REVIEW OF PREVIOUS RESEARCH AND NEED FOR THE PRESENT STUDY

Bone is a highly complex and dynamic living tissue continuously engaged in the process of modelling and remodelling i.e. building of new bone tissue and breaking down of old bone tissue. Bone mass continuously increases in concordance with skeletal growth in length and breadth during childhood and adolescence and the total skeletal mass peaks a few years after growth arrest. Bone mass accrual during the growing years is influenced by numerous factors. A variety of diseases and pharmaceutical interventions can result in suboptimal accrual or bone loss or both. Thus, clinical assessment of bone density during growing years, especially when a child is not well is essential. According to ISCD 2007 Pediatric Official Position Statement of the International Society for Clinical Densitometry, for the correct interpretation of bone status during childhood and adolescents, assessment of bone mineral density in accordance with ethnicity based reference database is essential (Gordon et al. 2008).

For more than a decade and a half, since Morrison et al. (1992) identified the association of Vitamin D Receptor gene polymorphism and PBM, several studies have attempted to establish the relationship of VDR gene and bone mass accrual, with conflicting results. Especially, studies in children and adolescents examining the association of bone mass accrual and VDR gene polymorphisms are very limited (Arabi et al. 2010; Abrams et al. 2005; Loretzon et al. 2001). Identifying polymorphisms associated with low bone mineral density during growing years would go a long way in devising additional strategies to improve PBM.

Adequate calcium intake is crucial for optimizing bone mass accrual. With high prevalence of low calcium intake during childhood and adolescence (Salamoun et al. 2005; Rozen et al. 2001; Ahmed 1998; Puri et al. 2008; Sanwalka et al. 2010), the risk for low bone mass accrual and thereby osteoporosis is higher.

The following review emphasizes the need to assess bone status during childhood and adolescence and develop ethnicity based reference percentile curves for Indian children and adolescents. The review also highlights the link between VDR gene and bone density and illustrates the importance of calcium in diet of children and adolescents.
2.1: Bone

The human skeleton consists of 206 bones and is divided into the axial (e.g. vertebrae and the pelvis) and the appendicular skeleton (long bones). Bone as an organ is made up of various tissues – bone or osseous tissue, cartilage, dense connective tissues, epithelium, adipose tissue and nervous tissue (Tortora and Grabowski 2003).

2.1.1: Composition and Histology of Bone

Bone or osseous tissue is the hardest tissue in the human body. It is defined as a special form of connective tissue with a collagen framework impregnated with calcium and phosphate salts, particularly hydroxyapatites (Ganong 2003). Bone tissue is made up of an organic (30%) and an inorganic (70%) component. The organic component consists primarily of cells, extracellular matrix of collagen and non-collagenous proteins and mucopolysaccharides. The inorganic component consists chiefly of bone mineral hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ comprising of calcium, phosphate and carbonate and other minerals (Heaney 2005; Tortora and Grabowski 2003).

Bone tissue is made up of 4 different types of cells: osteogenic cells, osteoblasts, osteocytes and osteoclasts (Figure 2.1). Osteogenic cells (genic = producing) are specialized stem cells that undergone cell division to form osteoblasts. Osteoblasts: (blasts = buds or sprouts) are bone-building cells which synthesize and secrete collagen monomers and proteoglycans. As osteoblasts surround themselves with matrix, they become trapped in their secretions to form osteocytes and are responsible for metabolism i.e. exchange of nutrients and wastes with the blood. Osteoclasts (clasts = break) are the exclusive bone-resorbing cells that are usually found in close association with bone surface and are characterized by their ruffled border. Osteoclasts secrete lysosomal enzymes and acids that digest the protein and mineral components of the underlying bone matrix. This process is known as resorption and is a part of the normal development, growth, maintenance and repair of bone (Tortora and Grabowski 2003).
2.1.2: Mechanism of Bone Calcification

The collagen monomers secreted by osteoblasts, rapidly polymerize to form collagen fibers and the resultant tissue is called osteoids. After formation of osteoids, calcium salts begin to precipitate on the surfaces of collagen fibers to from hydroxyapatite crystals. Initially calcium salts deposit as amorphous compounds (non-crystalline), a mixture of salts such as \( \text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \), \( \text{Ca}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O} \), etc. Thereafter, these salts are converted to crystalline hydroxyapatite salt \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \) over a period of weeks or months by processes of substitution and addition of atoms or reabsorption and re-precipitation. Some salts permanently remain in the amorphous form to facilitate rapid absorption of calcium when extra calcium is required to maintain extracellular fluid homeostasis (Guyton and Hall 2006).

2.1.3: Skeletal Modelling and Remodelling

Bone is a highly metabolically active tissue which is continuously being deposited by osteoblasts and constantly been broken down by osteoclasts. The breakdown of matrix by osteoclasts is called bone resorption. During resorption, an osteoclast attaches tightly to the bone surface at the endosteum or periosteum and forms a leak proof seal at the edges of its ruffled border. Osteoclasts then release protein – digesting lysosomal enzymes and several acids such as citric acid and lactic acid that digest collagen fibers and other organic substances and dissolve the bone minerals. The degraded bone proteins and matrix materials, mainly calcium and phosphorus, enter an osteoclast by endocytosis, cross the cell in vesicles, and undergo
exocytosis on the side opposite the ruffled border. Once in the interstitial fluid, the products of bone resorption diffuse into nearby capillaries. Once a small area of bone has been resorbed, osteoclasts are replaced by osteoblasts for bone mineralization to take place (Tortora and Grabowski 2003).

The term, bone modeling is used for growth of the skeleton until mature height is achieved. In modeling, the process of formation of new bone tissue and resorption of old tissue does not occur at the same site. Bone modeling is typically completed in girls by 16 to 18 years of age and in boys by age of 18 to 20 (Beyer 2000).

Once the bones have reached final length, the process of bone absorption and deposition is known as bone remodeling. Unlike bone modeling, during remodeling, bone is absorbed first by osteoclasts followed by rebuilding by osteoblasts at the same site. Remodeling normally serves two purposes: i) it renews bone tissue deterioration ii) it redistributes bone matrix along lines of mechanical stress. Remodeling also heals injured/ fractured bones (Tortora and Grabowski 2003; Heaney 2005).

To achieve homeostasis in bone, the bone-resorbing action of osteoclasts must balance the bone-making actions of the osteoblasts. If osteoblasts form too much new bone tissue, the bones become abnormally thick and heavy and may interfere with movement at joints. Alternatively, a loss of too much calcium or inadequate formation of new tissue weakens bone tissue and may result in osteoporosis.

2.1.4: Osteoporosis

The term “Osteoporosis” was coined in early 1820s’ in France to describe a pathological condition of the bone (Schapira and Schapira 1992). In 1993, osteoporosis was defined as a “disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk”. The NIH Consensus Development Panel on Osteoporosis in 2001 defined osteoporosis as “a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture” (Szulc and Bouxsein 2010).

Osteoporosis is the second most prevalent disease in adults in the world and an osteoporotic fracture is estimated to occur every 3 second worldwide (Johnell and Kanis 2006). According to a review by International Osteoporosis Foundation, 1 in 3
women and 1 in 5 men over 50 years of age are prone to suffer from an osteoporotic fracture and an estimated 75 million people are prone to suffer from osteoporosis in America, Europe and Japan (IOF 2011).

In 2003, in India, an estimated 26 million people were estimated to be suffering from osteoporosis and the numbers are projected to increase to 36 million by 2013 (Osteoporosis Society of India 2003). According to a recent review, osteoporotic fractures are very common in both the genders in India and occur at a younger age as compared to their Western counterpart (Malhotra and Mithal 2008).

Professor Charles E. Dent described senile osteoporosis to be a pediatric disease. The underlying assumption is that peak bone mass (PBM) i.e. the amount of bony tissue present at the end of the skeletal maturation achieved is a major determinant of the risk for future osteoporosis (Bonjour et al. 1994). Up to 90% of PBM is acquired by the age of 18 years in girls and 20 years in boys (National Institute of arthritis and musculoskeletal and skin disease 2009). During the subsequent years, bone mass remains fairly constant until, bone resorption begins predominating bone deposition during old age. Lower accrual of PBM would result in thinner bones during this period of rapid resorption. Thus, a compromise in bone mass accrual during childhood and adolescence would increase the risk of osteoporosis and fracture (Davies et al. 2005). It is therefore, essential to optimize bone mass accrual during childhood and adolescents.

2.2: Childhood and Adolescence – Period of Rapid Bone Growth

Childhood and adolescence are the period of rapid growth including bone growth. Childhood is defined as the period from after toddlerhood (3 years of age) until an individual enters puberty (Lucas 2000). Adolescence is the period of life beginning with the appearance of secondary sex characteristics and ending with the cessation of somatic growth (Spear 2000).

The bones of the skeleton are formed prenatally as cartilage models which is subsequently and gradually replaced by bony tissue by a process involving invasion of cartilage by blood vessel followed by bone calcification by osteoblasts and removal of cartilage tissue by osteoclasts. Thereafter, bones throughout the body grow in
thickness by appositional growth and in length by additional deposition of bone on the diaphyseal side of the epiphyseal plate.

A significant gain in height is seen during childhood and adolescences especially during the pubertal spurt. The gain in bone length contributes to 98% gain in height (Pipes 1988; Rees and Mahan 1988). The gain in bone mass accrual during adolescence is more a function of maturational stage than chronological age as two individuals with same chronological age may have different levels of maturation (Malina & Bouchard 2003). Increased levels of growth hormones and sex steroids during puberty accentuate mineralization and bone growth (Ollsson et al. 1998; Frank 1995). Also, the onset of puberty involves the activation of the hypothalamic–pituitary–gonadal axis with activation of hormones such as IGF-1 which further accelerates the growth rate of the skeleton (Kanbur et al. 2005).

As seen in Figure 2.2, peak bone mineral accretion occurs at the age of 12.5 years in girls and 14.0 years boys respectively (Bailey et al. 1999). A gender difference in bone mass accrual becomes evident at puberty. This difference appears to be due principally to a more prolonged bone maturation period in males than in females (Malina & Bouchard, 2003). In adolescent females, the gain in bone mass declines rapidly after menarche, and no considerable gains are observed two years after menarche (Bonjour et al. 2003). In adolescent males, bone mineral accrual accelerates particularly from 13-17 years and remains significant between 17-20 years at some sites (Bonjour et al. 2003).

![Total Bone Mineral Velocity Curve](image)

**Figure 2.2: Total Body Bone Mineral Content Velocity during Adolescences**
(Source: Bailey et al. 1999, Page No. 1675)
2.2.1: Health and Bone Mass Status during Childhood

Bone mineralization measured as bone mineral density (BMD) is affected by various illnesses and health conditions during the growing years. (Antoniazzi et al. 2011; Mager et al. 2011; Hill et al. 2011; Lims et al. 2011; Tasdemir et al. 2001; Bianchi et al. 2003; Mun˜ oz and Argente 2002; Shouman et al. 2010). Children suffering from osteogenesis imperfecta (autosomal, recessive disease causing extreme bone fragility) are shown to have low BMD at lumbar spine and hips as compared to normal children (Davie and Haddaway 1994; Antoniazzi et al. 2011; Kaur et al. 2011). In a study conducted in children aged 2.7 – 13.5 years from California, the mean BMD in children suffering from osteogenesis imperfecta was 76.6% of that of controls (Zoints et al. 1995).

Various inflammatory diseases are associated with low BMD in children. Celiac disease (an auto-immune disorder characterized by inability to digest gluten present in wheat, rye or barley) has also been associated with low BMD in children (Blazina et al. 2010; Mager et al. 2011). Mager et al. (2011) demonstrated that prevalence of low BMD (Z score < -1) at total body and lumbar spine increased from 10 – 20% at diagnosis to 30 – 32% in a period of one year in children aged 3 – 17 years suffering from celiac disease. In a recent review, on Crohn’s disease (disease causing inflammation of the intestine) and bone density in children, Hill et al. (2011) highlighted the prevalence of altered bone geometry in patients with Crohn’s disease. These micro-architectural changes have shown to increase the risk for fractures in children suffering from Crohn’s disease (Hill et al. 2011). In a study conducted in 80 children suffering from systemic lupus erythematosis (an auto-immune disorder causing inflammation of various parts of the body), 15% of children were found to have low BMD at lumbar spine (Lims et al. 2011).

Similarly, other health disorders like anorexia nervosa (Mun˜ oz and Argente 2002), congenital hypothyroidism (Demartini et al. 2007), growth hormone deficiency (de Boer et al. 1994), neuromuscular disorders [such as spinal cord injuries (Moynahan et al. 1996), or cerebral palsy (Tasdemir et al. 2001; Henderson et al. 2002), Duchenne muscular dystrophy (Bianchi et al. 2003)] are reported to affect BMD.
Gluco-corticoids (GC) are widely used in pediatric practice for treatment of various disorders and have also been shown to affect bone density (Basiratnia et al. 2006) which may due to decreased bone formation because of decreased osteoclast differentiation and activity; and increased bone resorption caused by an increased osteoblast and osteocyte apoptosis (Shouman et al. 2010).

Under nutrition also has an effect on bone mass in adolescents. Children and adolescents especially from the lower-socio-economic stratum who have low intake of nutrients and short stature are reported to have lower bone mineral density than the children from upper socio economic stratum who are well-fed (Arabi et al. 2004; Marwaha et al. 2005; Khadilkar et al. 2007).

These studies thus illustrate that chronic diseases and under-nutrition have a negative influence on bone mineral density in children and adolescents. Therefore, clinical diagnosis of decreased bone mineral density in children would assist in planning and implementing the preventive and curative line of treatment.

Various non-invasive methods such as single photon absorptiometry (SPA), dual photon absorptiometry (DPA), single X-ray absorptiometry (SXA), dual-energy X-ray absorptiometry (DXA), quantitative computerized tomography (QCT), quantitative ultrasound (QUS), radiographic absorptiometry (RA) and magnetic resonance imaging (MRI) have been developed and used over the years to analyze bone mineral density. Of these, DXA is the gold standard used to assess BMD in children and adolescents.

2.3: Dual Energy X- Ray Absorptiometry

Dual-energy X-ray absorptiometry (DXA) is the most commonly used bone densitometry technique for children throughout the world and preferred over other techniques because of its speed, precision, safety, low cost and easy availability (Gordon et al. 2008). A DXA scan measures areal BMD (g/cm³) defined as the integral mass of bone mineral per unit project area (Blake and Fogelman 1997). The first commercial DXA scanner was introduced in 1987.
2.3.1: Technical Principle and Sites of Measurement

The physical principle employed in DXA is the measurement of the transmission through the body of x-rays with high- and low-photon energies. The x-ray attenuation coefficient depends on the atomic number and photon energy, measurement of the transmission factors at two different energies and thus enables the areal densities (i.e, mass per unit projected area) of two different types of tissue to be inferred :i) bone mineral (hydroxyapatite) ii) soft tissue respectively. DXA measures bone density at the total body, lumbar spine, femurs and forearms (Blake and Fogelman 1997; Fogelman and Blake 2000).

2.3.2: Source of X-Ray, Radiation Dose and Precision

The X-ray sources can be of the pencil beam or fan beam (Figure 2.3). Pencil beam DXA scanners used a pinhole collimator producing a pencil beam coupled to a single detector in the scanning arm. Fan beam DXA scanners use a slit collimator to generate a fan beam coupled to a linear array of detectors. Fan beam systems have higher image resolution and lesser scan time. However, the amount of radiation exposure is more for a fan beam DXA scanner as compared to a pencil beam DXA scanner (Blake and Fogelman 1997; Fogelman and Blake 2000).

Figure 2.3: Fan Beam and Pencil Beam as Produced by DXA Scanner

(Source: Blake and Fogelman 1997, Page No. 217)
Radiation dose to the patient is very low (1–10 μSv) in pencil beam DXA machine and is comparable with the average daily dose from natural background radiation of 7 μSv (Blake and Fogelman 1997). Precision errors that reflect the reproducibility of a diagnostic technique ranges from 1-2 % for a DXA scan (Fogelman and Blake 2000).

DXA machines are manufactured by various companies such as Hologic, Lunar, Prodigy and Norland. Studies have shown that absolute bone mineral density (BMD) measurements from different manufacturers of DXA machines, although highly correlated, are significantly different as there are significant differences in the assignment of “normality” for BMD by different manufacturers as the technique for BMD assessment is different (Pocock et al. 1997; Pocock et al. 1992). Also, each manufacturer assigns normality of DXA measured BMD based on reference percentile curves calculated using scans performed by densitometry machines of the same make (Hologic/ Lunar/ Prodigy/ Norland) and type (fan beam/ pencil beam). There may be significant errors in interpreting DXA results if one uses measurements from one densitometry to predict the change in BMD using the scanner of the other manufacturer (Pocock et al. 1997). Thus, it is advised to get follow up scans on machine by the same manufacturer and use of reference data curves specific to the manufacturer.

2.3.3: Interpretation of DXA Measured Bone Density

For the interpretation of DXA measured BMD, World Health Organization (WHO) in 1994, recommended an arbitrary definition of osteoporosis based on “T-score”. T-score is the number of standard deviations of measurement above or below the mean for a healthy 30 year old adult of the same sex and ethnicity as the patient. A patient is termed to be suffering from osteopenia if the T-score >-1 and < -2.5 and osteoporosis is the T-score > -2.5 (WHO 1994).

However, use of T score in children is inappropriate as they are still in the growing phase and have not reached their peak bone growth. Thus, Z score which is the number of standard deviations of measurement above or below the mean for the patient's age and gender are recommended for use for interpretation of DXA measured bone mineral in children. A child having Z-score < -2 for a given bone parameter is to be considered to have low bone mass status. The DXA machine
calculates a Z-score based on the in-built reference database on White/ Black/ Asian (other than India) or Hispanic populations.

2.3.4: Need for Ethnicity Specific Reference Database

Ethnicity and race has a significant effect on bone mass status on an individual (Pollitzer and Anderson 1989). In a study conducted in 2339 women with a mean age of 46.2 years, to assess the effect of ethnicity on bone density, Finkelstein et al. (2002) found that on adjustment for various covariate factors, African-American women had the highest lumbar spine and femoral neck BMD, followed by Chinese and Japanese women whereas Caucasian women had the lowest BMD. In another study in 13091 adults, mean BMD was found to be significantly higher in Hispanic as compared to non-Hispanic backs (Looker et al. 2009). Indian men and women are shown to have significantly lower BMD as compared to Caucasians (Makker et al. 2008). Studies in children have also demonstrated a difference in bone status from different ethnic background. African American and Hispanic children have higher BMD at forearms as compared to Caucasian children (Wetzsteon et al. 2009). Yanovski et al. (1996) have demonstrated that black girls have significantly higher BMD as compared to White girls aged 7 – 10 years. Adolescent Indian girls are also shown to have lower BMD as compared to their Caucasian counterparts (Khadilkar et al. 2010). Thus, for the correct interpretation of DXA measured bone status in children, the use of ethnicity specific reference database to calculate Z scores is essential.

Kalkwarf et al. (2000) analyzed DXA measurements at total body, lumbar spine, left femoral neck and forearm in 1554 (761 boys) healthy children who were a part of the Bone Mineral Density in Childhood study in United States. BMC and BMD were significantly higher in Black children at all skeletal site as compared to non-black children and therefore, they computed different percentile curves for Blacks and Non-Blacks.

Moolgard et al. (1997) measured BMC and BMD using Hologic DXA scanner in 5 – 19 year olds from Copenhagen, Denmark and to construct percentile curves for assessment of bone mineralization. Even though not significant, a difference was noticed in the mean BMC of children from Denmark in comparison to those from
American and Canadian children. Total body BMC were within 267 g below and 173 g above that observed in Canadian children with no systemic age dependent difference. As there were no age and sex-specific reference database for evaluating as measured by Hologic DXA scanner in Caucasian children in UK, Ward et al. (2006) published reference percentile curves for total body BMC, lumbar spine and femoral neck bone mineral apparent density based on bone measurements by Hologic QDR DXA scanner in 442 (239 boys) healthy Caucasian children.

Boot et al. (1997) measured BMC using a Lunar DPXL DXA scanner in 500 (205 boys) children from different ethnic groups (Caucasian – 444; black - 21 children and Asian – 35) residing in Rotterdam, Netherland. The lumbar spine density was higher than that found in Finnish children where as it was comparable with that in Spanish children. Thus illustrating that bone mineralization in children differs in different parts of Europe (Boot et al. 1997).

de Rio et al. (1994) have published reference database for L2-L4 lumbar spine using Lunar DPXL DXA scanner in 3 months to 21 – year old Mediterranean Spanish children and adolescents. Pludowski et al. have published reference values for skeletal and muscle status as measured by Lunar DPXL scanner in healthy Caucasian children from Poland.

Maynard et al. (1998) published reference percentile curves for White children from Ohio based on total bone densitometry as assessed using Lunar DPX scanner and found that there was a difference in the BMC of children in their study as compared to the Canadian children analyzed by Faulkner et al. (1996) using Hologic DXA scanner. Children in their study had higher BMC as compared to Argentinean children as measured by Zanchetta et al. (1995)

Makker et al. (2008) have published reference percentile curves for BMD measured using Lunar Prodigy DXA scanner at femurs, lumbar spine and left forearm for adult Indian men and women aged 20 – 86 years and compared the prevalence of osteopenia and osteoporosis using the newly developed Indian reference curves as against Caucasian database. Caucasian database tends to estimate a larger proportion of Indian subjects as osteoporotic and osteopenic as compared to Indian reference database (Makker et al. 2008). However, the adult reference database cannot be used
for assessing the bone status in Indian children and adolescents and there is no Indian pediatric reference database for assessment of bone status in Indian children, thereby emphasizing the need to develop a reference database for same.

2.3.5: Methods for Normalization of DXA Measured Bone Mineral Density

Bone mass depends on both the size and density of skeletal bone, and a difference in bone mass may reflect a difference in either bone size or bone density. True BMD is a function of BMC per volume of bone; that is volumetric BMD (vBMD). Due to technological limitations, DXA calculates a two-dimensional projected area; hence, the output is expressed as areal BMD, i.e. bone mineral content per bone area. In adults, areal BMD is believed to be a good substitute for vBMD, as there is little change in bone size, and the major change occurring is related to the decline in BMC (Mazess et al. 1994). However, in children and adolescents, growth leads to a much greater change in bone volume than in bone area (Carter et al. 1992). Thus, use of areal BMD as measured by DXA alone may not be appropriate and hence, various methods have been suggested for normalization of BMD as measured by DXA.

i) Calculation of vBMD or BMAD: Bone mineral apparent density (BMAD) is a mathematical method to calculate vBMD at lumbar spine and femoral neck with the basic assumption that each lumbar spine vertebra or femoral neck is a cylindrical body and hence the depth can be measured and thereby, calculation of vBMD is possible (Kröger et al. 1992).

ii) Molgaard Approach: Low BMD as calculated by DXA, may be a reflection of small skeleton (that is small bones) and not just low mineralization. It is thus important to know whether a small bone area or a low BMC corrected for bone area is the cause of low BMD. Thus, Molgaard et al. (1997) introduced the concept of “Bone Mineral Content (BMC) adjusted for Bone Area (BA)” which is more useful than “areal BMD” as it is a measure of bone mineral that is not correlated with bone area. They described mineralization in growing children in terms of three components BMC adjusted for BA (BMC for BA), BA adjusted for height (BA for height) and height adjusted for age (height for age).
iii) **Crabtree Approach:** There is a strong influence of muscle mass on bone mineralization. Thus, to assess if low BMD as measured by DXA is an effect of reduced muscle mass, Crabtree et al. (2004) have suggested another approach to assess bone mass in children in relation to lean body mass (LBM). They recommended a 4-step approach for assessment of bone status: BMC adjusted for age, height adjusted for age, LBM adjusted for height and BMC/LBM relative to height.

### 2.3.6: LMS Method – Technique for Development of Reference Percentile Curves

Conventionally, population mean plus and minus twice the standard deviation are used as reference standard to calculate Z-scores for growth parameters during childhood and adolescence. However, with the advancement in science, techniques to develop reference percentile curves have been developed by clinicians and researchers to observe clinical measurements of an individual patient in the context of population values. Reference percentile curves are used widely in medical practice for anthropometric parameters such as height, weight and body mass index.

Like anthropometric reference percentile curves, reference percentile curves for bone density help identify children who are at risk of poor bone status. If the population centile corresponding to the child’s bone mass value is atypical, it indicates an underlying pathological condition. The curves also provide a background to compare the bone measurement as it changes with time.

While constructing percentile curves, it is important to transform the data to a Gaussian distribution to account for skewness of the data (Van’t Hof et al. 1985). The estimates of parameter obtained for percentiles by transformation are much more efficient than those obtained by a non-parametric technique (Healy, 1974). Also, properly transformed data allows for the use of standard deviation (SD) scores, which are helpful in the evaluation of individual development or comparison (Karlberg et al. 1976).

In 1988, Professor Cole suggested a method, LMS to generate smooth percentile curves. He assumed that the distribution at each covariate value is summarized by three parameters, the Box-Cox power $\lambda$, the mean $\mu$ and the coefficient of variation $\sigma$, and the initials of the parameters give the name to the LMS
method. LMS method describes a variable ‘y’ as a semi-parametric regression function of a time-dependent variable ‘t’, so that the distribution of ‘y’ changes gradually when plotted against ‘t’ (Cole 1988). In this method, the three parameters are constrained to change smoothly as the covariate changes, and can, like the centiles, be plotted against the covariate. Thus, advantage of the LMS method is that the three curves, L, M and S, completely summarize the measurement's distribution over the range of the covariate. A key assumption of the LMS method is that after a suitable power transformation, the data are normally distributed (Cole and Green, 1992).

2.4: Vitamin D Endocrine System

Vitamin D or the sun-shine vitamin is a pro-hormone responsible for regulating and maintaining intra and extra-cellular calcium levels. Pre-vitamin D is synthesized subcutaneously on exposure to sun which is then converted to the active form in 2 sequential hydroxylation reactions. Pre-vitamin D is converted to 25-hydroxy vitamin D [25(OH)D] in the liver (storage form of vitamin D) which is further hydrolysed to the active form 1,25 dihydroxy vitamin D [1,25(OH)2D] in the kidney (Gallagher 2000). Vitamin D accomplishes this goal through regulating calcium and phosphorus metabolism in the intestine and the bone (Holick 2005). Vitamin D functions like a steroid hormone in the body, i.e. it binds with VDR protein which than attaches itself to specific vitamin D response elements (VDRE) on specific gene and initiates transcription for messenger ribonucleic acid (mRNA) of several proteins essential to stimulate intestinal calcium and phosphate absorption, increases bone resorption and renal calcium/ phosphate re-absorption (Gallagher 2000; Guyton and Hall 2000).

Vitamin D-VDR protein complex affects bone metabolism by influencing actions of both osteoclasts and osteoblast. Vitamin D directly increases differentiation and activation of NF-kβ ligand (RANKL) in osteoclasts and indirectly increases differentiation and activation of osteoclasts (Khosla 2001). On the other hand, osteoblasts have receptors for vitamin D - VDR protein complex. Vitamin D – VDR protein complex also enhances bone mineralization by influencing alkaline phosphatase activity and gene expression of osteocalcin and osteopoitin which are essential for bone mineralization (Hollick 2005).
2.5: Vitamin D Receptor Gene

Bone mass accrual is under strong genetic control. Several epidemiologic and twin studies suggest that up to 80 - 85% of the variability in BMD can be explained by genetic factors (Stewart and Ralston 2000). With the help of various kinds of genetic studies such as gene-mapping, linkage analysis studies, linkage disequilibrium studies and association studies, various genes have been identified as candidate genes relevant to bone metabolism. Candidate genes are the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis (Zhu and Zhao 2007).

As, vitamin D receptor protein plays a very important role in maintaining bone mass status, the gene encoding for vitamin D receptor protein, the vitamin D receptor gene (VDR) is the most extensively studied candidate gene for bone mineralization.

2.5.1: Location and Structure of VDR Gene

Vitamin D Receptor (VDR) gene located at chromosome 12q.13.11 was the first gene to be identified which has shown to be associated with bone metabolism (Greene et al. 2009). It is a large gene of about 100kb and has an extensive promoter region capable of generating multiple tissues – specific transcripts and lies just downstream from the collagen type II α 1 (COL2A1) gene (Uitterlinden et al. 2004). VDR gene is similar to other nuclear receptor genes i.e. each of the two zinc fingers is encoded by separate exons (II and III), and the 5´ end of the gene exhibits some complexity in the form of alternate splice and/or translation start sites (Haussler et al. 1998). VDR gene is composed of 9 exons and 8 introns (Arababadi et al. 2001). The functional protein product is encoded by exon 2–9 while exon 1 is spliced in a tissue specific manner under the control of promoters (Crofts et al. 1998).

2.5.2: VDR Gene Polymorphisms

Polymorphisms are defined as variations of deoxyribonucleic acid (DNA) sequence that are present in more than 1% of the population. Most polymorphisms are single nucleotide polymorphisms (SNP’s) where as individual can differ from others
genetically by roughly 100,000 polymorphic sites and can occur in the non-coding part of the gene thereby having no effect in the protein product (Greene et al. 2009).

As seen in Figure 2.4, several SNP’s of VDR gene have been identified. Of these, polymorphisms of Bsm1, Taq1, Apa1 loci at intron 8 and Fok1 loci at exon 2 are most extensively studied. Bsm1 tends to over shadow the action of Taq1 and Apa1 loci polymorphisms as they are located at the same exon and thus, Bsm1 loci polymorphism is most widely studied along with Fok1 loci for their association with bone density in adolescents (Uitterlinden et al. 2004)

Figure 2.4: Exon – Intron Structure of VDR Gene and Known-Polymorphisms

The association of VDR receptor gene polymorphisms of Bsm1 loci with BMD was established for the first time by Morrison et al. (1994). The Bsm1 polymorphism is caused by G->A transition in intron 8 at nucleotide position 243 of the VDR gene. They analyzed 125 pairs of twins (70 monozygotic) for association of VDR gene polymorphisms of Bsm1 loci with BMD at lumbar spine and proximal femur and found that polymorphism of Bsm1 loci (BB genotype) was the strongest predictor of BMD at both the sites. They further studied 311 unrelated healthy women and found similar results in them, thereby confirming the association of VDR gene polymorphism of Bsm1 loci with BMD (Morrison et al. 1994). Morrison et al. (1994) demonstrated twins and unrelated post-menopausal women with BB genotype had significantly lower BMD at lumbar spine and femoral neck. Several studies including those conducted in Indian post-menopausal women have confirmed this association with BB genotype having significantly low bone density as compared to bb genotype (Tokita et al. 1996; Spector et al. 1995; Mitra et al. 2006) where as other studies have shown no such correlations (Garnero et al. 1995; Hansen et al. 1998; Jorgensen et al.
1998). Some studies have also shown an inverse association with BB genotype having significantly higher bone mass as compared to bb genotype (Salamone et al. 1996; Uitterlinden et al. 1996).

Two years after Morrison et al. published their findings, Gross et al. (1996) described the Fok1 polymorphism and its association with BMD. The Fok1 polymorphism is a T->C transition at 2nd nucleotide position of exon 2 of the VDR gene. They studied Fok1 polymorphisms in 100 postmenarchal Mexican American Caucasian women and found that women with ff genotype had 12.8% lower BMD at lumbar spine as compared to women FF genotype, thereby describing the association of BMD with Fok1 polymorphisms for the first time (Gross et al. 1996). Similar results were seen in post-menopausal women from Italy (Gennari et al. 1999), France (Lucotte et al. 1999), Korea (Choi et al. 2000) and India (Yasovanthi et al. 2011; Mitra et al. 2006) with ff genotype having significantly lower BMD as compared to FF genotype. On the other hand, Zmuda et al. (1999) did not find any association between VDR gene polymorphism and BMD in African-American women.

2.5.3: Studies in Children and Adolescents on VDR Gene Polymorphisms of Bsm1 and Fok1 Loci

Effect of age on association of VDR gene polymorphisms and BMD was recently demonstrated by a study done in Lebanese population (Arabi et al. 2010). Over past few years very few studies have focused on analyzing the associations of VDR gene polymorphisms of Bsm1 and Fok1 loci and bone mass accrual during the growing years and the results have been varied.

In a cross-sectional study on Chinese children aged 0 – 6 years, Yu et al. (2011) discovered that ff genotypes of the VDR Fok1 variant was significantly associated with a decreased total body (TB) BMD in Chinese children. Similarly, cross sectional study in Japanese girls aged 13.4 ±0.9 years revealed that girls with ff genotype of Fok1 loci were at significant risk of acquiring low BMD (Katsumata et al. 2002). In another study in 72 children from Houston, aged 7 – 12 years, Fok1 polymorphism was significantly associated with TBBMD and calcium absorption with FF genotype having significantly 8.2% higher BMD and 45% higher calcium
absorption than the ff genotype (Ames et al. 1999). Thus, ff has a negative association with BMD.

Laaksonen et al. (2004) demonstrated a gender difference in VDR gene polymorphisms and BMD in Finnish children aged 14 – 16 years. They found no significant association of Fok1 polymorphisms with BMD at radius, ulna or calcaneum in girls however, Finnish boys of the same age with Ff genotype had significantly higher BMD at radius and ulna and calcaneum (Laaksonen et al. 2004). However, in a longitudinal study on Fok1 polymorphisms and bone accrual at TBBMD, LSBMD or FNBMD in boys aged 16.9 ± 0.3 years, FF genotype was found to be significantly associated with bone mass accrual at lumbar spine and total body but not femoral neck (Strandberg et al., 2003).

Ferrari et al. (1998) analyzed the association of Bsm1 polymorphisms and TBBMD, LSBMD and FNBMD in 143 prepubertal and 54 peri- and postpubertal Caucasian adolescent girls from Switzerland and found that BB genotype had significantly lower LSBMD. Trends for a similar difference were also detected at the FNBMD (Ferrari et al. 1998).

Lorentzon et al. investigated association of VDR gene polymorphism and TBBMD, LSBMD and FNBMD values adjusted for age, height, weight and physical activity in healthy postmenarchal Caucasian girls. They found that VDR gene polymorphism of Bsm1 locus defined by Bb genotype had significantly higher LSBMD as compared to other 2 genotypes whereas there was the Fok1 locus did not show any association with BMD at any site. As opposed to this, Chinese children with Bb genotype were found to be at significant risk of decreased TBBMD (Yu et al. 2011).

In a cross sectional study, Fok1 locus polymorphism of VDR gene failed to show an association with TBBMD in Danish girls aged 11 – 12 years even after adjustment for dietary and environmental factors (Cusack et al. 2006). Also, Arabi et al. (2010) demonstrated that VDR gene polymorphisms of Bsm1 and Fok1 variants had no association with TBBMD, LSBMD or FNBMD in both Lebanese boys and girls aged 10 – 17 years. Similarly, in a longitudinal study on bone mass accrual in children, Nelson et al. (2000) found no association of VDR gene polymorphisms as
defined by \textit{Bsm1} with TBBMD either at baseline at 9 years or at follow up at 11 years of age.

The differences in the results of the associations of VDR polymorphisms with BMD may be due to the demonstrated ethnic differences in the prevalence of genotypes of VDR gene as demonstrated by Nelson et al. (2000). They analyzed polymorphism of \textit{Bsm1} loci in 35 African-American (16 children) and 49 White (25 children) residing in the Detroit, USA and found that BB genotype was absent in the African-American children whereas 24\% of the Whites had BB genotype.

Thus, VDR gene polymorphisms have different effects on BMD depending on age, gender, ethnicity, site and other lifestyle factors.

\textbf{2.5.4: Need for Analysis of VDR Gene Polymorphisms in Indian Adolescent Girls}

Bone mineral density in healthy Indian adolescent girls was found to be significantly lower than that in children from UK (Khadilkar et al. 2010). Studies in India have also shown the bone mineral density is lower in girls as compared to boys (Kadam et al. 2009) and a gender bias exists in India towards favorable allotment of calcium rich foods to boy over girls (Sanwalka et al. 2010; Puri et al. 2008; Marwaha et al. 2005). Thus, keeping in mind the ethnic differences, identifying polymorphisms associated with bone mass accrual during childhood and adolescence especially in girls will help focus and direct basic research on the mechanisms underlying bone mass accrual, and create short-cuts to the development of new tools, markers and techniques to maximize bone mass accrual.

\textbf{2.5.5: Methods for Analysis of Polymorphisms – Technical Principles}

The single nucleotide polymorphisms of the VDR gene can be identified by either Restriction Fragment Length Polymorphism – Polymerase Chain Reaction (RFLP-PCR) or Quantitative Real Time Polymerase Chain Reaction (qPCR)

Restriction Fragment Length Polymorphism - Polymerase Chain Reaction (RFLP) can be defined as a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and then
analyzing the size of the resulting fragments by gel electrophoresis (Greene et al.
2009).

Quantitative Real Time Polymerase Chain Reaction (qPCR) is a more
sensitive and specific method for identifying SNP. qPCR monitors the increase in
fluorescence during each cycle which indicated the increase in the synthesis of
amplicon. The fluorescent signal is either given by a Taqman probe labelled with a
reporter dye at the 5´ end and a quencher at the 3´ or else by a non-specific dye such
as SYBR Green that fluorescence when bound to double stranded DNA (Wittwer et
al. 2001). qPCR is a quicker, cheaper and much precise method of identifying SNP as
compared to RFLP-PCR. The RFLP approach is prone to generate false positives due
to partial or lack of DNA digestion; in fact this may be a common problem depending
on DNA purity and the restrictase used. In qPCR, an extra primer is used in the allele-
specific amplification to avoid false positives (Quesada et al. 2004).

Hence in the present study, qPCR technique was used for assessing Bsm1 and
Fok1 polymorphisms in adolescent girls. The present study would identify linkages of
Bsm1 and Fok1 with BMD. The loci that are identified to be associated with accrual
in young adolescent Indian girls may also serve as starting points for launching
studies of the interaction between genotype and environmental factors and gene by
gene interactions that affect bone mass accrual. It would also be useful in devising
genotype-specific interventions to promote bone mass accrual (Deng and Becker
2004).

2.6: Importance of Dietary Calcium in Bone Mass Accrual

Nutrition is an important modifiable factor in the development and
maintenance of bone mass. Calcium, phosphorus, proteins, vitamin D, magnesium,
znic, copper, iron, fluoride, and vitamins D, A, C, and K are some of the most
essential nutrients required for adequate bone health (Tucker et al. 2003; Ahmadieh
and Arabi 2011; Heaney 2005). Of these, calcium is the most vital nutrient required
for adequate bone growth and mineralization.

Calcium is a building block of bone and a deficiency of calcium is associated
with low mineralization and increased risk of fracture. The high velocity of bone
mineral accumulation during puberty especially requires a greater intake of calcium
compared to childhood and young adulthood (IOM 1997). To cope with the increased of calcium during growing years, there is an increase in the plasma levels of 1,25(OH)\textsubscript{2}D that stimulates the renal tubular reabsorption of calcium and inorganic phosphate (Bonjour et al. 2003). The action of 1,25(OH)\textsubscript{2}D seems to be mediated through the action of insulin like growth factor–1 (IGF-1) (Bonjour et al. 2003). The plasma levels of IGF-1 increase during pubertal maturation and peak around puberty. The increase in IGF – 1 levels stimulate the hydroxylation of 25(OH)D to 1,25(OH)\textsubscript{2}D. The increased 1,25(OH)\textsubscript{2}D also increases calcium and phosphorus absorption (Bonjour 2003).

The increased calcium absorption during growing years especially during puberty, is essential for optimal accrual of PBM (Bonjour et al. 2003). A low calcium intake during growing years has thus been associated with low bone density and increased fracture risk (Manias et al. 2006). Children with low calcium intakes (around 440 mg/d) are found to be shorter and have significantly lesser BMD at hips, lumbar spine and radius as compared to children with adequate calcium intake (around 1200 mg/d) (Blake et al. 2002). Children who avoid drinking milk and have low dietary calcium intakes are shown to have increased prevalence of fracture (Goulding et al. 2004). Thus, optimal calcium is required for adequate bone health and prevention of fractures.

2.6.1: Calcium Requirements in Children and Adolescents

The first Indian recommended dietary allowances (RDA) for calcium were published in 1989; these have been recently revised by the expert group of Indian Council of Medical Research (ICMR 2009) (Table 2.1). These recommendations are based on amalgamation of 3 approaches: 1) Calcium balance studies, 2) A factorial model using calcium accretion based on bone mineral accretion data 3) clinical trials investigating the response of change in calcium balance or BMC/BMD or fracture rate varying on calcium intake. Using these 3 approaches, an ICMR expert committee has defined the minimum amount of calcium needed to accrue enough BMC for good bone health during childhood and adolescence (ICMR 2009).

The World Health Organization Expert group has suggested that studies from developed countries should not be used for estimating calcium requirements of
developing countries as calcium requirement is directly affected by intake of animal protein and sodium which is relatively less in developing countries (ICMR 2009; WHO 2004). As there is limited data on calcium balance studies in Indian children, an ICMR expert group has used a factorial approach for estimating the calcium requirement for Indian children. Assuming that faecal excretion (50 mg/d) + urinary excretion (48 mg/d) + excretion through sweat (30 mg/d) + 125 mg of calcium retention, the total calcium requirement per day was calculated to be 253 mg/d. Assuming a retention rate of 40%, calcium requirement of 600 mg/day was set for young children (ICMR 2009). Based on the supplementation study by Shatrugna et al. (2006), the mean calcium requirement for 7 – 15 year old girls and boys was estimated to be 192 mg/d [123 mg/d calcium retention + 30-40 mg/d (faces) + 40 – 50 mg/d (urinary losses)]. Assuming a retention rate of 25% during the growing years, calcium requirement of 800 mg/day was set for adolescents in India (ICMR 2009). Recommendations for dietary calcium during childhood and adolescences by FAO/WHO and ICMR are given in Table 2.1.

Table 2.1: Recommendations for Dietary Calcium during Growing Years

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<tr>
<td></td>
<td>Populations from developed countries with high protein intake</td>
<td>Populations from developing countries with low protein</td>
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<tr>
<td>1 – 3 years</td>
<td>500</td>
<td>500</td>
<td>400</td>
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<tr>
<td>4 – 6 years</td>
<td>600</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>7 – 9 years</td>
<td>700</td>
<td>700</td>
<td>400</td>
</tr>
<tr>
<td>10 – 18 years</td>
<td>1300</td>
<td>1000</td>
<td>600</td>
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</tbody>
</table>

(Adapted from ICMR 2009, Page No. 179)
2.7: Calcium Intake in Children and Adolescents

Children and adolescents are observed to have large amounts of fast foods and sweets in their diets leading to nutritional imbalances. Dietary calcium deficiency is observed in adolescents from across the world.

2.7.1: International Studies

Analysis of dietary calcium intake using data from the National Health and Nutrition Examination Society, Bucholz et al. (2011) demonstrated that 40.2% children aged 3-5 years in America did not meet their RDA. Dietary calcium intake was found to be around 280 mg/d in Chinese children using the 5 day diet recall method (Lee et al. 1994). In 8.3 – 11.9 year old Gambian children, calcium intake has been reported to be around 300 mg/d which is about one fourth of the recommended intake in American children (Dibba et al. 2000).

Mean dietary calcium intake was found to be 1260 mg/d in Jewish and Arabs girls aged 14.5 years in Israel using a semi-quantitative FFQ. Of the 2000 girls surveyed, 20.4% Jewish girls and 19.8% Arab girls consumed less than 800 mg/d of calcium whereas 6.4% Jewish girls and 7.3% Arab girls consumed less than 500 mg/d of calcium (Rozen et al. 2001).

In a cross sectional study in 1- 16 year old children in Turkey, calcium intake was found to be significantly less than the recommended intake especially in children older than 8 years of age (Akman et al. 2011). Using a semi-quantitative FFQ to assess dietary calcium intake in 386 Lebanese children aged 10 – 16 years, mean calcium intake was found be around 673 – 786 mg/d in girls and 873 – 939 mg/d in boys. Only 12% of the children were found to meet their recommended intakes for calcium (1300 mg/d) (Salamoun et al. 2005).

Thus, these studies illustrate the prevalence of low dietary calcium intake in children and adolescents from various developed and developing countries across the world.
Dietary calcium deficiency is omnipresent in children and adolescents from all walks of life in India. The National Nutrition Monitoring Bureau (NNMB) of India analysed the dietary intakes in 5 rural villages each in 16 states of India from the year 2000-2001 using 24 hour diet recall. Calcium intake was less than the RDA in children from rural villages in all states in India. The mean dietary calcium intake was 278±238 mg/d in 7 – 9 year old. Calcium intake was 326±283 mg/d and 304±251 mg/d in 10 – 12 year boys and girls respectively whereas calcium intake was 407±361mg/d and 355±267 mg/d in 13 -15 year boys and girls. Calcium intake in 16 – 17 year old boys from rural India was 437±315mg/d whereas that in girls was 415±465 mg/d (NNMB 2002).

Dietary calcium analysis using a 24 hour recall in preschool children from 17 tribes in rural India from Bihar (Central India) revealed that calcium intake was around 50% of the RDA (Yadav and Singh 1999). Dietary calcium was found to be 211 ± 158mg/d in adolescent girls from lower socio economic stratum (LSES) from Lucknow, Uttar Pradesh, North India (Sahu et al. 200). Calcium intake analysed using 5- 7 day diet recall was found to be less than the RDA in children living in urban (293±6mg/d) as well as the rural (277±6 mg/d) areas of Tirupati, Andhra Pradesh, South India (Harinarayan et al. 2008).

In a cross sectional study to analyse the calcium intake in 200 adolescent boys and girls from 2 different socio-economic strata from Pune, Maharashtra, Western India, aged 14 – 15 years using a food frequency questionnaire (FFQ), median calcium intake was less than the Indian RDA in higher socio economic stratum girls (HSES) [764 mg/d (541-959)], LSES girls [506 mg/d (380-674)] and LSES boys [767 mg/d (585-1043)] whereas, calcium intake was adequate in HSES boys (Sanwalka et al. 2010).

Dietary analysis of calcium intake using 24 hour recall and a FFQ in 6 – 18 year old children from Delhi, North India, illustrated that calcium intake was less than the current RDA in both LSES (454±187 mg/d) and HSES (685±184 mg/d) children (Puri et al. 2010). Similarly in another study from Delhi, calcium intake was found to
480.8 (191.4) mg/d in LSES children and 707.3 (162.9) mg/d in HSES children (Marwaha et al. 2010).

Majority of these studies exhibited plant based vegetarian diets with low intake of milk and milk products (NNMB 2002). This might have resulted in low calcium intake.

2.7.3: Sources of Calcium in the Diet of Children and Adolescents

According to Nutritive value of Indian foods database, major dietary sources of calcium and their calcium content are given in Table 2.2. Dairy sources such as milk, curd, cheese etc. are rich sources of calcium with very calcium bioavailability. In developed countries most of the calcium requirement is met by adequate dietary intake of milk and milk products (Subar et al. 1998; Iuliano-Burns et al. 1999).

However, diets of children from developing countries like India, Pakistan and Bangladesh on are deficient in milk and milk products (Ahmed et al. 1998; Hakeem et al. 2002; Tupe and Chiplonkar 2007; Sanwalka et al. 2010; Puri et al. 2008). ICMR has recommended a minimum dietary milk intake of 500 ml/day to meet the dietary calcium requirements in Indian children and adolescents (Krishnaswamy 2003). Yet, according to the NNMB report (2002), dietary milk intake was 24% and 20% of the RDA for milk in rural Indian children aged 1-3 years and 4-6 years respectively. On the other hand, mean milk intake was reported to be merely 48-58 ml in children aged 7 – 18 years in rural India.

Even in urban cities like Delhi and Pune, the mean milk intake has been reported to be very low. Dietary analysis revealed that milk intake was 211 ml in LSES children and 362 ml in HSES children in Delhi, India (Puri et al. 2008). In another study from Pune, researchers found that milk contributed only 43.4 – 180 mg/d to the total calcium intake in girls diets and 200 – 271 mg/day in boys diets (Sanwalka et al. 2010).
<table>
<thead>
<tr>
<th>Foods</th>
<th>Calcium (Mg/100 G Raw Weight)</th>
<th>Foods</th>
<th>Calcium (Mg/100 G Raw Weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk And Milk Products</strong></td>
<td></td>
<td><strong>Foods</strong></td>
<td></td>
</tr>
<tr>
<td>Milk (Buffalos)</td>
<td>210</td>
<td>Parsley Leaves</td>
<td>390</td>
</tr>
<tr>
<td>Milk (Cows)</td>
<td>120</td>
<td>Turnip Greens</td>
<td>710</td>
</tr>
<tr>
<td>Curd (Cows)</td>
<td>149</td>
<td>Drumstick Leaves</td>
<td>440</td>
</tr>
<tr>
<td>Cheddar Cheese</td>
<td>790</td>
<td>Fenugreek Leaves</td>
<td>395</td>
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<tr>
<td>Whole Milk Powder</td>
<td>950</td>
<td>Curry Leaves</td>
<td>830</td>
</tr>
<tr>
<td>Skim Milk Powder (Cows)</td>
<td>1370</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Legumes And Split Pulses</strong></td>
<td></td>
<td><strong>Nuts And Oilseeds</strong></td>
<td></td>
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<tr>
<td>Bengal Gram Whole</td>
<td>202</td>
<td>Almonds</td>
<td>230</td>
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<tr>
<td>Horse Gram Whole</td>
<td>287</td>
<td>Coconut Dry</td>
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<tr>
<td>Moth Beans</td>
<td>202</td>
<td>Gingelly Seeds</td>
<td>1450</td>
</tr>
<tr>
<td>Kidney Bean</td>
<td>260</td>
<td>Garden Cress Seeds</td>
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</tr>
<tr>
<td>Soybean</td>
<td>240</td>
<td>Niger Seeds</td>
<td>300</td>
</tr>
<tr>
<td><strong>Cereals And Millets</strong></td>
<td></td>
<td><strong>Spices And Condiments</strong></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>240</td>
<td>Finger Millet</td>
<td>344</td>
</tr>
<tr>
<td>Amaranth</td>
<td>397</td>
<td>Amaranthus Flour</td>
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</tr>
<tr>
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<tr>
<td><strong>Green Leafy Vegetable</strong></td>
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<td><strong>Spices And Condiments</strong></td>
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<td>Colocasia Leaves</td>
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<tr>
<td>Knoll-Knol Greens</td>
<td>740</td>
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(Adapted from Gopalan, 2002)
Other dietary sources of calcium such as cereals, millets, pulses and green leafy vegetables contribute largely to the total calcium intake in the diet of children and adolescents from developing countries (Sanwalka et al. 2010). Analysis of the dietary pattern of 10 – 16 year old girls shows that the diet of adolescent girls in India are either pearl millet based, rice based, wheat based or sorghum based and milk, vegetable and fruit intakes are less than 30% of the recommended intakes (Tupe and Chiplonkar 2009).

Puri et al. (2008) found that non-dairy sources contributed to almost 45-50% of the total dietary calcium intake of LSES children and around 30% the total dietary calcium intake of HSES children. Green leafy vegetables contributed to 134- 210 mg/d of calcium whereas cereals, pulses and other vegetables each contributed anywhere between 41 – 78 mg/d of calcium in the diets of adolescents (Sanwalka et al. 2010).

In addition to low calcium intake, there is a significant gender bias prevailing in regards to unequal distribution of food resources in developing countries especially from Asia (Subramaniam 1996). Food allocation analysis in 105 Nepali households using diet recall and observation methods revealed that while staple food items (i.e., rice, lentil soup, bread, etc.) are distributed fairly equally between men and women, side dishes which are a good source of calcium (i.e., meat and yogurt) are preferentially allocated to men or small children (Gittelsohn et al. 1997).
In India, a gender bias in feeding practices is prevalent right from infancy. According to the results of the National Family Health Survey (NFHS 1999) the median duration of breastfeeding for a boy child is nearly two months longer than that for a girl child. In a study on 400 households from Punjab in India, Gupta found consistent differentials in milk and fat allocation between boys and girls, while adolescent girls and boys had roughly similar calorie intakes, the former were given more cereals while the latter had more milk and fats with their cereal (Gupta 1987). In another study, adolescent girls especially from the lower socio-economic stratum were shown to have significantly lower calcium intake from dairy products as compared to boys from the same socio-economic stratum while calcium intake from cereals and pulses was comparable in the 2 groups (Sanwalka et al. 2010). Thus, there is a need for increasing calcium absorption in Indian children and adolescents especially adolescent girls, who may be at a greater risk of calcium deficiency.

2.7.4: Factors Affecting Calcium Absorption

As shown in Figure 2.5 intestinal calcium absorption occurs via 2 routes: active transcellular and passive paracellular pathways.

**Figure 2.5: Schematic Representation of Calcium Absorption in Intestine**

(Source: Cashman 2003, Page No. 24)
The paracellular pathway is passive and non-saturable pathway for calcium absorption and involves calcium transport through the tight junction. The saturable, transcellular pathway is a multi-step process, involving the entry of luminal calcium across the microvillar membrane into the enterocyte, then movement through the cytosol (i.e., translocation to the basolateral membrane), followed by active extrusion from the enterocyte into the lamina propria and, eventually, into the general circulation (Cashman 2003).

Vitamin D enhances synthesis of calcium binding proteins in the intestine and influences the paracellular pathways of calcium absorption (Weaver and Heaney 2005). Carbohydrates such as lactose, improve calcium absorption by augmenting its passive diffusion across villous membranes (Buzinaro et al. 2006).

Cereals and millets which are a major source of calcium in the diets of adolescents from developing countries like India are very rich in phytates (Sanwalka et al. 2010; Ahmed et al. 2005). Phytate is a hexa phosphate of inositol. Phytates form insoluble complexes with calcium and reduce calcium bioavailability (Gopalan et al. 2002). In a study on calcium absorption, absorption was found to be significantly less from low phytate soybean as compared to soybean with high phytate content (Heaney et al. 1991). In another in-vitro study to analyses calcium absorption from vegetables, legumes and seeds, Kamchan et al. (2004) demonstrated that calcium absorption was negatively correlated with the phytate content of food. White and black sesame seeds which had phytate content of around 1200 – 1400 mg/100 g had calcium dialysibility of around 3.5 – 4.2% whereas celery (5.2 mg/100g) and kale (38.9 mg/100g) which had low phytate content had high calcium dialysibility of around 36 – 39% (Kamchan et al. 2004).

Another non-nutritional factor affecting calcium absorption is oxalates. Oxalates are dicarboxylic acid or its salts. They are found abundantly in green leafy vegetables and some pulses (Gopalan et al. 20025). Calcium absorption from spinach which is very high in oxalates is found to be less (around 5%) as compared to calcium absorption from milk (27.6%) (Heneay et al.1988). Kamchan et al. (2004) demonstrated that foods like Amaranth, betel leaves and pak-paw which are very high in oxalate but low in phytates also have very low calcium dialysibility of around 2.5 – 6.5%.
Food processing methods like leavening and malting have also been shown to decrease the non-nutritional factors in plant foods and increase calcium absorption.

Leaven is derived from a Latin word “levre” which means to raise or to make light by aeration. Overnight fermentation and use of yeast are some of the most commonly used fermentation techniques in India. The term fermentation refers to breakdown of carbohydrates and carbohydrate like materials under either aerobic or anaerobic conditions (Srilaxmi 2003). Navert and Sandstorm (1985) prepared 20 breads with varying time of leavening ranging from 0 hours to 120 hours. When baked after 2 hours of fermentation, phytic acid content had reduced to 40% of the raw dough which further reduced to 15% if the bread was leavened for 2 days (Navert and Sandstorm 1985). Fermentation of sorghum flour for over 72 hours to prepare Injera (a traditional Ethiopian bread) has been shown to decrease phytate content from 317.6±13.5 mg/100g in sorghum flour to 286.7±4.3 mg/100 g in Injera (Mohammed et al. 2010). Weaver et al. (1991) demonstrated that calcium absorption was higher from leavened bread labelled with $^{45}$Ca as compared to non-leavened bread.

Malting is a process involving germination and drying of cereals. The objective of malting is to promote development of hydrolytic enzymes that are not active in raw cereals (Dewar et al. 1997; Sastri 1939). Phytates are also broken down during the process of germination thereby increasing calcium absorption from malted foods (Gopalan et al. 2002). Malt pre-treatment of sorghum flour has shown to increase calcium extractability to 102 – 103% which is a result of reduction in the phytin content (Idris et al. 2005; Idris et al. 2007).

For many years, steatorrhea has been associated with decreased intestinal calcium absorption as fat are said to form insoluble soaps with calcium (Haderslev 2000). However, recent in-vitro studies using CaCo-2 cells suggest otherwise (Jewell and Cashman 2003; Jewell et al. 2005). When human intestinal Caco-2 cells were exposed to conjugated linoleic acid (CLA) and medium chain fatty acids to measure transepithelial and transcellular transport of $^{45}$Ca, fluorescein transport (a marker of paracellular Ca transport) and transepithelial electrical resistance (an indicator of permeability), paracellular calcium transport across Caco-2 cells was significantly increased (Jewell and Cashman 2003; Jewell et al. 2005). Oleic acid has shown to increase calcium absorption by an increased activation of the calcium binding proteins.
calmodulin and increased calcium permeability through calmodulin-dependent protein-kinase calcium channels (Kobayashi et al. 1996). Medium chain fatty acids like decanoic acid has shown to enhance calcium absorption through increased permeability of intracellular tight junction (Lindmark et al 1998). Fermentation and malting also enhances fatty acid content of foods (Achinewhu 1986; Aseidu et al. 1993)

In the last few years, use of prebiotics to enhance calcium absorption has increased tremendously. Prebiotics defined as non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon are also shown to increase calcium absorption (Gibson and Roberfroid 1995; Cashman 2003). Various prebiotics like inulin, oligofructose, galacto-oligosaccharide, and inulin have been shown to increase calcium absorption in adolescents (Abrams et al.; Griffin et al. 2002). Supplementation with 8 g inulin type fructan in pubertal adolescent girls has significant benefit on calcium absorption and bone mass accrual as compared to those receiving maltodextrin as placebo (Abrams et al. 2005). In a randomised, cross-over design, pubertal girls were supplemented with either 8g/d of placebo (sucrose) or oligofructose or a mixture of inulin+oligofructose. Calcium absorption was significantly higher in the group receiving inulin+oligofructose as compared to the placebo group, however there was no significant difference between the placebo and oligofructose group (Griffin et al. 2002).

The proposed mechanism for increase in calcium absorption after prebiotic supplementation is as follows: there is an increased production of lactic acid and short chain fatty acids due to stimulated microbial growth and fermentation by the presence of prebiotics in the large intestine which increase solubility of calcium and thus enhance passive calcium absorption. Also, the short chain fatty acids may act on transcellular calcium absorption by increasing the exchange of cellular H+ for luminal Ca²+. There may also be an increased production of butyrate or polyamines in the intestine, which in turn may (a) induce cell growth and thus increase the absorptive surface area of the gut, and/or (b) increase mucosal calbindin D9K levels in the large intestine (Cashman et al. 2003)
However, studies analysing combined effect of prebiotic fortification with food processing method like malting and fermentation on calcium absorption are lacking.

2.7.5: Methods for Assessing Calcium Absorption

The bioavailability of an orally administered exogenous compound is defined as the fraction of the dose that reaches the systemic circulation. True calcium absorption is most accurately measured using an oral calcium source intrinsically labelled with a suitable calcium isotope and quantifying the tracer that appears in blood, urine or body compartments after absorption is complete. However, assessing absorption of calcium from food sources using isotope methods may not be possible as labelling of foods with isotopes can be difficult (Heaney 2003).

Thus, for assessing the relative oral bioavailability of calcium especially from food sources, the pharmacokinetic method and urinary calcium excretion is a much more convenient method as it is simpler and faster (Heaney 2003; Hanzlik et al. 2005; Guéguen et al. 2000). Using the pharmacokinetic method, bioavailability is determined by comparing the dose-normalized area under the curve (AUC).

AUC is defined the area under the plot of plasma concentration of drug against time after drug administration. The area under the curve is conveniently determined by the “trapezoidal rule”. Delta AUC (ΔAUC) assess the increment in area under the curve after adjusting for baseline levels of the parameter being measured. ΔCmax which is the magnitude of elevation in a blood parameter after supplementation assessed by subtracting the zero-time (pre-dose) value from the maximum value observed post-dose is another important pharmacokinetic parameter that has been used by researchers to assess calcium bioavailability (Hanzlik et al. 2005).

For assessing calcium absorption, ΔAUC and ΔCmax for serum ionized calcium and parathyroid hormone (PTH) are assessed. Although extracellular fluid calcium concentration is tightly regulated, calcium absorption nevertheless does produce a measurable, if small, degree of calcemia that can be captured by AUC (Heaney 2003). Various time frames have been used to analysis AUC after supplementation with calcium salts [6 hours (Heller et al. 2000; 4.5 hours (Hanzlik et al. 2005); 24 hours (Heaney 2003)]. Analysis of calcium absorption from 500 mg of
calcium carbonate in 12 men at various time points over a period of 24 hours illustrated that best prediction given by the AUC for the increment in serum calcium calculated is over 9 hours (Heaney 2003).

Along with calcium, serum PTH levels are also assessed for measuring calcium absorption. Even with a small and transient change in serum ionized calcium levels there is a fall in the serum concentrations of PTH (Heller et al. 1999; Heller et al. 2000; Heaney et al. 2001). The decrease in PTH levels is proportional to efficiency of absorption and thus assessing AUC for PTH would be a very useful method of assessing calcium absorption along with AUC for serum ionized calcium.

Urinary calcium excretion is a simple and fast method of analysing calcium absorption which can be obtained within 3 – 4 hours of ingestion of a test meal. The urinary calcium excretion reflects instant absorbability of calcium and depends on several dietary factors that affect losses of calcium (Guéguen et al. 2000). Urinary collection has been used as a method to assess calcium absorption from calcium supplements as well as food sources like milk (Mortensen and Charles 1996).

**To summarize, the available evidence suggests that:**

- Adequate bone mineralization during the growing years is crucial for bone health throughout the life cycle
- Dual energy X ray absorptiometry (DXA) is the gold standard for assessing BMD in children and adolescents and there is a need to develop Indian reference database to accurately interpret DXA results
- Vitamin D receptor gene polymorphisms of Bsm1 and Fok1 loci may have an influence on bone mass accrual
- Analysis and understanding of VDR gene polymorphisms in adolescents may provide an opportunity to devise and implement additional preventive measures to promote accrual
- Dietary intake of bio-available calcium is essential for optimal mineralization of bones during childhood and adolescence
- Calcium intake is significantly less than the Indian RDA in children and adolescents in India
Unlike adolescents from the developed countries, the main source of calcium in Indian diets is cereals, pulses and vegetables which have low calcium bioavailability.

Strategies need to be devised to enhance intake of absorbable calcium in the diet of Indian children and adolescents.

Prebiotic fortification has a great potential to improve calcium absorption.

Therefore aim and objectives of the present study are:

**Aim:** To evaluate bone status of apparently healthy Indian youth, examine association of genetic make-up with bone mass and study factors influencing calcium absorption.

**Principle Objectives:**

- To generate a normative reference database for bone parameters in healthy children aged 5 – 17 years from Pune city, India.
- To study the association of Vitamin D Receptor gene polymorphisms of *BsmI* and *FokI* loci on bone status of post-menarcheal girls.
- To formulate calcium rich non-dairy based foods products and select processing methods which will enhance calcium absorption.
- To study the effect of prebiotic fortification on calcium absorption from non-dairy based food products naturally high in calcium in young girls aged 15 – 18 years.