant remodelling in which continuous bone formation and resorption occurs. In a typical remodelling cycle, resorption takes 7–10 days, whereas formation requires 2–3months that accounts for 10% of bone being replaced each year. Coupling should not to be confused with balance, which means that the amount of bone that is removed is completely replaced.[30]

After the age of around 35–40 years, every time a remodeling cycle is completed there is a net loss of bone because the amount of bone formed is less than the amount removed by resorption. Postmenopausally the estrogen deficiency and other

abnormalities of skeletal regulation will greatly increase the rate of remodeling and accentuate this imbalance.[30]

In this complex interplay of bone remodelling osteocytes, osteoblasts and osteoclasts work together. Figure (1) shows the role of these cells in the bone remodelling. Osteoclasts cause bone resorption and bone mineralization is done by osteoblasts, which replace bone by forming an osteoid protein matrix. Osteocytes and their canalicular network act as sensors and respond to mechanical stimuli, hormones and cytokines as shown in Figure(1).[31]

Osteoblasts express the "receptor activator of nuclear factor- k B ligand" (RANKL) on their surface as shown in Figure (2). At the molecular level the bone turnover is regulated by RANK/RANKL interaction and osteoprotegerin. The preosteoclasts have the receptor called the "receptor activator of nuclear factor- k B" (RANK) on their surface.[31]These preosteoclasts differentiate into the mature and activated osteoclasts upon binding of RANK to its RANK Ligand. Osteoprotegerin is a soluble decoy receptor that are secreted by the osteoblasts and acts as a physiologic regulator of bone turnover by blocking the RANK/RANKL interaction.[32]

Bone turnover rate increases in menopause, which is mainly a consequence of oestrogen deficiency. Normally, there is a balance maintained between bone formation and resorption, known as the remodelling balance. Postmenopausally, the bone formation rate is less than the resorption rate, which results in a negative remodelling balance, and it is related to aging and oestrogen deficiency. In oestrogen deficiency, rapid changes are seen mainly in trabecular bone, and the weakening of cortical bone follows it. In this

case, bone resorption and bone formation in trabecular bone are eight times more rapid than cortical bone. Usually after the fourth decade, trabecular bone resorption exceeds formation by a rate of 0.7% per year. This resorption rate increases after menopause, where 5% of trabecular bone is lost each year. This accelerated bone loss continues for 10–15 years, and around 20 years after a woman attains menopause, she would have lost almost 50% of her trabecular bone and around 30% of cortical bone.[33]

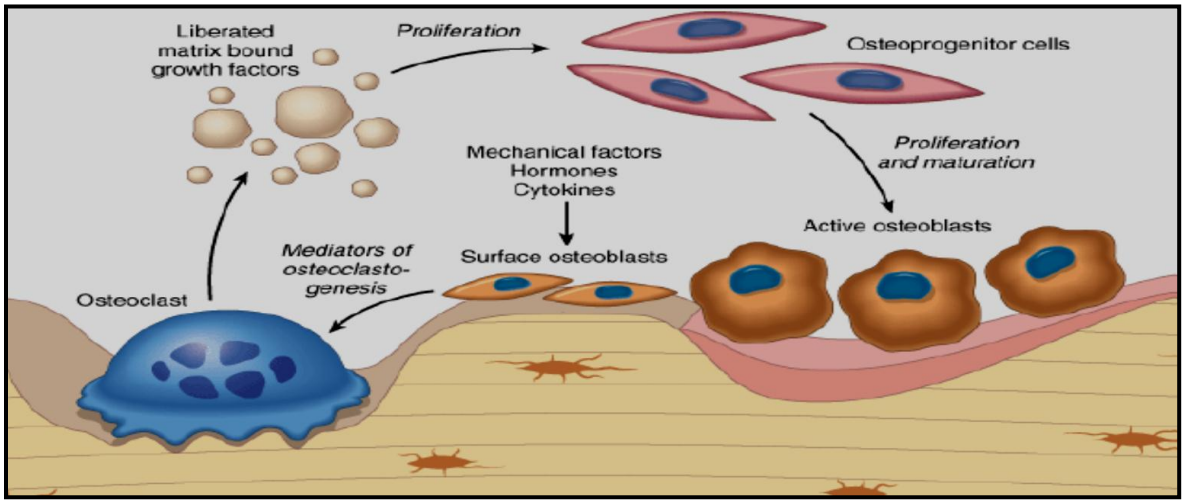


FIGURE (1) BONE RESORPTION AND FORMATION ARE COUPLED PROCESSES

Courtesy: Kumar, Cotran, Fausto. Bones, joints and soft tissue tumors. In: Robbins and Cotran eds.Pathological basis of diseases, 7th ed. Elsevier 2008:p.1282-1284.

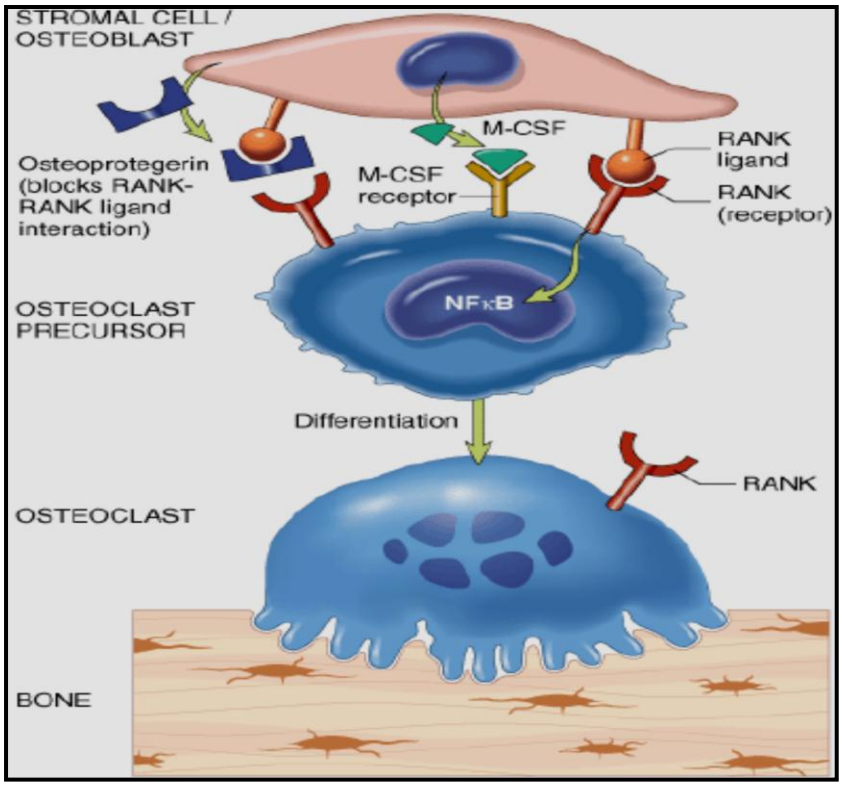


FIGURE (2) OSTEOCLASTS RESORBING BONE

Courtesy: Kumar, Cotran, Fausto. Bones, joints and soft tissue tumors. In: Robbins and Cotran eds. Pathological basis of diseases, 7th ed. Elsevier 2008:p.1282-1284.

RISK FACTORS FOR OSTEOPOROSIS

A number of risk factors predispose certain group of people to develop these osteoporotic changes much before the rest of the population.These include factors such as:

- Family history of osteoporosis - History of osteoporotic fracture in first degree relative increases the chances of a person to suffer from osteoporosis themselves.[34]

- Gender- Men attain higher peak bone mass in their adult life compared to women.As the initial peak bone mass itself is lower in women, they are more prone for increased bone loss.[34]

- Early menopause – Subjects who attained menopause at age < 45 years are more prone for such bony changes.[34]

- Prolonged amenorrhoea.[34]

- Low calcium diet- Peak bone mass may be impaired by inadequate calcium intake during growth of bone. The other nutritional factors that contribute to bone health are calories, protein, and other minerals. The associated nutritional deficiencies thereby lead to increased risk of osteoporosis later in life. Total daily calcium intakes of <400 mg are considered detrimental to the skeleton.[34]

- Vitamin D deficiency- Vitamin D insufficiency leads to compensatory secondary hyperparathyroidism which is a major factor causing osteoporosis and fractures.[34]

- Cigarette smoking - Chronic cigarette consumption causes toxic effects on osteoblasts and also modifies estrogen metabolism. Chronic cigarette smokers reach menopause 1–2 years earlier than non-smokers. Smoking also produces effects that can modulate skeletal status, including frailty, decreased exercise, poor nutrition, and the need for additional medications (e.g., glucocorticoids for lung disease).[34]

- Alcohol consumption.[34]

- Lack of exercise and immobility - Inactivity, such as in prolonged bed rest or paralysis, results in significant bone loss.[34]

- Body mass index- Bone tissue is sensitive to decrements in body weight as well. Previous studies have shown that even 10% weight loss can induce almost 2% bone loss. The impact can be higher in individuals exposed to more intense and rapid weight loss compared to moderate decrement in weight, for longer time.[35]

- History of anorexia or bulimia- Due to poor nutritional state, anorexic and bulimic patients suffer from various deficiencies like calcium, vitamin C etc.[34]

TABLE (2) CLASSIFICATION OF OSTEOPOROSIS [36]

A) **Primary osteoporosis**	B) **Secondary osteoporosis:**
i) Postmenopausal osteoporosis ii) Senile osteoporosis iii) Idiopathic osteoporosis	**1. Endocrine disorders:** a. Hyperparathyroidism b. Diabetes mellitus c. Cushing's disease d. Hypo-hyperthyroidism, e. Addisons disease f. Pituitary tumours g. Hypogonadism.

	2. Malignant diseases a. Carcinomatosis b. Multiple myeloma c. Leukemia. **3. Gastro-intestinal disorders** a. Malnutrition b. Malabsorption c. Hepatic insufficiency d. Vitamin C and Vitamin D deficiencies. **4. Rheumatologic disease** **5. Drug Induced** a. Alcohol b. corticosteroids c. Chemotherapy d. Anticoagulants e. Anticovulsants. **6. Immobilization** **7. Osteogenesis imperfecta** **8. Homocystinuria** **9. Anaemia**

A. PRIMARY OSTEOPOROSIS

i. POSTMENPAUSAL OSTEOPOROSIS

The age-related rise in bone resorption is attributed to the postmenopausal decline in plasma estrogen levels and consequent increase in sensitivity of the bone to bone-resorptive agents. The most likely explanation for the further increase in bone resorption causing accelerated osteoporosis is the malabsorption of calcium which is also a feature of this disorder.[37] Other factors like increased cytokine levels and expression of RANKL also contributes to the bone loss (Figure 3).

ii. SENILE OSTEOPOROSIS

Age-related changes in bone cells and matrix have a strong impact on bone metabolism. The replicative activity of cells reduces with age as shown in Figure (3). Osteoblasts from elderly individuals have reduced replicative and biosynthetic potential when compared with osteoblasts of younger individuals. Proteins bound to the extracellular matrix such as growth factors, which are mitogenic to osteoprogenitor cells and stimulate osteoblastic synthetic activity lose their biologic potency over time. The end result is a skeleton populated by bone forming cells that have a diminished capacity to make bone. This form of osteoporosis is categorized as a low turnover variant.[36]

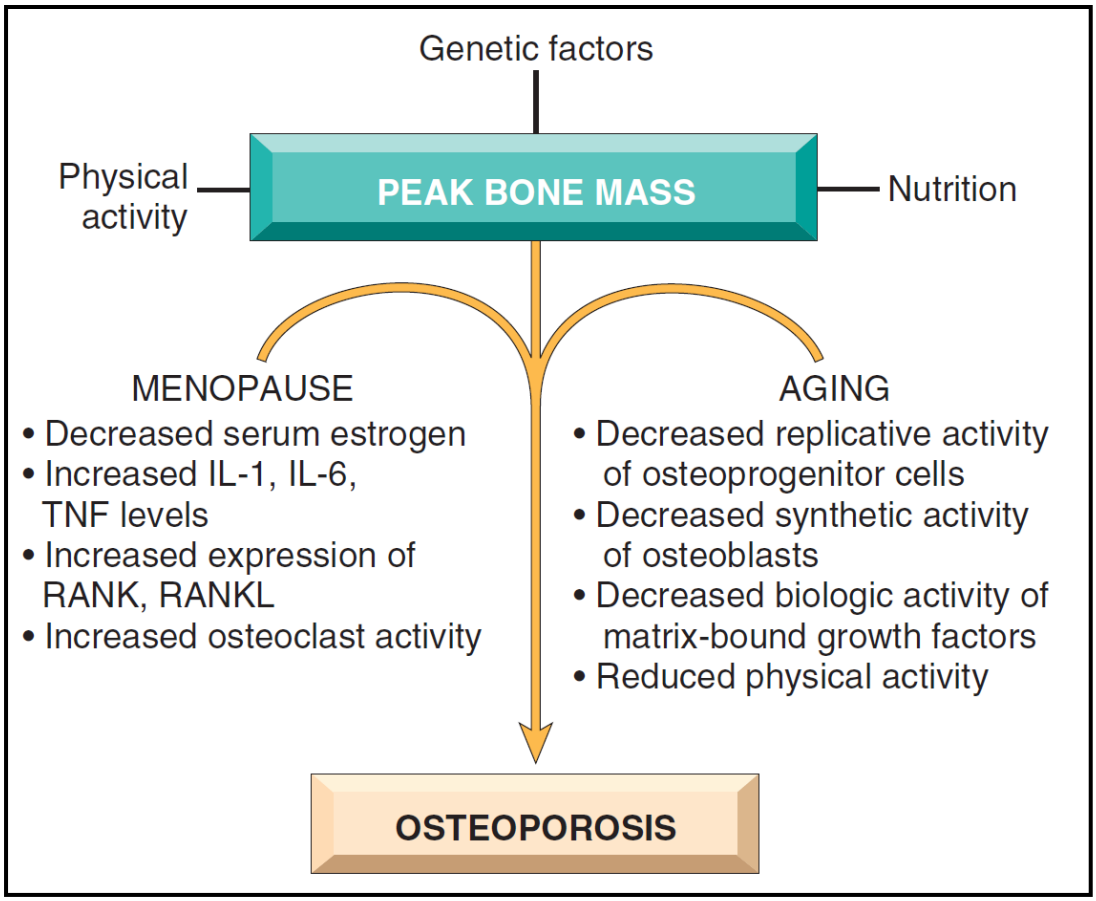


FIGURE (3) PATHOPHYSIOLOGY OF POSTMENOPAUSAL AND SENILE OSTEOPOROSIS

Courtesy: Kumar, Cotran, Fausto. Bones, joints and soft tissue tumors. In: Robbins and Cotran eds. Pathological basis of diseases, 7th ed. Elsevier 2008:p.1282-1284.

iii. IDIOPATHIC OSTEOPOROSIS

Osteoporosis with no obvious secondary cause in premenopausal women or younger men is called idiopathic osteoporosis. Some patients have a transient, self limited condition, where as others have a progressive and disabling disease. Idiopathic osteoporosis can be associated with nonspecific inflammatory changes, and these cases may be caused by abnormal cytokine activity. [36]

B. SECONDARY OSTEOPOROSIS

1. ENDOCRINE DISORDERS

Hyperparathyroidism, Diabetes mellitus, Cushing's disease, Hypo and hyperthyroidism, Addison's disease, Pituitary tumors, and hypogonadism contribute to development of osteoporosis.

a. HYPERPARATHYROIDISM

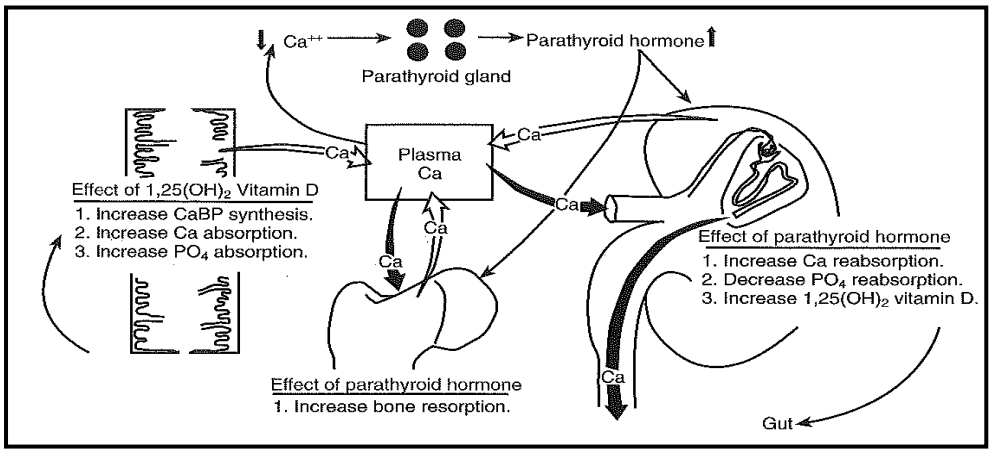


FIGURE (4) EFFECT OF PARATHYROID HORMONE

Courtesy: Endres DB, Robert K.Disorders of bone. In: Burtis CA, Ashwood ER, Bruns DA, eds. Tietz text book of clinical chemistry and molecular diagnostics, 6th ed. New Delhi: Elsevier Co., 2008; 729.

The PTH responds within seconds to a decrease in serum calcium.[38] The increased secretion of PTH could be a normal physiological response to hypocalcemia (secondary hyperparathyroidism) or due to primary hyperparathyroidism in which hyper secretion of PTH from the gland is the principal abnormality.As evident from Figure (4), the PTH increases distal tubular reabsorption of calcium in kidneys and increases the activity of the 25-hydroxyvitamin D l α-hydroxylase enzyme in proximal tubular cells. In bone,

PTH receptors are present on cells of osteoblastic origin. A gradual rise in plasma calcium occurs due to bone resorptive changes.[39]

Patients with Insulin-Dependent Diabetes Mellitus (IDDM) often have low bone mass and diminished bone formation, perhaps because they lack an anabolic effect of insulin. BMD is usually normal or high, but fracture incidence is increased suggesting that there is an independent effect of diabetes on bone fragility and or on the frequency of falls. Poorly controlled Non-Insulin Dependent Diabetes Mellitus (NIDDM) patients have a negative calcium balance which might result in accelerated bone resorption and loss of bone. Also hyperglycemic postmenopausal women show significantly increased bone activity as compared to normal postmenopausal women.[40]

b. DIABETES MELLITUS

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. Factors contributing to hyperglycemia include reduced insulin secretion, decreased glucose utilization, and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems. This multisystemic disease is the cause of enormous burden on the individual health with diabetes and also on the health care system.

PREVALENCE OF DIABETES MELLITUS

By the year 2011, globally the burden of type 2 Diabetes mellitus was around 366 million. It is expected to rise to 552 Million by the year 2030.[41] The prevalence of

Diabetes is around 2.7 % in rural India, and 14 % in urban India. [42] Total percent of new and old cases of Diabetes in Karnataka is 19.8 % in the year 2012.[43]

The two broad categories of DM are type 1 and type 2

1. Type 1 diabetes is the result of complete or near-total insulin deficiency.
2. Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production.

TABLE (3) CLASSIFICATION OF DIABETES [44]

Diabetes mellitus is classified as different types as follows.

Type 1 diabetes mellitus • Immune mediated • Idiopathic
Type 2 diabetes mellitus
Other specific type of diabetes mellitus Genetic defects of islet β- cell function • Genetic defects in insulin action diseases of exocrine pancreas • Endocrinopathies • Drug-and chemical- induced diabetes • Infections • Uncommon forms of diabetes • Other genetic syndromes
Gestational diabetes mellitus (GDM)

Type 2 diabetes has two hallmark features: 1) Insulin resistance, defined here as an impaired ability of the hormone to suppress hepatic glucose output and to promote peripheral glucose disposal. 2) Compromised function of pancreatic β cellsuch that insulin secretion is insufficient to match the degree of insulin resistance.[45]

The prevalence of obesity and type 2 DM is increasing rapidly. Both these diseases are associated with metabolic alterations, such as elevated plasma fatty acids and a reduced ability of insulin to suppress lipolysis, which may lead to the accumulation of intramyocellular lipid. This accumulation of lipid within muscle cells has been linked to the development of insulin resistance.[45]

CRITERIA FOR THE DIAGNOSIS OF DIABETES MELLITUS[46]

The National Diabetes Data Group and World Health Organization have issued diagnostic criteria for DM:

- Symptoms of diabetes plus random blood glucose concentration ≥ 11.1 mmol/L (200 mg/dL) *or*
- Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL) *or*
- Two-hour plasma glucose ≥ 11.1 mmol/L (200 mg/dL) during an oral glucose tolerance test.

EFFECT OF DIABETES ON BONE

The impact of diabetes on bone is still incompletely understood or clear. Bone turnover is regulated by local cytokines, cell matrix interactions and systemic hormones,

and hyperglycemia may affect any of these micro-environments that regulate bone turnover.[47] Osteoporosis is reported as a potential complication of type 1 diabetes. It has also been shown that older women with diabetes have increased risk of fractures.[48]The effects of type2 diabetes on bone mass have shown conflicting results in previous studies. Studies conducted by Kwon DJ et al have shown postmenopausal diabetic women to have reduced bone mineral density (BMD).[12] On the contrary several cross sectional studies done by Oz SG et al, Van Daele PL et al, and Akin O et al respectively have found diabetics with elevated bone mass.[11, 13, 14]

TYPE 1 DIABETES MELLITUS

Although the loss of bone mass has not been classically considered as one of the major complications of type 1 diabetes mellitus, evidence accumulated in recent years shows that diabetic patients have lower BMDs than their controls. The exact pathogenesis is not clear. The pathogenesis appears complex, as it involves hormonal, vascular, and mechanical factors. Evidence suggests that the development of osteopenia in diabetics could be related to a number of factors, such as the inhibition of collagen matrix formation, alterations in protein synthesis, decreased proteoglycan synthesis in the development of cartilage and bone, and decreased skeletal deposition of calcium.[49, 50, 51]

In type 1 diabetes, the deficiency of insulin and IGF-1, which is present since the diagnosis (in adolescence or childhood), leads to impaired bone formation, abnormal mineralization, abnormal bone micro architecture, increased fragility of the bone and reduced peak bone mass.[52]

In addition to insulin, pancreatic β cells produce other osteotropic factors, such as islet amyloid polypeptide (IAPP, also called amylin) and preptin, both of which are members of the calcitonin-gene-related peptide family. Production of these peptides is abolished in patients with type 1 DM. Amylin, a 37-amino-acid peptide, is secreted with insulin. Figure (5) shows pancreatic β-cell destruction in patients with type 1 DM prevents secretion of insulin, amylin and preptin, thereby reducing their effects on the RUNX2 gene. This reduction decreases proliferation and differentiation of mesenchymal stem cells into osteoblasts and their resistance to apoptosis. This leads to reduced osteogenesis. Also the reduced insulin secretion in patients with type 1 DM prevents stimulation of osteoblasts to produce osteocalcin, which stimulates β-cell proliferation and acts on the testes to produce testosterone, a hormone known to increase bone formation.[53]

PATHO PHYSIOLOGY OF INCREASED FRACTURE RISKS IN DIABETICS

Hyperglycemia

Hyperglycemia has adverse effects on bone metabolism in both poorly controlled type 1 and type 2 DM. Glucose is the principle energy source for osteoclasts and dose-dependently enhances avian osteoclast activity in vitro. Hyperglycemia also leads to nonenzymatic glycosylation of various bone proteins including type I collagen, which may impair bone quality [Figure (6)].[10]

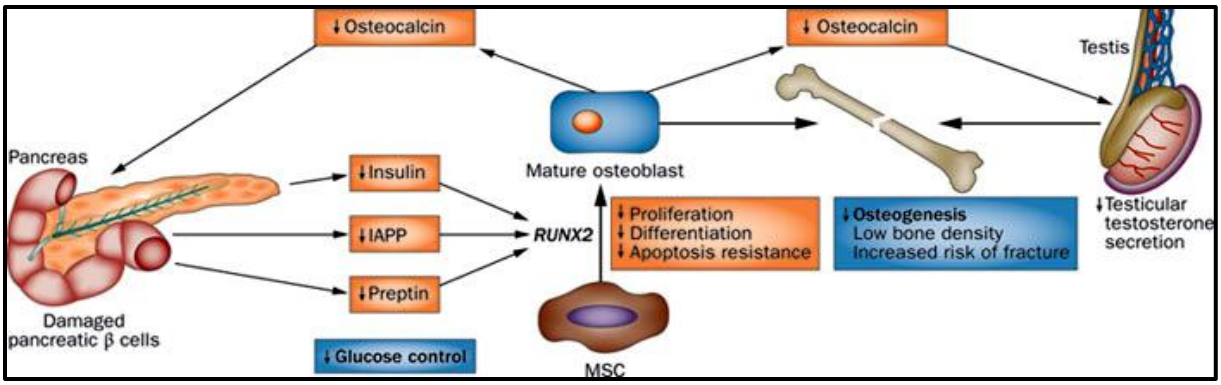


FIGURE (5) IMPAIRED OSTEOGENESIS IN TYPE 1 DIABETES MELLITUS.

Courtesy: Hamann C et al. Bone, sweet bone—osteoporotic fractures in diabetes mellitus. Nat. Rev. Endocrinol. 2012 doi:10.1038/nrendo.2011.233

Insulin

Insulin is an anabolic hormone which acts on bone through insulin receptors expressed by osteoblasts: IRS-1 and IRS-2 (IRS, insulin-like substrate). Stimulation of IRS-1 affects bone turnover, while stimulation of IRS-2 shifts the balance between bone formation and resorption towards the former. Insulin stimulates osteoblast proliferation, inactivates p27 (responsible for osteoblastogenesis), promotes collagen synthesis and increases glucose uptake.[52]

Insulin has an anabolic effect on bone.[10] Insulin like growth factor-1(IGF-1), structurally similar to insulin plays an important role in childhood growth and has anabolic effects in adults. Osteoblasts have receptors for both insulin and IGF-1. A positive correlation has been found between insulin, IGF-1and BMD. IGF-1 serum levels in patients with type 1 diabetes are known to be lower than those in type 2 diabetics and nondiabetics. In the untreated insulin deficient state decreased bone strength, deficit in

mineralized surface area, decrement in the rate of mineral opposition, decreased osteoid surface, decreased osteoblast activity and fewer osteoclasts have been observed.[54]

Advanced glycation end products (AGEs)

Hyperglycemia generates high concentration of AGEs in collagen that decreases bone strength, promote osteoblast apoptosis and increase osteoclast mediated bone resorption.[54] Studies conducted by Takagi et al, reported that advanced glycation end products (AGEs) stimulate production of interleukin-6, a bone resorbing cytokine, in human as well as mouse osteoblast-like cells in culture. However, as the formation of AGEs is considered irreversible, this mechanism cannot fully explain the rapid reduction in bone resorption.[55]

Phosphorus and calcium balance

Hypercalciuria is a potential risk factor for osteoporosis in poorly controlled type 1 and type 2 diabetes but glycemic control can reduce hypercalciuria.[54]

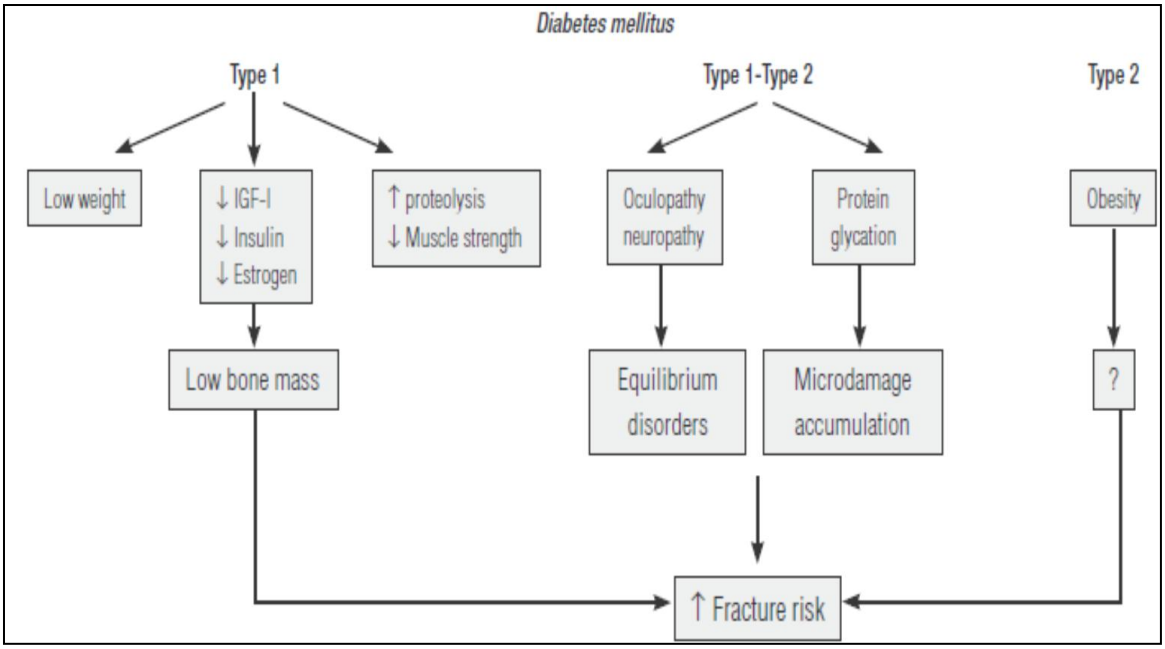


FIGURE (6) PATHOPHYSIOLOGY OF THE INCREASED FRACTURE RISK IN TYPE 1 AND TYPE 2 DIABETES MELLITUS

Courtesy: FransiscoPaula, Clifford Rosen. Obesity, diabetes mellitus,and last but not least osteoporosis.Arq Bras endocrinolmetab 2010; 54:150-157.

Adipokines

Adipokines are soluble factors released from adipocytes. They have mixed effects on human bone metabolism.

Leptin

Leptin is a protein hormone that plays a key role in regulating energy expenditure**.** A few studies in non diabetics have shown that leptin has a positive correlation with BMD. It may also reduce osteoclastogenesis. Whereas negative correlation with BMD has been shown in Diabetics in some studies.[54]

Adiponectin

Adiponetin receptors are present on both osteoblasts and osteoclasts. It suppresses osteogenesis in cultured osteoprogenitor cells. But in the presence of insulin this suppression was blunted. Adiponectin levels negatively correlate with BMD.[54]

Amylin

Amylin is an osteotropic factor secreted by pancreatic β cells and absent in Type 1 DM. In a rat model of DM in which streptozotocin selectively destroys pancreatic β cells, the administration of amylin maintained bone mass, inhibited biochemical markers of bone resorption, and elevated biochemical markers of bone formation.[10]

Resistin

Resistin is an adipocytokine that was discovered while screening for substances that are down-regulated in response to insulin-sensitizing anti-diabetic drugs. Resistin seems to play a role in glucose homeostasis, insulin resistance and inflammation, but very few studies have reported on the correlation of resistin to bone metabolism. It seems to stimulate osteoclastogenesis without affecting osteoblasts; however Oh KW et al have stated that further studies are required to confirm its exact role in bone. [56]

Peroxisome proliferator activated receptor gamma

Peroxisome proliferator -activated receptor gamma (PPARγ) is a transcription factor involved in adipocyte differentiation. PPARγ induces adipogenesis over osteoblastogenesis in pluripotent cells. Increased PPARγ expression has been detected in

type 1 diabetic mice contributing to bone loss due to fewer mature osteoblasts and more adipose accumulation. Thiazolidinediones given in diabetes activate PPARγ.[54]

c. CUSHING'S SYNDROME

The most common form of secondary osteoporosis is that induced by exogenous glucocorticoids manifesting as Cushing's syndrome. Cushing's syndrome, caused by an excess of endogenous glucocorticoids, is less common but may also involve osteoporosis at presentation. Though fragility fractures can occur in any patient receiving moderate to high doses of glucocorticoids, glucocorticoid-induced osteoporosis is particularly common in postmenopausal women, presumably because they also have primary osteoporosis.[25]

Glucocorticoid-induced osteoporosis is a result of both increased bone resorption and decreased bone formation. Increased resorption may be in part indirectly caused by decreased calcium absorption and the resulting secondary hyperparathyroidism. Decreased bone formation is probably caused by direct inhibition of osteoblasts, which are highly sensitive to glucocorticoids.[25]

d. Thyroid disorders

Both hormone excess and deficiency may increase the risk of fractures. Hyperthyroidism produces bone loss but the increase in bone formation is usually adequate in young persons and if the disease is treated early the resultant bone mass changes are small.[25]

e. Pituitary tumors

Loss of growth hormone may play a role in osteoporosis of pituitary tumors. Osteoporosis has been seen in patients with growth hormone deficiency and can respond to growth hormone replacement. A large pituitary tumor may cause gonadotropin deficiency and bone loss.[25]

f. Hypogonadism

There are multiple causes for hypogonadism in both men and women. Patients with primary hypogonadism related to ovarian or testicular failure or secondary hypogonadism related to hypothalamic or pituitary disease lose bone rapidly and often have fragility fractures. The hypogonadotropic group includes patients with anorexia nervosa, athletic amenorrhea, prolactinoma, or lesions of the pituitary gland or hypothalamus, including tumors. Under nutrition and hypercortisolism may also contribute to bone loss in anorexia nervosa and athletic amenorrhea. Certain drugs can also induce hypogonadism.[25]

2. Malignancy

The clinical picture of multiple myeloma and other lymphoproliferative malignancies can mimic that of primary osteoporosis. It is particularly important to exclude myeloma in patients with rapidly progressive vertebral crush fracture syndrome. Myeloma may cause rapid bone loss because the malignant cells produce both stimulators of resorption and inhibitors of formation.[25]

3. Gastro-intestinal disorders

The incidence and severity of osteoporosis are increased in patients with chronic hepatic and intestinal disorders, not only for nutritional reasons or because these patients often receive glucocorticoids or other drugs that affect the skeleton, but also because of increased cytokine production. Impaired absorption of calcium and vitamin D also occurs in celiac disease and it is likely that impairment of vitamin D function in hepatic and intestinal disease would cause osteoporosis.[25]

4. Other causes

People with severe alcoholism can also have osteoporosis; however, lower intakes of ethanol may be associated with increased bone mass and a decreased fracture risk. Many drugs are known to cause osteoporosis. Heparin stimulates bone resorption and inhibits bone formation and can cause osteoporosis. Patients receiving anticonvulsants, including phenytoin, barbiturates, and carbamazepine, often have low bone mass. Immunosuppressive agents, such as cyclosporine and FK506, are associated with bone loss. GnRH analogues, which decrease production of gonadal hormones and aromatase inhibitors, which block formation of estrogen from androgen, can lead to osteoporosis.[25]In these causes an impairment of vitamin D metabolism is possible mechanism.

EVALUATION OF BONE MINERAL DENSITY

The evaluation of bone mineral density and management of osteoporotic fractures is done usually by the protocol defined in the Figure 7.

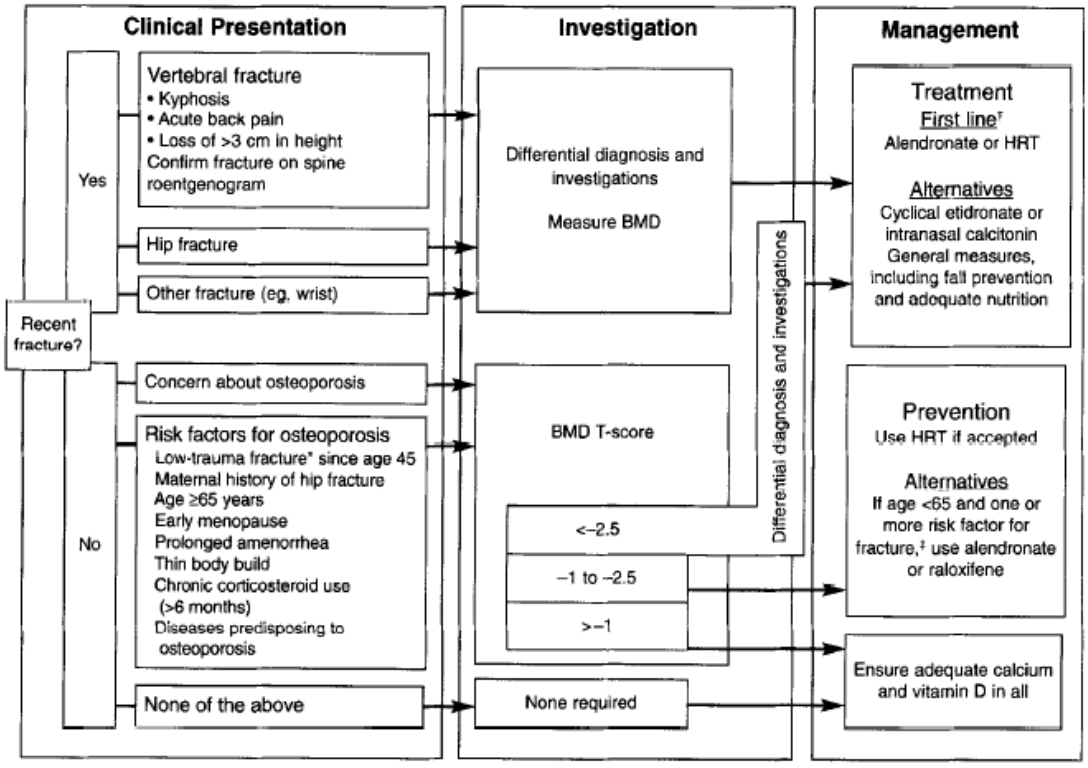


FIGURE (7) ALGORITHM OF BONE MINERAL DENSITY EVALUATION.

Courtesy: Meunier PJ, Delmas PD, Eastell R, McClung MRet al.Diagnosis and Management of Osteoporosis in postmenopausal Women: Clinical Guidelines. Clinical therapeutic 1999;21:1025 -1028

There is wide range of investigations that can be done in order to assess the bone status of a subject, including biochemical and non biochemical parameters. Each has its own advantage and disadvantage.

NON BIOCHEMICAL INVESTIGATIONS

i) Radiology

Identification of trauma induced fracture of the hip or distal forearm due to osteoporosis is straight forward. The more reliable finding on a given radiograph to support the diagnosis of osteoporosis is presence of a deformed vertebra. It is a common mistake to take X ray only in the painful area. This misses asymptomatic fractures in other parts of spine. The anteroposterior view is usually used to identify the level of fracture and exclude other causes of deformities like malignancy. Some deformities mimic fracture like epiphysitis which need to be excluded. Even though slight changes of osteoporosis may be identified on supine radiographs, but these changes are unreliable, and the suspicion of osteoporosis must be confirmed by BMD measurement.[57]

ii) Bone mineral density measurement

Bone mineral density measurement is more reliable and has become widely available over the last 10 years. The techniques include:

a. Dual-energy X-ray Absorptiometry (DEXA)

DEXA provides a precise and accurate method of measuring changes in bone mineral density (BMD) at various skeletal sites. This includes exposure to X-rays in low doses. Two energy peaks of X-ray are absorbed to different extent by bone and soft tissue and the density is calculated in g/cm^2 using simultaneous equations. The T score and Z score are measured by comparing values with two different reference ranges. This method has become a standard method for BMD calculation. Usually measurements are

taken at two sites spine and total hip. Even single energy X-ray absorptiometry is available that is less expensive but clinically less useful.[57]

b. Quantitative computed tomography (QCT)

This method allows a three dimensional measurements of BMD of the lumbarspine. This technique also allows measurement of trabecular bone alone, which is usually lost first in the development of osteoporosis. However, Quantitative CT is more expensive, less precise and involves a higher radiation dose than DEXA.[57]

c. Quantitative ultrasound measurements

Quantitative ultrasound measurements are usually made on the calcaneum bone. The ultrasound signal used for BMD measurement has a lower frequency (200-600 kHz) compared to that used in obstetrics (>mHz). The attenuation of the signal may reflect both the density and the architecture of bone, and the velocity of the signal reflects the density and bone properties (elasticity). This ultrasonography is currently used only in research but if studies of its predictive ability in osteoporosis are confirmed, it could be considered as an established technique.[57]

iii) Bone biopsy

Histomorphometric analysis of bone biopsies is usually done to assess bone turnover. It is probably the most reliable method available at present. However bone biopsy is an invasive procedure and yields information only on a restricted area of bone thus, it may not be representative of the entire skeleton.[58]

iv) Bone turnover

Bone turnover by formation and resorption is a continuous process. Biochemical parameters, both for formation and resorption are measured to reflect these changes and may be used as indicators of effectiveness of any therapeutic regimens.

Biochemical markers of bone turnover could be valuable in assessing the fracture risk than changes in BMD. They could be valuable in assessing the response of individual patient to antiresorptive treatments of osteoporosis. They are attractive because even slight bone changes become evident by these changes and these occur comparatively early (within weeks), whereas it may take a year or longer to observe changes with bone densitometry.[59]

PITFALLS OF DUAL ENERGY X RAY ABSORPTIOMETRY (DEXA) SCAN

Even though many technologies are available for measuring bone mineral density but DEXA has been reported by many investigators as gold standard for measurement of bone mass density.[60]

The measurement of Bone mineral density done by the standard method of DEXA has its own disadvantages. The mineral density changes with the positioning of the subject, due to rotation of the bone structure. The BMD can be miscalculated by the machines if some anatomic variations are present like floating ribs. Artefacts like coins, clips should be excluded before analysis. Any repetition of bone analysis needs to be done preferably in the same DEXA scan machine.[61]

By the time bone density changes are detected by X-rays or DEXA scans a considerable amount of bone loss would have already occurred.

BONE MARKERS

Several factors influence the concentration of bone turnover markers in blood or urine including age, sex, fasting or non-fasting state, the body circadian rhythms, menstrual cycle, exercise history and medical history. They are also affected by a variety of physiological and pathological factors. Age exerts maximum effect on bone turnover markers. Concentrations are higher in children and adolescents than in adults. There is a significant increase in markers during growth spurts. In females, bone turnover markers reach a plateau between 20 and 25 years of age, and in males between 25 and 30 years of age reflecting their peak bone mass.[16]

Biochemical markers help in the early and dynamic measurement of the body bone metabolism, thus they are clinically useful for the prediction of postmenopausal osteoporosis. Bone turnover markers are helpful to assess the response to any nutritional intervention on the bone. These bone markers have an advantage over Bone mineral density measurement in that, they give information about the mechanism of the bone change and also that the changes are observed much faster or rapidly.[62] While BMD provides a static picture of the skeleton, the biochemical markers of bone turnover provide dynamic measures of bone remodelling and useful in predicting the course of changes in bone mass. If preventive measures are to be initiated prior to the onset of excessive bone loss, measurement of bone turnover through these markers could form a tool available to assist health care professionals to predict fracture risk.[26]

TABLE (4) BIOCHEMICAL MARKERS OF BONE TURNOVER[63]

Formation markers

Plasma alkaline phosphatase (ALP) (total or bone specific)

Osteocalcin (OC)

Procollagen I carboxy-terminal extension peptide (PICP)

Procollagen I amino-terminal extension peptide (PINP)

Resorption markers

Plasma

Cross-linked C-telopeptide of type I collagen (ICTP)

N-telopeptide of collagen cross-links (NTX)

C-telopeptide of collagen cross-links (CTX)

Tartrate-resistant acid phosphatase (TRAP)

Urine

Hydroxyproline

Hydroxylysine

Pyridinoline (total and free)

Deoxypyridinoline (total and free)

N-telopeptide of collagen cross-links (NTX)

C-telopeptide of collagen cross-links (CTX)

HYDROXYPROLINE

Hydroxyproline, an imino acid present in collagen, was first used in the 1960s as a marker of bone resorption in both clinical practice and research.[64] Hydroxyproline is a major component of fibrillar collagen of all types, comprising approximately 14% of the total amino acid content. It is produced by the post-translational modification of proline by the enzyme prolyl hydroxylase. [65]

Collagen is made of repeating amino acid structure, represented as $(Gly\text{-}X\text{-}Y)_n$, for the formation of the triple helix. X positions are occupied by amino acid proline and Y positions are filled by hydroxyproline. Hydroxyproline confers rigidity to the collagen molecule. This mainly occurs by the crosslinks formed .[66]

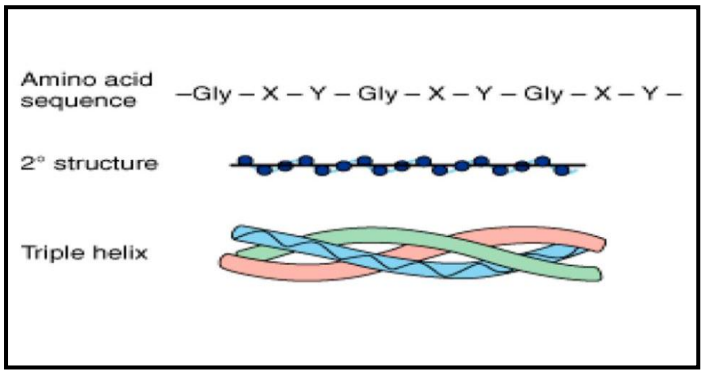


FIGURE (8) COLLAGEN STRUCTURE

Courtesy: Kenelly PJ, Rodwell VW. Proteins: Higher orders of structure.In: Murray R, Granner D, Mayes P, Rodwell V, eds. Harpers illustrated biochemistry, 28th ed, United states, McGraw hill.p. 56

SYNTHESIS OF HYDROXYPROLINE

Collagen is initially synthesized as a larger precursor polypeptide, procollagen. Numerous prolyl and lysyl residues of procollagen are hydroxylated by prolyl hydroxylase and lysyl hydroxylase, enzymes that require ascorbic acid (vitamin C). Hydroxyprolyl and hydroxylysyl residues provide additional hydrogen bonding for stabilizing the mature collagen protein. Hydroxyproline arises from hydroxylation of proline but only after these amino acids have been incorporated into peptides.

Hydroxylation of peptide bound prolyl residue is catalysed by prolyl-4 hydroxylase. The hydroxylases are mixed function oxygenases (mono-oxygenases). They require substrate, molecular O_2, ascorbate, Fe^{2+}, and α-ketoglutarate for every mole of proline hydroxylated.[66]

SOURCE OF HYDROXYPROLINE IN BODY

The formation occurs due to degradation of various types of collagen in the body. Most of the collagen in the body is in the bones, thus the urinary hydroxyproline is considered as a bone resorption marker. Only 1-2% of hydroxyproline in the urine is present as the free amino acid, the rest being in peptide form. About 10 % of hydroxyproline released is excreted in urine mainly as small dialyzable peptides.[65]

CALCIUM

The human skeleton contains 99 % of body calcium. The total body calcium in a human adult is approximately 1kg.[65] Its mainly present in the body in three forms, the

free or ionized form which constitutes around 50 % of total calcium, protein bound form, and the anion bound form. The free form is responsible for the biological activity. Calcitriol works in concert with parathyroid hormone in Ca^{2+} homeostasis regulating Ca^{2+} in blood and the balance between Ca^{2+} deposition and Ca^{2+} mobilization from bone. Acting through nuclear receptors, calcitriol activates the synthesis of an intestinal Ca^{2+} binding protein essential for uptake of dietary calcium.[67]

Apart from being a major constituent of the bone, calcium also is known to regulate the nerve conduction and muscle function. The absorption of calcium requires calbindin. Regulation of the calcium levels in the body is by hormones like parathyroid hormone, calcitonin and vitamin D. The toxicity of calcium occurs by excess absorption due to hypervitaminosis D or hypercalcemia due to hyperparathyroidism or idiopathic hypercalcemia. The main sources of calcium is dairy products, beans, leafy vegetables.[68]

Serum total calcium as a bone formation marker

During the growing phase, bony skeleton strengthens by mineralisation and attains its peak bone mass. With age this peak mass keeps reducing.Attainment of the peak bone mass may be impaired by inadequate calcium intake during growth phase among other nutritional factors there by leading to increased risk of osteoporosis later in life.[69]

PHOSPHORUS

Phosphorus forms a major constituent of bones, teeth, ATP, phosphorylated metabolic intermediates like phospholipids, phosphoproteinsand nucleic acids.[68] In blood

most of the organic phosphate esters are in the cell. Inorganic phosphate is the major component of hydroxyapatite in bone.[70]

Phosphorus accounts for about 1% of the weight of the elementary composition of the human body. The bulk of phosphorus (85%) is in the skeleton and teeth (largely in the form of hydroxyapatite) and 14% is located within the cells of the soft tissues. Within different cell types, the phosphorus content may vary from around 300 to 1300 mmol/kg.[39]

Only 1% of total body phosphorus is present in extracellular fluids.The inorganic moiety,which is measured routinely in laboratories. It exists predominantly in the form of the $HP0_4^{2-}$ and H_2P0^{4-} in a ratio 4: 1. The main sources of phosphate transfer to and from the plasma pool are the intestine, bone, soft tissues and the kidneys. Under steady-state conditions, the net intestinal phosphorus absorption equals the net urine excretion.[39] ^{Serum} levels are regulated by kidney reabsorption. Low serum Ca^{2+}: P ratio stimulates secondary hyperparathyroidism; and it may lead to bone loss. [68]

ALKALINE PHOSPHATASE (ALP)

ALP is present in most organs of the body. It exists in multiple forms some of which are true isoenzymes coded by different genetic loci. The main four isoforms are

a) Bone (osteoblast)

b) Liver

c) Placenta

d) Proximal convoluted tubules of kidney and mucosa of small intestine

The bone, liver, and the kidney isoforms have the same genetic loci, but they differ mainly in their carbohydrate content.[70] Post translational modification produces the tissue specific isoforms. Estimation methods for ALP do not distinguish between the different isoforms.[71]

Bone isoform of ALP (BAP)

This isoform is derived from osteoblasts. It is mainly found in the matrix in the form of buds derived from the cell membrane. They are an excellent marker for overall bone formation.[70] BAP has longer half life and less diurnal variation when compared to osteocalcin. Thus, measurement of BAP is considered to be better than osteocalcin. As clearance of BAP is not dependant on kidneys, it is considered to be better in individuals with impaired renal function.[71]

ALP Level variation

The level is higher in males when compared to females. In both sexes, it is found to increase with age. Bone alkaline phosphatase (BAP) is increased in bone diseases like rickets, hyperparathyroidism, renal osteodystrophy, thyrotoxicosis, acromegaly, bone metastasis, glucocorticoid excess, Paget's disease and other conditions with increased bone formation. In Paget's disease, total ALP or BAP can be estimated for diagnosis and monitoring the therapy. Children also show especially higher levels during the growth spurt .[71]

MARKERS OF HYPERGLYCEMIA

GLYCATED HEMOGLOBIN

Glycation of hemoglobin is by the nonenzymatic addition of a sugar residue to the amino group of proteins. Human adult haemoglobin usually consists of HbA (97%), HbA_2 (2.5%) and HbF (0.5%). Chromatographic analysis of HbA identifies several minor hemoglobins namely HbA_{1a}, HbA_{1b} and HbA_{1c} which are referred to as glycated hemoglobins.[72]

HbA_{1c} is the major fraction, constituting approximately 80% of HbA_1. It is formed by the condensation of glucose with the N-terminal valine residue of each β-chain of HbA to form an unstable Schiff base (aldimine).

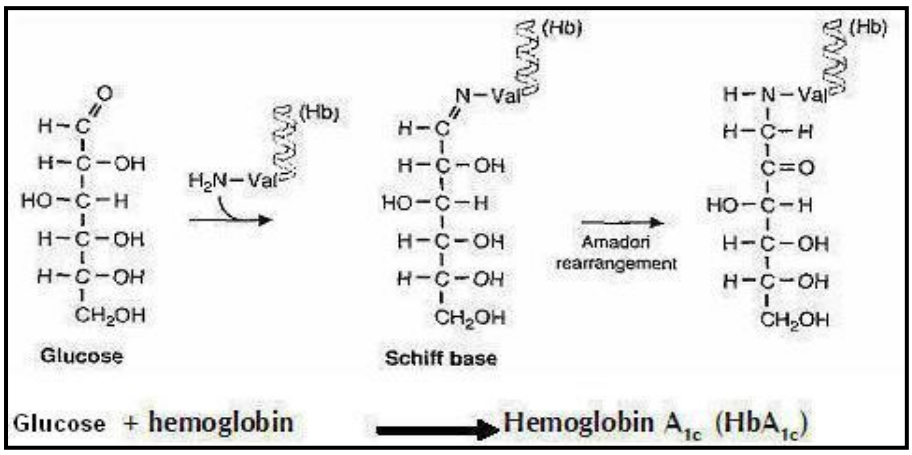


FIGURE (9) FORMATION OF GHb [72]

Formation of glycated hemoglobin (GHb) is essentially irreversible and the concentration in the blood depends on both the life span of the red blood cells (average 120 days) and the blood glucose concentration. Measurement of glycated hemoglobin is

effective in monitoring long term glucose control in people with diabetes. It provides a retrospective index of the integrated plasma glucose values over the preceding 6-8 weeks and is not subject to the wide fluctuations observed when assaying blood glucose concentrations. In addition glycated hemoglobin is a measure of the risk for the development of complications of diabetes mellitus.[72]

HbA_{1c} amounts to 5-8% of total HbA_1 in healthy individuals and ranges from 8-30% in patients with diabetes mellitus, depending on the degree of control of blood glucose concentration. In conditions of sustained hyperglycaemia the proportion of haemoglobin that is glycated is increased substantially.

The estimation by HPLC method is considered to be gold standard method when compared to other methods. The advantage is that this test does not require any dietary preparations and has low sensitivity but high specificity.

SOURCE OF DATA:

Ours is a hospital based observationalstudy carried out over a period of one year with 60 subjects. Study started in February 2012 at R.L.Jalappa Hospital and Research Centre attached to Sri Devaraj Urs medical college a constituent of SDUAHER, Kolar, a deemed to be university.

I. STUDY GROUP

Consisted of 60 postmenopausal women (n= 60).

Cases (Group I) – 30 postmenopausal women with clinically diagnosed type 2 diabetes mellitus, n =30.

Controls (Group II) – 30 postmenopausal women without diabetes mellitus, n=30.

INCLUSION CRITERIA

Postmenopausal women with clinically proven type 2 diabetes mellitus as cases and postmenopausal women without diabetes as controls were selected. Non surgical, physiological menopausal women were included.

EXCLUSION CRITERIA

1. Women more than 70 years of age.
2. Postmenopausal women on hormone replacement therapy.
3. Subjects with hyperthyroidism or hyperparathyroidism.
4. Subjects on steroid therapy, heparin or drugs that alter calcium and phosphate levels

5. Chronic renal failure patients.

6. Chronic alcoholics or tobacco users.

7. Subjects with malignancies or other systemic diseases.

II. METHOD OF COLLECTION OF DATA:

After obtaining written informed consent from the subjects, 5 ml of blood was drawn under aseptic conditions. Serum was separated. Twenty four hour urine sample was collected after a 24 hour gelatin free diet as Gelatin rich /protein rich food is known to alter the urine hydroxyproline levels. The twenty four hour urine sample was collected with 6 N Hydrochloric acid as preservative. Clinical data was obtained from each case with respect to name, age, clinical history, diabetes mellitus, hypertension, habit of alcohol consumption and history of any drug intake. Internal and external quality control was strictly followed through out the study.

III. PARAMETERS MEASURED:

In our study the following parameters were estimated:

PARAMETER	METHOD	INSTRUMENTATION
Urinary hydroxyproline	Modified Neuman and Logan method.	Colorimetry
Serum Calcium	Arsenazo III	Vitros 250 Dry chemistry auto analyzer based on reflectance spectrophotometry
Serum Phosphorus	Phosphomolybdate reduction	Vitros 250 Dry chemistry auto analyzer based on reflectance spectrophotometry
Serum Alkaline phosphatase	p-nitrophenyl phosphate	Vitros 250 Dry chemistry auto analyzer

		based on reflectance spectrophotometry
Random blood glucose	Glucose oxidase/ Peroxidase	Minitecno semi auto analyzer
Glycated hemoglobin	Cation exchange chromatography	Colorimetry

IV. METHODS OF ESTIMATION

A. ESTIMATION OF BLOOD GLUCOSE [73]

GLUCOSE OXIDASE-PEROXIDASE METHOD

PRINCIPLE: Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The hydrogen peroxide formed reacts, under catalysis of peroxidase (POD), with phenol and 4-aminophenazone (4AAP) to form a red violet quinonemine dye as indicator. The oxidation of sample glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction is followed by an oxidative coupling catalyzed by peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured by reflected light.

$$\text{Glucose} + \tfrac{1}{2}\,O_2 + H_2O \xrightarrow{\text{Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

INSTRUMENT: SEMI AUTO ANALYZER based on Beer-Lambert's law

REAGENTS AND MATERIALS

- Enzyme reagent- Phosphate buffer 50 mmol/L, Phenol 15 mmol/L, 4AAP 2.5mmol/L, GOD 18 KU/L, POD 2.5 KU/L
- Glucose standard- 100 mg/dL
- Preservative: Sodium azide (0.02%)

Safety Precautions and Warnings

- Reagent is for *in vitro* use only
- Reagent contains sodium azide (0.02%) as a preservative. In a dry state it may react with copper or lead plumbing to form explosive metal azides. Upon disposal flush with large amounts of water to prevent azide build up.
- **SAMPLE:** Plasma.

Stability: 7 days at 2-8^0 C.

- **REAGENT PREPARATION:** Reagent is ready to use.
- **STABILITY:** Reagent is stable till expiry when stored at 2-8^0 C. Store protected from light.
- **LINEARITY:** The method is linear upto a concentration of 400mg/dL (200mmol/L). Dilute samples above this concentration 1:1 and multiply by 2.

- **AUTOMATED PARAMETERS**

- Wavelength: 505nm (490-550nm), Measurement: End point, Cuvette: 1 cm path length, Reaction temperature: 37^0 C, Reaction direction: against reagent blank, Sample/reagent ratio: 1:100, Incubation: 05 min,Maximum blank absorbance: 0.300

- Linearity at 37^0 C: 400 mg/dl

MANUAL PROCEDURE

	BLANK(B)	STD(S)	SAMPLE(T)
SAMPLE	-	-	10µl
STANDARD(STD)	-	10µl	-
REAGENT	1000µl	1000µl	1000µl

Mix & incubate for 05min at 37^0 C or 15 min at room temperature (RT). Measure absorbance of sample (T) and standard (S) against reagent blank(B) at 505 nm. The color is stable for 30 min at room temperature.

CALCULATION

TOTAL GLUCOSE(mg/dl) = (T-B/ S-B) X Concentration of standard (100 mg/dl)

REFERENCE INTERVAL

Serum or plasma: 60-110mg/dl, Urine: <0.5 g/dl

INTERFERENCES

Hemoglobin: No interference upto 1000 mg/dl.

Free bilirubin: No interference upto 46mg/dl

Conjugated bilirubin: No interference upto 11mg/dl

Lipemia: No interference from lipemia, measured as triglycerides upto 2000 mg/dl

Ascorbate: No interference from ascorbateupto 6.5 mg/dl

B. GLYCOHEMOGLOBIN[74]

WEAKLY BINDING CATION EXCHANGE RESIN METHOD

PRINCIPLE:

Whole blood is mixed with lysing reagent to prepare a hemolysate. This is then mixed with a weakly binding cation exchange resin. The non-glycosylated hemoglobin binds to the resin leaving GHb free in supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb.

REAGENTS AND MATERIALS:

1. Resin reagent: 8mg/ml Cation-exchange resin buffered at pH 6.9
2. Lysing reagent: 10 mg Potassium cyanide surfactant added

3. Glycohemoglobin Standard: 10% Glycohemoglobin

4. Serum separators

PREPARATION OF REAGENTS

1. Glycohemoglobin lysing reagent: Bring the contents to room temperature.

2. GlycohemoglobinCation-exchange resin: Bring contents to room temperature, swirl and gently invert for a minimum of 10 times, swirl the bottle after addition of each tube.

REAGENTS STORAGE & STABILITY:

All reagents are stable at 2-8^0 C till expiry date. Do not freeze.

PHYSICAL OR CHEMICAL INDICATIONS OF INSTABILITY:

Alterations in the physical appearance of the reagents or values of control sera outside the manufacturer's acceptable range (4-20%) may be indications of reagents instability.

INSTRUMENT: Spectrophotometer or colorimeter set at 415 nm.

SPECIMEN COLLECTION AND PREPARATION:

Special preparation of the patient is unnecessary. Fasting specimens not required. No special additives or preservatives other than the anticoagulants are required. Collect venous blood with EDTA using aseptic technique.

MATERIALS NEEDED: 20 µl and 100µl micropipettes, 500µl, 3ml and 5ml pipettes or dispensers, 13 x 100 mm glass tubes, Glass or plastic test tubes to hold 0.6 ml and 5 ml, Rocker or rotator, Glycohemoglobin controls: Normal level, Elevated level.

PROCEDURE OUTLINE:

STEP A. **Hemolysate preparation:**

- Dispense 500 µl Lysing reagent into tubes labeled: Standard (HS), Control(HC), Sample(HT).
- Place 100 µl of the well mixed blood sample- Standard or control into the appropriately labeled tube. Mix well.
- Allow to stand for 5min.
- Plastic or glass tubes of appropriate size are acceptable.

STEP B. **Glycohemoglobin preparation**

1. Dispense 3.0 ml of GlycohemoglobinCation exchange resin into 13 x 100 mm glass tube labeled Standard(GS), Control(GC), Sample(GT).

   NOTE: Before use, mix the resin by inverting atleast 10 times, swirl the bottle after addition to each tubes.

2. Add 100 µl of the prepared hemolysate (from Step A)
3. Position the filter separators in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.

4. Place the tubes on the rocker or rotator and mix continuously for 5min.

5. Remove the tubes from the rocker or rotator.

6. Push the filter separator into the tubes until the resin is firmly packed.

7. Supernatant may be poured into another tube or directly into a cuvette for absorbance measurement.

8. Adjust the instrument to zero absorbance at 415 nm with deionized water as the blank (Wavelength range: 390-420 nm)

9. Read and record the absorbance values for Standard (GS), Control(GC), Sample(GT). These readings are glycohemoglobin.

STEP C. **Total Hemoglobin fraction:**

1. Dispense 5.0 ml deionized water into tubes labeled Standard (TS), Control (TC), Sample (TT).

2. Place 20 µl of the prepared hemolysate (from Step A) into appropriately labeled tube. Mix.

3. Adjust the instrument to Zero absorbance at 415 nm with deionized water as the blank.

4. Read and record the absorbance values for Standard (TS), Control (TC), and Sample(TT). These readings are for total hemoglobin.

   Plastic or glass tubes of appropriate size are acceptable.

QUALITY CONTROL:

Glycohemoglobin Control: Normal, Elevated.

CALCULATIONS:

Results for unknowns and controls are calculated as follows:

$$\text{Absorbance of Std} = A\ 1 = \frac{\text{Absorbance of Std GHb (GS)}}{\text{Absorbance of Std THb (TS)}}$$

$$\text{Absorbance of sample} = A2 = \frac{\text{Absorbance of Sample GHb (GT)}}{\text{Absorbance of Sample THb (TT)}}$$

$$\%\ \text{GHb in sample} = \frac{A2}{A1} \times 10$$

Std Concentration =10

LIMITATION OF PROCEDURE:

Sample from patients with hemoglobinopathies or decreased erythrocytes survival times may show incorrect values.

EXPECTED VALUES:

Non-diabetics	4.5-8.0 %
Good control	8.0-9.0 %,
Fair control	9.0-10.0 %
Poor control	10.0 % and above

LINEARITY:

Glycohemoglobin assay shows linearity for glycohemoglobin level in the range of 4.0-20.0 %. Blood samples with total hemoglobin more than 18 g/dl should be diluted x 2 with deionized water before assay.

PRECISION:

Within run: The intra assay precision was established by assaying bloods with normal and elevated glycohemoglobin levels.

CORRELATION:

A comparative study of the glycohemoglobin procedure and another widely used commercial method showed correlation 1 of 0.96.

SENSITIVITY:

This glycohemoglobin procedure has a sensitivity of 0.02% glycohemoglobin per 0.001 units of absorbance.

INTERFERING SUBSTANCES:

Samples that are severely lipemic may cause elevated results. Fetal hemoglobin (HbF) has resin binding characteristic similar to Glycohemoglobin if present. Glycosylated HbS and HbC bind more tightly than HbA1 and produce lower values. Other hemoglobinopathies (e.g beta thalassemia and hemolytic anemia) also produce lowered results.

C. ESTIMATION OF URINARY HYDROXYPROLINE[75]

Method : Modified Neuman and Logan method[75]

Principle: Hydroxyproline is treated with copper sulphate and hydrogen peroxide in an alkaline solution. This 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 a pink colored complex, measured at 540 nm.

REAGENTS:

1. Copper sulphate (0.01M) – Dissolve 0.159 gm of $CuSO_4$ in 100 ml of distilled water.

2. Sodium hydroxide (2.5N) – Dissolve 10gm of Sodium hydroxide in 100ml of distilled water.

3. 6% hydrogen peroxide.

4. Sulfuric acid (3N)

5. P-dimethylaminobenzaldehyde (5% solution in n-propanol)

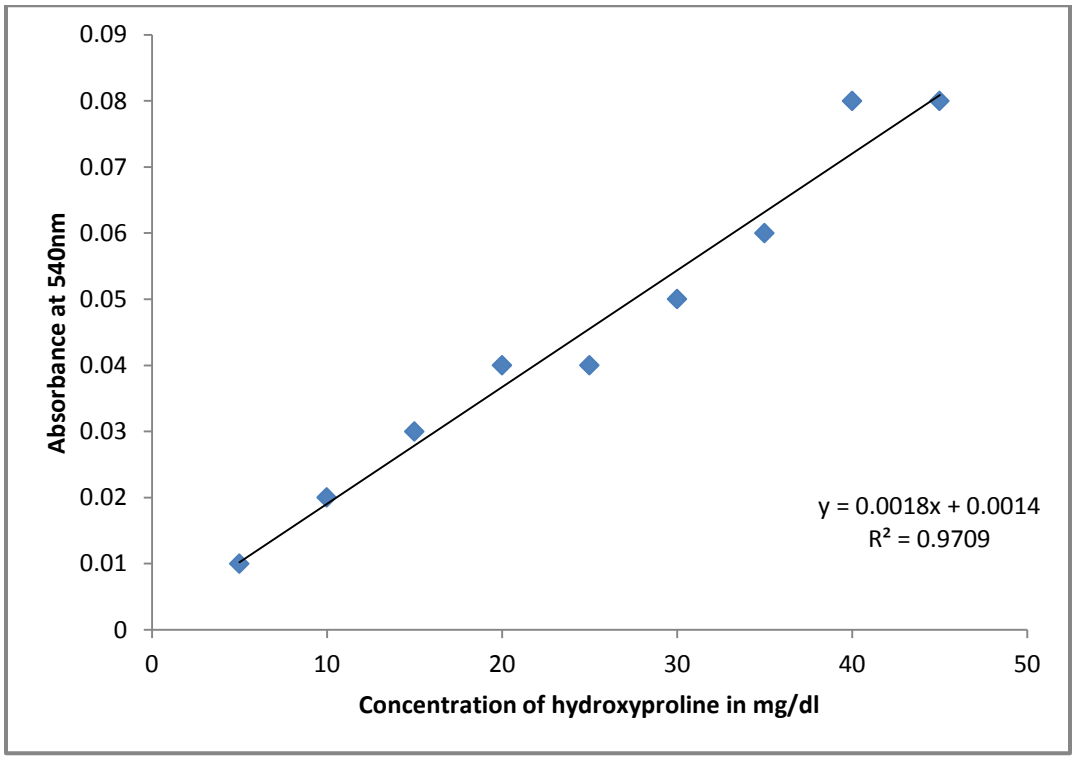
6. Stock hydroxyproline standard: prepared by dissolving 20mg of hydroxyproline in 100ml of distilled water.

7. Working hydroxyproline standard: (2mg/100ml) prepared by dissolving 10ml of stock standard made upto 100ml with distilled water.

Procedure: Mix reagents in test tube and wait for 5 minute. Place test tubes in a water bath at 80°C for 5 minute. Cool it in ice which is followed by keeping in water bath at 70°C for 16 minutes. Take readings at 540 nm.

REAGENT	**TEST (T)**	**STD (S) (2mg/100ml)**	**BLANK (B)**
URINE	1 ml	-	-
STANDARD	-	1ml	-
DISTILLED WATER	-	-	1ml
$CuSO_4$ (0.01 M)	1ml	1ml	1ml
NaOH (2.5 N)	1ml	1ml	1ml
6.6 % H_2O_2	1ml	1ml	1ml
H_2SO_4 (3 N)	4ml	4ml	4 ml
P-dimethylaminobenzaldehyde	2ml	2ml	2ml
MIX AND WAIT FOR 5 MINUTES			
WATER BATH AT 80 ^{0}C FOR 5 MINUTES			
COOL IT IN ICE, KEEP IN WATERBATH AT 70 ^{0}C FOR 16 MINUTES			
TAKE OD VALUES AT 540 nm			

STANDARD GRAPH OF HYDROXYPROLINE



Urinary hydroxyproline (mg/dl) = [(T-B/ S-B) X Concentration of Std (2 mg/dl)]

REFERENCE RANGE

18-25 years - 14-38.7 mg/day

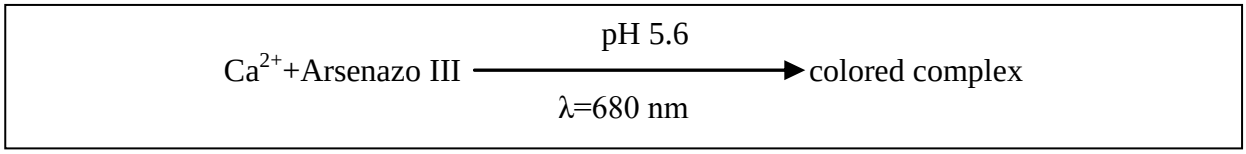
20-77 years - 11.8- 42.5 mg/day

D. SERUM CALCIUM[76]

INSTRUMENT: Vitros 250 dry chemistry auto analyzer based on the principle reflectance photometry

PRINCIPLE:

The VITROS Ca Slide is a multilayered, analytical element coated on a polyester support. A drop of patient serum sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The bound calcium is dissociated from binding proteins, allowing the calcium to penetrate through the spreading layer into the underlying reagent layer. There, the calcium forms a complex with Arsenazo III dye, causing a shift in the absorption maximum. After incubation, the reflection density of the colored complex is measured spectrophotometrically. The amount of colored complex formed is proportional to the calcium concentration in the sample.



Incubation time: 5 min

Temperature: 37^0 C (98.6^0 F)

Wavelength: 680 nm

Reaction sample volume: 10µL

SAMPLE: Serum

REAGENTS – Reactive Ingredients per cm^2 of slide

Arsenazo III dye 60µg

Other Ingredients

Pigment, binders, surfactants, buffer, cross-linking agent and mordant

PRECAUTIONS

The slide cartridge must reach room temperature, 18–28 °C (64–82 °F), before it is unwrapped and loaded into the slide supply.

1. Remove the slide cartridges from storage.

2. Warm the wrapped cartridge at room temperature for 30 minutes when taken from the refrigerator or 60 minutes from the freezer.

3. Unwrap and load the cartridge into the slide supply.

Note: Load the cartridges within 24 hours after they reach room temperature, 18–28 °C.

STABILITY

VITROS Ca Slides are stable until the expiration date on the carton when they are stored and handled as specified. Not to be used beyond the expiration date.

INTERFERING SUBSTANCES:

- Blood from patients receiving Hypaque radiographic contrast agent cannot be used.
- Suramin, an antiparasitic drug, has been reported to cause a bias of approximately 10% in calcium results at a suramin concentration of 300 µg/mL.

DETECTION LIMIT: 1-14 mg/dl

REFERENCE INTERVAL: 8.4-10.2 mg/dl

QUALITY CONTROL: BIO-RAD internal quality assurance scheme (IQAS) and External quality assurance system (EQAS).

AUTOMATED PARAMETERS

Type of reaction: End point,Wavelength: 650 nm, Incubation time: 5min

LINEARITY:

The method is linear upto a concentration of 16 mg/dl.

REFERENCE VALUES:

Serum: 8.8-10.2 mg/dl

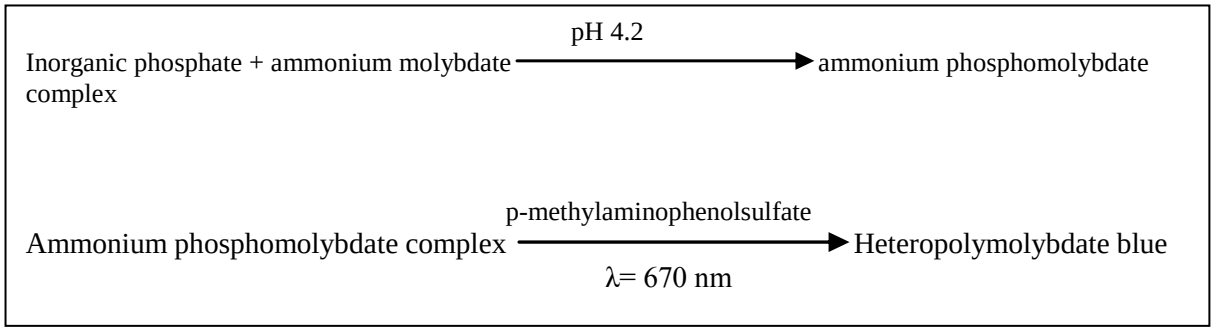
E. SERUM PHOSPHORUS[77]

INSTRUMENT: Vitros 250 dry chemistry auto analyzer based on the principle reflectance photometry

PRINCIPLE:

The VITROS PHOS Slide is a multilayered, analytical element coated on a polyester support. The analysis is based on the reaction of inorganic phosphate with ammonium molybdate to form an ammonium phosphomolybdate complex at acidic pH, similar to Fiske and Subbarow method of inorganic phosphorus estimation.p-Methyl aminophenol sulfate, an organic reductant reported by Gomori, reduces the complex to form a stable heteropolymolybdenum blue chromophore.

A drop of patient sample is deposited on the slide and is evenly distributed by spreading layer to the underlying layers. Phosphorus in the specimen forms a complex with ammonium molybdate. This complex is reduced by p-methyl aminophenol sulfate to give a blue complex. The concentration of phosphorus in the sample is determined by measuring the heteropolymolybdenum blue complex by reflectance spectrophotometry. The absorbance is directly proportional to the phosphorus concentration in the sample.



Incubation time: 5 min, Temperature: 37^0 C (98.6^0 F), Wavelength: 670 nm, Reaction sample volume: 10µL

SAMPLE: Serum

REAGENTS-

Reactive Ingredients per cm^2 of slide

p-methyl aminophenol sulfate 350 µg; and ammonium molybdate 340 µg.

Other Ingredients: Pigment, binders, surfactants, buffer and stabilizers

PROCEDURE -Reflectance from the slide is measured at 670 nm or 680 nm after the fixed incubation time. Once a calibration has been performed, phosphorous concentration

in unknown samples can be determined using the software-resident endpoint colorimetric math model and the response obtained from each unknown test slide.

PRECAUTIONS: The slide cartridge must reach room temperature, 18–28 °C (64–82 °F), before it is unwrapped and loaded into the slide supply.

1. Remove the slide cartridges from storage.

2. Warm the wrapped cartridge at room temperature for 30 minutes when taken from the refrigerator or 60 minutes from the freezer.

3. Unwrap and load the cartridge into the slide supply.

4. Handle and store specimens in stoppered containers to avoid contamination and evaporation.

5. Mix samples by gentle inversion and bring to room temperature, 18–28 °C (64–82 °F), prior to analysis.

Note:

Load the cartridges within 24 hours after they reach room temperature, 18–28 °C.

SAMPLE: Serum

STABILITY:

VITROS PHOS Slides are stable until the expiration date when they are stored and handled properly.

AUTOMATED PARAMETERS

Type of reaction: End point

Wavelength: 670 nm

SAMPLES NOT SUITABLE: Do not use hemolyzed specimens. Hemolysis produces elevated phosphorus values due to the inorganic phosphates and phosphatases present in red blood cells.

INTERFERING SUBSTANCES

Acetaminophen >5 mg/dL, Ethanol >300 mg/dL, Acetylsalicylate >30 mg/dL, Intralipid>800 mg/dL, Ampicillin >5 mmol/L, L-Dopa >2.63 mmol/L, Ascorbic Acid> 4 mg/dL, Beta-carotene> 0.6 mg/dL, Mannitol>638 mg/dL, Bile acid >6 mg/dL, Penicillin >4 U/mL, Bilirubin >40 mg/dL, Phenytoin> 2 mg/dL, Biliverdin>3 mg/dL, Salicylic acid> 35 mg/dL, Chlorothiazole> 3 mg/dL, Total Protein >5 g/dL, Chlorpromazine> 0.15 mmol/L, Total Protein >10 g/dL, Dextran >1000 mg/dL

DETECTION LIMIT: 0.5-13 mg/dl

REFERENCE INTERVAL: 2.5-4.5 mg/dl

QUALITY CONTROL: BIO-RAD internal quality assurance scheme and EQAS external quality assurance system.

REFERENCE VALUES:

Serum: 2.5–4.5 mg/dl

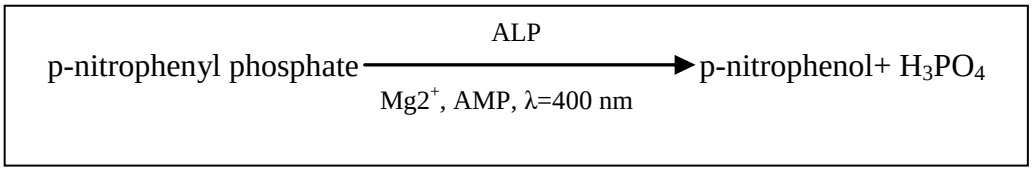
F. SERUM ALKALINE PHOSPHATASE[78]

INSTRUMENT: Vitros 250 dry chemistry auto analyzer based on the principle reflectance photometry.

PRINCIPLE:

VITROS Chemistry Products "ALKP Slides" quantitatively measure alkaline phosphatase (ALKP) activity in serum and plasma. The VITROS ALKP Slide is a multilayered, analytical element coated on a polyester support.

A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The spreading layer contains the p-nitrophenyl phosphate substrate and other components needed for the reaction. The ALP in the sample catalyzes the hydrolysis of the p-nitrophenyl phosphate to p-nitrophenol at alkaline pH. The p-nitrophenol diffuses into the underlying layer, and it is monitored by reflectance spectrophotometry. The rate of change in reflection density is converted to enzyme activity.



Incubation time: 5 min, Temperature: 37^0 C (98.6^0 F), Wavelength: 400 nm, Reaction sample volume: 11µL

SAMPLE: Serum

REAGENTS:

Reactive Ingredients per cm^2 of slide

p-nitrophenyl phosphate 55 µg; 2-amino-2-methyl-1-propanol (AMP) 0.1 mg; and magnesium sulfate 1.6 µg. Other Ingredients -Pigment, binders, buffers, surfactants, cross-linking agent and stabilizer.

PRECAUTION: The slide cartridge must reach room temperature, 18–28 °C

(64–82 °F), before it is unwrapped and loaded into the slide supply.

1. Remove the slide cartridges from storage.

2. Warm the wrapped cartridge at room temperature for 30 minutes when taken from the

refrigerator or 60 minutes from the freezer.

3. Unwrap and load the cartridge into the slide supply.

Note:

Load the cartridges within 24 hours after they reach room temperature, 18–28 °C.

STABILITY:

VITROS ALKP Slides are stable until the expiration date when they are stored and handled as specified.

INTERFERING SUBSTANCES:

Bilirubin >20 mg/dL (342 μmol/L)

Methotrexate> 200 μg/mL (440 μmol/L)

Nitrofurantoin>40 μg/mL (168 μmol/L)

DETECTION LIMIT: 20-1500 U/L

REFERENCE INTERVAL: 38-126 U/L

QUALITY CONTROL: BIO-RAD internal quality assurance scheme and EQAS external quality assurance system.

STATISTICAL ANALYSIS

- The data collected was tabulated and analyzed using descriptive statistical tool.
- Mean and standard deviation was calculated for urinary hydroxyproline, serum calcium, phosphorus, alkaline phosphatase, random blood sugar and HbA1c individually for cases and controls.
- The mean and standard deviation of cases and controls was compared using independent‘t’ test.
- Correlation of urinary hydroxyproline with serum calcium, phosphorus and alkaline phosphatase was done using Pearson’s correlation.
- Correlation of HbA_{1C} with urinary hydroxyproline, serum calcium, phosphorus and alkaline phosphatase was done using Pearson’s correlation.
- SPSS package version 14 was used for all analysis
- ‘p’ value of < 0.05 was taken statistically significant.

Our study included postmenopausal women aged within the range of 50 to 70 years. Group I comprised of 30 Diabetic postmenopausal women and Group II had 30 Non-diabetic postmenopausal women. The average age of postmenopausal women with Diabetes (Group I) was approximately 62 years (61.9 years) and for postmenopausal non diabetics (Group II) it was around 64 years.

In our study the postmenopausal women with and without Diabetes mellitus had significant differences in the RBS values, with a p value <0.001. The mean RBS in Group I was 231.43±113.69 mg/dl, i.e. above the normal range. The lowest RBS value in Group I was 75mg/dl, and the highest value was 483mg/dl. In non diabetics it was 104.7±18.4 mg/dl table (5) and Chart (1). The Glycated hemoglobin also showed significant difference. Chart (2) shows that the Group I had a mean GHb value of 9.54±1.89 %. The Group II had a mean GHb of 5.68±0.82 % which very well lies in the normal range.The GHb shows significant difference between the two groups with a p value of < 0.001. The group I GHb suggests that they were under “fair control ” of their diabetic status.

Chart (3) shows that bone resorption marker, the 24 hour urinary hydroxyproline levels in the diabetics was higher, compared to non diabetic postmenopausal women. The meanlevels of 24 hr urinary excretion of hydroxyproline in Group I women was 37.12±6.5 mg /day and in Group II was 33.55±8.2 mg/day i.e. within the reference range for the age group. However there was no significant difference observed in 24 hour urinary hydroxyproline levels with p value of 0.068.

The mean serum calcium values were 8.34±0.78 mg/dl and 8.58±0.62 mg/dl in Group I and Group II respectively as shown in Chart(4). However, the calcium values in both groups were towards the lower limit of the reference range. The decreased dietary calcium intake or calcium deficiency condition such as post menopausal osteoporosis and their lower socioeconomic status could be the reason for such a finding.

The mean phosphorus values in both groups are within the reference range and the difference is not statistically significant. The Chart (5) shows that the mean values in Group I was 3.75±0.81 mg/dl, and in Group II was, 3.64±0.75 mg/dl.

The bone formation marker alkaline phosphatase was higher in Group I, compared to the Group II as evident in Chart(6). GroupI had mean ALP of 120.16±33.71 U/L and Group II had a mean value of 116.26±35.93U/l, both within reference range. However clearly the levels in group II were less than group I. ALP is independent marker for bone formation. Clearly inspite of the diabetic status of Group I there is no statistical significance in the difference of ALP values.

Within the Group I the Pearson's correlation between urinary hydroxyproline with other parameters are shown in table (6). Since intraparametric correlation was done the value r was considered for statistical analysis. The value r represents the sample correlation coefficient. Its values range from -1 to + 1.The value r = 0, implies there is no correlation between the variables. A value of r = 1, implies that a linear equation describes the relationship between the two variables. As the value of one variable increases, even the other variable value increases. A value of r = −1 implies inverse relation between the variables, as one decreases the other variable value increases.

The formula for calculating r is:

$$r = \frac{\sum_{i=1}^{n}(X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2}\sqrt{\sum_{i=1}^{n}(Y_i - \bar{Y})^2}}$$

Where X and Y are the two variables, n is the number of observations made, and Σ represents the sum of values.

It is evident from the Chart(7), that serum calcium showed a positive correlation with the urinary hydroxyproline values in diabetics, with a r value of 0.369, and a p value of <0.05. This means that as the urinary excretion of hydroxyproline increased, the serum calcium levels also increased. This could be due to the bone resorption causing release of calcium from the bone to the circulation. The Chart (8) shows correlation of hydroxyproline with serum calcium in non diabetics.

The Chart (9) shows phosphorus and hydroxyproline have a r value of 0.065. Chart (10) shows correlation between phosphorus and hydroxyproline in non diabetics.

The r value of ALP was in the negative side (r = - 0.062) as shown in Chart (11), showing negative correlation with urinary hydroxyproline in diabetic group. However the p value is not significant (p =0.746). Similarly Chart (12) depicts the correlation of ALP with urinary hydroxyproline in the non diabetic group.

Correlation of GHb with bone turnover markers in postmenopausal females with diabetes mellitus is shown in Chart(13) GHb Vs serum calcium, Chart (15) showing GHb Vs serum phosphorus, Chart (17) showing GHb Vs ALP and Chart (19) showing GHb Vs hydroxyproline.The p value in all these charts is not significant.

Correlation of GHb with bone turnover markers in postmenopausal women without diabetes mellitus is depicted in chart (14) GHb Vs serum calcium, Chart (16) GHb Vs serum phosphorus, Chart(18) GHb Vs ALP and Chart (20)GHb Vs hydroxyproline. The GHb was correlated in order to see the effects of the severity of diabetes on the various bone turnover parameters. Correlation of urinary hydroxyproline with other bone turnover markers in postmenopausal non diabetic women is clear from charts numbered (8), (10) and (12).

From the Charts and tables it is clear that the RBS and GHb are elevated in the diabetic postmenopausal group, compared to the non diabetic group. The urinary hydroxyproline shows positive correlation with the serum calcium levels in diabetic group. Rest of the biochemical bone parameters do not show significant changes in the diabetics.

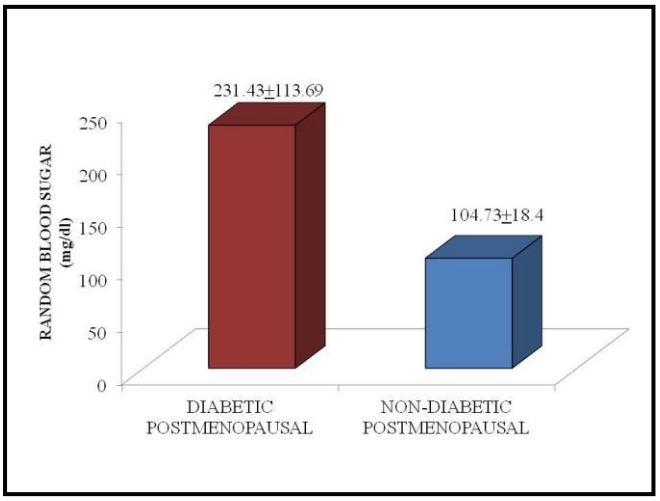
TABLES AND CHARTS

TABLE (5): COMPARISON OF PARAMETERS BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN

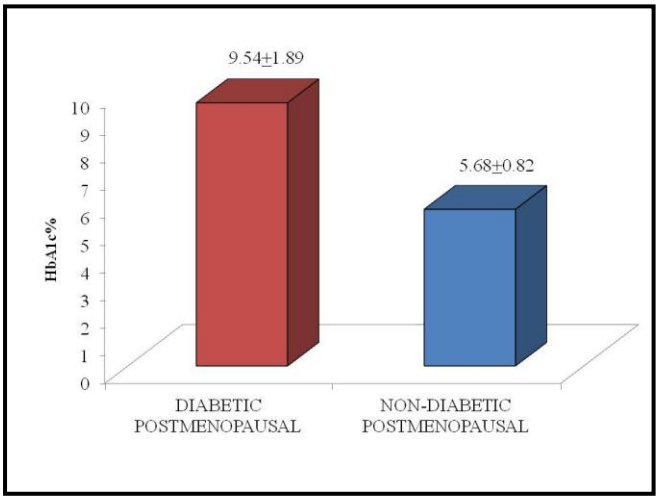
Parameter	**Diabetic postmenopausal women n=30 (group 1) Mean±SD**	**Non-diabetic postmenopausal women n=30 (group 2) Mean±SD**	**'p' value**	**Reference range**
Random blood sugar (mg/dl)	231.43±113.69	104.73±18.4	<0.001**	75-140
HbA1c%	9.54±1.89	5.68±0.82	<0.001**	4-8
Urinary hydroxyproline (mg/day)	37.12±6.52	33.55±8.21	0.068	11.8- 42.5
Calcium (mg/dl)	8.34±0.78	8.58±0.62	0.206	8.8-10.2
Phosphorus (mg/dl)	3.75±0.81	3.64±0.75	0.584	2.5-4.8
Alkaline phosphatase (U/L)	120.16±33.71	116.26±35.93	0.666	100-290

** Statistically significant p values <0.001, *Statistically significant p value <0.05

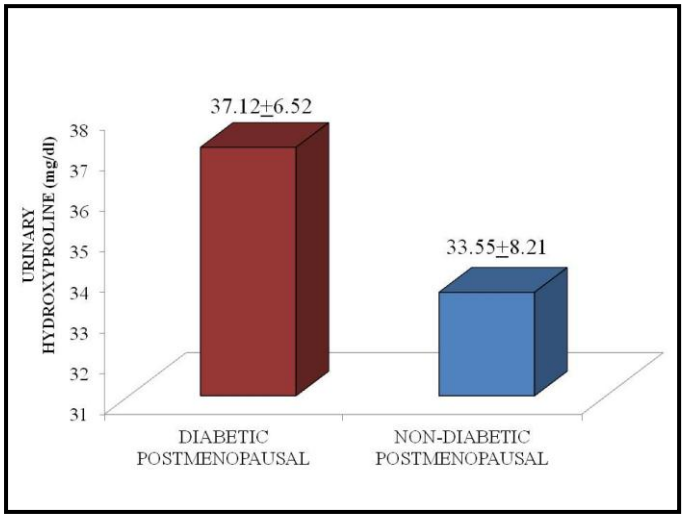
CHART (1): COMPARISON OF RANDOM BLOOD GLUCOSE BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN



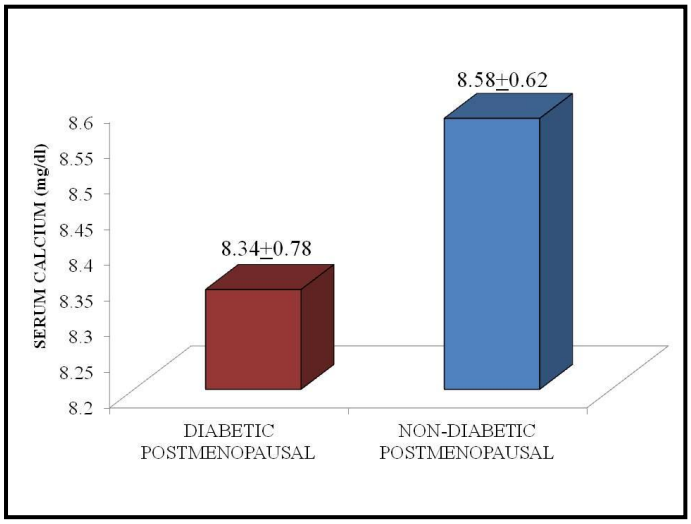
CHART(2): COMPARISON OF HbA_{1C} BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN



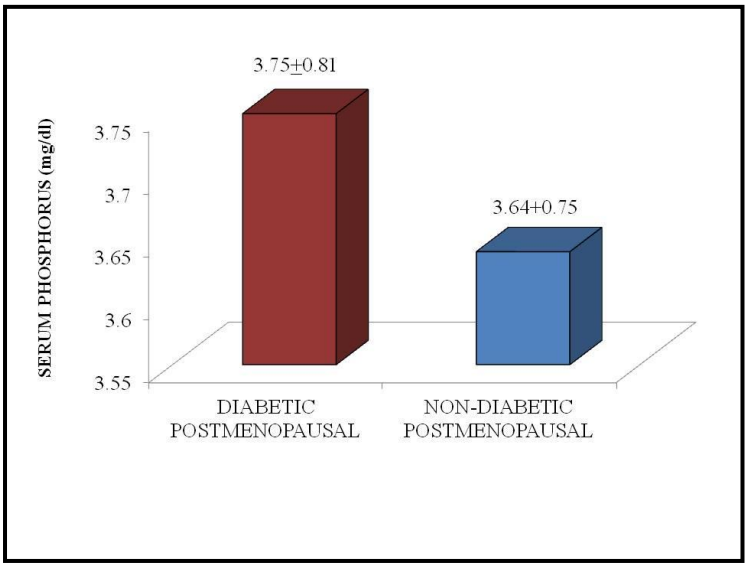
CHART(3):COMPARISON OF URINARY HYDROXYPROLINE BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN



CHART(4): COMPARISON OF SERUM CALCIUM BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN



CHART(5): COMPARISON OF SERUM PHOSPHORUS BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN



CHART(6): COMPARISON OF SERUM ALKALINE PHOSPHATASE BETWEEN DIABETIC AND NON-DIABETIC POSTMENOPAUSAL WOMEN

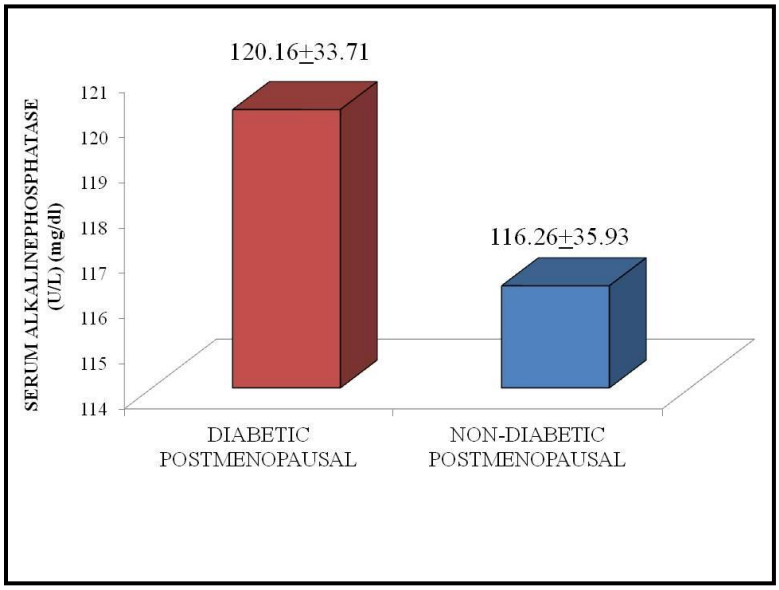


TABLE (6): CORRELATION OF URINARY HYDROXYPROLINE WITH BONE RELATED BIOCHEMICAL MARKERS – CALCIUM, PHOSPHORUS & ALKALINE PHOSPHATASE

Correlated Parameter with Urinary Hydroxyproline	**'r' value**	**'p' value**
Calcium (mg/dl)	0.369	<0.05
Phosphorus (mg/dl)	0.256	0.172
Alkaline phosphatase (U/L)	-0.062	0.746

CORRELATION OF URINARY HYDROXYPROLINE WITH OTHER BONE TURNOVER MARKERS IN POSTMENOPAUSAL DIABETIC AND NON DIABETIC WOMEN

CHART (7): PEARSON'S CORRELATION OF URINARY HYDROXYPROLINE WITH SERUM CALCIUM IN POSTMENOPAUSAL DIABETIC WOMEN SHOWING SIGNIFICANCE WITH $p < 0.05$

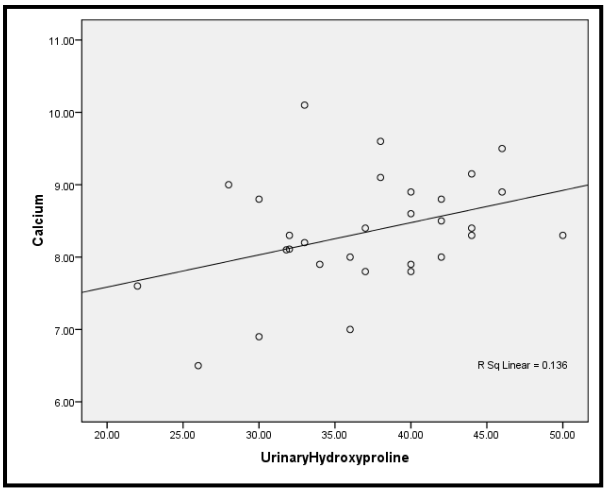


CHART (8): PEARSON'S CORRELATION OF HYDROXYPROLINE WITH CALCIUM IN POSTMENOPAUSAL NON DIABETIC WOMEN

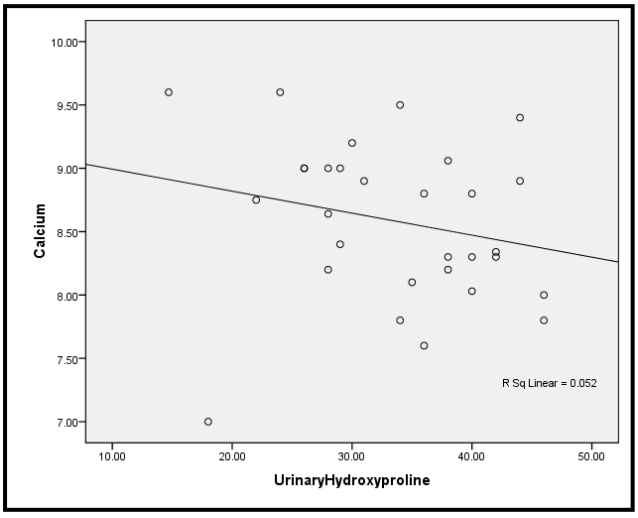


CHART (9):PEARSON'S CORRELATION OF URINARY HYDROXYPROLINE WITH SERUM PHOSPHORUS IN POSTMENOPAUSAL DIABETIC WOMEN

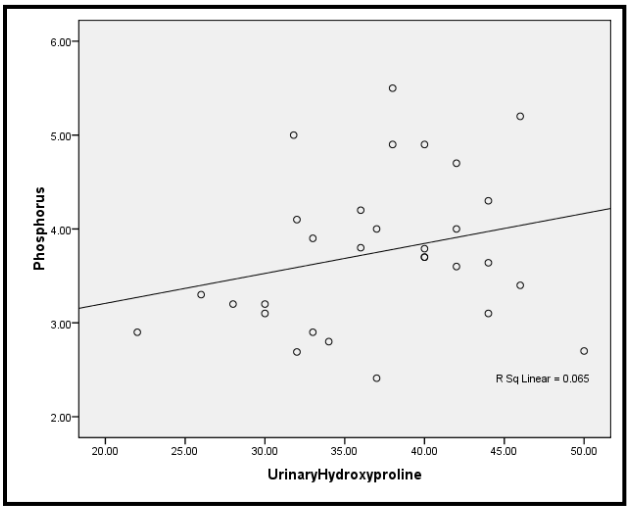


CHART (10): PEARSON'S CORRELATION OF HYDROXYPROLINE WITH PHOSPHORUS IN POSTMENOPAUSAL NON DIABETIC WOMEN

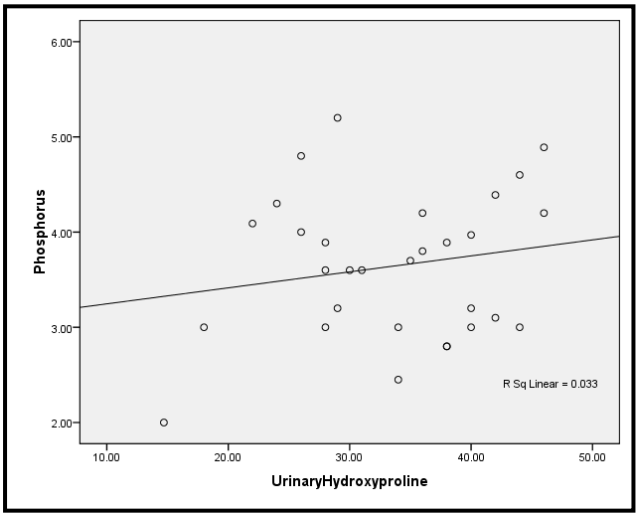


CHART (11):PEARSON'S CORRELATION OF URINARY HYDROXYPROLINE WITH SERUM ALKALINE PHOSPHATASE IN POSTMENOPAUSAL DIABETIC WOMEN

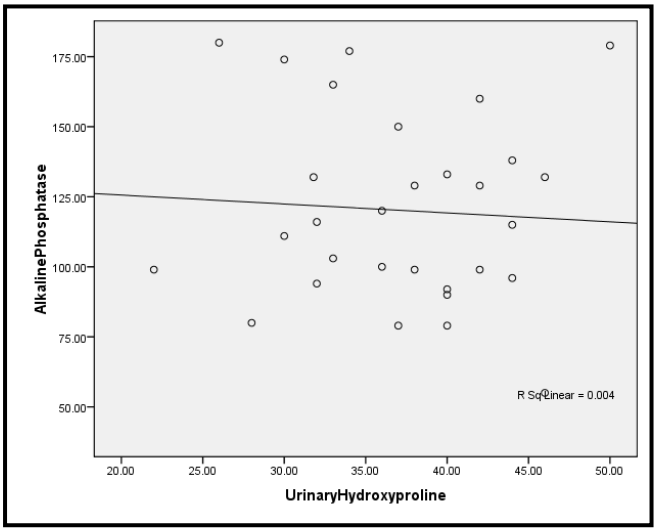
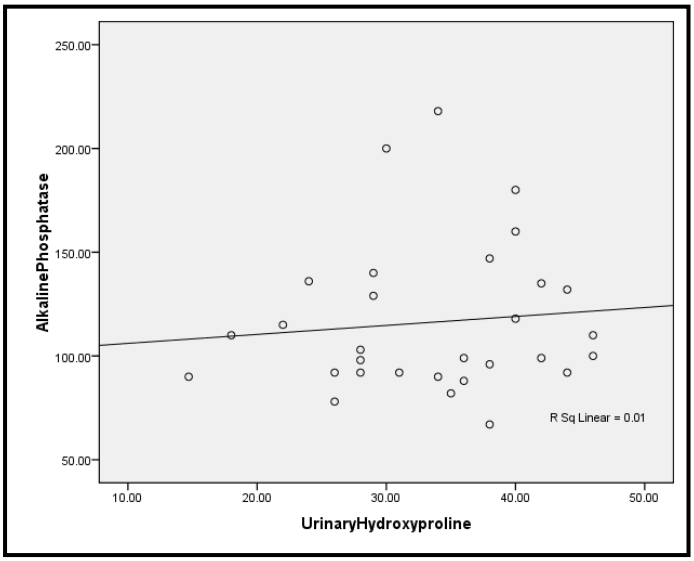


CHART (12): PEARSONS CORRELATION OF URINARY HYDROXYPROLINE WITH ALP IN POSTMENOPAUSAL NON DIABETIC WOMEN



CORRELATION OF GHb WITH BONE TURNOVER MARKERS IN POSTMENOPAUSAL DIABETIC AND NON DIABETIC GROUPS

CHART (13): PEARSON'S CORRELATION OF GHb WITH SERUM CALCIUM IN POSTMENOPAUSAL DIABETIC WOMEN

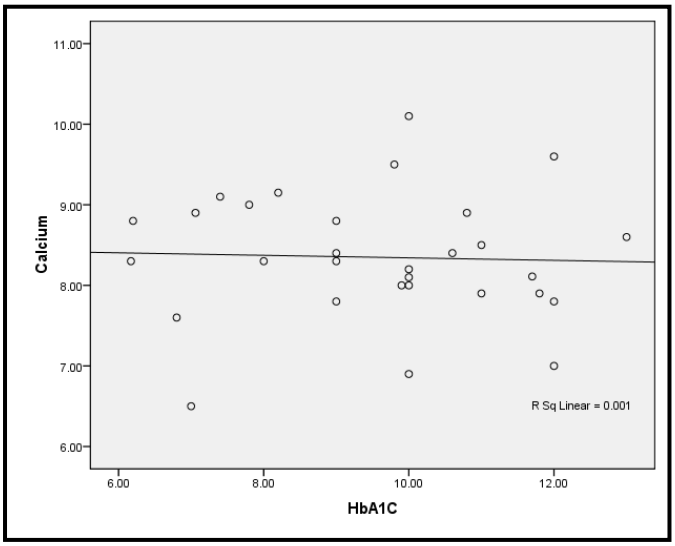


CHART (14): PEARSON'S CORRELATION OF GHb WITH CALCIUM IN POSTMENOPAUSAL NON DIABETIC WOMEN

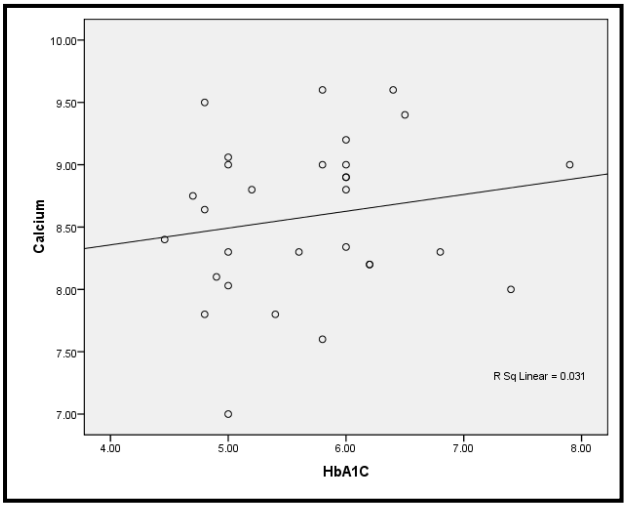


CHART (15): PEARSON'S CORRELATION OF GHb WITH SERUM PHOSPHORUS IN POSTMENOPAUSAL DIABETIC WOMEN

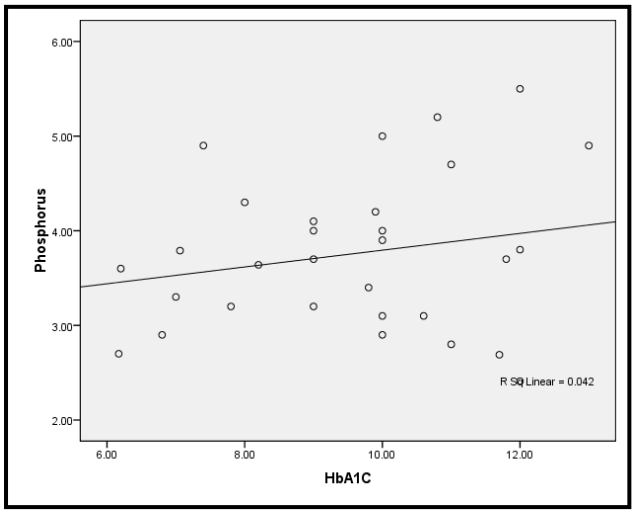


CHART (16): PEARSON'S CORRELATION OF GHb WITH PHOSPHORUS IN POSTMENOPAUSAL NON DIABETIC WOMEN

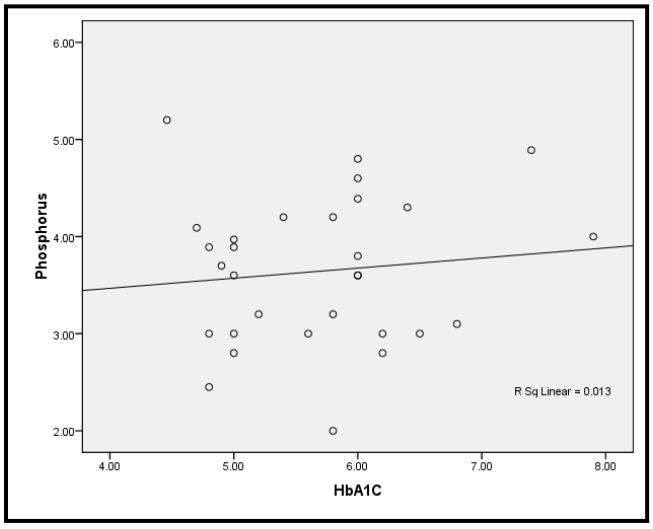


CHART (17): PEARSON'S CORRELATION OF GHb WITH SERUM ALKALINE PHOSPHATASE IN POSTMENOPAUSAL DIABETIC WOMEN

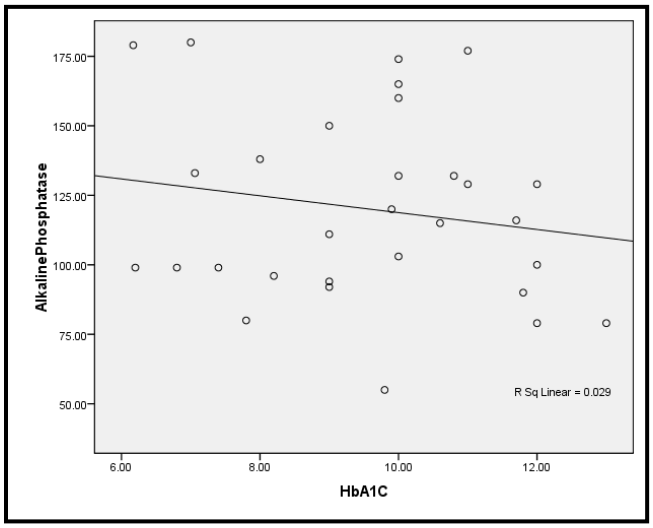


CHART (18): PEARSON'S CORRELATION OF GHb WITH ALP IN POSTMENOPAUSAL NON DIABETIC WOMEN

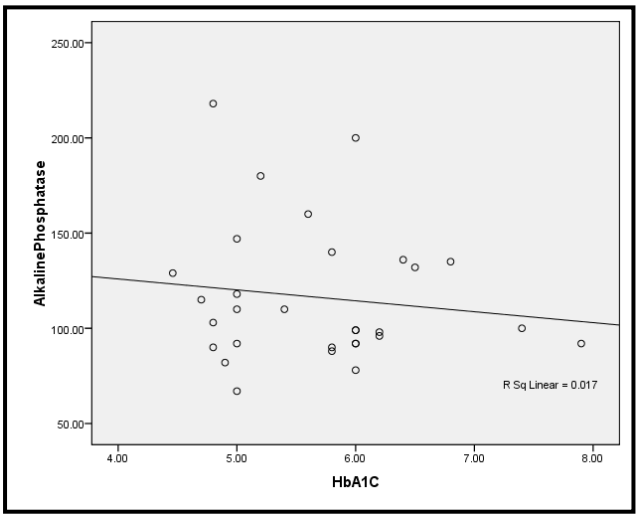


CHART (19): PEARSON'S CORRELATION OF HbA_{1C} WITH URINARY HYDROXYPROLINE IN POSTMENOPAUSAL DIABETIC WOMEN

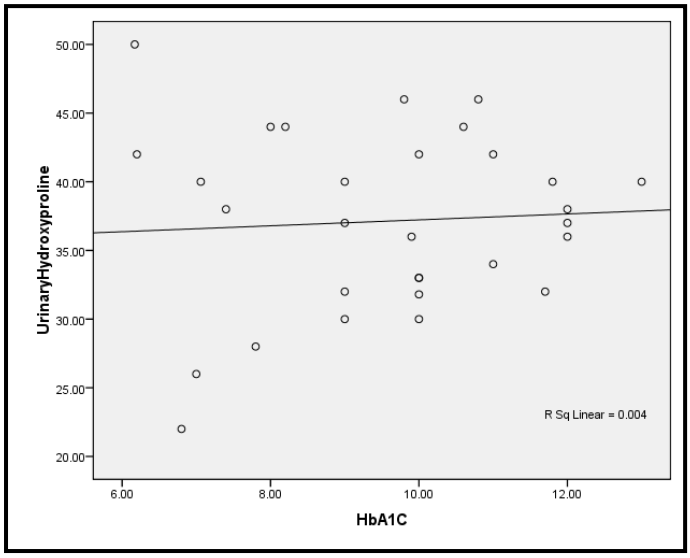
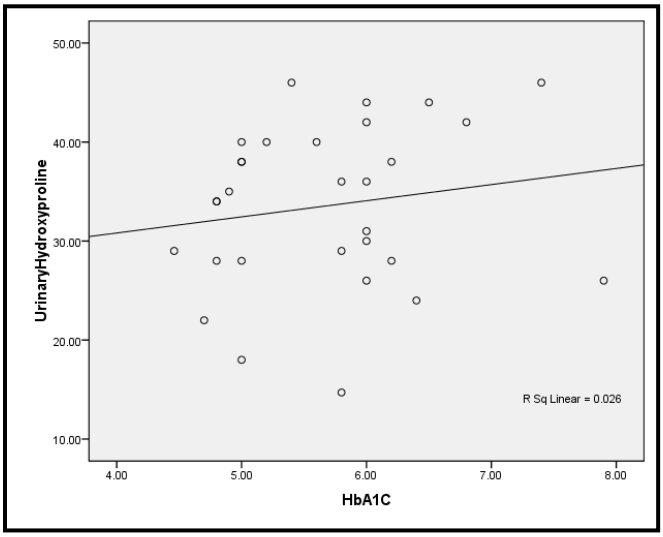


CHART (20): PEARSON'S CORRELATION OF GHb WITH HYDROXYPROLINE IN POSTMENOPAUSAL NON DIABETIC WOMEN



Osteoporosis, a dreaded complication of menopause is characterized by low bone mass and increased susceptibility to fractures. Until a fracture occurs, the disease usually remains silent. Osteoporosis commonly affects older women, particularly postmenopausal women. This bone disease leads to significant morbidity and mortality as it leads to pain, deformity and disability.

Like osteoporosis, even type 2 diabetes mellitus is highly prevalent in post menopausal women. With ongoing scientific advances in medical sciences and treatment modalities, the demographic trend worldwide is towards longer life expectancy. Thus, more number of women are entering the menopausal age group. Modern amenities have changed the lifestyle pattern nowadays. With lesser physical activity and more consumption of high energy foods, there is an increase in incidence of Diabetes and Osteoporosis.

Studies in patients with Diabetes have revealed an increased fracture risk.[9,11]Thus the older population, especially the diabetics, should be routinely screened for risk factors of developing osteoporosis as a part of their disease management. There have been conflicting reports with regard to the bony changes in Type 2 diabetes mellitus. Studies conducted by Oz SG, Van Daele PL and Akin O et al respectively have found diabetics to have elevated bone mineral density.[11,13,14.] In contrast studies done by Kwon DJ et al show reduced BMD in diabetic women.[12] Thus for better assessment of risk of fractures, parameters other than BMD, like biochemical bone markers should also be included.

The effects of type 2 diabetes mellitus on bone metabolism are less clear. Very few studies have assessed the effect of diabetes on bone, particularly in postmenopausal

women. Our study sought to assess the levels of bone metabolism markers in postmenopausal diabetic women, and assess if they were at a higher risk of developing osteoporosis.

The postmenopausal women included in Group I in our study had Diabetes mellitus for an average of around 6years. Significant difference was found between the two groups when RBS and glycated hemoglobin were considered with p values < 0.001. The Group I had a mean GHb value of 9.54±1.89 % whereas Group II had mean GHb of 5.68±0.82 %.These findings are consistent with the findings of Sosa M et al and Chen HL et al.[79,80.] Chen HL et al found GHb, and fasting blood glucose to be higher in the diabetic group with p value <0.05. In our study the diabetic postmenopausal women had their diabetic status under fair control according to the GHb reference range (9.0-10.0%). Group I subjects were clinically diagnosed to be type 2 Diabetics, and were on oral hypoglycemic drugs or insulin therapy.

In our study Group I had higher bone resorption marker levels than Group II, however this difference was not statistically significant (p=0.068 for urinary hydroxyproline). Similar results for urinary hydroxyproline were found in studies done by Sosa M et al and Chen HL et al with p value > 0.05. These findings suggest that the diabetic state doesn't alter the resorption rate in bone.[79, 80]

No significant difference was found between the two groups when other bone markers such as serum alkaline phosphatase, serum calcium and serum phosphorus were considered. This finding correlated with studies done by Sosa M et al. Their study also included postmenopausal diabetic and non diabetic women in the age group

58.8± 8.5 years in control group and 61.3± 7 years in NIDDM group. They had assessed the bony changes by biochemical and radiological parameters. Bone turnover markers included formation markers such as Osteocalcin and ALP. Bone resorption markers included urinary hydroxyproline and TRAP. But they could not find any difference in bone mass between two groups when assessed by highly popular methods of BMD estimation like DEXA and CT scan. They measured the BMD at the lumbar spine and hip, and concluded that the bone mass was normal in non insulin dependent diabetes mellitus patients.[79]

With respect to the serum calcium, the mean values we observed were found to be lesser in diabetics (8.34±0.78 mg/dl) than non diabetics (8.58±0.62 mg/dl) but with a non significant p value of 0.206. Studies done by Chen HL et al also observed similar findings. They found significant decrease in the serum calcium levels in type 2 diabetics. However their study was done in males of ≥60 years of age with type 2 diabetes.[80]Studies by Verma M et al in type 2 DM postmenopausal women and postmenopausal women without diabetes as controls, a significant decrease ($p < 0.001$) in the serum calcium and phosphorus level was observed. They suggested that poorly controlled type 2 DM patients have relative hypercalciuria probably caused by osmotic diuresis associated with glycosuria thereby contributing to lowered serum calcium levels, as found in our study.[40]

Studies conducted by Sosa M et al and Hamad NA et al have shown unaltered calcium levels in diabetics compared to non diabetics.But Hamad et al have suggested that the relative availability of dietary calcium sources (milk, dairy products), even for those with limited income could compensate for any deficiency associated with diabetes.[79,81]

The mean phosphorus levels observed in Group I of our study were marginally higher than Group II with a p value of 0.584. Studies done in Brazil by Cutrim DMSL et al found similar non significant elevation in mean serum phosphorus values.[82]In their study the poorly controlled diabetic group had higher phosphorus values. They found no significant difference in urinary deoxypyridine (resorption marker) between the diabetic and non diabetic groups. Even in our study the difference in resorption marker hydroxyproline was not significant between the two groups. This showed that hyperglycemia is probably not associated with increased bone resorption in type 2 diabetes mellitus and that BMD is not altered in type 2 diabetes.Patients with type 2 DM had normal IGF1 levels even in the presence of poor chronic control. IGF1 is an important stimulator for osteoblasts.They also found that subjects with poorly controlled type 2 DM presented with significantly lower osteocalcin levels than the non diabetic group. Type 2 DM is associated with obesity, hyperandrogenism, elevation of estrogen levels, normal IGF1, and frequently hyperinsulinemia, which are factors causing increase in BMD. However they did not find any significant change in either the bone mass or the bone remodeling parameters like PTH and vitamin D in the two groups. Thus they suggested that hyperglycemia does not seem to be a mechanism involved in the induction of osteopenic activity.[82]

Cakatay et al and Hamad et al found similar alkaline phosphatase levels between type 2 DM patients and controls.[83,81]These findings tally with our study where ALP values are not significantly different between the groups. According to Hamad et al the multiple sources of ALP in the body could be the cause of such a finding.[81]

Studies by Cakatay et al showed no statistically significant difference in the serum calcium and phosphorus levels was found as well. In spite of the decrease in the Osteoclacin levels in the diabetic group no difference was observed in deoxypyridine levels. It was postulated that this could be due to Diabetes affecting only the formation phase, while the resorption phase remaining unaltered.[83]

Studies by Maghbooli et al showed no significant difference in the prevalence of osteopenia and osteoporosis between Type 2 diabetic women and non-diabetic subjects.[84]According to them the prevalence of diffused osteoarthritis at the vertebral site in diabetic women is higher than non-diabetic women and this can lead to falsely elevated BMD at that site.[84]This could possibly explain the elevated BMD found in Diabetic women when compared to non-diabetics as observed by Oz SG, Van Daele PL and Akin O et al found in some studies done previously.[11,13,14]

Our findings are on par with a cross sectional study by Qorbani M et al, where they did not observe a correlation between plasma glucose levels and the biochemical parameters of bone formation and resorption, thus showing no association between DM and BMD. They did a large scale cross sectional study with 300 subjects, assessing their diabetic, thyroid and bone status. Using DEXA scans they concluded that there was no significant correlation between the diabetic status and bone loss.[85]

Most diabetics involved in our study were under almost full or at least partial control of their diabetic status due to oral hypoglycemic drugs and insulin therapy.Thus the effect of chronic hyperglycemia could have got relatively canceled.

Considering the conflicting findings further studies need to be done in larger population groups and with the help of more specific markers to draw a line in conclusion about any association between type 2 Diabetes and bone turnover.

- Urinary hydroxyproline in diabetic postmenopausal women was higher when compared to non diabetic group but in reference range. Its value correlated positively with serum calcium levels, showing that as bone resorption occurred, the calcium from bone got released, thereby increasing serum calcium levels. Hydroxyproline being a collagen degradation product can be used as an inexpensive and reliable tool for assessing bone loss.

- Serum calcium was found to be towards the lower side of reference range in both groups indicating a need for supplementation of calcium in women of this age group.

- The study was inconclusive to comment on effect of diabetes on bone, and further studies need to be conducted.

- A possible factor contributing to these results is that all patients involved in this study were under partial control so that the effect of chronic hyperglycemia is relatively cancelled.

Our study is an observational comparative study done in RLJH & RC, Tamaka, Kolar to estimate the levels of bone related biochemical markers urinary hydroxyproline, serum calcium, phosphorus, alkaline phosphatase in postmenopausal women with and without diabetes mellitus. The individual measurement of single biochemical marker of bone turnover has limited utility. Thus, a combination of bone related biochemical markers were used.

Urinary hydroxyproline was elevated in postmenopausal women with diabetes mellitus when compared to non-diabetics. Serum calcium was decreased in postmenopausal women with diabetes mellitus when compared to non-diabetics. Serum phosphorus and alkaline phosphatase were elevated in postmenopausal women with diabetes mellitus. However, these bone related biochemical markers were within the reference intervals.

Estrogen in women contributes to glucose homeostasis by making body cells sensitive to insulin. In postmenopausal diabetic women estrogen secretion is either absent or inadequate which alters the glucose milieu resulting in elevated random blood sugar.

Hyperglycemia affects the bone turnover regulation probably by changing the local microenvironment of cell-cell, cell-matrix interactions, cytokine production and as well as systemic hormones.

Early assessment of these parameters may help in decreasing the osteoporotic fractures as their levels may serve as simple marker to identify patients with increased bone resorption. They may also help in predicting the outcome and in effective management of these patients. DEXA and CT scan may not be available everywhere,

whereas biochemical parameters can be estimated at any basic biochemistry lab and are cheaper. Hence I conclude my study to state that biochemical parameters are better, but if available DEXA and CT have to be used as an adjunct to assess the osteoporosis in postmenopausal women with and without diabetes mellitus.

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ANNEXURE 1

TITLE OF THE STUDY: A COMPARATIVE STUDY OF BONE RELATED BIOCHEMICAL MARKERS IN POSTMENOPAUSAL WOMEN, WITH AND WITHOUT DIABETES MELLITUS

CASE HISTORY OF THE PATIENTS

Case No:

Name: Mrs. OP No:

Age: IP No:

Ward:

Date: Occupation:

Weight:

Address:

CHIEF COMPLAINTS:

HISTORY OF PRESENTING ILLNESS:

PAST HISTORY:

Hypertension : yes/no if yes , duration:

Diabetes : yes/no if yes , duration:

Tuberculosis : yes/no if yes , duration:

Heart diseases : yes/no if yes , duration:

Liver diseases : yes/no if yes , duration:

Drug ingestion : yes/no if yes , duration & details:

Others :

Thyroid disorders:

Cancer:

FAMILY HISTORY:

Diabetes : yes/no if yes , duration:

Hypertension : yes/no if yes , duration:

Osteoporosis : yes/no if yes , duration:

PERSONAL HISTORY:

Economic status : Below poverty level/Above poverty level

Diet: Vegetarian / mixed/non-vegetarian

Tobacco consumption : yes/no if yes, duration:

Alcohol : yes/no if yes , duration:

MENSTRUAL HISTORY :

Duration of menopause:

Type of menopause: Surgical/Non-surgical

GENERAL PHYSICAL EXAMINATION:

Ht: Wt: BMI:

Built : normal / below normal / well built / obese/athletic

Nourishment : well / poor nourished

Oedema : Icterus :

Pallor : Clubbing :

Cyanosis : Lymphadenopathy :

Blood pressure : Pulse rate :

SYSTEMIC EXAMINATION :

CVS :

RS :

CNS :

PER ABDOMEN :

CLINICAL DIAGNOSIS :

INVESTIGATIONS :

BLOOD :

RANDOM BLOOD GLUCOSE: mg/dl

WHOLE HbA1c: %

SERUM CALCIUM: mg/dl

SERUM PHOSPHORUS: mg/dl

SERUM ALKALINE PHOSPHATASE: mg/dl

HEMOGLOBIN : g/dl

URINE:

URINARY HYDROXYPROLINE:

OTHERS:

ANNEXURE (2)

Patient details:

Name: **Age:** **Hospital No.**

TITLE OF THE STUDY: A COMPARATIVE STUDY OF BONE RELATED BIOCHEMICAL MARKERS IN POSTMENOPAUSAL WOMEN, WITH AND WITHOUT DIABETES MELLITUS

INFORMED CONSENT

I understand that I remain free to withdraw from this study at any time.

I have read or had read to me and understand the purpose of this study and the confidential nature of the information that will be collected and disclosed during the study.

I have had the opportunity to ask my questions regarding the various aspects of this study and my questions have been answered to my satisfaction.

I, the undersigned agree to participate in this study and authorize the collection and disclosure of my personal information as outlined in this consent form.

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Participant's Name & signature Date

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Signature of the witness Date

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Signature of the principal investigator Date

Master Chart – Postmenopausal Diabetic Women (Group I)

SI No.	AGE	BMI	DURATION	INSULIN	RBS	GHb	Ca^{2+}	P	ALP	Hydroxyproline
1	65	20	4	Y	132	8.2	9.15	3.64	96	44
2	65	25	10	O	348	10.8	8.9	5.2	132	46
3	65	21	6	O	114	7.4	9.1	4.9	99	38
4	60	27	8	O	208	10	10.1	2.9	165	33
5	55	30	3	Y	109	9	8.8	3.2	111	30
6	65	25	4	Y	395	10.6	8.4	3.1	115	44
7	56	28	10	Y	295	11	8.5	4.7	129	42
8	60	22	6	Y	307	10	8.1	5	132	31.8
9	65	20	5	O	268	11.7	8.11	2.69	116	32
10	70	28	8	Y	230	9.8	9.5	3.4	55	46
11	65	33	10	O	432	11.8	7.9	3.7	90	40
12	70	29	7	Y	483	12	7.8	2.41	79	37
13	60	24	4	O	124	6.17	8.3	2.7	179	50
14	65	22	6	O	130	7.8	9	3.2	80	28
15	70	21	4	Y	200	9	7.8	3.7	92	40
16	73	27	2	O	380	9.9	8	4.2	120	36
17	65	32	10	O	110	6.8	7.6	2.9	99	22
18	70	25	3	Y	108	7	6.5	3.3	180	26
19	60	28	8	Y	280	10	6.9	3.1	174	30
20	65	24	4	Y	141	9	8.3	4.1	94	32
21	55	28	6	O	220	12	9.6	5.5	129	38
22	70	25	8	Y	330	13	8.6	4.9	79	40
23	68	26	7	O	75	10	8.2	3.9	103	33
24	70	22	6	O	100	9	8.4	4	150	37
25	52	21	10	Y	240	11	7.9	2.8	177	34
26	65	20	5	Y	301	6.2	8.8	3.6	99	42
27	55	23	2	Y	77	8	8.3	4.3	138	44
28	65	25	1	O	346	7.06	8.9	3.79	133	40
29	50	28	4	Y	260	10	8	4	160	42
30	70	24	8	Y	200	12	7	3.8	100	36

Master Chart – Postmenopausal Non-diabetic Women (Group II)

SI No.	AGE	BMI	RBS	GHb	Ca^{2+}	P	ALP	Hydroxyproline
1	65	22	110	5.8	9.6	2	90	14.7
2	76	28	92	5	9	3.6	92	28
3	65	26	118	6	8.9	3.6	92	31
4	65	19	93	4.8	9.5	2.45	218	34
5	52	26	123	5.2	8.8	3.2	180	40
6	60	29	100	5	7	3	110	18
7	55	20	152	6.2	8.2	2.8	96	38
8	66	18	91	6	9.2	3.6	200	30
9	63	22	151	7.9	9	4	92	26
10	65	19	69	4.9	8.1	3.7	82	35
11	50	19	120	5	8.3	2.8	147	38
12	85	20	98	5.6	8.3	3	160	40
13	75	21	90	5.8	9	3.2	140	29
14	65	24	98	6	8.9	4.6	92	44
15	60	20	86	4.8	7.8	3	90	34
16	70	22	110	6	8.34	4.39	99	42
17	66	18	114	5	9.06	3.89	67	38
18	65	24	106	5	8.03	3.97	118	40
19	65	19	85	4.7	8.75	4.09	115	22
20	68	20	101	4.8	8.64	3.89	103	28
21	58	25	126	6	8.8	3.8	99	36
22	65	22	119	6.8	8.3	3.1	135	42
23	55	26	88	4.46	8.4	5.2	129	29
24	55	20	112	6.4	9.6	4.3	136	24
25	58	25	102	6.5	9.4	3	132	44
26	70	23	98	5.4	7.8	4.2	110	46
27	65	22	112	6.2	8.2	3	98	28
28	58	30	100	7.4	8	4.89	100	46
29	70	26	82	5.8	7.6	4.2	88	36
30	70	24	96	6	9	4.8	78	26