SUMMARY AND CONCLUSIONS

Bone mass accrued during the rapid period of growth during childhood and adolescence serves as a reservoir to prevent osteoporotic fractures in later life (Bonjour et al. 1994). However, certain medical and environmental factors such as malnutrition may have a deleterious effect on bone mineralization, necessitating measurement of bone status during 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; Munoz and Argente 2002; Shouman et al. 2010).

To assess bone status in children, dual energy X-ray absorptiometry (DXA) is used as the gold standard and comparison with ethnicity, age, gender and height specific reference is essential for immaculate evaluation of DXA measurements (Gordon et al. 2008). In absence of an Indian reference database for bone mass in children, the present study was undertaken to measure bone mass at the total body, lumbar spine and femoral neck in healthy Indian children and adolescents aged 5 to 17 years studying in schools and colleges in Pune city, Maharashtra, Western India and to develop percentile curves for bone mass status in children and adolescents.

Polymorphisms of vitamin D receptor (VDR) gene located on chromosome 12q13.1 influences peak bone mass accrual and have been shown to be associated with bone mineral density (BMD) in elderly women from various countries including India (Morrison et al. 1994; Tokita et al. 1996; Spector et al. 1995; Lorentzon et al. 2001; Mitra et al. 2006; Yasovanthi et al. 2011; Mitra et al. 2006)). As association of VDR gene polymorphism with VDR gene is age dependent (Arabi et al. 2010), the present study examined the association of VDR gene polymorphisms and bone mass status in adolescent girls.

Calcium is the most essential nutrient affecting bone mineral density. Various studies in India and other developing countries have highlighted the wide spread calcium deficiency amongst children and adolescents from all socio-economic strata (Puri et al. 2008; Sanwalka et al. 2010; Salamoun et al. 2005; Rozen et al. 2001; Ahmed 1998; NNMB 2002). The major sources of calcium in the diet of children and adolescents are oxalate and phytate rich cereals and pulses which have low calcium extractibility (Puri et al. 2008; Sanwalka et al. 2010). Food processing methods like
malting and leavening (fermentation) decrease the amount of inhibitory factors in foods and increase calcium extractability (Idris et al. 2007; Weaver et al. 1991). Thus, to improve the calcium intake in adolescents, non-dairy based calcium rich food products were developed in the current study using processing methods like malting and fermentation.

Supplementation with prebiotics has been shown to increase calcium absorption via an increase in the intestinal short chain fatty acid production and decrease in the intestinal pH (Cashman 2003). Studies have demonstrated an increase in fatty acids like palmitoleic acid, stearic acid, oleic acid, arachidonic acid during fermentation and malting (Achinewhu 1986; Aseidu et al. 1993). However, studies on effect of prebiotic fortification on fatty acid profile and pH of food during fermentation and malting and thereby on calcium absorption from non-dairy based calcium rich products are lacking. Thus, the current study evaluated the effect of prebiotic fortification on fatty acid profile and pH during fermentation and malting. The study also examined calcium absorption from a fermented –malted snack (plain or fortified with prebiotic) in comparison with a standard calcium supplement using area under the curve for serum iCa and PTH.

The ethical approval of the entire study protocol was obtained from the ethics committee of Hirabai Cowasji Jehangir Medical Research Institute and Jehangir Clinical Development Center, Pune.

To develop the percentile curves for bone mass, a cross sectional study was conducted in 920 children (480 boys) aged 5 – 17 years. Clinical examination was performed by a pediatric endocrinologist to assess health status of the children. Data were collected on anthropometry and socio-demographic factors. Pubertal staging was assessed using Tanner method. Bone mineral content (BMC), bone area (BA), bone mineral density (BMD) were measured for total body (TB), lumbar spine (LS) and left femoral neck (FN) using GE-Lunar DPX Pro (GE Healthcare, Wisconsin, USA, 2005) Pencil Beam DXA scanner. LS and FN bone mineral apparent density (BMAD) was calculated. Lean body mass (LBM) was measured for total body. Reference percentile curves were plotted using LMS method.
Study results indicate that the average increase in TBBMC and TBBA with age was of the order of 8 to 12% at each age group. Average increase in TBBMD with age was 2.9% in both boys and girls, which is lower than the reported rate of increase of around 4% in Caucasian children. Maximal increase in TBBMD occurred around the age of 13 years in girls and three years later in boys. Average increase in left FNBMD with age in boys and girls was 3.5% and 4% respectively with maximal increase around 10 years in both boys and girls. Average increase in LSBMD with age in boys and girls was of the order of 5.8% with maximal increase around 15 years in boys and around 11 years in girls. Increase in LSBMAD was 30% and 36% from age 5 to age 17 in boys and girls respectively. Increase in left FNBMA from age 5 to 17 years was 2% (boys) and 6% (girls).

Reference percentile curves were plotted to assess bone mineral status in children and adolescents. TBBMC for age, TBBA for age, TBBMD for age, LSBMAD for age and FNBMA curves were plotted. A significant gender difference was seen with flattening of the girls’ curves around the age of 15 years for TBBMC, TBBA and TBBMD, while in case of boys, there was a sharp increase till the age of 17 years. The percentile curves for left femoral neck BMAD by age were flatter than those for the LSBMAD by age.

To assess bone density using Molgaard et al.’s (1997) method, TBBA for height and TBBMC for TBBA curves were plotted. TBBA was more closely associated with height [boys (r = 0.961; P < 0.001), girls (r = 0.956; P < .01)] than with age [boys (r = 0.900; P < 0.001), girls (r = 0.871; P < 0.001)]. There was a strong association between TBBMC and TBBA values for boys (r = 0.981; P < .001) and girls (r = 0.982; P < 0.001). Application of Molgaard et al. approach was demonstrated with the help of a known case of osteogenesis imperfect.

To distinguish bone disease from muscle disease, using Crabtree et al.’s (2004) method, LBM for height and TBBMC for LBM curves were also plotted. A close association was observed between TBBMC and LBM values for boys (r = 0.953; P < 0.001) and girls (r = 0.939; P < 0.001). Application of Crabtree et al.’s approach was demonstrated with the help of a case of stunted, malnourished child.
To study the association of VDR gene polymorphism and bone mass in adolescent girls, data were collected on age, height, weight, socio-demographic factors, diet and physical activity in 120 girls aged 15 – 17 years. Prevalence of signs and symptoms of calcium deficiency and the amount of time spent in sun was evaluated. Serum ionized calcium (iCa), parathyroid hormone (PTH) and 25-hydroxyvitamin D [25(OH)D] were measured. BMC, BA, BMD were measured for total body, lumbar spine and femurs. LBM was also measured. Polymorphism of Bsm1 and Fok1 loci of VDR gene; were studied using real time quantitative polymerase chain reaction (qPCR) and restriction fragment length polymorphism (RFLP).

The overall prevalence of genotype for Bsm1 in this study was 33.3% Bb, 29.2% bb and 37.5% BB. The overall prevalence of genotype for Fok1 in this study was 44.2% Ff, 7.5% ff and 48.3% FF.

When classified according to Bsm1 or Fok1 genotype, there were no significant difference in the anthropometric, dietary, physical activity and blood parameters of the girls (p>0.1). Around 10% of the girls showed signs of genu varum and genu valgum while almost 57 – 60% of the girls complained of non-specific signs and symptoms of calcium deficiency such as aches and pains. Except for fat intake, nutrient intake was below the recommended dietary allowance (RDA) for all 120 girls. For Bsm1 genotypes, 14 (35%) Bb, 16(45.7%) bb, 16 (35.6%) BB had hyperparathyroidism (PTH > 6.4 pmol/L). Similarly, hypocalcaemia (iCa <1.12 mmol/L) was seen in 22 (55%) Bb, 16 (45.7%) bb, 23 (51.1%) BB. For Fok1 genotypes, 17 (32.1%) Ff, 4(44.4%) ff, 25 (43.1%) FF had hyperparathyroidism. Similarly, hypocalcaemia was seen in 26 (49.1%) Ff, 4(44.4%) ff, 31 (53.4%) FF. One hundred and eighteen (98%) girls had hypovitaminosis.

When classified according to Bsm1 genotype, BB genotype showed association with adjusted means [for 25(OH)D or LBM or diet and physical activity or prevalence of signs and symptoms of calcium deficiency or time spent in sun] of TBBMC, TBBA and LSBMC (p<0.05). However, Bsm1 genotype did not show any correlation with FNBMC or FNBA (p>0.1). On the other hand, when classified according to Fok1 genotype, Ff genotype tended to show association with FNBMC and FNBA when adjusted for 25(OH)D or diet and physical activity (p<0.1) Ff
genotype had significantly higher FNBA when adjusted for prevalence of deficiency signs (<0.05). However, Fok1 genotype did not show any association with adjusted means of TB or LS BMC or BA (p>0.1).

A dietary survey in boys and girls aged 6 to 19 years in schools and colleges revealed that milk intake was lesser than the recommended intake of milk of 300 ml/day in both boys and girls. Of 236 children, 36% boys and girls had no intake of milk in their diet and 86.2% were not consuming any milk products such as cheese. Dietary calcium intake was 64±34% and 54±25% of the Indian RDA (800 mg/day) (ICMR 2010) in boys and girls respectively. Calcium intake from milk was 46% and 35% of the total calcium intake in boys and girls respectively indicating the need to develop non-dairy based foods to improve dietary bioavailable calcium intakes.

Thus, 20 non-dairy based calcium rich products (NDBCRP) were developed using calcium rich foods and processing methods like malting and leavening. Of the 20 recipes, 14 recipes (having calcium content of > 200 mg) were analysed for calcium content using atomic absorption spectrophotometer and their acceptability was assessed using Hedonic rating scale. The recipes were compared with 12 dairy-based calcium rich products (DBCRP) for their calcium content.

The calcium content of 100g NDBCRP (337.5 ± 104.4 mg) was on par with 100g DBCRP (274.3 ± 127.8 mg) (p=0.199). Even after adjusting for calorie content, there was no significant difference in the calcium content of the two groups (p = 0.547). About 86% of RDA (800 mg/day) can be met by consuming one meal of 200 g of NDBCRP. Using the Hedonic rating scale, 10 out of 14 products were very well accepted by the panel members. Of the 14 products, finger-millet pancake with sesame-soybean dip was the most acceptable product and was selected to assess the effect of galacto-fructo-oligosaccharide (G-FOS) (prebiotic) on relative calcium bioavailability.

Sixty-one girls from the 120 girls enrolled for VDR study were randomly selected for the study and were again randomly divided into 3 equal groups; i) Group-P: fermented, malted finger millet pancake with soy-coconut dip (534mg calcium/serving), ii) Group-G: fermented, malted finger millet pancake with soy-coconut dip fortified with 8 g of Galacto-fructooligosaccharide(G-FOS) (prebiotic),
(534mg calcium/serving) iii) Group-C: low calcium snack fortified with calcium carbonate (500mg). Serum ionic calcium (iCa) and intact parathyroid hormone (PTH) were measured at 0 (before calcium load), 1, 3, 5, 7, 9 hours. Increment in area under the curve over baseline (ΔAUC) and the magnitude of the elevation (ΔCmax) was calculated to assess calcium absorption. Twenty four hour urinary calcium was measured. Fatty acid profile of the pancakes [fermented (plain and fortified with G-FOS) and unfermented] with sesame soybean dip was analysed using gas chromatography.

There was no significant difference in anthropometry or dietary intake of the 3 groups (p<0.1). Mean serum iCa and 25 OHD concentrations at baseline were also not significantly different amongst the three groups. Thus, the 3 groups were similar in terms of their characteristics.

Maximum rise in serum iCa of 9.1% was seen in group G, followed by group C (3.1%) and then group-P (1.4%). ΔAUC was 109.5 % more in group G as compared to group P which was statistically significant (p<0.05), indicating that calcium absorption is more than double on fortification of pancake with G-FOS. ΔCmax occurred relatively late at 5 hours post supplementation as compared to group C wherein ΔCmax was achieved by 3 hours indicating slow and sustained absorption of calcium from pancakes.

ΔAUC for serum PTH was 53.5 % more in group C as compared to group P which was statistically significant (p<0.05). ΔAUC for PTH was 39.7% higher for group G as compared to group P (p>0.1). ΔCmax was the highest in group C and it was significantly higher from the other two groups (p < 0.05). There were no significant difference in ΔCmax for group G and group P (p>0.1).

Urinary calcium was significantly higher in group C than in group G (113%) and P (46%) (p<0.05), similar results were seen by Mortsen et al. (1996). There was no significant difference in urinary calcium in group G and group P. Thus, the findings show that despite similar calcium load in all the 3 groups, calcium excretion in urine was higher in groups receiving calcium from tablets than that from food source.
pH of fermented-malted pancake (both plain and G-FOS fortified) was 5.9 and was less than the unfermented-malted pancake with sesame-soybean dip (pH = 6.3). G-FOS fortified pancakes had the highest total fatty acid content whereas unfermented had the lowest fatty acid content. Low pH combined with high fatty acid content may have resulted in highest calcium absorption for G-FOS fortified pancakes group.

Main conclusions:

2. Age, gender and Tanner specific reference data for total body, lumbar spine and left femoral neck bone status measured by Lunar DPX Pro DXA scanner, for Indian children and adolescents have been described for the first time in this study
2. Reference percentile curves for bone status at the total body, lumbar spine and left femoral neck are constructed which will facilitate assessment of bone health of Indian children
3. Bsm1 locus polymorphism is associated with total body and lumbar spine bone status of Indian adolescent girls whereas Fok1 locus polymorphism has a tendency for association with femoral neck bone status
4. Non-dairy based calcium rich recipes developed using processes like malting and leavening have the potential to increase absorbable calcium intake
5. Calcium absorption from fermented –malted food products naturally high in calcium is on par with standard calcium supplements and should be recommended to increase calcium intake in adolescents
6. Galacto-fructo-oligosaccharide (prebiotics) fortification during fermentation of foods increased the fatty acid content of food which further improves calcium absorption

Clinical and Public Health Implication of the current study can be stated as:

✓ The reference percentile curves developed in the present study would be useful in assessment of bone status of Indian children and adolescents and help in identifying children with low bone mass status in a clinical as well as research set-up

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✔ Analysis of VDR gene polymorphism of Bsm1 locus can be used as a tool to identify Indian adolescents at risk for low bone mineral density and additional measures can be taken to increase bone mass accrual in these individuals

✔ The new non-dairy based calcium rich products developed in the current study can be used as food-based interventions for vegetarian and vegan populations like Indians

✔ Processes like fermentation and malting can be easily taught and adopted at household level to increase calcium absorption from non-dairy calcium rich products

✔ Prebiotics increase fatty acid content of foods naturally high in calcium during fermentation and thus prebiotic fortification can be used to increase calcium absorption from cereal-pulse based foods

Further scope of research in this area includes:

❖ India is a huge country with diverse populations. Though studies have shown that anthropometric (height and weight) differences in the various regions in India are not statistically significant (Khadilkar et al. 2009), it may be worthwhile to generate a similar bone database through multicentre study to evaluate regional differences in bone status of children and adolescents. However the bone measurements must be with DXA machine of same make and company and investigating team should be the same to avoid bias.

❖ Further studies may be undertaken to investigate and confirm marked effect of Bsm1 genotype on bone mass than Fok1 genotype after accounting for environmental variation as also to evaluate age and gender differences

❖ Considering the diverse food habits in India and across the vegetarian population as a whole, more plant based recipes may be developed for increasing bioavailable calcium intake

❖ The effect of prebiotic fortification on calcium absorption from non-dairy based calcium rich products in terms of its utilization on bone mass accrual need to be examined with long term supplementation studies