TO OBSERVE THE RATE OF BONE LOSS IN ACUTE SPINAL CORD INJURY PATIENTS BY MEANS OF BONE BIOMARKERS

Dissertation submitted to the Tamil Nadu Dr M.G.R Medical University, Chennai, Tamil Nadu, in partial fulfilment of the requirements for the MD branch XIX (Physical Medicine and Rehabilitation) University Examinations in April 2016

CERTIFICATE

This is to certify that the thesis tilted **"To observe the change in serum bone biomarkers over a period of six months among patients with acute spinal cord injury"** is the bone fide work of **Dr Prince Thakkar**, candidate number **20132905** in fulfilment of the requirement of the Tamil Nadu Dr M.G.R Medical University, Chennai, Tamil Nadu for the MD branch XIX (Physical Medicine and Rehabilitation) University Examinations in April, 2015.

Dr Alfred Job Daniel
Principal
Christian Medical College
Vellore

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Guide:
Dr George Tharion
Professor and Head
Department of Physical Medicine and Rehabilitation
Christian Medical College Vellore

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Co Guide:
Dr Thomas Paul V
Professor
Department of Endocrinology
Christian Medical College
Vellore

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AIM: • To observe the change in serum bone biomarkers over a period of six months among

patients with acute spinal cord injury. 66

OBJECTIVES ? Compare the bone biomarker levels of

acute spinal cord injury patients with 66

age matched premenopausal female and post menopausal female group in the community, from another study at Endocrinology department. ? To observe the effect of Vitamin D on the bone biomarkers. INTRODUCTION Spinal cord injury (SCI) is a partial or total disruption of the structural or functional integrity of the spinal cord following non-traumatic or traumatic cause leading to temporary or permanent impairment of motor, sensory and/or autonomic functions. The spinal cord transmits and modulates the

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Dr Prince Thakkar
Vellore

CONTENTS

AIM

- To observe the change in serum bone biomarkers over a period of six months among patients with acute spinal cord injury.

OBJECTIVES

- Compare the bone biomarker levels of acute spinal cord injury patients with age matched control group of premenopausal female and vulnerable group of post menopausal female in the community.

- To observe the effect of Vitamin D on the bone biomarkers.

INTRODUCTION

Spinal cord injury (SCI) is a partial or total disruption of the structural or functional integrity of the spinal cord following non-traumatic or traumatic cause leading to temporary or permanent impairment of motor, sensory and/or autonomic functions. The spinal cord transmits and modulates the neural signals between the brain and the target organs in the body, also contains neural circuits that can independently control numerous reflexes. The disruption of these neurohumoral functions following spinal cord injury results in various acute and chronic secondary complications including osteoporosis. Sublesional skeletal tissue, i.e. below the neurological level, undergo significant metabolic changes due to mechanical unloading, loss of neurogenic control over blood vessels and hormonal changes following spinal cord injury.(1) The changes in bone metabolism have been shown to start within weeks of injury, in the form of bone loss which manifest later as osteoporosis, a state of reduced bone strength .(2) Osteoporosis seems to be an inevitable sequel in all complete spinal cord injury patients, which predisposes them to low trauma fractures. Often the diagnosis of osteoporosis is delayed in SCI patients and treated when present with pathological fractures. In view of the high morbidity and medical expenses following pathological fractures, there has been paradigm shift in management of osteoporosis. Currently the focus is to prevent bone loss in the acute phase and treat established low bone mass of chronic spinal cord injury.(3)(4) Literature reports that about 25% of bone loss takes place within first twelve months of spinal cord injury and thereafter it plateaus within next few years.(5)(6) Thus prevention of excess bone loss in the acute phase will

prevent osteoporosis and reduce chances of fracture. Bone biomarkers are the newer modalities which inform us about the metabolic changes related to remodelling in bone.(7) These are chosen over DEXA scan as they give us an idea about the dynamic changes in the bone over a period of time and throw light on the pathology of bone loss. This study observed the change in percentage of the bone biomarkers with respect to normal range and over a period of six months post spinal cord injury, which helped in understanding the nature and extent of bone loss in paraplegics during the acute phase of injury.

JUSTIFICATION OF THE STUDY

Incidence of sublesional fractures is high in Spinal cord injury patients. Recent prospective longitudinal study suggested that about 25 % SCI patients have fractures and very few had received prophylactic treatment.(8) Sublesional fractures increase the mortality and morbidity, especially the in old veterans who are at highest risk.(4) Thus it becomes essential to prevent fractures which affect the rehabilitation and quality of life in SCI patients. Fractures can be prevented by limiting bone loss during the acute phase. Osteoporosis and Osteopenia are inevitable complications following Spinal Cord injury. Independence in mobility puts them at a risk of low trauma fracture with fragile /weak bones; hence it becomes essential to have bone strength sufficient for weight bearing. Literature review suggests limited use of antiresorptive agents even though the incidence of low trauma fracture is high. This could be attributed to lack of use of objective measures to suggest significant loss in acute phase. Only patients with established osteoporosis or history of fractures are treated. Natural history of osteoporosis in Spinal Cord injury reveals 25 % bone loss occurring within a year of spinal cord injury.(2) Hence it is essential to prevent bone loss in early phase, rather than treating established loss in chronic phase. Routinely done Dexa Scan (2 SD) for osteoporosis will not be able to detect this early loss as changes in bone mineral density (BMD) are small over the years and reliable repeated BMD measurements are possible only after 1-2 years. (10) Bone biomarkers can detect change as early as 3-4 months, which help in better assessment and evaluation of response to therapy.(11) Thus there is a need to detect early bone loss and initiate

therapeutic agents at the appropriate time. This study looks at the level of bone biomarkers serially over a period of 6 months to detect early bone loss. The change in bone biomarkers to suggest significant bone loss (that is 30 % bone loss over 6 months) and the timing of peak bone loss will throw light on the point of initiation of treatment and selection of pharmacological agents for prevention of bone loss in the acute stage. Least significant change as assessed in previous studies has been used here for assessing percentage change in bone biomarkers over a period of six months.(12)

LITERATURE REVIEW

BONE AS A DYNAMIC ORGAN

Bone is the structural framework of the body, characterized by its rigidity and hardness. It has highly organised living material having a hierarchical structure and specialized metabolic functions, including the power of repair and regeneration.(Fig 1) It constantly undergoes modeling (reshaping) during life to help it adapt to changing biomechanical forces which determine its structure. Throughout life it continues remodeling, i.e. removal of old micro-damaged bone which in turn is replaced with new and mechanically stronger micro structure to preserve the bone strength.(13)

BONE STRUCTURE

The bones are covered by membrane outside and it has two components enclosed inside, these are called cortical and trabecular bones.

BONE MEMBRANES: PERIOSTEUM AND ENDOSTEUM

The periosteum is a double layered protective membrane, comprising of an outer dense fibrous connective tissue layer and inner osteogenic layers comprising of bone cells. The inner layer is richly supplied by nerve fibres, blood vessels and lymphatics which enter the bone via nutrient foramina. Sharpeys' fibers are the thick collagenous fibrous strands which tightly bind the periosteum and outer cortical surface together. The endosteum cover the inner surface of cortical and trabecular bone, Volkmann's canal. It is in contact with the bone marrow space and contains blood vessels, osteoblasts, and osteoclasts.

CORTICAL / COMPACT BONE

Cortical bone is dense and solid, surrounding the marrow space and composed of units called osteons, also called as Haversian systems. Haversian systems are cylindrical in shape, and form a branching network within the cortical bone. Concentric layers of lamellar bone form the walls of harvesian canals.

Cortical bone has an outer periosteal surface and inner endosteal surface. The endosteal surface has higher remodeling activity than the periosteal surface, most likely due to greater biomechanical strain or greater inflammatory exposure from the adjacent bone marrow. Bones normally increase in diameter with aging as bone formation exceeds bone resorption on the periosteal surface.

CANCELLOUS / TRABECULAR / SPONGY BONE

Trabecular bone is a honeycomb like network of plates and rods interspersed in the bone marrow compartment. Trabecular osteons are also called packets. The plates and rods on an average have 50 to 400 mm of thickness. Trabecular osteons are semilunar in shape, about 35 mm thick, and composed of concentric lamellae.

Cortical and trabecular bone both are normally formed in a lamellar pattern, in which collagen fibrils are laid down in an alternating fashion.

Woven bone

Woven bone is normally produced during primary bone formation. It is characterised by random arrangement of cells and connective tissue, seen in early callus formation. It is seen in high bone turnover states such as osteitis fibrosa cystica, and other hyperparathyroid state.

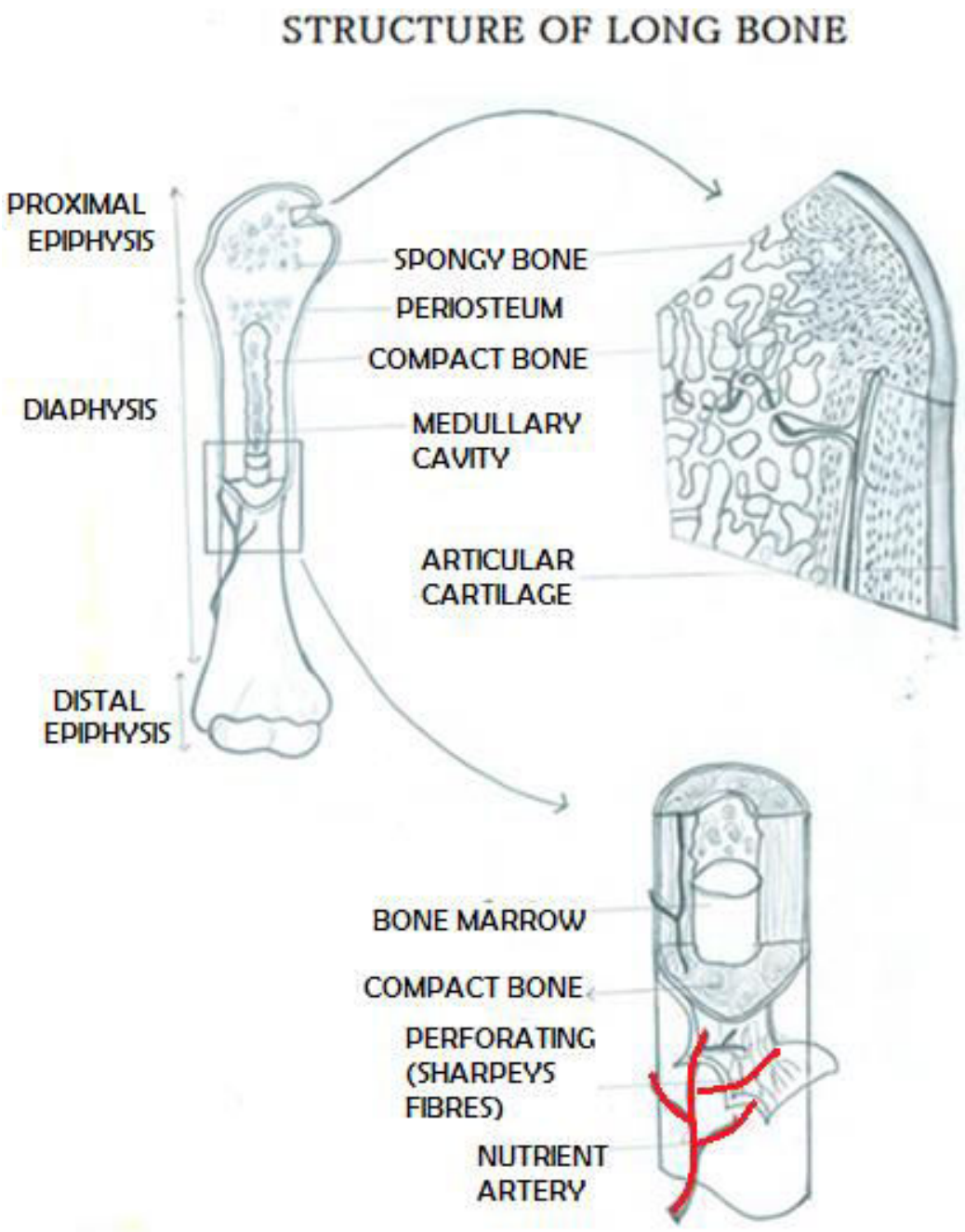


Figure 1: Structure and cross section area of long bone.

Lamellar bone

Orderly distribution of cells and proper orientation of collagen fibres is a typical feature of lamellar bone. Cortical and trabecular bone, both are normally formed in a lamellar pattern, in which collagen fibrils are laid down in alternating fashion. Alternating orientations of collagen fibrils gives significant strength to the lamellar bone, which is absent in the woven bone. Hence, lamellar bone has greater strength when compared to woven bone.

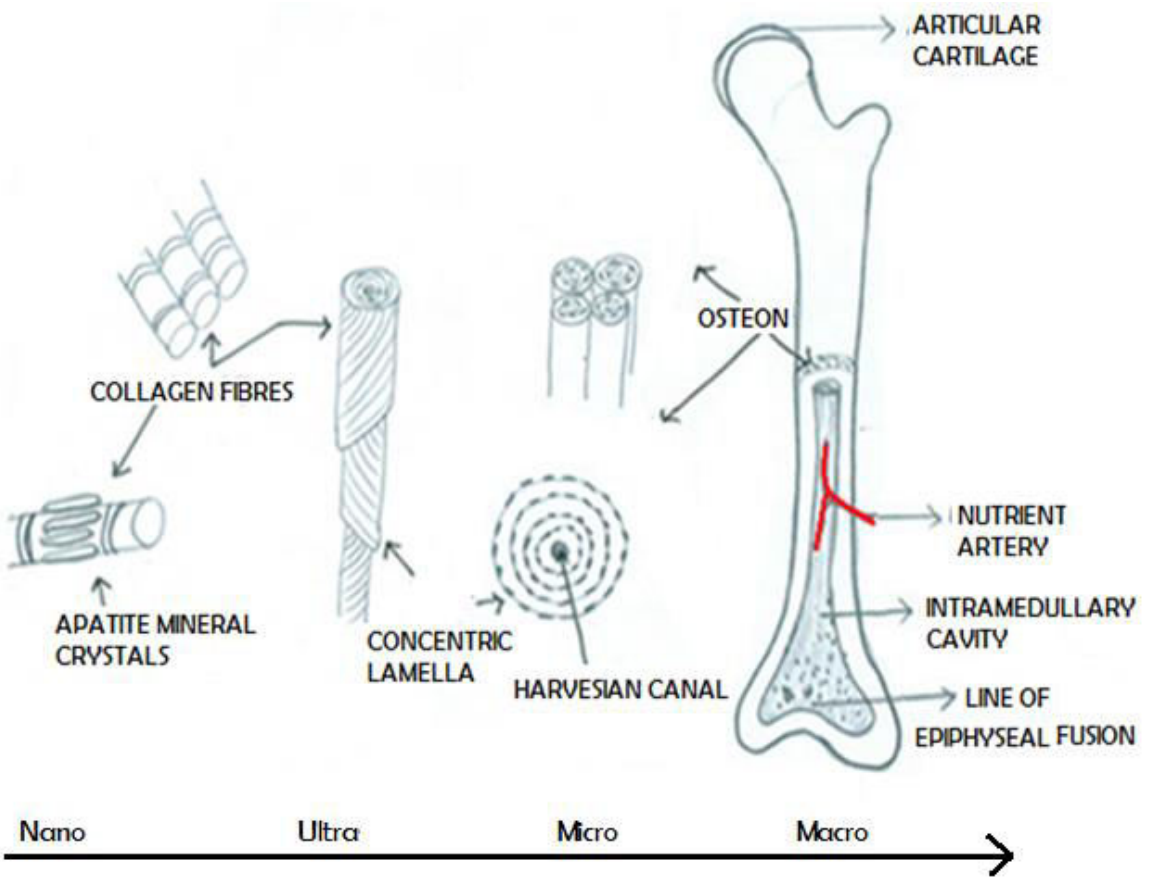


Figure 2: Hierarchical structure of bone from Nanostructure to Macrostructure (left to right)

BONE MICRO STRUCTURE AND CHEMISTRY

Bone tissue is composed of a mineral (inorganic) phase in an organic matrix mainly constituted by type I collagen fibril. It comprises of 50 to 70% mineral, 20 to 40% organic matrix, 5 to 10% water, 3% lipids. Bone mineral is structurally related to naturally occurring geological mineral hydroxyapatite [Ca 10(PO4)6(OH)2], differing in crystalline size, perfection and content of impurities (Mg+2, Sr+2, CO3 2-, H P O 4 2-). In human bone, mineral is composed of a poorly crystallized, Calcium-deficient and non stoichiometric apatite. It contains major elements, like Ca2+ (40 wt %), PO4 3− (18 wt %), C03 2− (6–7 wt %), minor elements as Mg2+ or Na+, and trace elements (Sr2+, F−). It is a reservoir of ions that can be stored or released by the means of remodeling to maintain phosphocalcic equilibrium.(14)

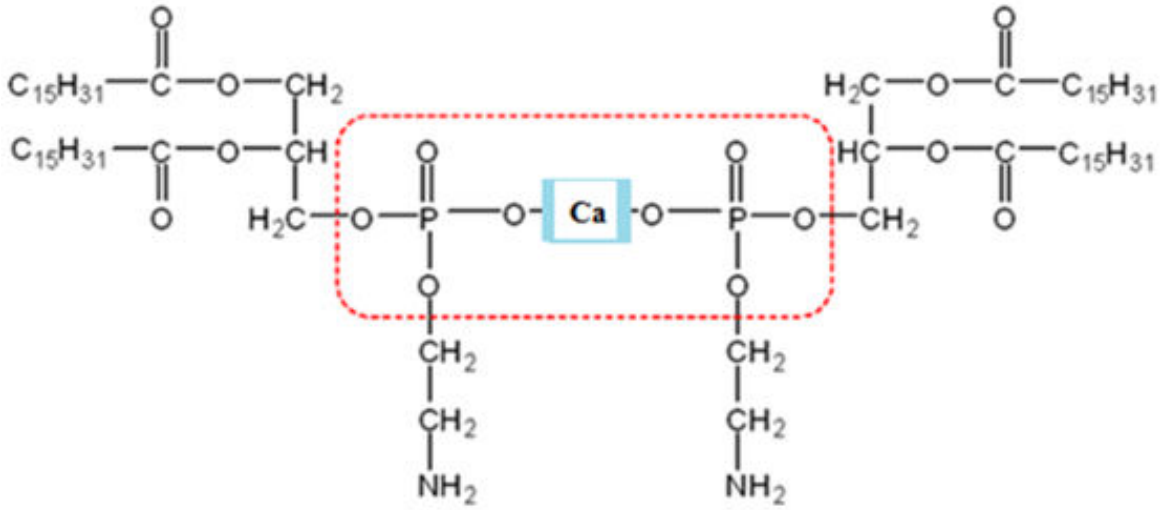


Figure 3: Hydroxyapatite crystal structure showing the calcium moiety and the phosphate with the carboxyl group attached

Mineral crystallites are nano-sized platelet-shaped (1–7 nm in thickness, 15–200 nm in length, and 10–80 nm in width). Their organization in bone tissue depends on the structural properties of organic matrix, which includes collagen
that acts as a template for mineral deposition and the distribution of other matrix proteins. Diet, age, mineral turnover, cell viability, health status, and the use of therapeutic modalities also affect the crystal size and mineralisation. The mineral crystals are arranged parallel to each other, and crystallographic c-axis of the apatite is oriented parallel to the longitudinal axis of the collagen fibrils in a staggered arrangement, with the first nucleation within the gap zones of collagen fibrils (Fig. 1). Crystal nucleation is triggered by collagen and by non collagenous proteins, which also regulate several steps of mineralization.

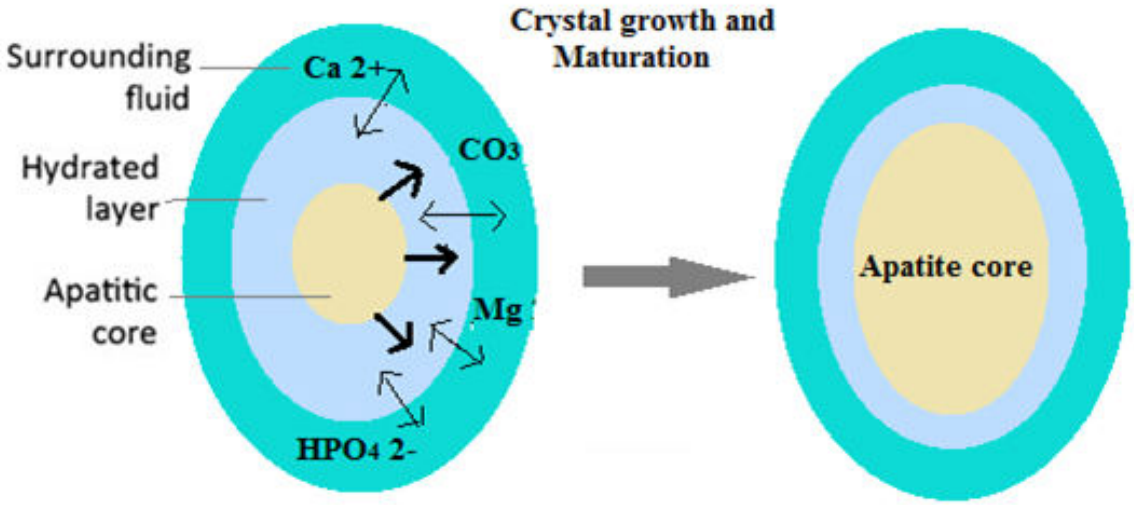


Figure 4: Evolution of the apatitic core and hydrated core via ionic exchange with surrounding fluid

The crystals are first elongated, typically in a plate-like shape, and then grow in thickness rather than in length.(15) Bone mineral provides mechanical rigidity and load-bearing strength. Organic matrix provides elasticity and flexibility. Bone crystals can be depicted in three compartments: Surrounding fluid; hydrated layer; and apatitic core, shown in figure 4.

Ions in the hydrated layer are very labile and reactive, and constitute the nonapatitic domains (HPO_4^{2-}, PO_4^{3-}, CO_3^{2-}). During mineral maturation, non apatitic domains exchange readily and reversibily with the apatitic domain which is associated with an increase in stable apatitic core. The presence of apatite enhances the tensile modulus and strength of collagen where as organic matrix directly acts on the proportions of loads transferred on mineral particles preventing mineral cracking. The "quality" of bone mineral can be described by its crystalline nature, maturity, and level of substitution. Crystallinity encompasses the size and perfection of the crystal lattice, i.e., the degree of order of the ions constituting the lattice and is therefore sensitive to the size and strain of crystals. The mineral maturity reflects the conversion of nonapatitic precursors into apatitic mineral.(16)

BONE CELLS AND PHYSIOLOGY

At the tissue level, bone is composed of bone structural units (BSUs)—the osteons in cortical bone and bone packets in trabecular bone. These BSUs correspond to the net production of bone tissue following a remodeling cycle. The remodeling process takes place throughout the skeleton at anatomically distinct sites termed basic multicellular units (BMUs). The Basic Multi cellular Unit is a team of cells that dissolves a pit in the bone surface and then fills it with new bone"(17). Osteoclasts remove old bone, then osteoblasts synthesize new bone. Old bone is replaced by new bone in quantized packets. (19)

OSTEOBLASTS

Osteoblasts originate from osteoprogenitor cells. Osteoblasts synthesize new bone matrix on bone-forming surfaces, the osteocytes within bone matrix, and the protective lining cells that cover the surface of quiescent bone as shown in Figure 5. Active mature osteoblasts synthesize bone matrix. Osteoblasts secrete type I collagen and matrix proteins which contributes toward bone surface formation.(13) Upon completion of bone formation, it remains on the surface of bone.

OSTEOCYTES

Osteocytes are the terminally differentiated osteoblasts, positioned between lamellae in a concentric pattern around the central lumen of osteons. Osteocytes are connected with each other and the surface via multiple cilia like cellular processes. They function in a co-ordinated fashion with syncytial network responsible for mechanosensory

property, structural support, and metabolism. The primary function of the osteocyte-osteoblast cellular syncytial network is mechanosensation.(20) Osteocytes transduce stress signals, like stretching of bone into biologic activity, by means of flow of fluid within the canalicular channels. This in turn induces a variety of responses within osteocytes. Transmission of information between surface osteoblasts and osteocytes may be through calcium fluxes via gap junctions or ion channels. Osteocytes live for decades in low remodelling states, acting as mechanosensors, but undergo apoptosis once there is disruption of intercellular networks or gap junctions.(21) Oestrogen therapy and mechanical loading of bone may help prevent osteoblast and osteocyte apoptosis.(22)

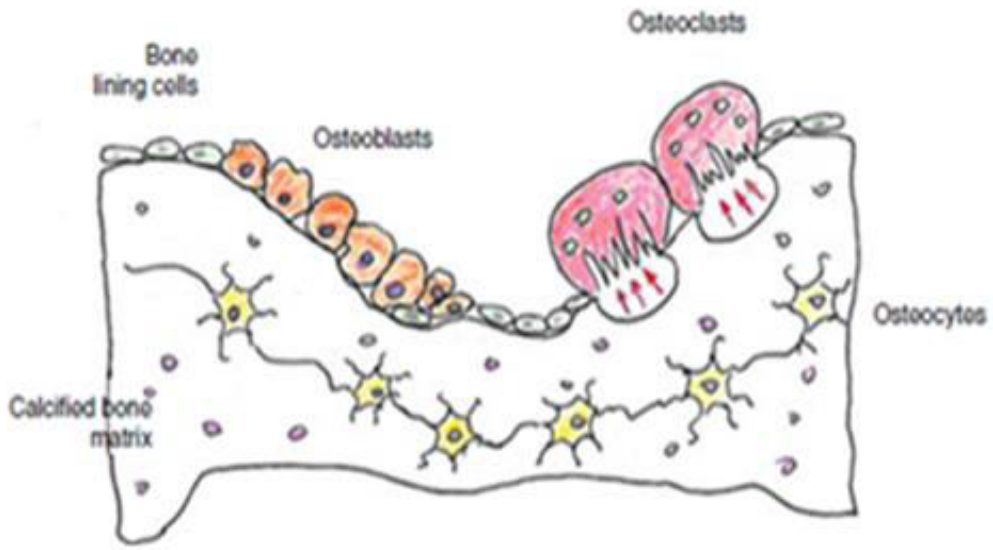


Figure 5: Basic multicellular Unit of bone, comprises od Osteocytes extensively connected with each other and surface, Osteoclasts, bone resorping cells and Osteoblasts, bone forming cells.

OSTEOCLASTS

Osteoclasts are the only cells capable of bone resorption. Mononuclear precursor cells of the monocyte macrophage lineage give rise to activated multinucleated osteoclasts.(23) Osteoclasts attach to bone matrix *via* integrin receptors on the osteoclast membrane linked by bone matrix peptides. Osteoclasts bind to the bone matrix and becomes polarized, with the bone resorbing surface developing a ruffled border. The ruffled border secretes H+ ions *via* H-ATPase and chloride channels and exocytosis of lysozymes and cathepsin K which degrades collagen in the acidified vesicles.(24) A sealing zone around the periphery of osteoclast attachment to the matrix is formed which isolates the acidified resorption compartment from the surrounding bone surface.(25)

FUNCTIONS OF BONE

Bone is involved in wide variety of functions ranging from structural support to hematogenesis and biochemical mineral balance.

Mechanical Support

- Frame work of the body
- Provides strength and stiffness
- Hollow cylinder: Strong and light
- Prevents fatigue fracture

Hematopoesis

- Development of blood cells
- Occurs in the marrow of bone
- These regions are mainly composed of trabecular bone
- (e.g. iliac crest, vertebral body, proximal and distal femur)

Protection of vital structures

- Flat bones in the head protect the brain
- Protects heart and lungs in chest
- Vertebrae in the spine protect the spinal cord and nerves

Mineral Homeostasis

- Primary store house of calcium and phosphorus
- Trabecular bone is rapidly formed or destroyed
- It response to shifts in calcium stasis without serious mechanical consequences

FACTORS REGULATING BONE ACTIVITY

- Estrogen and Testosterone
- PTH, Calcitonin, Vitamin D, Ca / Po4
- Cytokines and Interleukins
- Growth factors,
- Transforming growth factor-a and b

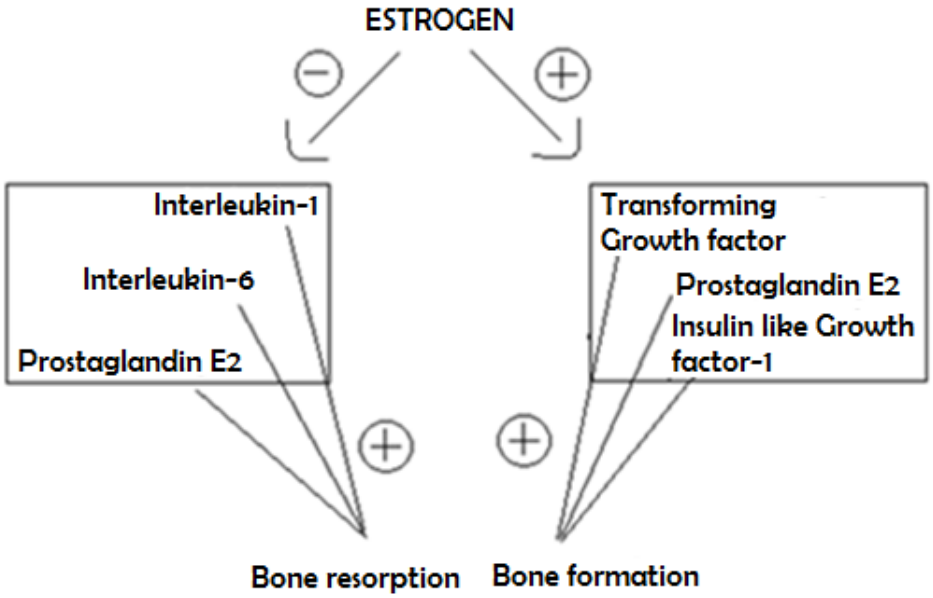


Figure 6: Influence of Estrogen on local mediators affecting bone formation and resorption.

ESTROGEN

Estrogen acts on bone via multiple mediators, the exact mechanism of which is not known. Inhibitory effect of estrogen on osteoclast is removed in menopausal period which leads to prolonged survival and activity of osteoclasts, with increased remodeling rates. Morphological pattern of bone loss in menopausal females is located in trabeculae and endocortical surfaces. This can be explained by hypothesis that it causes skeletal tissue to have optimal sensitivity to loading. This loss of sensitivity to loading results in a morphologic pattern of bone loss that closely resembles disuse. Estrogen deficiency promotes T cell activity which increases osteoclast and reduces osteoblast activity. The inflammatory mediators involved in bone resorption are TNF and IL 1 and 6, which are down regulated by estrogens.(26)(27)

PARATHYROID HORMONE

PTH is the primary regulator of calcium homeostasis. It acts on three important sites, bone, intestine and kidney, which maintains strict control of serum calcium levels. Maintenance of free calcium concentration of the ECF is the basic function of PTH. With respect to the skeletal tissue, it acts on the bone surface in a catabolic manner leading to bone resorption.

PTH acts on the kidney to increase calcium reabsorption and stimulate renal production of 1,25 (OH)2 Vitamin D to increase intestinal absorption of calcium and maintain the ECF concentration of calcium.(28)

Bone remodeling - While its exact mechanism and function is unclear, the net result is increase in the synthesis of collagen. Parathyroid hormone (PTH) does this by

affecting the non collagenous protein bone mineral process to raise the free calcium level in the ECF from the base level. PTH acts on bone to release calcium in two phases; immediate effect is to mobilize calcium from skeletal stores that are readily available and in equilibrium with the extracellular fluid. Later, PTH stimulates release of calcium by activation of bone resorption.

Osteoblasts, not the osteoclasts, express PTH receptors. PTH increases osteoclast activity indirectly through effects on RANKL and osteoprotegerin. (29) The net effect of PTH on bone varies according to the severity and chronicity of the PTH excess, ranging from osteopenia to osteitis fibrosa cystica. PTH when given intermittently promotes bone formation, but at high concentrations or when given continuously, it activates resorption and inhibits collagen synthesis. The positive effect of intermittent PTH on bone appears to be mediated through the PTH1R, in view of the equivalence of PTH (1-84) and PTH (1-34) in bone anabolism. It involves the induction of multiple target genes including IGF1, TGF-beta, RANKL, 1 alpha hydroxylase, and M-CSF; while sclerostin, an inhibitor of the wnt signalling pathway, is suppressed.(28)

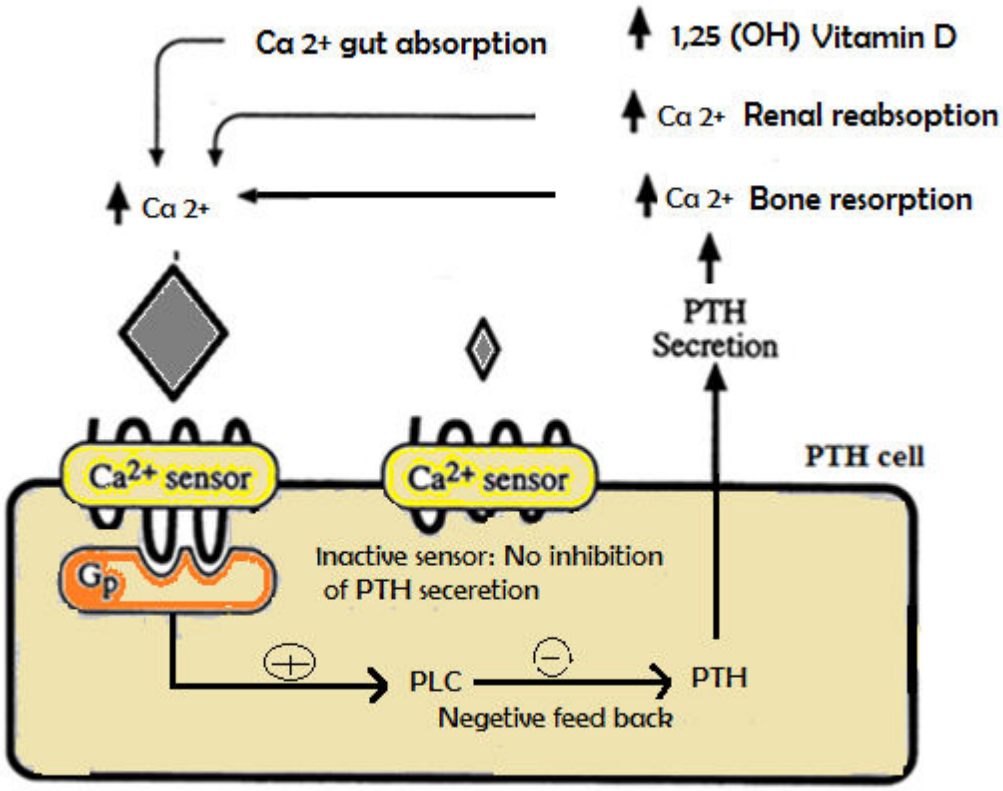


Figure 7: Regulation of serum calcium by PTH mediated via calcium sensing receptors

CALCITONIN

Calcitonin is a protein hormone secreted by parafollicular cells of thyroid. Ionized calcium regulates calcitonin secretion, increase in ionized calcium produces an increase in calcitonin secretion, which inhibits osteoclasts and therefore bone resorption in pharmacologic doses. However, its physiologic role is minimal in the

adult human skeleton. Its pharmacologic effects are transient, probably because of receptor down regulation. As a result, it is only transiently effective in treating hypercalcemia due to excessive bone resorption.

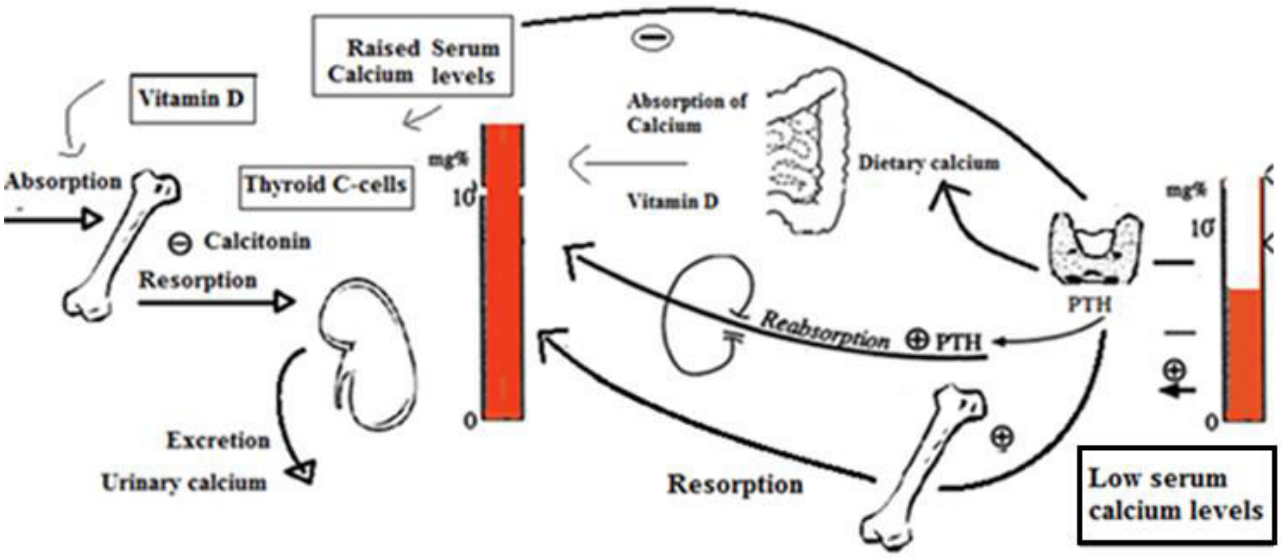


Figure 8: Overview of serum calcium regulation by PTH and calcitonin

VITAMIN D

Production, metabolism, and function

Vitamin D is truly a prohormone not a vitamin. 7-dehydrocholesterol, in the skin after sunlight exposure (ultraviolet B light) is converted to vitamin D3. Vitamin D 3 is hydroxylated in the liver to 25-hydroxyvitamin D3 (25(OH) Vitamin D 3), and further hydroxylated in the renal proximal tubule to 1, 25-dihydroxyvitamin D3 (1, 25(OH) 2 D3, Calcitriol), the principal active form. The production of 1, 25(OH)2 Vitamin D in the kidney is under tight control of parathyroid hormone (PTH) and inhibited by calcium, phosphate, and fibroblast growth factor 23 (FGF23). 1, 25(OH) 2 Vitamin D stimulates its own catabolism by induction of CYP24-hydroxylase, which avoids vitamin D toxicity. It is the first step in the catabolism of active Vitamin D metabolites.

Vitamin D receptor and mineralisation

VDR belongs to the steroid hormone nuclear receptor super family, and has been shown to regulate both gene transcription as well as rapid responses such as the opening of voltage-gated Ca 2+ channels, and stimulation of intestinal Ca 2+ absorption. Infusion of calcium and phosphate to vitamin-D–deficient rats induced growth and mineralization as effectively as vitamin D replacement.(31) Evidence indicates that the stimulation of intestinal calcium and phosphate absorption is the mechanism by which vitamin D increases bone mineralization, effects on matrix vesicles.(32)

Vitamin D-Regulated Bone Formation

Vitamin D deficiency causes rickets due to hypocalcemia and hypophosphatemia. Skeletal changes in vitamin D deficiency are due to hyperparathyroidism that develops in the vitamin D-deficient state. PTH has its own actions on remodelling activity which affects the bone and cartilage. Terminal differentiation of the hypertrophic chondrocytes and the calcification of the matrix are significantly impaired in vitamin D deficiency which leads to flaring of ends of the long bones and the rachitic rosary pattern of costochondral junctions, typical features of rickets. Adequate supply of calcium and phosphate may correct the defects in terminal differentiation and calcification. Vitamin D metabolites, $1,25(OH)_2D$ and $24,25(OH)_2D$, are known to exert distinct roles in endochondral bone formation.(33)(34)

Direct action on bone

Vitamin D directly plays a role in osteoblast formation, mineral apposition rate, and bone density. Studies show trabecular bone with osteopenia, decreased mineral apposition rates and reduced alkaline phosphatase expression, signifying reduced osteoblast number or activity.(33)(35) Endochondral bone formation is impaired in vitamin D deficiency associated with decrease alkaline phosphatase activity of the hypertrophic chondrocytes.$1,25(OH)_2D$ and $24,25\ (OH)_2D$ both are required for endochondral bone formation and proliferation of hypertrophic chondrocytes.(33) Vitamin D affects endochondral bone formation on the one hand and systemic calcium/phosphate homeostasis via changes in FGF23 production on the other.(33)

Involvement of vitamin D in collagen cross-linking-Collagen cross-linking is considered as one of the determinants of bone quality. Vitamin D treatment increases interconnections and formed plate like structures. It maintained bone formation in the endocortical perimeter, and stimulated bone formation in the periosteal perimeter(32). Anti apoptotic effects of vitamin D have been believed to be mediated mainly through osteoblasts, but osteocytes may also be involved(32).

Indirect action

$1,25(OH)_2D_3$ is mainly responsible for uptake of calcium and phosphate from the intestine and aid in mineralisation of bone, rather than a direct action on bone.(33) Both indirect and direct effects of vitamin D on bone are essential and required; as deficiency in one, can be partially compensated by the other.

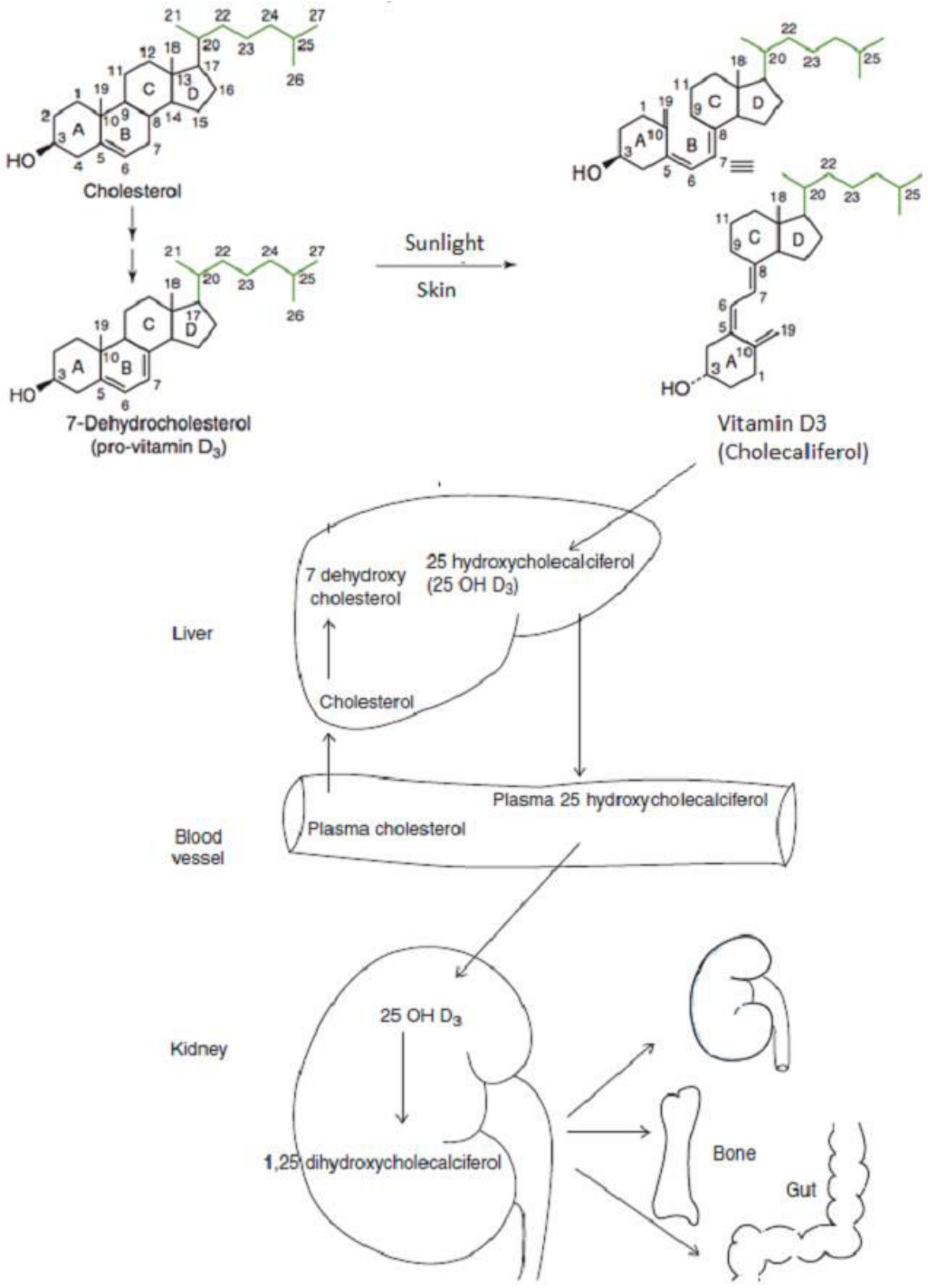


Figure 8: Vitamin D structure, site of hydroxylation and action

BONE FORMATION

Bone cells (Osteoblast) and the bone matrix are the two most crucial elements involved in bone formation. Two important processes involved in normal bone formation are Intra membranous and Endochondral ossification.(36)

Intramembranous ossification is the laying down of bone material into the mesenchyme, the primitive connective tissue resulting in the formation of bones. (Example - skull, mandible)

Endochondral ossification is where a cartilage acts as a precursor for ossification (e.g. humerus, radius, tibia, femur) It is the process which occurs during fracture healing when treated by cast immobilization.

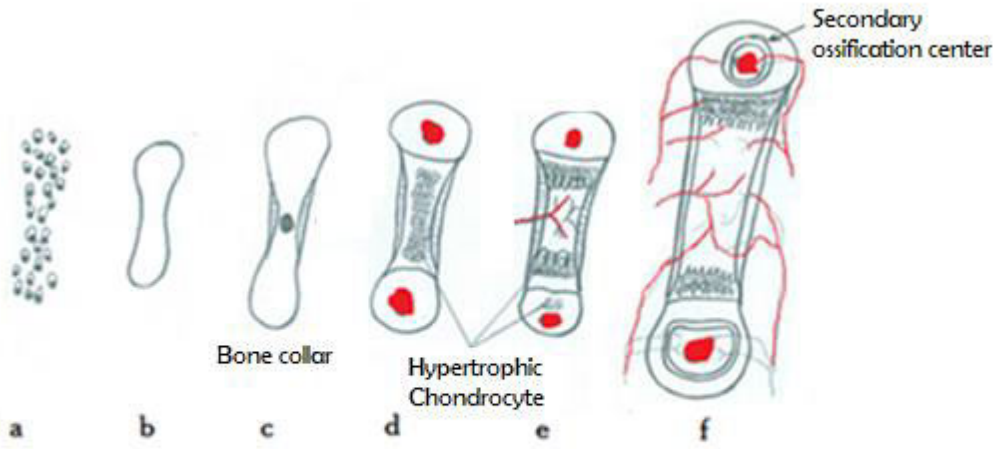


Figure 9 : Intracartilagenous ossification
(a) Aggregates of progenitor cells (b) Modal of hyaline cartilage (c) Pimary centre for ossification (d) Secondary centre for ossification (e) Bone with medullary cavity and epiphyseal ends (f) Mature bone with feeding vessals

CARTILAGE MODEL

Each long bone present as a rod of hyaline cartilage in early foetal life replaces a rod of condensed mesenchyme. The cartilaginous model is surrounded by a vascular condensed mesenchyme or perichondrium, with its deeper layers containing osteoprogenitor cells.

Growth of cartilage model- The cartilage model grows in length by continuous cell division of chondrocytes called as interstitial growth. The process of appositional growth occurs when the cartilage grows in thickness on the peripheral surface of cartilage that develops from the perichondrium. On reaching maturity, epiphyseal and metaphyseal ossification encroach upon the growth plate, followed by final bony fusion with cessation of growth.

Development of the primary ossification centre-

The primary centre of ossification lies in the middle of the shaft, which undergoes following events:

• *Formation of Periosteum* – As vascularity improves, the perichondrium becomes the periosteum. Undifferentiated osteoprogenitor cells on the periosteum later become osteoblasts.

• *Formation of Bone Collar* - The osteoblasts secrete osteoid against the shaft of the cartilage model (appositional growth). These acts as scaffold support for the new bone.

• *Calcification of Matrix* - Chondrocytes at the primary center of ossification undergo hyperplasia. Alkaline phosphatase secreted from the chondrocytes is essential for mineral deposition and calcification of the matrix. Following this, hypertrophic chondrocytes undergo apoptosis and creates cavities within the bone.

• *Invasion of Periosteal Bud* - The hypertrophic chondrocytes secrete VDGFs (vascular endothelial cell growth factor) that induces the sprouting of blood vessels from the perichondrium. Sprouting blood vessels form the periosteal bud and invade the formed cavity carrying the hemopoietic cells and osteoprogenitor cells. The hemopoietic cells form the bone marrow. Osteoclasts, formed from macrophages, break down spongy bone to form the medullary cavity.

• *Formation of Trabeculae* – Newly formed osteoblasts within the cavity secrete osteoid, which lays down over the calcified matrix acting as scaffold for bone trabecula.

Development of the secondary ossification center

At birth, a secondary ossification centre appears in each epiphysis of the long bones. Periosteal buds carry mesenchyme and blood vessels in which the primary ossification centre forms. The cartilage between the primary and secondary ossification centres is called the epiphyseal plate, and it forms new cartilage, which is replaced by bone, resulting in an increase in length of the bone. Growth continues until the cartilage in

the plate is replaced by bone or about 21 of year's age. The point of union of the primary and secondary ossification centres is called the epiphyseal line.

Formation of articular cartilage and epiphyseal plate:

The cartilaginous extremity (where an epiphysis usually forms) grows by appositional and interstitial mechanisms. When the whole bone is reaching maturity, epiphyseal and metaphyseal ossification gradually encroaches upon this growth plate, and final bony fusion occurs with cessation of growth.

BONE MODELING AND REMODELING

Bone undergoes radial and longitudinal growth, modelling, and remodeling during life. Modeling is a process by which bone adapts overall change in shape in response to mechanical forces it encounters during development. Bones normally widen with aging in response to periosteal apposition of new bone and endosteal resorption of old bone. During bone modeling, bone formation and resorption are not tightly coupled. Bone remodeling is removing and replacing packets of bone. It is the primary mechanism whereby bone is renewed and adapts to changes in load bearing. It is a useful process for decreasing skeleton size in the event of immobilization and for repairing the micro damage before they become clinically apparent. This is the process by which bone is renewed to maintain bone strength and mineral homeostasis is maintained. It prevents accumulation of bone micro damage. The bone remodeling unit is composed of osteoclasts, osteocytes and osteoblasts that are tightly regulated to carry out resorption of old bone and formation of new bone. Osteocytes play an important role in cell signaling, regulating osteoblast and osteoclast function, and sensing mechanical loading.(37) The mechanisms controlling remodeling are largely unknown, but mechanical loading has a significant effect. The number of active bone structural units created per unit time at any chosen surface locus is measured histomorphometrically as activation frequency and is a tissue-level measure of the rate of bone remodeling. The net amount of bone formed is termed bone balance. A negative bone balance occurs at menopause, immobilized patients and in astronauts in a microgravity environment.(38) Remodeling sites mostly develop in a random manner but are also targeted to areas that require repair.

TYPES OF REMODELLING

- Targeted/Stochastic remodeling- Targeted remodeling is responsible for the repair of micro damage. It is supposed to be the most important pathogenetic factor for increased bone fragility in osteoporosis(26). Increase / accumulation of micro damages leads to structural failures (i.e. micro fractures)

- Non targeted/Non Stochastic remodeling helps in maintaining the plasma calcium homeostasis.

Clinical significance of remodeling

It helps in assessment of bone activity over a period of time. Changes in bone remodeling can be identified over a relatively shorter time period (several days to months) before changes in BMD are detected. Change in remodeling markers, i.e. increased bone resorption markers correlate with fracture risk better than BMD. Clinical trials of antiresorptive agents have shown that reducing excess remodeling decreases fracture risk significantly.(39)

SEQUENTIAL PHASES OF REMODELING:

QUIESCENCE

It is a period of relative inactivity. After the tunnelling and refilling some osteoblasts become osteocytes, remain in bone and sense mechanical stresses on bone. Remaining osteoblasts become lining cells. Factors that activate the remodeling cycle are not yet known.

ACTIVATION

It occurs when bone experiences micro damage or mechanical stress, or randomly. A Bone Morphological Unit (BMU) originates and travels along the bone surface. This is followed by retraction of the bone lining cells (osteoblasts on surface) and digestion of the endosteal membrane by collagenase enzymes. This completes the first step towards activation. Activation of monocyte-macrophage system and osteoclast precursor cells from the circulation, results in interaction of osteoclast and osteoblast precursors.(40) This leads to the differentiation, migration, and fusion of the multinucleated osteoclasts which attach to the mineralized bone surface and initiate resorption.

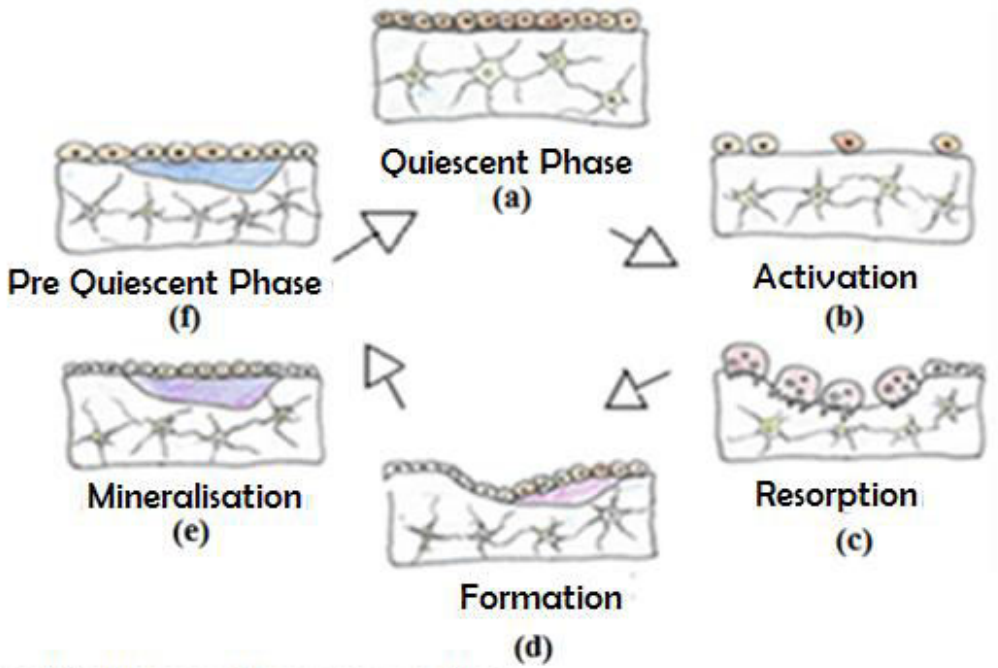


Figure 10 : Phases of bone remodelling
(a) Quiescent phase - flat bone cells lining the endosteal membrane
(b) Activation phase - cell retraction with resultant membrane resorption
(c) Resorption - activated osteoclasts resorbing the underlying bone
(d) Formation phase - Osteoclasts replaced by osteoblasts with new osteoid matrix
(e) Mineralization of osteoid matrix
(f)Formation of bone structure unit with progression to quiescent phase

RESORPTION

Newly differentiated osteoclasts are activated and begin resorption. Minerals are dissolved and the matrix is digested by lysosomal enzymes and hydrogen ions, particularly cathepsin K, which can degrade all the components of bone matrix, including collagen, at low pH. Osteoclastic resorption produces irregular cavities called Howship's lacunae and cylindrical Haversian canals in trabecular bone and cortical bone respectively. It takes 2–4 weeks for resorption during each remodeling cycle.

REVERSAL

It is the transition from osteoclastic to osteoblastic activity. Cavities formed at the end of resorption contain monocytes, osteocytes released from bone matrix recruited to begin new bone formation. The proposed coupling signal candidates linking the end of resorption to beginning of formation are IGF-1,2, bone morphogenetic proteins, and fibroblast growth factor.

FORMATION

It is transition from osteoclastic to osteoblastic activity. Osteoclasts are replaced by cells of the osteoblast lineage. The growth factors are liberated from the matrix which act as chemotactics and stimulate their proliferation. The preosteoblasts secrete cement like substance upon which the new tissue is attached and express *bone morphogenic proteins* (BMP), which is responsible for differentiation. Differentiated osteoblasts synthesize the *osteoid matrix* which fills the cavity perforated areas (41). Some osteoblasts continue synthesising bone until they transform to quiescent lining cells that cover the new bone surface and connect with the osteocytes in the bone matrix through canalicular network.

MINERALISATION

Bone tissue is composed of a mineral portion in an organic matrix mainly constituted by type I collagen fibrils. The degree of mineralization of bone and the characteristics of the mineral deposited (apatite crystals) are major determinants of bone strength.

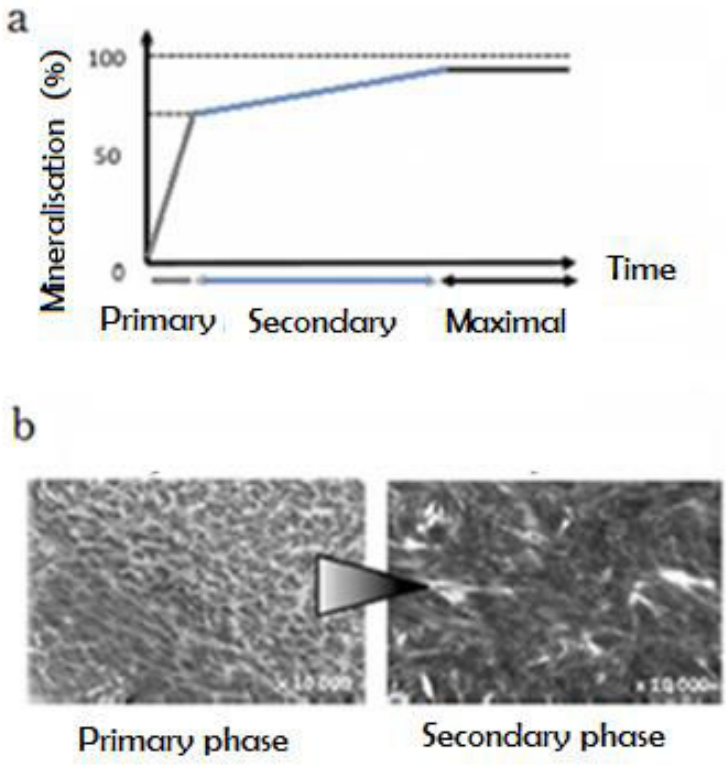


Figure 11: Steps of bone mineralization (a)During primary mineralization, bone matrix reaches a large propotion of its maximal mineralization. (b) Secondary mineralization consists of slow increase in crystal number, size and perfection (micrographs of same size during primary and secondary mineralization toward completion of mineralization) Picture Bala et al.

Mineralization involves not only the initial deposition of mineral in organic matrix but also its maturation until the upper mineral density in a given volume of matrix is reached. Mineralization is a multistep process during bone remodelling. (Fig.11). Newly formed organic matrix deposited by osteoblasts begins to mineralize 5–10 days after deposition by secondary nucleation, i.e., the crystals act as nucleation sites for the newer ones. This first step leads to a mineral content corresponding to 50–70 % of the maximal value.(42) After a few days, or weeks, the speed of mineralization decreases substantially and secondary mineralization begins. This latter process

corresponds to gradual increase in crystal size, number and/or perfection, occurring on the year-scale until the maximal (Mean degree of bone mineralisation) DMB is reached. Independent of bone mass and its distribution in space, the mineralization and the “quality” of the mineral play a crucial role in the elastic, plastic, and viscoelastic properties defining the mechanical behaviour of bones.

Mineral deposition happens at multiple discrete sites of the collagen fibers. This process, also, is regulated by the osteoclasts. After mineral maturation, once the cavity is full, the mineral crystals pack together, increasing the density of the new bone. The cycle continues and quiescent phase begins again. One cycle of remodelling equalises amount of bone formation and bone resorption.(43)

OSTEOPOROSIS

"Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality"(44). Clinical diagnosis of osteoporosis now depends on the presence of low-trauma fracture, defined as fracture resulting from trauma equal to, or less than, a fall from a standing height, excluding fractures of face, skull, or digits.

OSTEOPOROSIS - REDUCED BONE STRENGTH

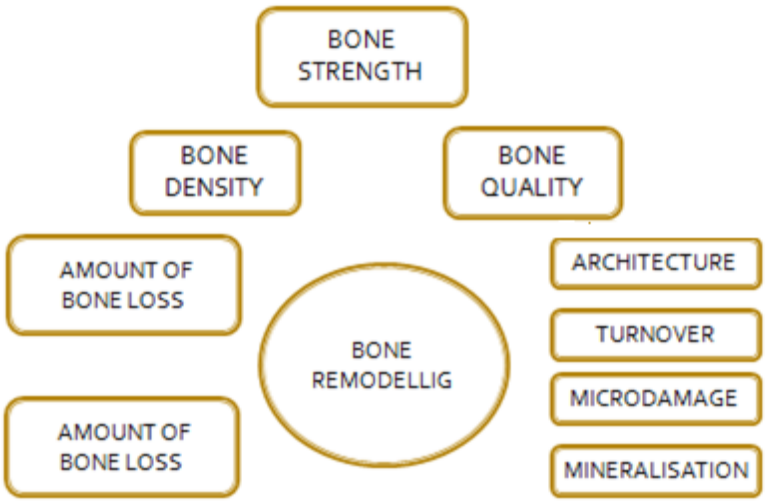


Figure 12 : Factors responsible for bone strength and their relation with bone remodeling.

Features of bone quality that contribute to skeletal fragility and risk of low-trauma fracture are defects in micro architecture of trabeculae, defective material properties of bone tissue, and incomplete repair of micro-damage. Defective repair of micro-damage results from physiologic loading occurring in normal daily life. These features

are also observed in age-related bone loss, and increased bone remodeling rates greater than those found in normal premenopausal women(45)(46).

BONE DENSITY

Peak bone mass – It determines 50 to 70% of bone strength. Bone mass reaches its peak at about 20 years of age in males and 18 years in females. Peak Bone Mass determines long-term fracture risk. Paediatric population are at high risk for fractures as they have not attained their peak bone mass.

BONE QUALITY

Micro architecture

It is essential for resistance to fracture. It comprises of amount, size, shape, and connectivity of trabecular bone and cortical bone tissue. In Osteoporosis there is a decrease in size and number of trabeculae. Thinner and rod like trabeculae, replacing the stronger plate like morphology that is seen in non osteoporotic bone(47). Excessive remodeling present in osteoporotics is likely to be the primary cause of these changes in micro architecture.

Turnover

Higher bone turnover influences bone strength by affecting mineralisation. When bone turnover is high, probability for a cortical or trabecular bone structural unit (BSU) to be resorbed before the completion of its secondary mineralization increases. This leads to greater proportion of younger and sub maximally mineralized bone.

An increased proportion of sub maximally mineralized bone matrix leads to an increase in the proportion of immature crystals with a low crystallinity. (46)

Micro damage

It occurs due to repeated sub maximal loading. It can transect lamellae and canaliculi and disrupt the communication of osteocytes. This process can trigger osteocyte apoptosis, which signals osteoclast precursors to differentiate into osteoclasts that target the microdamage for removal and repair by osteoblasts(48). Small cracks may coalesce, forming larger cracks, which, if left unrepaired, result in clinically apparent fractures. Once a trabecular surface is eliminated, subsequent formation does not happen at that location. Excess of weakened loci in trabeculae and an increase in micro damage outpaces the ability to repair. Micro damage increases with age, which can accumulate and result in structural failure (i.e. fractures).

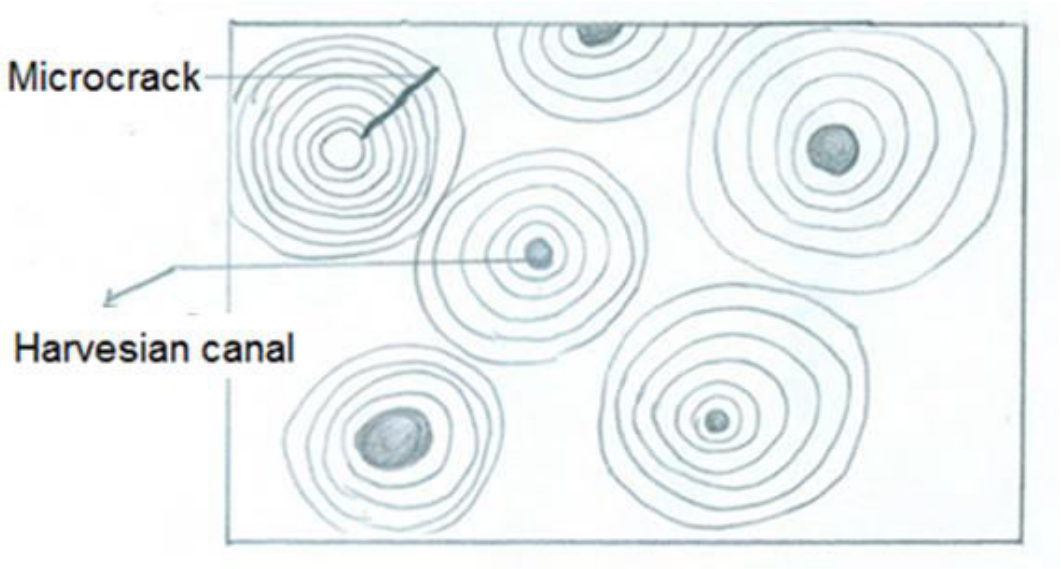


Figure 13 : Haversian canal with lamellar bone and microcrack

Mineralisation

Bone remodeling activity acts as a regulator of the degree of mineralization and of the distribution of mineral at the tissue level, directly impacting bone mechanical properties. Reduced bone turnover activity provide osteons and bone trabecular packets (BSU) with more time to complete their secondary mineralization before being resorbed in a further remodeling cycle. Therefore, a greater proportion of tissue is maximally mineralized not only increasing the mean tissue mineral content but also narrowing its distribution. Contrastingly when bone turnover is high, probability of BSU to be resorbed before completion of secondary mineralisation raises, this leads to younger optimally matured bone at tissue level. At crystal level, increased proportion of sub maximally mineralized bone matrix leads to an increase in the proportion of immature crystals with a low crystallinity i.e. suboptimal size and lattice structure, interface between collagen and crystal. (e.g. menopause). Decrease in remodelling rate leads to homogenous mineral characteristics (e.g., after antiresorptive treatment).(43) In rapid remodeling, bone strength may be compromised indicating that optimal bone strength rises from an optimal mineralization.

REMODELLING, BMD and FRACTURE – RISK

Factors which compromise the bone strength and skeletal fragility:

(1) Defects in micro architecture of trabeculae,

(2) Defective material properties of bone tissue,

(3) Incomplete repair of micro damage and

(4) Excess bone remodeling rates.

Remodelling appears to be the first detectable change following in skeletal tissue following SCI. Routinely done DEXA scan show changes few years when significant bone loss has occurred. Clinical studies of mechanical loading, estrogens deprivation, age related, disuse related bone loss and role of antiresorptive have improved the knowledge regarding factors affecting bone quality and mechanisms underlying them. Other aspects of bone quality not measurable clinically are probably more important for mechanical integrity of bone and resistance to fracture than the bone mineral density, especially for early detection of bone loss. It consists of the amount, size, shape, and connectivity of trabecular bone tissue as well as the amount and shape of cortical bone tissue. The trabeculae also became thinner and rod like in shape, replacing the stronger plate like morphology that is seen in non osteoporotic bone. The excessive remodeling present in most postmenopausal females is likely to be the primary cause of the changes in micro architecture, hence routine follow up of remodelling rates may suggest the dynamic changes in bone. (47). As bone turnover rates increase they affect mineralisation and later changes in BMD appear, as shown in figure 14. But as bone markers do not indicate the extent of established bone loss

both the parameters are required for appropriate management of osteoporosis. Anti resorptive treatment in Post Menopausal females increases the Bone Mineral Density, but more importantly reduces the remodelling rates. Change in BMD accounts for anti-fracture effect of antiresorptive agents, but facture risk reduction may not be related to changes in BMD. Low BMD is predictive of fracture in untreated patients whereas reduction of fracture risk in treated patients, has less to do with their BMD response to treatment.(49)

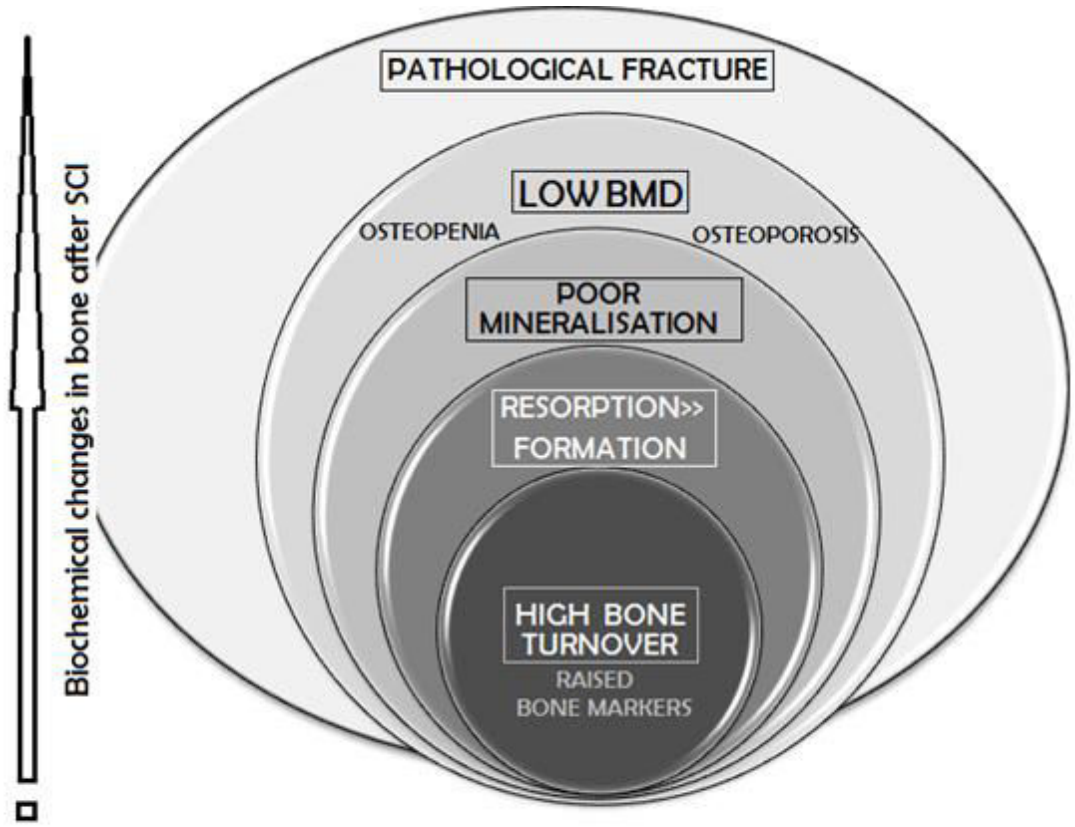


Figure 14 Remodeling is closely linked with mineralisation and fracture risk. Bone markers are elevated much earlier than changes noticed in BMD.

PATHPHYSIOLOGY OF BONE LOSS IN SCI

1. LOCAL FACTORS

- Mechanical unloading
- Reduced mechanical signals

2. DENERVATION

- Autonomic dysregulation
- Neuropeptide expression

3. HORMONAL DYNAMICS

- Suppression of PTH- Vitamin D axis following hypercalciuria in the acute phase of spinal cord injury.
- Low vitamin D levels and low sun exposure following injury.
- Increased cortisol levels due to stress.
- Steroids in the course of treatment.
- Inhibitory effect of Spinal Cord Injury on the synthesis and secretion of sex steroids contributes to the pathogenesis of SCI induced osteoporosis(26)(50)

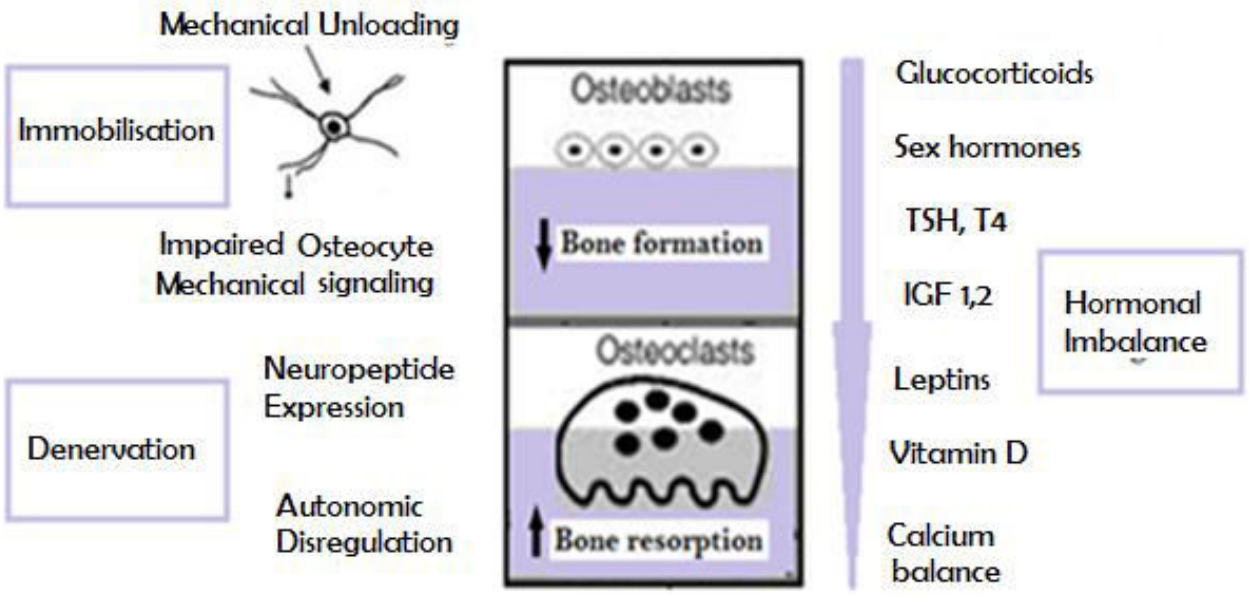


Figure 15: Factors affecting skeletal tissue following spinal cord injury

Multiple factors are responsible for skeletal changes following SCI. The major factors are immobilisation and mechanical unloading during the acute phase. Bone loss is significantly higher in SCI related immobilisation than other causes of immobilisation. This is probably where SCI patients need better evaluation and aggressive management for prevention bone loss in acute phase, osteoporosis and pathological fractures in later stages.

Mechanical loading play a significant role in maintaining the bone strength by mechanosensation mediated through osteocytes Mechanical loading leads to bone matrix deformation around the osteocytes, which creates perturbation of cell membrane (probably by stretching the glycocalyx present in the cell membrane) and bone fluid leading to shear stress, which contribute to mechanosensation and mechanotransduction. (37)

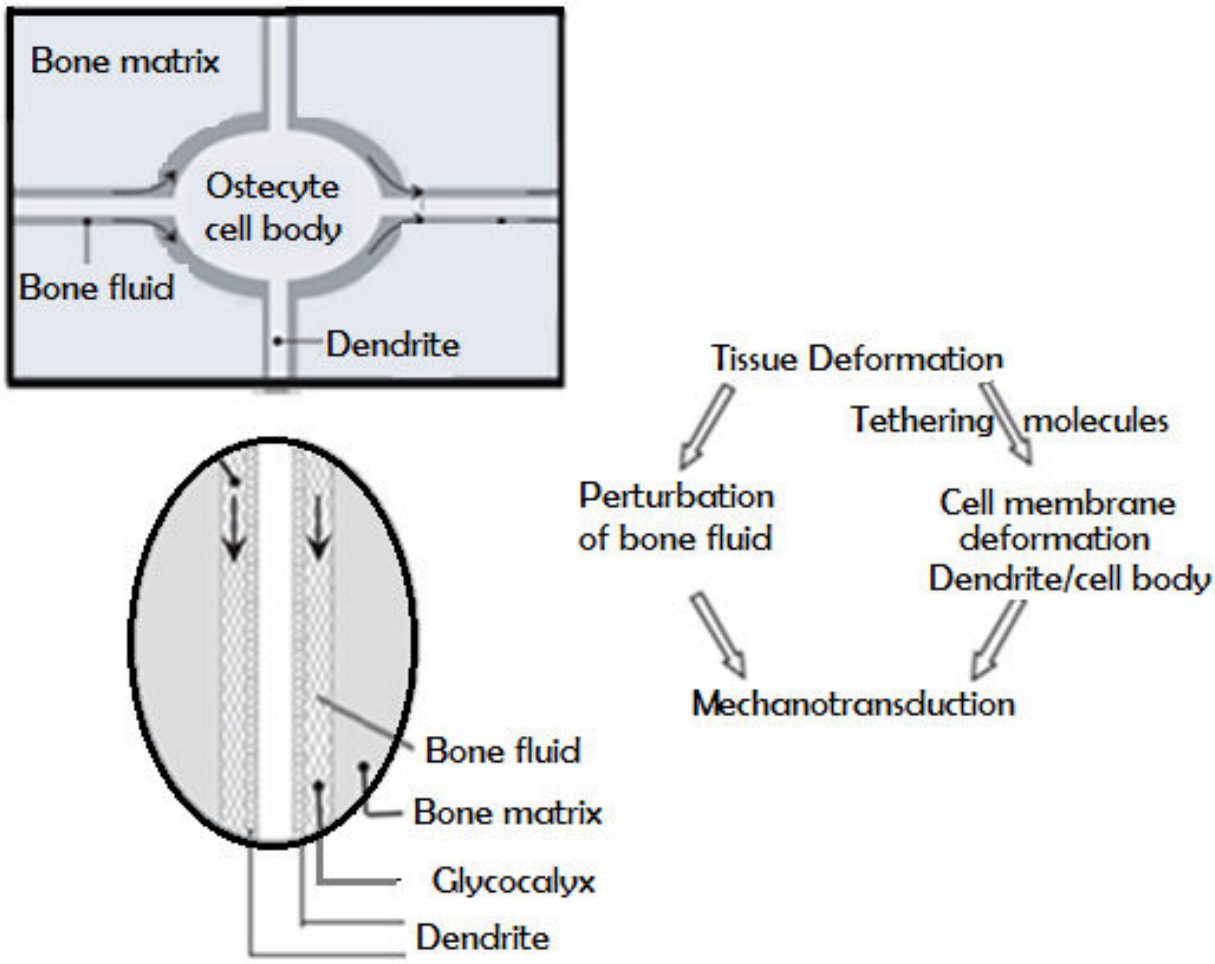


Figure 16: Mechanotransduction by tissue deformation in osteocytes

WNT THEORY OF BONE

WNTs are glycoproteins that regulate cellular activities, such as cell fate, proliferation, migration, survival and gene expression. They play a major role in osteoblasts activation and bone formation, which inturn inhibits agents associated with bone resorption. Reduced WNT signaling has been associated with increased bone loss.(51)

Wnts, glycoproteins undergo post-translational modification by addition of lipids, required for activation of bone formation. Wnt/β-catenin pathway acts as a critical regulator of osteocyte function, responsible for mechanical loading of bone. Fluid flow-induced shear stress stimulates gap junction-mediated intercellular release of prostaglandins. (37) PGE2 increases intracellular cAMP and activate Protein Kinase A. Connexins form gap junctions also called hemichannels which provide a mechanism for ATP and NAD+ release and increases intracellular Ca2+ levels. Hence, Prostaglandin (PGE2) released through hemichannels in response to shear stress has an autocrine effect on osteocytes through the EP2 receptor. (41)(26). Currently accepted paradigm states that Wnt binds to a co receptor complex involving Frizzled receptor and low density lipoprotein receptor-related protein (LRP)-5, present on osteoblasts. This binding stabilizes cytoplasmic β-catenin and translocates it to the nucleus. Translocation activates transcription of genes that promote osteoblast proliferation and differentiation, ultimately resulting in new bone formation.

Sclerostin, encoded by the sost gene, is produced primarily by osteocytes.. (52). Mechanical unloading cause's up-regulation of sclerostin, a potent inhibitor of bone formation and growth. It competitively inhibits the Wnt/β-catenin signaling in osteoblasts which is primarily responsible for bone formation. Sclerostin causes up-regulation of RANK Ligand and down-regulates OPG (Osteoprotegrin) expression by osteocytes, which increases osteoclast differentiation and activity, leading to bone resorption.

Sclerostin and Dickkopf-related protein 1 (Dkk1), inhibit the Wnt pathway by preventing the formation of the Wnt-Frizzled-LRP5 complex by promoting the internalization of the LRP5/6 coreceptor or by competitive binding to LRP5.(53)

PTH levels are suppressed following acute SCI probably due to the hypercalcemia that accompanies increased bone loss as. PTH inhibits sclerostin production where as intermittent PTH treatment has been proved to stimulate bone formation in humans. Thus if PTH act as negative feedback regulator of sclerostin, suppressed PTH in acute SCI would exacerbate sclerostin-mediated bone loss. However, these complex associations remain unsolved in acute SCI and needs further research.

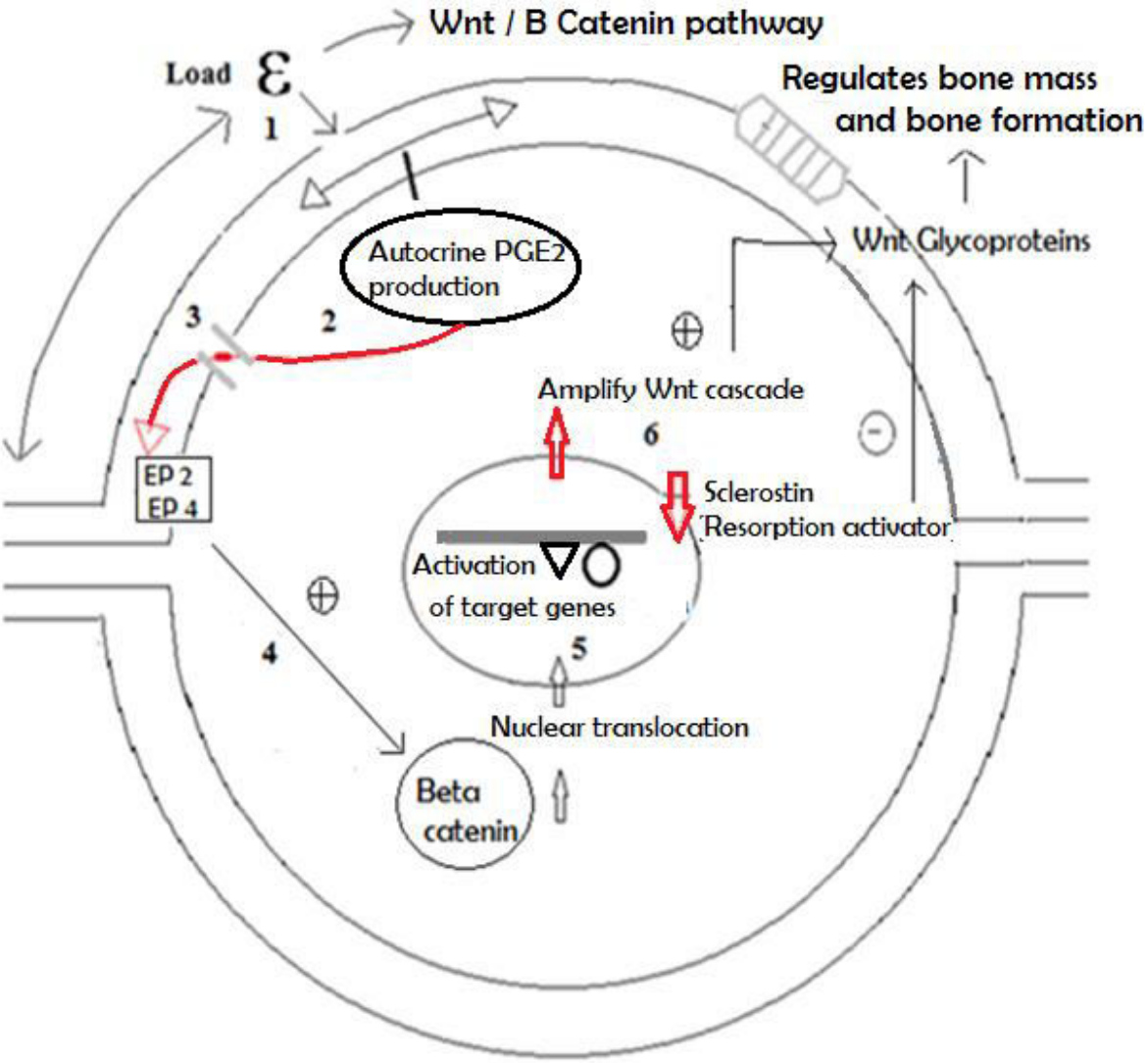


Figure 17: Wnt/ Beta catenin cascade in osteocytes on loading response.

1. Mechanical load over bone surface (E) percieved as flow of fluid wih in the canalicular system

2. Production of PGE2 due to the fluid sheer tension.

3. Autocrine effect of PGE2 on EP2 receptor after it passes the hemi channel.

4. Down grade inhibition of degradation enzymes leads to increased beta catenin accumulation.

5 Nuclear translocation of beta catenin and activation of target genes.

6. Amplification of Wnt cascade and reduction of sclerostin (resorption activator and competative inhibitor of Wnt ligand)

BONE LOSS IN SCI

In comparison to bone loss of other immobilizations (TBI, CVA) bone loss in spinal cord injury is rapid and severe which suggest additional aetiologies contributing to reduced bone strength(55). Identification of pathway related to mechanical loading has significantly improved our understanding regarding the loading mechanism and its regulation by mechanosensor osteocytes. Still the quest for therapeutic agents to prevent the bone loss on unloading remains unsolved; hence it becomes essential to explore the other causes responsible for bone loss. Neural and endocrinal factors affect the process of bone remodeling and hence the bone loss(56). A number of hormonal changes takes place following spinal cord injury which may explain the excess bone turnover (1). Suppression of PTH-Vitamin D axis following hypercalciuria in the acute phase of spinal cord injury may contribute to sclerostin induced bone loss. Low vitamin D levels and low sun exposure following injury and steroids in the course of treatment. Inhibitory effect of Spinal Cord Injury on the synthesis and secretion of sex steroids contributes to the pathogenesis of SCI induced osteoporosis (26)(50). Hypercalcemia following Spinal cord injury suppresses the PTH- Vitamin D axis as a result of which negative calcium balance persists in the acute phase. Recent studies reveal the role of PTH in suppression of sclerostin a protein molecule, supposed to be associated with SCI-induced bone loss. Sclerostin, is encoded by the sost gene, produced by osteocytes, Sclerostin reduces Wnt/β-catenin signaling in osteoblasts and reduced bone formation.(57)

Spinal cord injury mediated hormonal changes and inadequate Vitamin D levels may contribute to rapid bone loss and osteoporosis.(1) Vitamin D deficiency has been

reported to have high prevalence in acute spinal cord injury patients and may be a factor for rapid bone loss in acute phase of spinal cord injury.(58)

Studies have shown adequate Vitamin D replacement in spinal cord injury patients helps to reduce the bone loss by decrease in bone resorption markers.(59) These findings make us explore the equation between vitamin D and rate of bone loss in spinal cord injury patients. Remodeling comprises of bone formation and resorption, the bone loss in spinal cord injury is explained by the fact that bone formation shows minimum rise whereas bone resorption significantly increases(61). Formation and resorption biomarkers give some idea about the rapid turnover and therefore throw light on the mechanism and quantity of bone loss. This bone loss in later stage presents as osteoporosis and predisposes to low trauma fractures such as fall during transfers, from wheel chair or from standing height.(58)(62) Bone turnover is rapid during the acute phase and thereafter plateaus to continue at slower rate for few years.(63) Prevention of bone loss beyond the fracture risk or fracture threshold levels can largely avoid the low trauma fractures. In spite of significant literature, suggesting early bone loss in spinal cord injury, none quantifies the loss and its progression with time. This study observes the bone resorption and formation markers over a period of six months and compares with bone markers of pre and post menopausal females from the community.

OSTEOPOROSIS IN SPINAL CORD INJURY

Osteoporosis is an inevitable complication found in almost every individual following Spinal cord injury. Osteoporosis is often treated only when patient has obvious clinical fractures. As the treatment following fracture is quite difficult and non satisfactory, there has been paradigm shift towards the approach to problem: Prevention of acute bone loss in SCI (<1 year) and effective treatment of established low bone mass with long-standing SCI (≥ 1 year).(64)

Osteoporosis is defined by the World Health Organization as a condition characterized by "low bone mass and micro architectural deterioration of bone tissue leading on to increase in the bone fragility and predisposing to fracture(65). Among the various modalities currently available to diagnose osteoporosis, assessment of the bone mineral density by dual-energy X-ray absorptiometry is most commonly used in clinical practice. Despite being the current practice, BMD has several limitations in qualifying as an ideal diagnostic test for assessment of osteoporosis. Addition of clinical risk factors to the BMD value has led to the development of SCI FRAX-Spinal Cord Injury Fracture Risk Assessment Tool which helps in predicting the risk of fracture development.(66) However, both SCI FRAX and BMD assessment failed to incorporate one of the most important factor determining bone strength i.e. bone remodeling.(65) Moreover BMD do not give us correct idea about the bone loss when repeated within a period of 12 months. This has led to the development and use of bone turn over markers (BTM) which guides us about the bone loss over a period of time. Recent literature reports about 30% bone loss within one year of injury, hence it becomes essential to observe the change over a period of time, and prevent the bone

loss earliest possible.(67) Hence the bone markers are very important in identification of the peak phase of bone loss, which is difficult to detect with the DEXA scan. The most commonly used bone resorption marker is breakdown products of type 1 collagen (amino or carboxy terminal cross linking telopeptides, pyridinium cross links). This study will enable us to study the rate and pattern of bone loss in patients with acute spinal cord injury in comparison with age matched healthy controls (pre and postmenopausal females). This would help to take appropriate measures to prevent bone loss, osteoporosis and low trauma fractures during the chronic phase.

Magnitude of osteoporosis in the Indian SCI population

Bone loss is an inevitable complication of spinal cord injury. It is reported to be found in almost every individual at certain point of time following spinal cord injury. Hence primary prevention of the disease becomes essential both for the patient and the health care givers. The prevalence of osteoporosis is about 80% in spinal cord injury patients. A wide range of facture prevalence (1-35%) has been reported and it increases with increase in age.(3) After an initial phase of rapid bone loss, the bone remodeling rate plateaus down but do not touch the baseline and continue at the same rate thereafter.(68)(56) Immobilization, reduced sun exposure, inadequate nutritional status, hormonal imbalance all these risk factors put the spinal cord injury patients at high risk for osteoporosis. Hence it becomes essential to evaluate the bone loss in the acute phase and prevent the rapid bone loss, which ultimately will prevent osteoporosis.(69) No Indian literature is available correlating the biomarkers and bone loss in spinal cord injury patients.

BONE METABOLISM MARKERS

These are the newer modalities of assessment of dynamic activity of bone. They provide us information regarding the different phase of activity and are broadly classified as formation and resorption markers.

Marker of bone resorption

C Telopeptides (CTX)

The bone resorption markers are breakdown products of type 1 collagen.
They are derived from the carboxy terminus of the type 1 collagen as result of oosteoclastic activity. This can be measured both in the serum and urine. The C terminal telopeptide of type 1 collagen undergoes beta isomerization and racemization, which is an age dependent process and hence the ratio of alpha to beta CTX can differentiate recently broken down collagen to aged collagen and used as an index of rapid bone turnover. This ratio is more in patients with high bone turnover like Pagets disease and reduces with bisphosphonate therapy. These are not only useful in assessing the need for treatment and response to treatment, if these levels are higher in post menopausal women they would predict a higher risk of fracture(70).

Measuring ranges

Male 20-50 years – Normal range 142-584 pg/mL

Female premenopausal - Normal range 137-573 pg/mL

Female postmenopausal - Normal range 226-1008 pg/mL

The detection limit represents the lowest measurable analytic level other than zero. It is calculated as two standard deviations above the lowest standard (standard 1 + 2 SD) repeatability study.

Analytical sensitivity: Lower limits of measurement

Analytical specificity: No cross reactivity was detected with monoclonal antibodies for beta cross laps, parathyroid hormone and bone specific alkaline phosphatase.

Markers of Bone Formation

Bone formation markers are by products of osteoblasts expressed at different phases of osteoblast activity hence they reflect different osteoblast function and of bone formation.

Serum Total Alkaline Phosphatase (AP)

Alkaline Phosphatase is anchored to the cell surfaces of osteoblasts. The precise function of AP although not clearly known, it is supposed to play a role in osteoid formation and mineralization, by degradation of the mineralisation inhibitor pyrophosphate.(71).The advantages being its low intra-individual variability (<10%), minimal effect on renal function, minimal interaction with food, long half-life (1–2 days), sample stability and cheap rates. (72)(73) It has about 20% cross reactivity with liver isoforms. In adults with normal liver function, approximately 50% of the total Alkaline Phosphatase activity is derived from the liver, whereas 50% arises from bone. If hepatic dysfunction is ruled out, serum levels of total AP gives good impression regarding new bone formation and osteoblast activity. Variation with exercises is minimal, i.e. less than 25% of LSC. (least significant change).(74).From a

clinical perspective, detection of the bone-specific AP (BAP) isoenzyme is preferred because of higher specificity.(75)

Osteocalcin (OC)

Osteocalcin is a hydroxyapatite-binding protein synthesized by osteoblast and hypertrophic chondrocytes. It is also called as the bone gla protein and constitutes 15% of the non collagenous bone matrix. OC is an active molecule involved in the organization of the extracellular matrix. Previous studies suggest OC involvement in osteoid mineralisation, as it is expressed mainly during bone formation. OC is considered a specific marker of osteoblast.(76) Intact molecule of osteocalcin is unstable, having a short half-life of a few minutes. OC gene is regulated at transcriptional level by Vitamin K and 1,25-OH2 Vitamin D, which act cofactors for γ-carboxylation of OC resulting in increased affinity for calcium and hydroxyapatite(77). It has short half life, large inter-lab variation and is influenced by Vitamin K status, renal function and circadian rhythm.(78)

Measuring ranges

Male < 30 years – Normal range 24-70 ng/mL

Male 30-50 years- Normal range 14- 42ng/mL

Female premenopausal - Normal range 11-43 ng/mL

Female postmenopausal - Normal range 15-46 ng/mL

Relevance of measuring bone markers in acute spinal cord injury scenario

Bone markers can be tested early in the course of treatment (as early as 3 months).This will enable the treating physician to keep track on the dynamic changes in the bone, which is not significant with DEXA scan, check compliance and adequate response to therapy even before second DXA scan showing improvement in Bone Mineral Density. It is not an expensive (INR 200) investigation. If measurements fail to show an improvement, alternative medications or parenteral therapy (if patient was already on oral medications, ex: Bisphosphonates can be used for treatment.

The role of repeat BMD is not yet proven to correlate with anti fracture effects or reduction in fracture risks, rather some other non clinical parameters are thought to be better predictors of fracture risks.(22) (23) Increased bone loss and osteoporosis are inevitable complications of spinal cord injury.(1,58,68,79,80) Reduced bone strength predisposes them to low-trauma fractures.(58,66,81)(3) Fracture affects the freedom of mobility, activities of daily living and quality of life of persons and increases the duration and cost of hospitalisation with spinal cord injury**.**(63) Pitfalls **-** No ethnicity specific values. No studies in Indian population has been done to assess the rate of bone loss in acute spinal cord injury patients, especially in the acute phase where literature worldwide are also few.(82) Prevention of bone loss beyond the fracture risk or fracture threshold levels can largely avoid the low trauma fractures.(66) This can be done by routine assessment of risk factors and secondary causes of osteoporosis among spinal cord injury patients. Currently there is no evidence to suggest Vitamin D levels correction to therapeutic levels may prevent bone loss (81),(83), but there are promising results with antiresorptive agents which may prevent osteoporosis and low

trauma fractures.(77)(78) This study will enable us to compare bone loss in spinal cord injury patients with the control/ high risk groups; pre and post menopausal females. This will help to identify early bone loss and emphasize on early treatment in acute phase to prevent osteoporosis and low trauma fractures in chronic spinal cord injury patients.

Bone markers in the assessment of fracture risk

After long duration research and clinical trials, Bone Turn over Markers (BTM) are now recognised as tools for clinical management of metabolic bone diseases. Some of the markers lack clinical correlation and are limited by the high biological and individual variability; few like C Telopeptide represent more realistic applications of bone markers in clinical studies. Large epidemiologic studies have shown that bone remodeling and its products the bone turnover markers are independent contributors of fracture risk and are correlate well with therapeutic management**.**(86)

VARIABILITY OF BONE MARKERS IN DIFFERENT INDIVIDUALS

Intra subject variability poses a major difficulty in practical use of bone bio markers. Whenever a change is observed in bone marker levels, in an individual patient (e.g. following an intervention), this must be interpreted against the background of possible variability associated with the particular marker. Therefore, knowledge regarding sources of variability and different strategies to cope with them are essential for the meaningful interpretation of results.

A number of factors need to be considered when serial measurements of biochemical markers are assessed to determine changes in bone turnover. To minimize limitations linked with analytical variability, standardized sampling and sample handling methods are mandatory for obtaining reliable results.

In addition to parameters of assay performance factors such as the choice of sample (i.e. serum versus urine), correct handling, processing and storage of specimens should be considered. All these steps are important as the technical sources of variability are controllable and modifiable. Some bone markers are temperature sensitive, some are sensitive to haemolysed sample which result in values that are either too high or too low. Osteocalcin is usually more sensitive, but variations have been described in other serum markers also.

Intra-individual (i.e. biological) variability is difficult to control than the technical aspects of variability. Age, gender, ethnicity are the non modifiable biological variations. All these factors should be considered while interpreting the results of bone bio markers. Serum and urinary concentrations of bone markers are high during

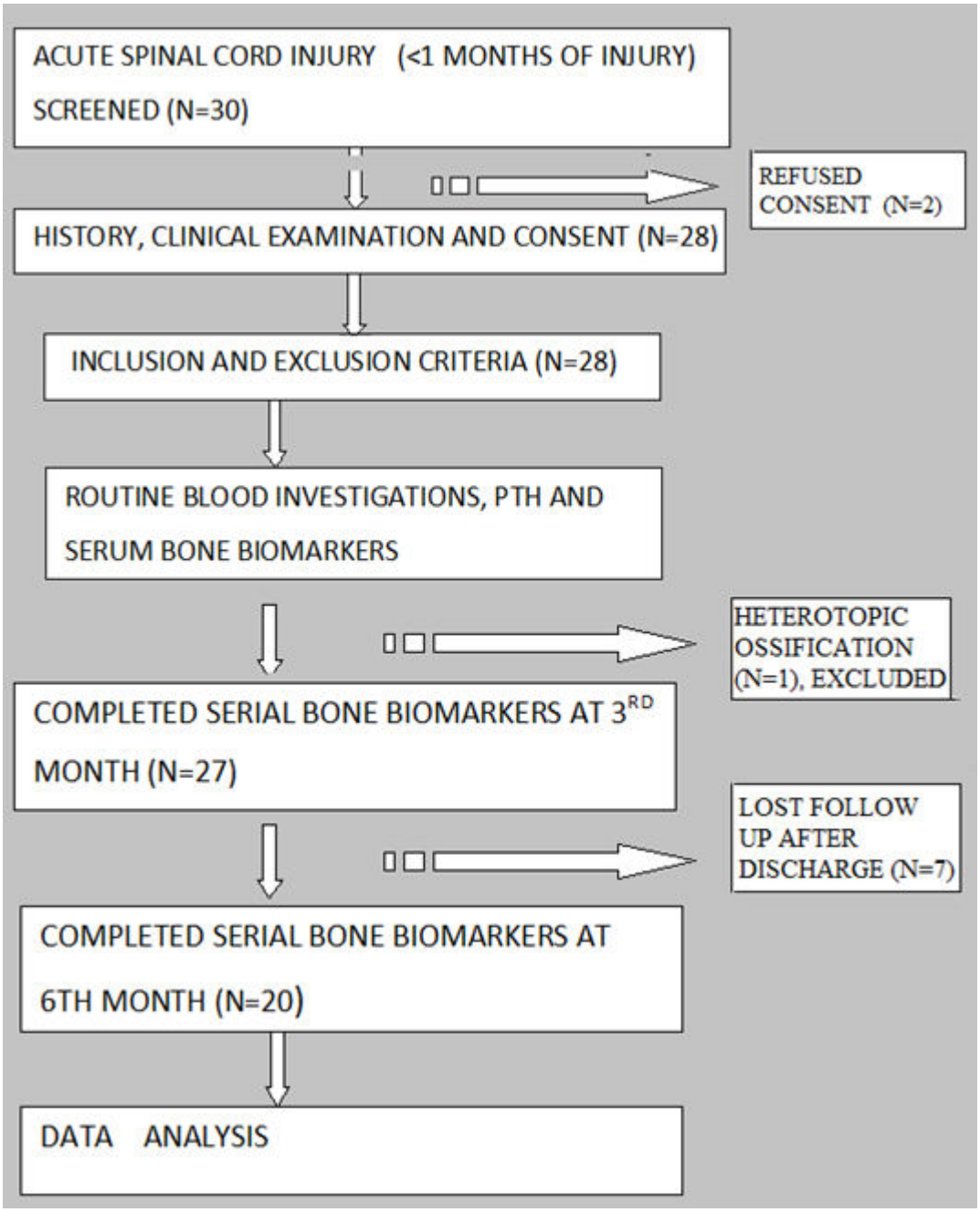
normal puberty and growth phase, but declines as the growth subsides. These are usually stabilised by the 3rd decade in healthy men, and practically remain more or less unchanged until 70 years. Spinal cord injury is associated with a substantial increase in bone turnover, evidenced by a 50–70% increase in bone resorption markers. Some biomarkers of bone are excreted via kidney (i.e. OC, C Telopeptide), change in glomerular filtration may significantly affect urinary and serum concentrations. Hence serum creatinine levels are monitored to look at the variations.

MATERIAL AND METHODS

MATERIAL AND METHODS

This is a prospective study of bone biomarkers in patients with acute SCI enrolled within 1 months of sustaining injury. This study was conducted in the Department of Physical Medicine and Rehabilitation. Acute spinal cord injury patients aged between 18-45 years, who met the inclusion and exclusion criteria were enrolled for a period of 6 months from April 2014 to June 2015. Thirty patients were enrolled for the study; twenty acute spinal cord injury patients completed the study. Baseline demographic parameters, age, sex were assessed. Serum calcium, phosphorus, creatinine, albumin and alkaline phosphatase were assessed as part of routine clinical evaluation. Fasting samples of Serum C Telopeptide and Osteocalcin, bone resorption and formation markers respectively were collected. Patients with hyperthyroidism, hyperparathyroidism, hepatic or renal dysfunction, malabsorption or on medications for osteoporosis were excluded. Enrolled patients were followed up with serial estimation of bone biomarkers at 1, 3 and 6 months. Data was collected to observe the change in bone biomarker levels among acute SCI patients over a period of six months and statistically analysed. In addition, serum bone biomarkers of the patients were compared with age matched individuals from the community, from another study conducted at Endocrinology Department of our institution.

DIAGRAMMATIC ALGORITHM OF THE STUDY



PARTICIPANTS

Inclusion Criteria:

- Patients < 1 months of traumatic spinal cord injury
- Age group 18 -45 years.

Exclusion Criteria:

- Patients with non traumatic spinal cord injury.
- Patients with renal failure (Contracted kidneys in the ultrasound and serum creatinine >1.5 mg%)
- Patient with bone disease (Including Heterotopic Ossification)
- Patient already on other treatment for osteoporosis.
- Patient taking drugs regularly for other diseases, which are likely to develop osteoporosis (e.g. steroids, anti-inflammatory agents etc.)
- Patient who is not able to give informed consent.

STATISTICAL METHODS

All baseline variables were expressed in terms of mean ± SD, if they are continuous variables. All categorical variables were reported using frequencies and percentages. The distribution of CTX and Osteocalcin was checked by plotting the histogram and QQ plot. If the distributional assumption satisfies normality, then the measurements will be expressed as mean and standard deviations. If not normally distributed, Shapiro Wilk's test was performed to check for normality assumption and then median with inter quartile range was reported. The comparison of the two groups was done using t-test for the change in bone markers at 1^{st} and 3^{rd} and 3^{rd} and 6^{th} month of SCI, if assumption of normality held good. If not, Mann Whitney U test will be done to compare between the groups. Bonferroni correction was used to adjust the p value. ANOVA was used to compare more than two groups. All the analysis was done using SPSS (version 16).

RESULTS

RESULTS

During six-month study period, 30 subjects were enrolled among which 20 completed the study. Two patients refused to participate, one was detected to have Heterotopic ossification (HO) and seven patients were lost to follow after discharge. Most of the patients were from remote areas and were unable to continue participation. There were 19 males and 1 female in the study. Mean age at the time of SCI was 29.3 years (18–45 yr). Among the 30 patients enrolled, two refused consent, one had Heterotopic ossification (HO) and seven were lost to follow up after discharge. Two had lower limb deep venous thrombosis and Heterotopic Ossification (HO) both simultaneously. Among two patients with HO, in one patient it was detected at 4 month duration, and was not included in the study. One patient lost to follow up after 3rd month later presented with HO.

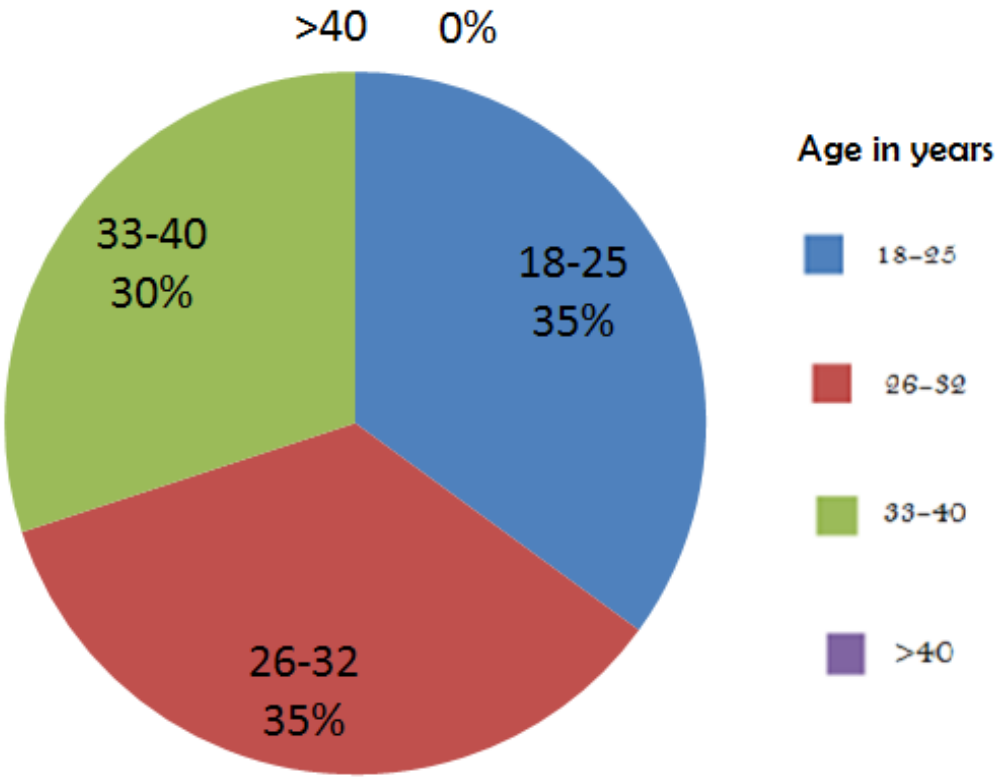


Figure 1 : Distribution of patients according to age (years)

The severity of the injury was assessed by ASIA score (American Spinal Injury Association Score: Classified from A to E) A indicates the maximum severity and poor prognostication and E indicates the best prognosis similar to normal. In this study patient with ASIA A-C has been included as they are most likely to be affected with spinal cord injury related bone loss. 80% (n=15) of the population was ASIA-A, 5 % (n=1) ASIA-B, and 15 % (n=3) ASIA-C. Patients with ASIA-A had highly raised bone resorption marker C Telopeptide, in comparison to ASIA –B and ASIA- C. but was statistically not significant (p value - 0.069) where as no correlation was observed with bone formation marker Osteocalcin. As patients with ASIA-B and ASIA-C were few it was difficult to comment on its statistical association with the bone markers.

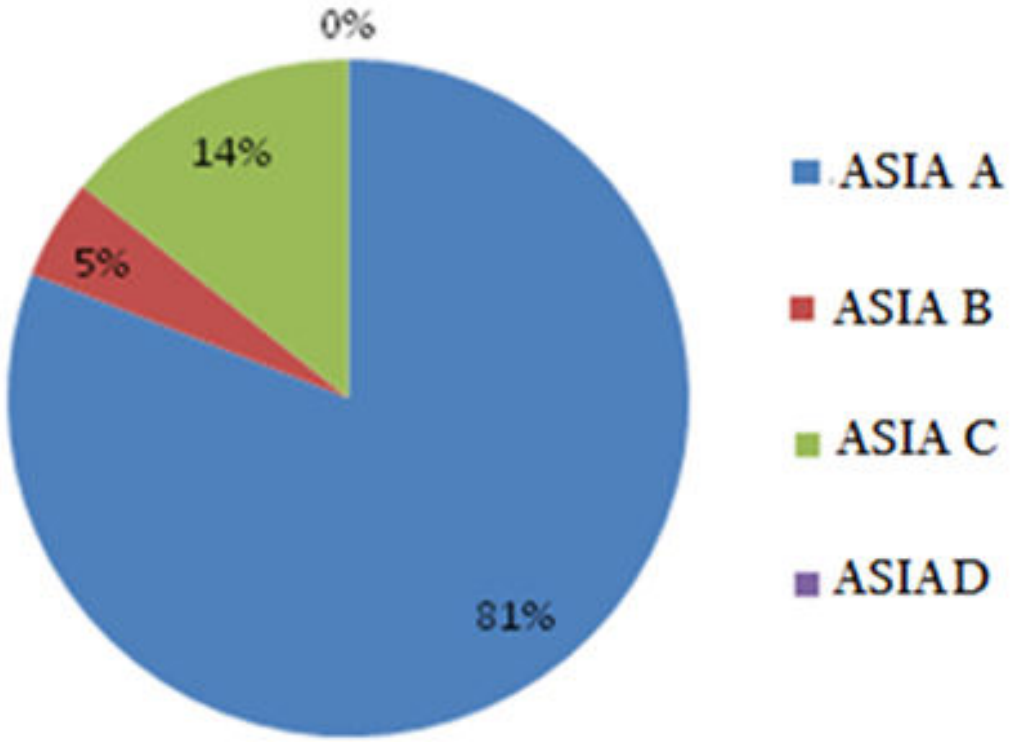


Figure 2: Distribution of Patients accoring to ASIA grading

Biochemical data

Serum calcium levels were within normal range (8.3-10.4 mg/dl) at one month of the study. PTH levels were at the lower normal range (8-74 pg/ml) with the mean value being 15. 2pg /ml (shown in Figure 3 below) this can be probably explained by the fact that immobilisation related hypercalciuria causes suppression of serum PTH and 1, 25 (OH) Vitamin D axis.

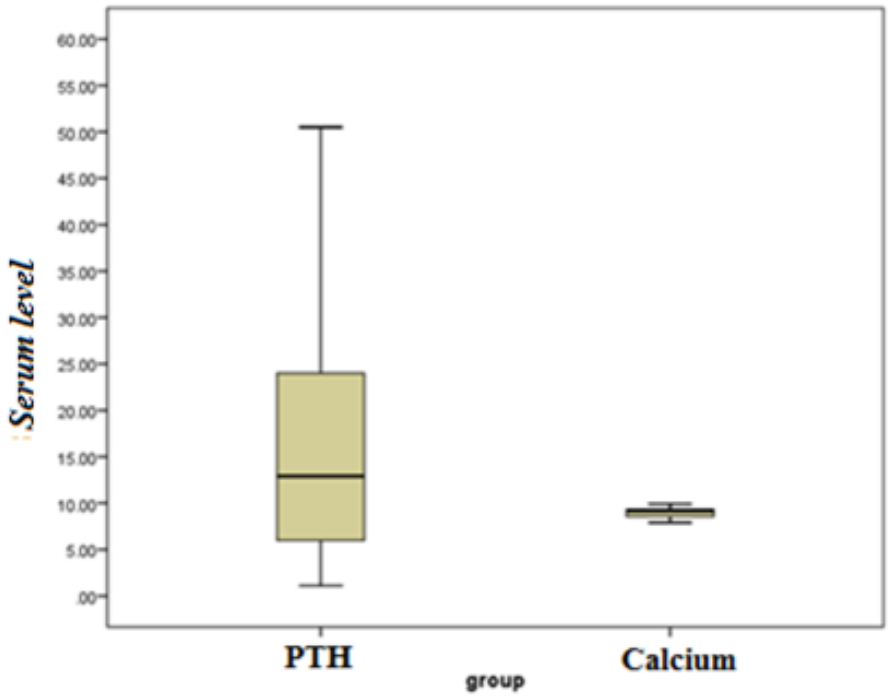


Figure 3: Mean serum levels of Calcium and PTH at 1 month of SCI

The mean vitamin D level among study population was 14.9 IU (Normal 20-30 IU). This can be probably due to poor nutritional intake, inadequate sunlight exposure or as a response to hypercalcemic status during early phase of injury. It was observed that there was no correlation between serum vitamin D levels and bone biomarkers namely C Telopeptide and Osteocalcin (Figure 4a and 4b). Bone-specific alkaline phosphatase constitutes 50% of total AP, and in absence of liver disease, it reasonably reflects bone

formation in our subjects. Total Alkaline phosphatase (tAP) was marginally raised at one month of study and not significantly greater than those at baseline.

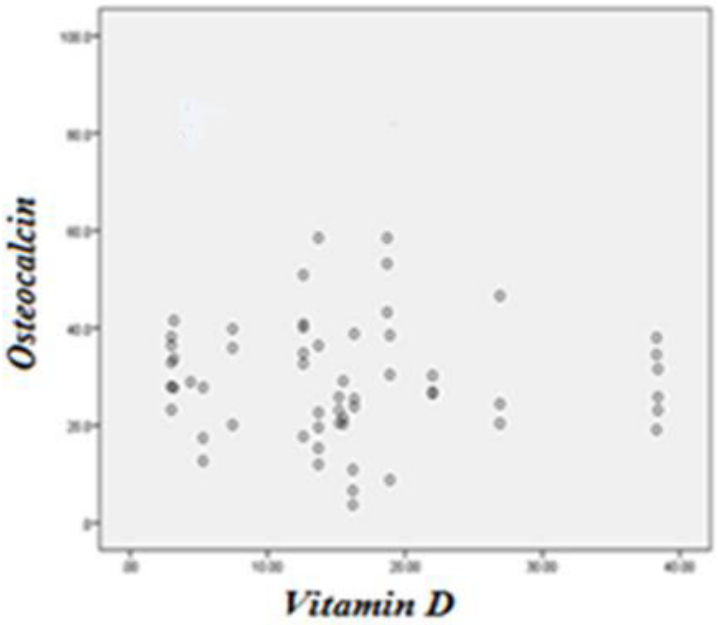


Figure 4a: Scatter plot graph to observe the association between bone formation marker osteocalcin and Vitamin D

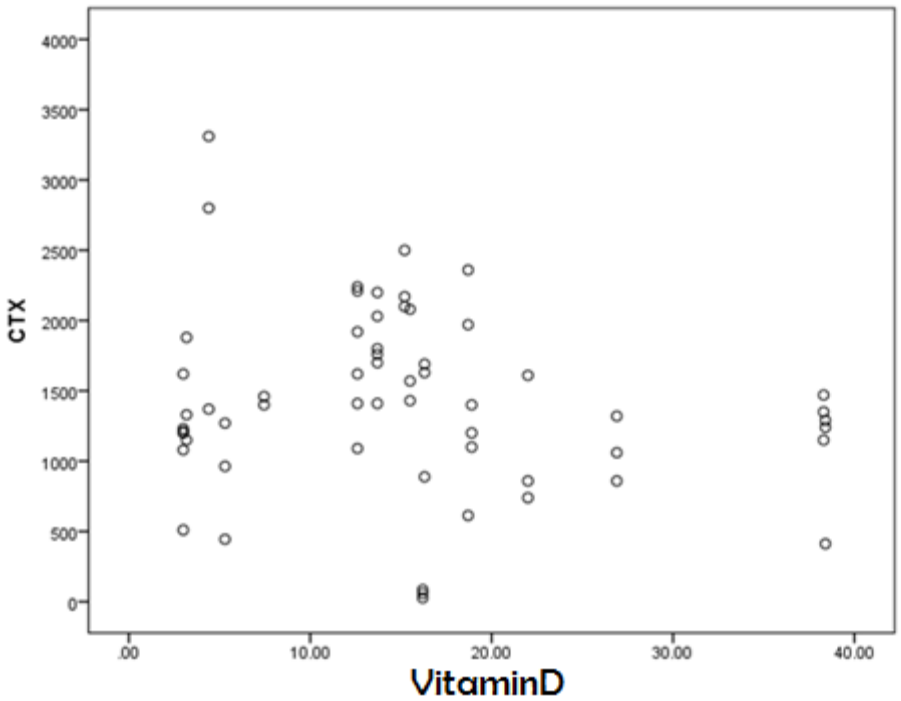


Figure 4a: Scatter plot graph to observe the association between bone resorption marker C telopeptide and Vitamin D

Bone formation marker, Serum Osteocalcin levels were assessed at 1, 3, 6 month. It was observed that there was a marginal rise at the 3rd and 6th month. Serum Osteocalcin levels of these patients were compared with Osteocalcin levels of pre and post menopausal females from the community. It was noticed that there was modest rise in Osteocalcin levels among SCI patients, but was statistically insignificant in comparison to post menopausal females. (Figure 5, 6) Osteocalcin levels were measured from morning fasting blood sample. The upper normal range of premenopausal women as taken from our reference laboratory values was 30ng/dl. In this study pre menopausal females represented the normal population and the post menopausal women represented the group vulnerable to osteoporosis.

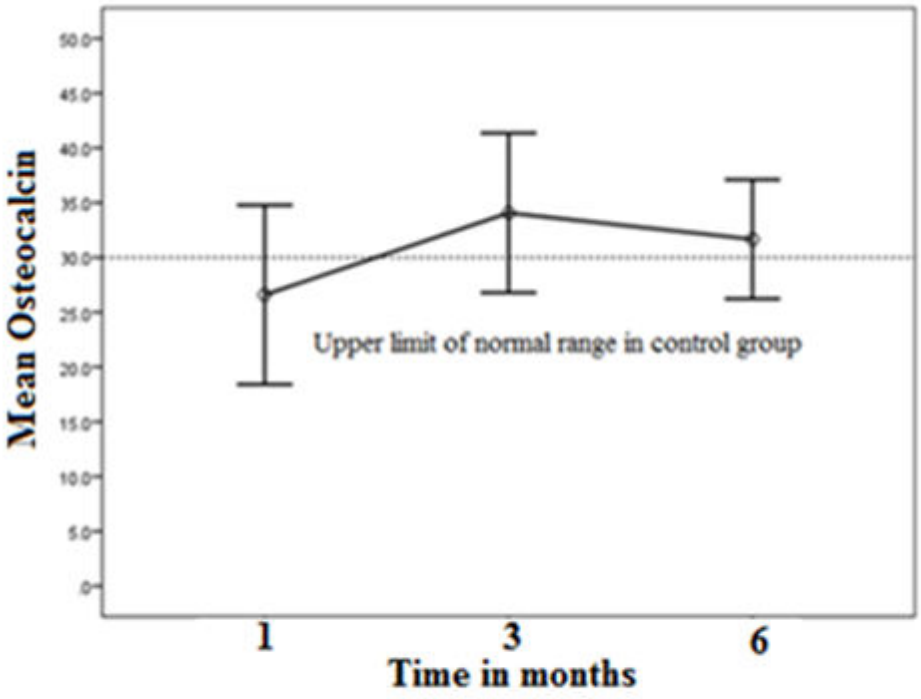


Figure 5: Bone formation marker Osteocalcin after acute SCI. The dotted line represents the upper normal limit for adult pemenopausal females. The data are represented as mean SE+/-95CI

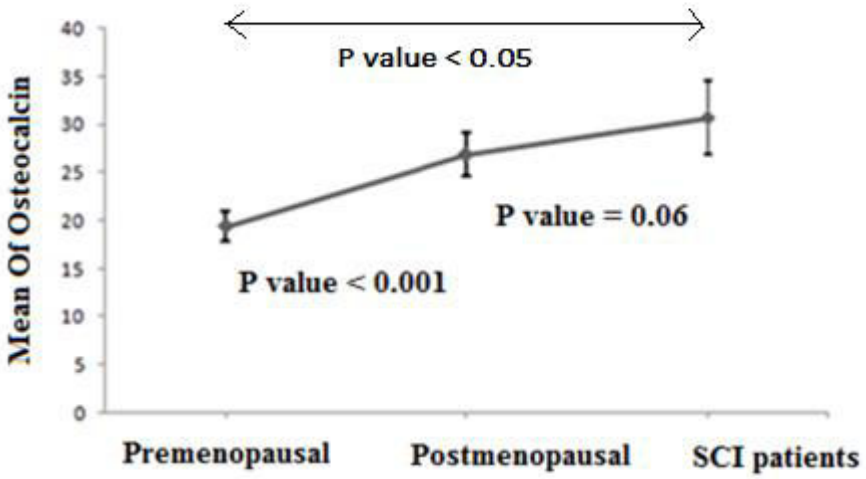


Figure 6: Comparison of serum Osteocalcin levels in premenopausal, post menopausal and acute SCI patients. Serum levels of Osteocalcin show significant rise n acute SCI patients

Bone resorption marker, Serum C telopeptide levels were assessed at 1, 3, 6 month. It was observed that there was significant rise in bone resorption markers in comparison to normal C telopeptide levels. Maximum rise was observed at 3 to 4 month interval following which there was a statistically significant drop at 6 month, but the CTX levels were still persisted significantly higher than the base line values, suggesting ongoing bone loss. Serum C Telopeptide levels of these patients were about 3 times increased in comparison to the mean values of C telopeptide levels of Pre and post menopausal females.(Figure 7 and 8) In this study pre menopausal females represented the control group and the post menopausal women represented the group vulnerable to osteoporosis.

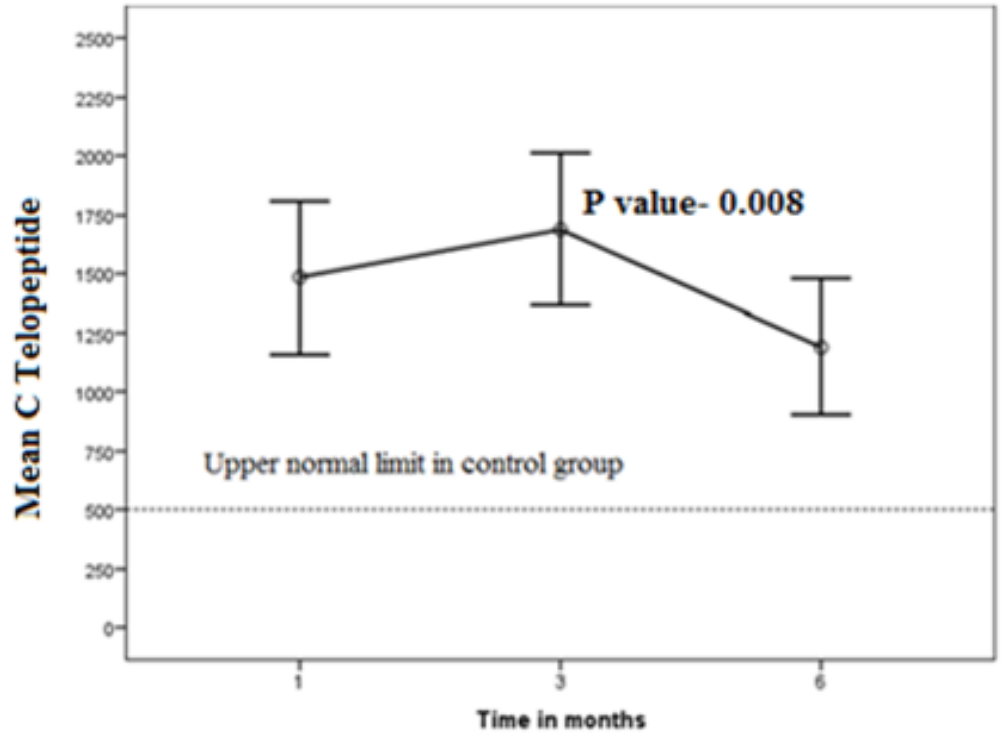


Figure 7: Bone resorption marker C telopeptide after acute SCI. The dotted line represents the upper normal limit for adult premenopausal females. The data are represented as mean SE+/- 95CI

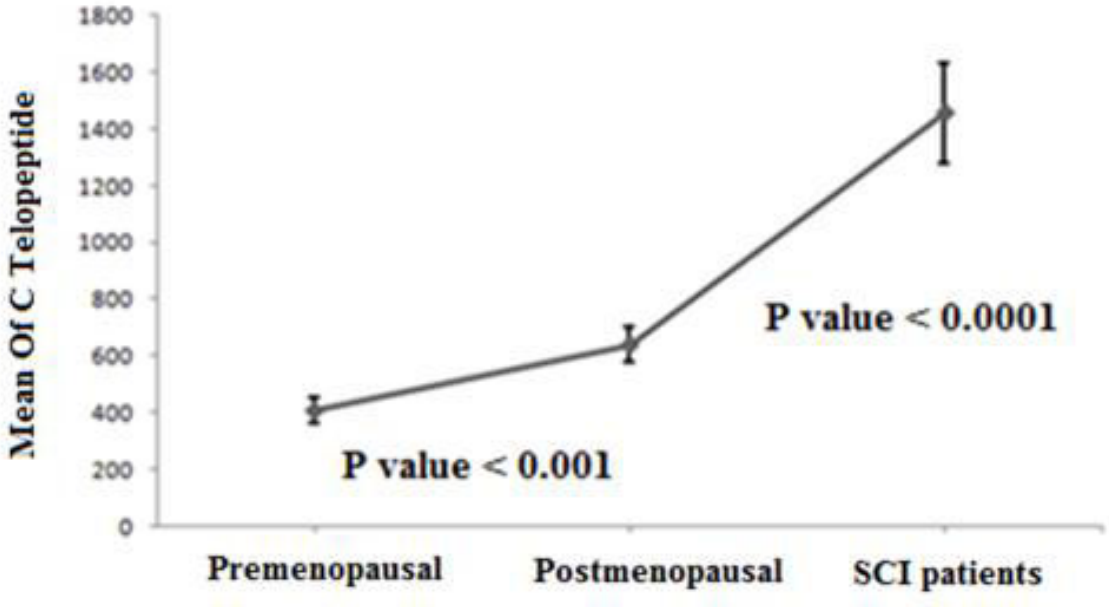


Figure 8: Comparison of serum C Telopeptide levels in premenopausal, postmenopausal and acute SCI patients. Serum levels of C Telopeptide show significant rise in acute SCI pateints

Mean values of serum Osteocalcin and Ctelopeptide were compared among patients with flaccid paralegia and patients with spastic paraplegia.There was no significant corelation between tone of the muscles (spasticity or flaccid) and bone markers as shown in figure 9 below.

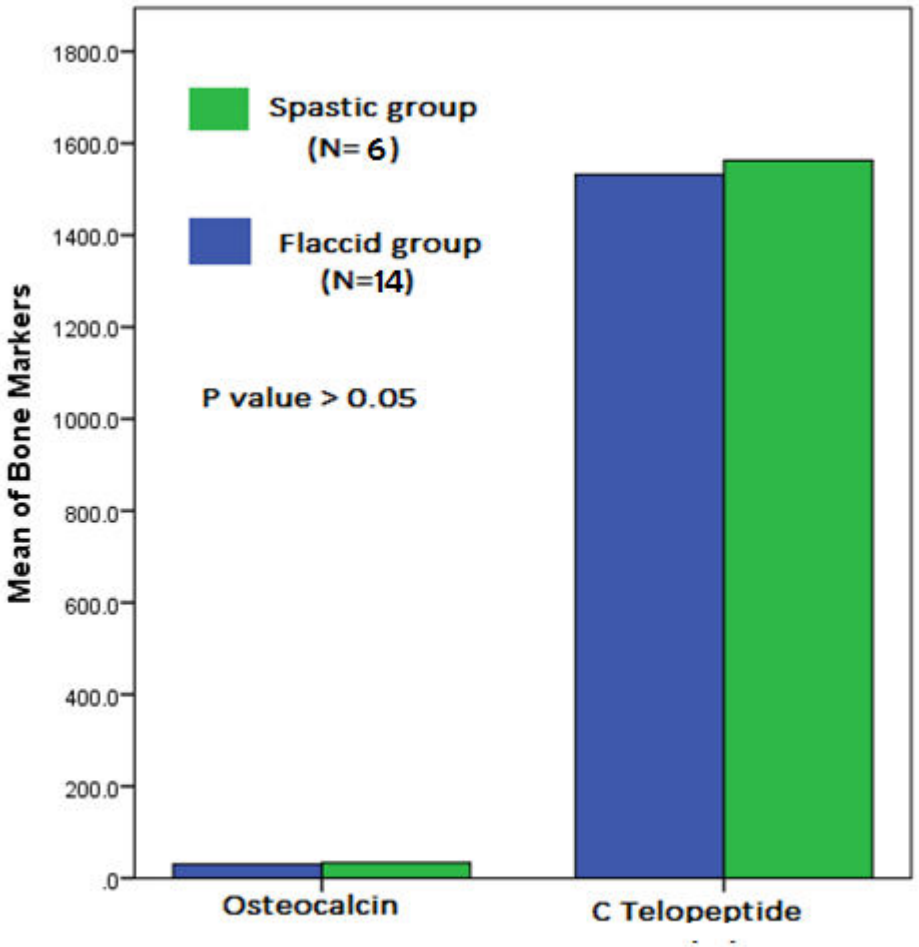


Figure 9: Correlation between mean values of bone markers and muscle tone (spasticity and flaccid tone) among SCI patients

DISCUSSION

DISCUSSION

This is a longitudinal study observing the bone activity among acute SCI patients by means of bone markers. These bone turn over markers show ongoing metabolic changes during the period of study. Acute SCI causes significant neuromechanochemical changes in the body, as a result of which sublesional skeletal system undergo rapid remodelling, i.e. increased rate of bone resorption and bone formation. In this study the most striking feature noticed was the bone resorption activity (indicated by serum C Telopeptide levels), was about 3 times higher than its upper normal limit. In contrast, there was a modest rise in bone formation marker, Osteocalcin and tAP, marginally raised than the reference values. This indicates gross uncoupling of remodelling cycle in favour of bone resorption, where bone formation lags behind significantly.

Significant rise in resorption markers were observed at 4 weeks of SCI. Highest values were noticed around 12–16 weeks, followed by a statistically significant (p value - 0.008) dip at 24th week. Although there was a significant drop observed at sixth month, the resorption markers remained significantly higher than normal base line values. Literature suggest raised bone markers due to increased bone turnover activity after acute SCI, which return towards baseline by 6–14 months, maximum bone loss being at 4 months post injury.(2)(87) In this study bone markers value have not reached the baseline at 6 month interval, suggesting ongoing resorption activity and bone loss. As revealed from a study, 30% of the post menopausal females lose bone at faster rate than the normal; similarly each spinal cord injury patient may behave

differently according to their level and grade of injury.(71) Thus serial monitoring of bone markers helps to appropriately judge the bone activity.

Bone markers of premenopausal and post menopausal females were taken from another study (Conducted by the Department of Endocrinology) at our institute as control values. Premenopausal females were taken as base line controls and postmenopausal females were taken as vulnerable group to bone loss and osteoporosis. Bone markers of both these group were compared with mean values of bone marker in acute SCI patients. As observed in this study, bone resorption marker, C Telopeptide levels were significantly higher than post menopausal females. This suggests that multiple factors other than hormonal imbalance are responsible for acute bone loss. Prolonged lack of a loading response during the acute stage probably down regulates the mechanosensory feed back at the cellular level (Osteocytes), which is responsible for promoting bone formation and inhibiting the resorption promoting agents like sclerostin. Normal muscle tone is under the influence of CNS (Brain and spinal cord) and is regulated by feedback mechanism from the stretch reflex. Specialised sensory organs, muscle spindles act as receptors to “stretch” and carry afferent impulses via dorsal root to spinal cord, where it activates the alpha motor neuron to cause contraction of extrafusal muscle fibres. Hence, stretch increases afferent firing and activates motor neurons to contract the muscle and reduce afferent firing. Acute SCI presents with a state of spinal shock, resulting in flaccid muscle tone due to neuronal disruptions within the cord. Flaccid muscle tone may further minimise the mechanosensation, in addition to mechanical unloading, at the cellular level and

aggravate the bone loss by rapid recruitment of resorption activators which results in early and significant bone loss. However in this study comparison of bone markers among patients with spastic and flaccid muscle tone after SCI did not show any significant correlation. Further studies comparing bone markers with spasticity in large number of patients may provide better insight regarding the association of bone markers with muscle tone.

At 4 weeks, the cohort had calcium values within normal range (8.5-10.5 mg/dl.). An interesting observation was of serum PTH levels, which were at the lower normal range (Normal 8-80 ng/dl) in most of the SCI patients. The mean value of serum PTH level among these patients was 15.2ng/dl. Only four patients had PTH values > 20 ng/dl, among which one patient had Heterotopic Ossification (HO), two were of ASIA B and two of ASIA C, none with ASIA A. The dynamics of PTH with neurological severity, bone resorption and HO needs to be further evaluated with serial PTH monitoring. In this study no correlation was observed between PTH and bone markers. Lower distribution of PTH levels may probably be due to mobilization of skeletal calcium because of SCI related immobilisation hypercalciuria. Studies have shown that PTH remain suppressed for about 1–4 months post-SCI and then return towards normal range.(88) None of the subjects in the study developed clinically significant hypercalcemia.

Most of the study patients (n=16) had Vitamin D levels < 20 IU and were started on supplementation with oral cholecalciferol. No association was observed between

Vitamin D and bone markers, suspecting its role in acute bone loss. Moreover, reliable dietary details were not obtained hence it was difficult to comment on its effect on bone biomarkers. Inpatients in rehabilitation centre spend time outdoors with adequate sunlight exposure which will help to prevent hypovitaminosis D. As secretion of Osteocalcin is dependent on Vitamin D, supplementation of vitamin D may have influenced Osteocalcin results.(89)(90) Statistically no correlation was observed between Vitamin D and serum bone biomarkers.

Bone densitometry was not a part of this study as change in DEXA scan becomes noticeable only after 6-12 months of duration usually in the lower limbs. BMD is preserved at the lumbar spine in SCI patients, probably due to loading of vertebral column on attaining the sitting posture whereas distal femur BMD is depleted by 22% at 3 months, 27% at 4 months, and 32% at 14 months.(87) BMD of entire lower limbs, instead of a part of lower limb decreases the error as larger area is being measured. Review from previous studies show significant difference in BMD at the lower end of femur and proximal tibia as the earliest one to involved with maximum change noticeable in this region.(2) BMD of the lower limbs probably at one or two year interval until the biomarkers reach the baseline (state of normal bone turn over activity); may be a good option for knowing the residual density at that point of time. Bone biomarkers reflect the bone activity, it does not comment on the bone mineral density (BMD) as standardised by Z- Score of - 2.5 and -1.5 for osteoporosis and Osteopenia respectively. Z scores at the baseline remodelling rates will indicate no further risk of active bone loss, where as Z scores with increased resorption rates will

indicate higher risk for fracture. Hence both together can prognosticate the fracture risk better than being guided by individual parameters.

Source of potential bias was the varying degrees of mobilisation (wheel chair/ Crutch ambulation) and duration of mechanical loading depending up on their neurological levels. All the participants continued the routine therapy during their stay in rehabilitation centre, which included wheel chair ambulation, therapeutic standing and ambulation with walker and elbow crutches, as per there neurological levels. To avoid heterogeneity of the population, SCI patients with quadriplegia were not included in the study.

Sublesional fractures are more common in paraplegics than in quadriplegia population. This may be due to increased independence in mobility in paraplegics which makes them prone to trivial trauma, while ambulation or transfers. Prospectively looking at spinal cord injury population, admitted during the year 2014-2015 revealed 17 SCI patients having lower limb fractures. Most of them are paraplegics performing transfers and ambulation independently. The history of fractures almost never pointed towards an event / trauma pertaining towards fracture. This suggests trivial trauma at the back ground of sublesional fractures. Long term prospective studies including larger numbers of subjects, with appropriate stratification OF neurological level of impairment (ASIA Grading), degree of immobility and presence or absence of spasticity will provide further insight in the mechanism of fractures. Recent prospective longitudinal study showed 25 % of

traumatic SCI had fractures. None of the factors correlated with fractures, except ASIA grading. ASIA A SCI patients had highest risk of fractures and were mostly observed after 6 years of injury.(8) In this study, analysis of ASIA A scores and bone biomarkers suggested that there was correlation between ASIA A and resorption markers, where as there was no correlation with bone formation markers. As there were only 5 patients with ASIA B and C statistical correlation was insignificant. This may be due to extensive paralysis seen in ASIA - A group of patients.

This was a longitudinal study of bone markers among acute SCI patients to detect early bone changes in patients following acute SCI. It showed significant rise, (about 3 times of upper normal limit) in bone resorption marker, C Telopeptide, suggesting ongoing rapid bone loss. Based on the results of this study, it would be interesting to notice the response of the biochemical markers when treated during the acute phase of SCI. It also would help to select appropriate medications which would prevent bone resorption or facilitate osteogenesis.

LIMITATIONS

LIMITATIONS

1. Although the results of bone resorption markers was concrete, other parameters and its correlation with the severity of injury (ASIA scale) was not clearly established due to small sample size..

2. It was a 6 month follow-up study, at which the bone markers did not reach the base line levels. A long term study (2 year follow up), combined with bone markers and Dexa Scan (one year interval) will probably provide better insight regarding dynamic bone activity and available density at a particular time interval.

3. Stratification of patients according to their neurological level and ASIA grading, tone of the lower limbs (spasticity / flaccidity), mechanical loading and participation in mobilisation activity varied in all the subjects according to their neurological status and their level of activity.

CONCLUSIONS

CONCLUSIONS

1. Biochemical markers of bone turnover observed longitudinally in a cohort of acute SCI patients demonstrated a dramatic rise in bone resorption markers but only a modest rise in bone formation markers.

2. When compared to age matched premenopausal females, taken as control group and post menopausal females as vulnerable group there was significant rise in bone resorption markers.

3. Bone markers show significant changes as early as one month after SCI, much before DEXA Scan detects Osteopenia and Osteoporosis. The resorption markers started declining after 4 months of SCI but did not return to base line until 6 months suggesting ongoing bone loss at significantly higher levels.

4. It was observed that there was no correlation between serum vitamin D levels and bone biomarkers

.

FURTHER RESEARCH RECOMMENDATIONS

Fracture prevention should be the goal in people with SCI as it increases the morbidity significantly. Hence would suggest longitudinal studies of bone biomarkers until these returns to base line and its relation with BMD of lower limbs. This would help to initiate appropriate treatment sufficiently early to prevent pathological fractures among SCI patients. Further studies to observe the response of bone biomarkers on treatment with different pharmacological agents that influence bone remodeling will be the next step ahead in minimising early bone loss and preventing pathological fractures.

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ANNEXURE

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDI
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

April 22, 2014

Dr. Prince Thakkar
PG Registrar
Department of Physical Medicine and Rehabilitation
Christian Medical College, Vellore 632 004

Sub: **Fluid Research grant project:**
To study the rate of bone loss in acute spinal cord injury patients and the effect of vitamin D replacement on the rate of bone loss by means of assessment of bone biomarkers.
Dr. Prince Thakkar, PG Registrar, Physical Medicine and Rehabilitation, Dr. George Tharion, Physical Medicine and Rehabilitation, Dr. Thomas V. Paul, Dr. Sahana Shetty, Endocrinology, Diabetes and Metabolism, Dr. Joe Fleming, Clinical Biochemistry.

Ref: IRB Min No: 8734 [OBSERVE] dated 06.03.2014

Dear Dr. Prince Thakkar,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
Vice - Principal (Research) - Reg. No. 43983
Christian Medical College, Vellore - 632 004.

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Cc: Dr. George Tharion, Physical Medicine and Rehabilitation, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDI
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 50,000/- (Rupees Fifty Thousand only) will be granted for 1 year.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

NOT TO BE MINISTERED UNTO BUT TO MINISTER
CHRISTIAN MEDICAL COLLEGE
VELLORE
INDIA
Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
Vice - Principal (Research) - Reg. No. 43963
Christian Medical College, Vellore - 632 004.

Cc: Dr. George Tharion, Physical Medicine and Rehabilitation, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

PATIENT INFORMATION SHEET:

You are being requested to participate in the study. A study, regarding the strength of the bone following spinal cord injury.3 samples of blood will be collected and the samples will be analyzed after 1 year. A correlation between study results and the bone strength or bone loss will be made.

1. WHAT IS THE PURPOSE OF BLOOD COLLECTION?

In this study, three samples of blood (5 ml each time) at 1, 3, and 6 month interval following spinal cord injury will be collected. The blood sample will be analyzed for routine investigations and bone biomarkers for the first sample and second and third one for bone biomarkers only.

These blood test results will help the patient to know their routine blood parameters after spinal cord injury. Moreover the study of biomarkers over six months will give some information regarding the bone changes.

2. WHO WILL COLLECT THE BLOOD? WHAT ARE THE POSSIBLE COMPLICATIONS?

   Blood sample will be collected by trained staffs only. The blood taking procedure will be sterile and done with all aseptic measures. The complications that can occur with blood collection are pain, swelling and hematoma. The pain is the most common and usually resolves within minutes. There are least chances of other complications .Even if they do occur they will be treated for it in the hospital.

3. WILL WITHDRAWL OR NON PARTICIPATION AFFECT THE USUAL TREATMENT?

The patient can withdraw from the study at any point of time and is also free to decide regarding his participation. Non-participation or withdrawal will not affect your usual treatment at any point of time during the hospital stay.

4. DOES THE PATIENT HAVE TO PAY FOR THE INVESTIGATIONS?

The patient included in the study will not have to pay for any of the additional blood investigations done for study purpose.

5. WILL MY BLOOD REPOTS BE KEPT CONFIDENTIAL?

The results of the study will be published in a medical journal but you will not be identified by any name in any public presentation of results. However your medical notes may be reviewed by people associated with the study, without any additional permission, should you decide to participate in the study.

For any further questions, contact: Dr. Prince Thakkar- 09843766039,

Department of Physical Medicine and Rehabilitation,

PMR -0416 2282158, 0416 5212158

Informed Consent form

1. **Study Title:** To observe the rate of bone loss in acute spinal cord injury patient's by means of bone biomarkers.

Study Number:

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age:_____

Please initial box

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/
Legally Acceptable Representative:_____

Date: _____/_____/_____Signatory's Name: _____

Signature/thumb of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: **Dr. Prince Thakkar (Ph no. 09843766039, 0416-2282158)**
Signature of the Witness (or Thumb impression): _____Date: __/_____/_____

Name of the Witness: _____

DATA SHEET (20 PATIENTS)

Durtn/Age	Hospi no.	DOI	ASIA scale	Sp/Fl	A-D	Vita-D	PTH	Ca	Alb	Creat	Alk P	Osteo 1	0stcl 3	Ostcl6	Ctx 1	Ctx 3	Ctx 6	SURGERY
28	904850f	11-05-2014	L 1 C	F	C	18.9	14	9.9	3.9	0.65	155	8.8	30.4	38.5	1200	1400	1100	yes
32	917852F	28-08-2014	L 3 B	F	B	38.4	21.2	9.3	3.8	0.6	80	25.8	23.1	31.6	1290	1240	412	NO
30	029309g	29-06-2014	T 10 A	F	A	13.7	1.1	8.7	2.5	0.7	232	12	36.4	58.5	1410	2030	1760	NO
24	884746f	14-05-2014	T 10 A	F	A	16.3	19.6	9.3	3.7	0.48	123	25.4	38.8	23.9	1690	1630	888	yes
18	909435f	30-05-2014	T 10 A	F	A	18.7	12.9	7.9	2.8	0.54	106	53.2	58.5	43.2	1970	2360	613	YES
34	836425f	03-03-2014	T 11 A	F	A	12.6	12.6	9.7	3.6	0.55	100	34.8	50.9	40.8	1090	1920	2210	yes
30	917303f	16-08-2014	T 11 A	F	A	38.3	13.8	8.1	3.3	0.7	104	19.1	34.5	38	1150	1470	1350	yes
37	087794g	29-09-2014	T 11 C	F	C	5.3	33.3	9.2	4.1	0.46	113	12.7	27.8	17.4	963	1270	444	yes
28	100305G	10.11.2014	T 12 A	F	A	22	10	9.4	4	0.72	113	26.9	30.2	26.5	739	1610	859	yes
32	114988G	21-11-2014	T 12 A	F	A->B	13.7	29.2	9.3	3.5	0.58	181	15.3	19.6	22.6	2200	1800	1700	yes
36	726942f	12-12-2013	T 10 A	F	A	4.4	17	8.6	4	1.07	57	30	33.8	29.8	3310	2800	1370	yes
23	909197f	17-04-2014	T 8 A	F	A	7.45	12.9	8.2	2.9	0.47	122	20.1	35.9	39.8	1400	3430	1460	NO
27	937418F	30-11-2014	T11 A	F	A	12.6	7.5	8.6	3.6	0.6	100	17.7	40.1	32.7	1620	1410	2240	yes
34	915376f	01-08-2014	T11 A	F	A	3	45	9.17	3.5	0.5	118	32.9	28	23.2	1230	1210	1080	yes
23	046862G	07-07-2014	T7 A	S	A	3	1.5	8.9	3.9	0.3	59	36.4	38.2	27.9	1620	1200	510	YES
40	013822g	14-07-2014	C 4 C	S	C	26.9	26.8	8.5	3.9	0.47	90	20.4	24.4	46.6	1060	1320	859	yes
23	942912f	25-01-2015	T 7 A	S	A	15.5	4.3	9.4	3.8	0.8	80	29.1	21.5	20.3	2080	1570	1430	yes
18	923444F	04-10-2014	T 8 A	S	A	15.2	2.4	9.2	4	0.6	99	25.8	23.1	20.5	2500	2170	2100	yes
19	114740G	20-11-2014	T 9 A	S	A	3.18	4.5	9.34	3.6	0.42	64	27.7	33.7	41.5	1150	1880	1330	yes
30	860265f	01-05-2014	T10 A	S	A	10.8	14.4	8.3	3.6	0.82	119	28.5	34.2	13.6	1100	727	725	yes

SOP's for Automated Chemistry analysers

BETA CROSS LAPS

Method to measure C-terminal telopeptide of type 1 collagen(β- cross laps,

ASSAY METHOD – SANDWICH IMMUNOASSAY

Immunoassay for the in vitro quantitative determination of degradation products of type 1 collagen in human serum.

The Elecsysβ-CrossLaps/ serum assay is specific for crosslinked isomerized type 1 collagen fragments, independent of the nature of the crosslink (e.g.pyrrole, pyridinolinesetc)

The assay specificity is guaranteed through the use of two monoclonal antibodies each recognizing linear β-8AA octapeptides (EKAHD-β-GGR).

Test Principle

Sandwich principle. Total duration of assay: 18 minutes.

- *1st incubation: 50µL of sample and a biotinylated monoclonal anti-β-CrossLaps antibody are incubated together; the antigen in the sample is liberated from the serum components.*
- *2nd incubation: Following addition of streptavidin-coated microparticles and a monoclonal β-CrossLaps-specific antibody labeled with a ruthenium complex, a sandwich complex is formed which becomes bound to the solid phase via interaction of biotin and streptavidin.*
- *The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.*
- *Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.*

Limits and ranges:

Measuring ranges

0.010-6.00 ng/mL or 10-6000 pg/mL (defined by the lower detection limit and the maximum of the master curve). Values below the detection limit are reported as < 0.010 ng/mL (< 10 pg/mL). Values above the measuring range are reported as > 6.00 ng/mL (> 6000 pg/mL).

Lower limits of measurement

Lower detection limit: 0.01 ng/mL (10 pg/mL).
The detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, repeatability study, n = 20)

ALKALINE PHOSPHATASE (ALP) *EC 3.1.3.1*

Date of introduction: March 1995
Review Date: annual
Analyser: Automated Chemistry analysers
Frequency: daily, Monday to Saturday
Analysis time: results available the same working day, OP up to 1930 hrs

Summary and explanation of the test

Colorimetric, kinetic, p-nitrophenyl phosphate with AMP buffer.
This is an optimised standard method confirming to the recommendation of the IFCC. The liberated phosphate group is transferred to water and the reaction rate is enhanced by certain amino alcohol buffers such as AMP (2 – amino – 2 methyl-1-propanol) which act as a phosphate group acceptor.

Clinical Indications for the test

Phosphatases catalyse the splitting of phosphate group from monophosphoric esters, those operating at a alkaline pH optimum are called ALP. ALP in the serum is a mixture of isoenzymes from liver, bone intestine and placenta, occurring particularly in osteoblasts (bone forming cells) and in the liver. It is increased in bone disease associated with increased osteoblastic activity and in any form of biliary obstruction, which induces enzyme synthesis in hepatocytes adjacent to the biliary canaliculi. It is a standard test for bone and liver disorders.

Specimen type, collection and storage

Serum is the preferred specimin but lithium heparin plasma is also suitable for this test.
Stable for 7 days at 40C 0% activity decreased
7 days at 20-250C 10% activity decreased

Principle of the method

The substrate is self indicating as the 4-nitrophenol is converted to 4-nitro phenoxide in alkali with increased in absorbance at 415 nm being proportional to the ALP activity.
ALP
p-nitrophenol phosphate + H20 ----------> Phosphate + p –nitrophenol

Source of the Method Protocol

Tietz NW, Rinker D, Shaw LM. IFCC methods for the measurement of catalytic concentration of enzymes

N-MID Osteocalcin

Intended use

Immunoassay for the in vitro quantitative determination of N-MID osteocalcin in human serum and plasma. The determination is used for the control of antiresorptives therapeutic efficiency, e.g. for patients with osteoporosis or hypercalcemia.

*The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on the Roche Elecsys 1010 and 2010 immunoassay analyzers.*

Summary*1–10

Osteocalcin, the most important non-collagen protein in bone matrix, is a bone-specific, calcium-binding protein which is dependent on vitamin K. It contains 49 amino acids and has a molecular weight of approx 5800 D. It contains up to three Ö-carboxyglutamic acid residues (bone-GLA-protein, BGP).

During bone synthesis osteocalcin is produced by the osteoblasts. Its production is dependent upon vitamin K (formation of Ö-carboxyglutamic acid residues) and is stimulated by vitamin D3. After release from the osteoblasts, osteocalcin is not only assimilated into the bone matrix but also secreted into the blood stream. Accordingly, the serum (or plasma) osteocalcin level is related to the rate of bone turnover in various disorders of bone metabolism, e.g. osteoporosis in particular, but also in primary and secondary hyperparathyroidism or Paget's disease.

Osteocalcin is therefore termed a bone turnover marker and is used for this purpose. By means of osteocalcin measurements it is possible to monitor therapy with antiresorptive agents (bisphosphonates or hormone replacement therapy, HRT) in, for example, patients with osteoporosis or hypercalcemia.

Both intact osteocalcin (amino acids 1–49) and the large N-Mid fragment (amino acids 1–43) occur in blood. Intact osteocalcin is unstable due to protease cleavage between amino acids 43 and 44 . The N-Mid-fragment resulting from cleavage is considerably more stable.

The Elecsys N-MID Osteocalcin assay uses two monoclonal antibodies specifically directed against epitopes on the N-Mid-fragment and the N-terminal fragment. The test is non-dependent on the unstable C-terminal fragment (amino acids 43-49) of the osteocalcin molecule and thus ensures constant measurement results under routine conditions in the laboratory.

***Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy){)*

Test principle*

Sandwich principle. Total duration of assay: 18 minutes

*_ **1st incubation**: 20 µl of sample, a biotinylated monoclonal N-MID osteo-calcin-specific antibody and a monoclonal N-MID osteocalcin- specific antibody labeled with a ruthenium complex** react to form a sandwich complex.*

*_ **2nd incubation**: after the addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.*

_ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemilumin-escent emission which is measured by a photomultiplier.

_ Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve pro-vided via the reagent bar code.

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Assay*

For optimal performance of the assay it is important to follow the dir-ections given for the analyzer used, and to check that the system's inventory of assay materials and other consumables is adequate. Resuspension of the microparticles before use and the reading in of the test-specific parameters via the reagent bar code take place auto-matically. No manual input is necessary. If in exceptional cases the bar code cannot be read, enter the 15-digit sequence of numbers.

*Elecsys 2010: Bring the cooled reagents to approx. 20°C and place on the reagent disk of the analyzer. Avoid the formation of foam. The system **automatically** regulates the temperature of the reagents and the opening/closing of the bottles.*

Elecsys 1010: Bring the cooled reagents to approx. 20–25°C and place on the sample/reagent disk of the analyzer (ambient temperature 20– 25°C). Avoid the formation of foam. Open bottle caps ***manually*** *before use and* ***close manually*** *after use.*

Calibrators*

Elecsys N-MID Osteocalcin was calibrated against in-house reference standards: osteocalcin in analyte-free human serum matrix.11

Every N-MID Osteocalcin reagent set has a bar-coded label containing the specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer by the use of Elecsys

N-MID Osteocalcin CalSet. 118

Calculation*

Elecsys 1010 and 2010 automatically calculate the N-MID osteocalcin concentration of each sample. The results are given in ng/ml.

Limitations – interference*11*

The assay is unaffected by icterus (bilirubin < 65 mg/dl), lipemia (Intralipid < 1500 mg/dl) and biotin < 100 ng/ml (criterion: recovery within ± 10% of initial value).

Hemolysis interferes. Erythrocytes contain proteases which degrade osteocalcin. In patients receiving therapy with high biotin doses (> 5 mg/day) no sample should be taken until at least 8 hours after the last biotin admin-istration.11

No influence was observed from rheumatoid factor up to 2200 U/ml. There is no high-dose hook effect at N-MID osteocalcin concentrations up to 4200 ng/ml.

In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.

For diagnostic purposes, the Elecsys N-MID Osteocalcin findings should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Measuring range- Measuring range- 0.500–300 ng/ml (defined by the lower detection limit and the maximum of the master curve).

		N-MID osteocalcin
Women, premenopausal	*200*	*31.2*
Women, postmenopausal, no HRT	*211*	*41.3*
Men, ≥ 50 years	*162*	*26.3*