Is caffeine a Risk Factor for Osteopenia of Prematurity?

By

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Abstract

Caffeine, the most common used medication in NICUs, has calciuric and osteoclasteogenesis effects.

Objectives: To examine the association of caffeine cumulative dose and duration of therapy and the Osteopenia of prematurity (OP)

Study design: A retrospective observational cohort study included premature infants less than 31 weeks and birth weight less than 1500 grams. OP was evaluated using chest X-rays on biweekly basis over 12 weeks hospital stay. Caffeine cumulative dose and duration of therapy, steroid dose and diuretic dose along with other maternal and neonatal risk factors were collected to examine their impact on OP.

Results: The cohort included 109 infants. 51% had OP and 8% had spontaneous rib fractures. Using the generalized mixed model, Caffeine dose and duration of caffeine displayed strong association with OP. Steroids and vitamin D had significant correlation with OP while diuretic use did not show statistical significant effect.

Conclusion: Caffeine cumulative dose and duration of therapy are associated with OP.

Keywords*:* premature infants, caffeine, osteopenia of prematurity, metabolic bone disease.

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Dedication

This work is dedicated to all the premature infants whose suffering, is starting from the first day of their life. As well as to all the parents who found out that their premature infant had a spontaneous bone fracture

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Chapter 1. Is Caffeine a Risk Factor for Osteopenia of Prematurity?

Neonatal bone health is a problem of growing interest and concern because of the increasing recognition of its impact upon childhood, adolescence and even adulthood. Osteoporosis in adulthood often has its roots in childhood. Some forms may be prevented by proper attention to neonatal and childhood bone health. A premature infant likely suffers lifelong decreased bone mineral density as a result of his or her early birth and the lack of adequate mineral stores that are typically present in full-term infants (Done, 2012). Caffeine is now one of the most commonly prescribed drugs in the NICU to treat apnea of prematurity. Recent studies in preterm infants confirmed the diuretic effect of caffeine, and revealed a significant increase in creatinine clearance and urinary calcium excretion. The effect of caffeine treatment on bone health of premature infants was not studied before. (Natarajan, Lulic-Botica& Aranda, 2007).

Chapter 2. Review of Literature

This review will include variable definitions of osteopenia of prematurity, then some reviews of the literature about the physiology and pathophysiology of osteopenia and the prevalence among the preterm infants. It also discusses long-term outcomes and the magnitude of the problems that extend throughout adulthood. The evaluation extends to cover the maternal risk factors that can affect premature infants' health such as maternal parity, vitamin D intake, and caffeine intake during pregnancy. The review of literature includes some of the neonatal risk factors that can predispose infants to osteopenia of prematurity. These risks include gestational age, weight gain, gender, parenteral nutrition days, medications received that may affect bone metabolism comprising of caffeine, steroids, and diuretics. Then finally this is followed by a short note about diagnosis and treatment of osteopenia of prematurity.

2.1. Definition

Osteopenia is the precursor of osteoporosis; it is simply defined as a reduction in bone mass. The structural basis of osteopenia is decreased thickness or number of trabeculae and/or decreased thickness of the bone cortex (Parfitt, 1990). Osteopenia of prematurity (OP), also known as metabolic bone disease of prematurity (MBD), is defined as postnatal bone mineralization that is less than the normal intrauterine bone density at a comparable gestational age (Steichen, Gratton& Tsang, 1980). Bone mineral content (BMC) (the mass of mineral per unit length) and bone mineral density (BMD) (the ratio of BMC to bone area) are both decreased. Osteoporosis refers to a decrease in BMD below 2.5 standard deviations from the norm in adults (Backstrom, Kuusela, Koivisto& Sievnen, 2005).

2.2. Physiology and Pathophysiology

The skeleton is a highly dynamic organ that has both structural and metabolic functions. Its structural function is critical for locomotion, respiration, and protection of internal organs. While metabolic function acts largely as a storehouse for calcium, phosphorus, and carbonate, it can contribute to buffering changes in hydrogen ion concentration. There are two major types of bone: the cortical bone and the trabecular (cancellous) bone. The cortical bone composes 80 percent of the skeletal weight. Although its major function is to provide mechanical strength and protection, but it can also participate in metabolic responses, particularly when there is severe or prolonged mineral deficiency. The other category is the trabecular bone. It is found inside long bones, particularly at the ends, throughout the bodies of the vertebrae, and in the inner portions of the pelvis and other large flat bones. Trabecular bone is an important contribution to mechanical support, particularly in the vertebrae. It is also more metabolically active than cortical bone and provides the initial supplies of mineral in acute deficiency states (Robey, 1996). The skeleton is composed of an orderly collagen matrix on which calcium and phosphate are deposited in the form of hydroxyapatite. Collagen is accumulated in a lamellar fashion and strengthened by multiple crosslinks, both within and between the triple-helical collagen molecules. These crosslinks are pyridinolines that are resistant to degradation. They are released during bone resorption either as a free or peptide form that can be measured in serum and urine (Robey, 1996).

The calcium-binding proteins, including osteocalcin (bone Gla protein) and matrix Gla protein, contain gamma carboxyglutamic acid and are vitamin K-dependent, similar to many clotting factors. These proteins may delay mineralization and allow the bone matrix to mature. Although osteocalcin is the most specific protein product of osteoblasts, knocking out the osteocalcin gene does not impair skeletal growth and mineralization (Ducy, Desbois, Boyce &

Pinero, 1996). To the contrary, according to a study done by Diaz and his colleagues in 1999, skeletal mass was increased in mice where osteocalcin was removed as a result of increased bone formation. Bone sialoprotein and osteopontin bind both calcium and collagen and may play a role in the adherence of osteoclasts to the bone surface. The bone mineral is composed of complex, often incomplete, crystals of hydroxyapatite. The crystals might contain carbonates, fluoride, and a variety of trace minerals, depending on the environment in which the skeleton grows. They are relatively small deeming them appropriate for a structure that can undergo strain with minimal micro damage. Mineralization is probably limited not only by the packing of the collagen fiber, but also by the substances present on the bone crystal surface such as pyrophosphate.

The bone remodeling cycle begins with osteoclast generation and recruitment to a particular site. Under physiological conditions, such site may be in need of repair. After osteoclastic resorption is completed, there is a reversal phase in which mononuclear cells, possibly of monocyte/macrophage lineage, appear on the bone surface. These cells could prepare the surface for new osteoblasts to begin bone formation. A layer of glycoprotein-rich material is laid down on the resorbed surface, the so-called "cement line," to which the new osteoblasts can adhere. The formation phase follows, in which successive waves of osteoblasts lay down bone until the resorbed bone is completely replaced and a new bone structural unit is fully formed. The newly formed osteoid begins mineralization after approximately two weeks. This process involves accumulation of matrix molecules. Mineralization occurs rapidly at first, and then gradually slows down. It takes several years for a bone structural unit to become fully mineralized (Diaz, El-Hajj Fuleihan & Brown, 1999). While the primary ossification centers from between 8 and 12 weeks of gestation in the vertebrae, the period of skeletal mineralization

is mainly during the third trimester (Kovacs, Chafe, Fudge & Friel, 2001). The increased fetal demand for calcium during this period is met by maternal increased calcium absorption from the gut. The fetal plasma Ca2+ concentration primarily determines skeletal mineralization in utero, which is dependent on the placental Ca2+ transfer and fetal calcitropic hormones. Both parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) is elevated in fetal blood in response to low plasma Ca2+ concentration and appears to regulate the transfer of Ca2+ across the placenta (Kovacs, Chafe, Fudge & Friel, 2001).

During fetal development an adequate placental transfer of calcium and minerals is necessary to ensure appropriate growth and mineralization of its skeleton. The human fetus requires approximately 30 g of calcium during development for adequate skeletal formation to occur. Fetal Ca2+ concentration is maintained at a higher level compared to the maternal serum by active transport across the placenta from approximately 20 weeks of gestation (Forestier, Daffos & Rainaut, 1987). Fetal PTH increases Ca2+ resorption by the kidney and possibly from bone. Maternal PTH does not cross the placenta, but will influence fetal PTH indirectly by altering maternal Ca2+ concentration and thus, the pool of calcium available to the fetus (Kovacs, 2003).

PTH and PTHrP also directly influence fetal bone linear growth, mediated through an effect on chondrocyte differentiation. The importance of this is clearly demonstrated by changes in PTHrP expression or receptor function, leading to short-limbed dwarfism in animal models and forms of chondrodysplasia (Weir, Philbrick & Amling , 1996). The level of Ca2+ transport across the placenta is strongly regulated by plasma membrane Ca2+ ATPase (PMCA) gene expression (Glazier, Atkinson, Thornburg & Sharpe, 1992). Furthermore, PMCA3 expression in the placenta has been positively correlated with offspring whole body bone mineral content

(BMC) at birth (Martin, Harvey, Crozier & Poole, 2007). PMCA gene expression regulation has not been fully characterized, although there is some evidence, in animal models, suggesting it might be influenced by 1,25-dihydroxyvitamin D [1,25(OH)2D] (Kip & Strehler, 2004). This would therefore be consistent with the notion that intrauterine vitamin D exposure might modify placental calcium transport and bone mineral accrual.

Epigenetics refers to changes in gene expression caused by mechanisms other than sequence changes in the underlying DNA. Epigenetic changes are essential in determining when and where genes are expressed. They are heritable as they last through multiple generations (Curtis, Moon & Dennison, 2014). Data indicate that perinatal events play a powerful role in influencing later susceptibility to certain chronic diseases such as osteoporosis. Recent data suggested that epigenetic processes are responsible for tissue-specific gene expression during differentiation, playing a possible key role in adaptive responses to nutritional and environmental factors during fetal and neonatal life. Thus, epigenetic mechanisms may underlie the processes of developmental plasticity. Developmental plasticity is the ability of a single genotype to give rise to several different phenotypes, allowing the organism adapt to future generations to prevailing environmental conditions. This phenomenon was termed “programming” and defined as “persisting changes in structure and function caused by adverse environmental influences at a critical stage of early development” (Barker, 1995).

Maternal or environmental factors during embryonic or fetal development may disrupt the patterns of DNA methylation, which in turn will alter gene expression during adult life and potentially leading to the development of disease. These mechanisms could be affected by the roles of maternal calcium and vitamin D status of offspring bone development. The actions of vitamin D are mediated through the heterodimerization of 1,25(OH)2D with the vitamin D

receptor (VDR) and the retinoid X receptor alpha (RXRA). Consequently, it acts on vitamin D response elements in target genes, initiating gene transcription. Vitamin D response elements are found in the promoter region of vitamin D regulated genes, some of which are calcium transporters. Indeed, methylation status at 4 sites of the RXRA in umbilical cord tissue has been negatively associated with size-corrected whole body BMC at 4 years of age (Gicquel, El-Osta & Le Bouc , 2008). Furthermore, maternal free 25-hydroxyvitamin D [25(OH)D] index was also negatively associated with percentage methylation at one of these sites (Harvey, Sheppard & Godfrey, 2014), suggesting that vitamin D could have a role in epigenetic regulation.

Upon cutting the umbilical cord, important postnatal adaptations of the skeletal system to extrauterine conditions result in a rapid adjustment in the regulation of mineral homeostasis occurring over hours and days. The neonate becomes dependent on intestinal calcium intake, skeletal calcium stores, and renal calcium reabsorption to maintain normal blood calcium at a time of continued skeletal growth (Land &Schoenau, 2008). The (physical) bone mineral density of long bones decreases by about 30% during the first months of life (Rauch &Schoenau, 2001). This mostly is due to a rapid increase in bone marrow size, which increases faster than the cross-sectional area of the bone cortex. This process has often been called as ‘physiological osteoporosis of infancy’ (Stettner, 1931).

The maintenance of calcium and phosphate homeostasis involves changes in intestinal, bone, and renal function. Regulation of intestinal function is important because the absorption of Ca2+ and phosphate is incomplete. This limitation is due both to the requirement for vitamin D and to the formation of insoluble salts in the intestinal lumen, such as calcium phosphate, calcium oxalate, and magnesium phosphate. The minute-to-minute regulation of serum ionized calcium is exclusively regulated through PTH, maintaining the concentration of this cation

within a narrow range, through stimulation of renal tubular calcium reabsorption and bone resorption (Diaz, El-Hajj Fuleihan & Brown, 1999).

A decrease in serum ionized calcium of as little as 0.1 mg/dL (0.025mmol/L) results in a large increase in serum PTH concentration within minutes. Conversely, an equally small increase in serum ionized calcium rapidly lowers the serum PTH concentration. Parathyroid cells contain vitamin D receptors, and the PTH genes contain vitamin D-response element. Calcitriol, by binding to the vitamin D receptor, inhibits PTH gene expression and therefore PTH synthesis. Calcitriol also inhibits parathyroid-cell proliferation (Naveh-Many, Friedlaender & Mayer, 1989).

Approximately 80% of bone mineralization takes place during the third trimester of pregnancy because of the high rate of intrauterine growth (Specker, 2004). Thus, preterm infants bereft of that period and eventually are born with less BMC. On the other hand, physiological adaptation of bone to extrauterine life leads to an increase in bone resorption. This process occurs earlier in preterm than in term infants can be accompanied by an increased risk of bone fragility and fractures (Pieltain, de Halleux, Senterre& Rigo, 2013).

High bone turnover appears to be more important than decreased bone formation in the pathogenesis of osteopenia of prematurity (Tsukahara, Takeuchi, Fujisawa, Miura, Hata& Yamamoto, 1998). Parathyroid hormone (PTH) concentrations increase immediately after birth as plasma calcium declines, and increases several-fold during the first 24–48 h. It can be expected that in newborn infants this PTH increase, in response to a decline in serum calcium, is necessary to maintain calcium homeostasis by increasing osteoclastic metabolic activity. The recruitment, activation, and fusion of osteoclastic precursors provide an important mechanism to ensure that longer-term calcium requirements are met. Although the mechanism coupling bone

resorption to new bone formation is unclear, the increase in osteoclastic activity stimulated by PTH following delivery should be matched by appropriate new bone formation. As a result, the increase in shaft diameter of long bones outweighs the loss in bone mineral mass in the early postnatal phase, thus maintaining (or improving) bone strength (Cooper &Anast, 1985).

Since calcium and phosphorus levels are among the most tightly regulated electrolytes, rapid sources of calcium are available from the skeleton where 99% of the body's stores exist. This is most prominent in areas where metabolic activity has been already significant and osteoclasts are readily available for the work of resorbing bone in the metaphysis. It is unknown whether demineralization after birth and physiological osteopenia of prematurity are secondary or primary, however, primary osteopenia is unusual in neonates (Done, 2012). Some evidence indicates that the placenta has a role in BMC because 25 OH Vitamin D is converted to 1,25-dihydrocholecalciferol in the placenta, which is important in the transfer of phosphate across the placenta to the fetus (Arash & Holick, 2013).

The regular fetal kicks against the uterine wall represent an intrauterine form of resistance training and, as a result, the mechanical stimulation is expected to be higher in utero. After birth, the infant's movements typically occur without much resistance or do not occur at all because of sedation use. Recent evidence shows that passive range-of-motion exercises lead to an increase in bone mineral mass and may help to attenuate the above-mentioned postnatal decline in bone mineral density in preterm babies (Moyer-Mileur, Brunstetter& McNaught, 2000).

2.3. Epidemiology

Almost 10% of babies are born preterm worldwide, representing more than 15 million births every year. The incidence and severity of osteopenia of prematurity increases as the birth

weight and gestational age decrease (Backström, Kuusela & Mäki, 1996). In 2009–2010, the Canadian in-hospital preterm birth rate was 8.0%. Preterm birth rates were also significantly higher for women with a pre-existing condition, compared to women whose conditions developed during pregnancy (Pieltain, de Halleux, Senterre&Rigo, 2013).

Holland et al. (Holland, Wilkinson &Diez, 1990) described a higher incidence of postnatal rickets in babies with intrauterine growth restriction, suggesting that chronic damage to the placenta may alter phosphate transport. In preeclampsia, the transfer of calcium to the infant is impaired because of placental calcification, which is associated with neonatal rickets (Bosley, Verrier-Jones & Campbell, 1980).

Recent advances in neonatal care significantly increase the survival rate in preterm and particularly in extremely low birth weight infants (ELBW infants). Nutrition is becoming one of the most challenging issues in order to improve short and long term health and developmental outcomes. Known predictors of osteopenia risk are genetic predisposition and environmental influences such as diet and exercise. However, a significant portion of BMD variance remains unexplained. It is proposed that this remaining variation results from the programming of systems controlling skeletal growth trajectory during critical growth periods (Gale, Martyn, Kellingray, Eastell& Eastell, 2001).

2.4. Long Term Sequelae and Magnitude of the Problem:

Until a few decades ago, pediatric health care in western societies focused on the prevention of rickets and malnutrition due to low caloric intake. Nowadays, the urgent issues are an atrophic musculoskeletal system caused by low motor activity and malnutrition due to high caloric intake. In addition, the improved care of chronically ill children introduced the issue of secondary musculoskeletal diseases. Therefore, knowledge and experience in the evaluation of

muscle–skeletal interactions will become more of an issue in pediatrics (Schoenau & Fricke, 2008). Therefore, the most critical property of bone for survival is now thought to be its strength rather than its weight. Since fracture of a bone would have resulted in death in the wild, it is logical that humans would develop mechanisms during the evolutionary process that would encourage bone development to produce bones of optimal strength (Parfitt , 1994). Exercise and nutrition are key environmental factors known to affect muscle and bone development. Exercise acts directly through muscle action and indirectly through endocrine regulation; during growth, exercise is thought to influence bone modeling (Schoenau & Fricke, 2008). The risk of osteoporotic fracture ultimately depends on two factors: the mechanical strength of bone and the forces applied to it. Bone mass (a composite measure, including contributions from bone size and from its volumetric mineral density) increases throughout childhood and early adulthood to reach a peak in early adulthood. Peak bone mass (PBM) is a major determinant of later osteoporosis risk, accounting for half of the variance in bone mineral density (BMD) at age 70. (Hui, Slemenda & Johnston, 1990). On the other hand, other studies showed that bone mineral density in adulthood depends predominantly on growth and mineralization of the skeleton and the resulting peak bone mass achieved and then, to a lesser extent, on the subsequent loss. Longitudinal studies of females suggested that this peak is reached at about 30 years of age (Recker, Davies, Hinders, Heaney, Stegman& Kimmel, 1992). For each standard deviation decrease in bone mineral density, fracture risk doubles in girls, similar to the risk in postmenopausal women. It has vast public health consequences due to the morbidity and mortality of the resulting fractures and the associated healthcare expenditure. More recent work has demonstrated that peak bone mass (PBM) is six times a more powerful predictor of age of onset of osteoporosis than the rate of bone loss or age of menopause. (Hernandez, Beaupre &

Carter , 2003). As there is no cure, it is important to identify early life influences on later bone mineral density, which may aid the development of interventions to optimize bone health and reduce osteoporosis risk (Claire, Wood, Alexander, Wood, Harker& Nicholas, 2013). The contributions of physical exercise both in utero and childhood, cigarette smoking and caffeine intake during pregnancy and lactation, diet and endocrine status in childhood, are strongly linked to early life exposures and later peak bone mass in childhood (Bonjour, Theintz, Buchs, Slosman& Rizzoli, 1991;Valimaki, Karkkainen&Lamberg-Allardt, 1994). Bone mineral density shows strong tracking during childhood and adolescent growth and into adulthood. A reduced peak BMD in childhood is associated with increased fracture risk and has been proposed as one of the best predictors of later life fracture risk in females (Rigo, Pieltain, Salle &Senterre, 2007). These studies showed highly significant relationships between weight at 1 year and adult bone area at the spine and hip ($p < 0.005$); the relationships with bone mineral content (BMC) at these two sites were weaker but remained statistically significant ($p < 0.02$) (Cooper, Fall & Egger, 1997). A retrospective study involving term infants demonstrated independent effects of birth weight and weight at one year on bone size and strength during the sixth and seventh decades after adjustment for confounding lifestyle factors (Oliver, Jameson, Sayer, Cooper & Dennison, 2007).

Preterm infants are known to have a lower bone mass, BMD and BMC (Bowden, Jones & Ryan, 1999) at the corrected age of term, as well as a lower weight and Ponderal index (Ahmad, Nemet & Eliakim, 2010). A study of 7- year-old boys showed greater measures of cortical thickness, whole body BMC, and hip BMD in term compared to preterm boys after adjustment for weight, height and age. These differences remained after adjustment for birth weight, length of neonatal hospital stay, and current activity level (AbouSamra, Stevens, Binkley

&Specker, 2009). A study by Fewtrell et al., in 2000 found former preterm infants who were followed up at around 10 years of age were shorter, lighter, and had lower BMC than controls (Fewtrell, Prentice, Cole, Lucas, 2000).

In a study by Backstrom et al., individuals who were born preterm were assessed with computerized tomography as young adults. Lower bone strength was demonstrated at the distal tibia and radius compared to age and sex matched controls. This effect was more pronounced in males and remained after adjustment for potential confounders (Backstr¨om, Kuusela, Koivisto&Siev¨anen, 2005). In a study by Cooper et al., those who were lightest at one year of age had the lowest BMC (Cooper, Westlake, Harvey, Javaid, Dennison & Hanson, 2006). In a further study, weight gain during the first two years of life predicted BMD at ages 9–14 years (Bhopal, Mann, Embleton, Korada, Cheetham& Pearce, 2011). As this cohort of survivors reaches middle age the impact of preterm birth on long-term metabolic outcomes, such as decreased bone mineral density, will become increasingly important. It is one of the most prevalent skeletal disorders; with approximately 30% of women and 12% of men over the age of 50 being affected by it (Melton, 1995).

Candidate genes associated with osteoporosis in adults have been evaluated for MBD in VLBW infants. An association has been identified between MBD and thymidine-adenine repeat (TA) polymorphism of the estrogen receptor (ERα) gene, with a higher number of (TA)n repeats seen in infants who do not have MBD. The locus interaction between vitamin D receptor (VDR) and collagen I α1 (COLIA1) genes also has been identified as being protective against the development of bone disease in preterm infants (Vachharajani, Mathur, & Rao, 2009).). In the short-term, osteopenia has been implicated in myopia of prematurity (Pohlandt, 1994), impaired

respiratory function (Glasgow & Thomas, 1977) and, in the longer-term, with poor growth during childhood (Lucas, Brooke & Baker, 1989).

2.5. Maternal Risk Factors

Several maternal factors are known to have a negative impact on neonatal growth and skeletal mineralization in term infants. Examples are shorter maternal height, high parity, high caffeine intake, smoking during pregnancy, low fat stores (Harvey, Javaid& Arden, 2010)& (Yajnik & Deshmukh, 2008), and low vitamin D exposure (Rigo, Pieltain, Salle &Senterre, 2007; Cooper, Westlake, Harvey, Javaid, Dennison & Hanson, 2006; Innes, Greenberg, 2002). Vitamin D deficiency is highly prevalent in pregnant and lactating women and produces adverse consequences in these women, their fetuses, and ultimately, their growing infants and children (Dawodu, Agarwal, Hossain, Kochiyil & Zayed, 2003; Ginde, Sullivan, Mansbach& Camargo, 2010). Several factors are responsible for this epidemic, including increased awareness of the injuries associated with sun exposure, insufficient vitamin D intake, and the rising prevalence of obesity (Arunabh, Pollack, Yeh&Aloia, 2003). There is a strong relationship between maternal and fetal (cord blood) 25(OH)D concentrations. 25(OH)D readily crosses the placenta and is metabolized to 1,25-dihydroxycholecalciferol by the placenta as early as the first trimester and by the fetal kidney as early as 24 weeks of gestation. At birth, neonatal serum 25(OH)D concentration is 50%-70% of maternal serum 25(OH)D concentration (Hollis & Wagner, 2004). In addition, lower ionized calcium concentration in umbilical venous serum also predicted reduced childhood bone mass. Around 31% of the mothers had insufficient and 18% had deficient circulating concentrations of 25(OH) vitamin D during late pregnancy (<50 and <25 nmol/L, respectively). Lower concentrations of serum 25(OH) vitamin D in mothers during late pregnancy, were associated with reduced whole-body and lumbar spine BMC in children at the

age of 9 years. Maternal vitamin D status was also statistically significant with its association with childhood bone area and BMD. Adjunctive evidence supporting a role for maternal vitamin D status was obtained in the Southampton Women's Survey where maternal vitamin D concentrations were again correlated with neonatal bone mass. These findings suggested that vitamin D supplementation of pregnant women, especially during winter months, could lead to long-lasting reductions in the risk of osteoporotic fracture in the offspring (Harvey, Javaid & Poole, 2008). Circulating maternal 25(OH)-vitamin D concentrations in this cohort were relatively high and were not associated with childhood skeletal measures. Thus, in populations during nutritional transition where maternal sunlight exposure is sufficient to maintain adequate vitamin D status, the availability of calcium becomes a more critical determinant of fetal and childhood bone mineral accrual.

Holroyed et al., showed that maternal balanced diet was found to be associated with greater bone size and BMD in the offspring .The observed effect was independent of social class, education, maternal height, maternal smoking status and late pregnancy vitamin D levels as well as childhood height, weight and exercise (Holroyd, Harvey & Dennison, 2012).

However, in multi-ethnic populations, the prevalence of vitamin D deficiency is often much higher. Importantly, the fetus is dependent on the mother as a source of 25(OH)D. 25(OH)D will readily cross the placenta and umbilical cord venous and neonatal 25(OH)D strongly correlates with maternal 25(OH)D. Breast milk contains low levels of vitamin D (Javaid, Crozier, & Harvey, 2006), and therefore an exclusively breast fed infant born to a vitamin D deficient mother is at high risk of vitamin D deficiency unless supplemental vitamin D is provided. Maternal vitamin D deficiency in pregnancy has been associated with early rickets

and neonatal hypocalcemia (Orbak, Karacan & Doneray, 2007), but these outcomes remain extremely rare outside of dark-skinned populations.

A number of studies have demonstrated associations between maternal 25(OH)D and offspring bone variables in the neonatal period. A study of Korean neonates identified both greater whole body BMC in the summer compared with winter born neonates and positive associations between neonatal 25(OH)D at delivery, which was strongly correlated with maternal 25(OH)D status and whole body BMC across the cohort (Namgung, Tsang, Lee & Han, 1998). Curtis et al found that whole body and femur BMC relative to body weight was significantly greater in 27 neonates with a cord blood 25(OH)D >37.5 nmol/L compared with 23 infants with a 25(OH)D <37.5 nmol/L (Curtis, Moon & Dennison, 2014).

The Avon Longitudinal Study of Parents and Children (ALSPAC) cohort study found no association between maternal vitamin D status in pregnancy and offspring bone mass at 9–10 years of age. However, positive associations between maternal 25(OH)D concentration and childhood bone mass or BMD are not seen universally (Lawlor, Wills, Fraser & Sayers , 2013).

Given the lack of intervention studies, it is not surprising that guidance on vitamin D supplementation in pregnancy has been rather conflicting. Both Cochrane (Mahomed & Gulmezoglu, 2000) and National Institute for Clinical Excellence (NICE) (National Institute for Health and Care Excellence (NICE), 2008) reviews concluded that there was insufficient evidence on which to base a firm recommendation. However, more recently NICE and the UK Department of Health have recommended supplementation with 400 IU cholecalciferol daily in pregnancy. Outcomes from randomized controlled trials of vitamin D in pregnancy, including the Maternal Vitamin D Osteoporosis Study (MAVIDOS) study (Harvey, Javaid & Bishop, 2012), which primarily aims to assess offspring bone mass at birth and in early childhood in women

randomized to either 1000 IU cholecalciferol or placebo from 14 weeks gestation, will help to determine whether this recommendation is optimal.

The human fetus requires a total of 30 g of calcium for bone development, most of which is acquired during the third trimester via active transport across the placenta, resulting in greater calcium concentration in the fetus than maternal plasma. Fetal calcium needs are primarily met by increased maternal intestinal calcium absorption during pregnancy, and therefore, very low maternal calcium intakes may be a risk for lower bone mass in neonates. (Namgung & Tsang, 2003). Further studies have confirmed that independent predictors of greater neonatal whole-body bone area and BMC include greater maternal birth weight, height, parity, fat stores (triceps skinfold thickness) and lower physical activity in late pregnancy. Maternal smoking was statistically significant (and independently) associated with lower neonatal bone mass. These relationships were observed in both male and female offspring (Harvey, Poole & Javaid, 2007).

Two large observational studies conducted in Europe by Heppe et al (Holland) and Tobias et al (UK) assessed whole body less head BMC and BMD by DXA at 6 and 9 years of age, respectively, in approximately 3000 children, both studies showed that higher maternal calcium intake was associated with higher whole body BMC. There was no significant association between maternal calcium intake and offspring bone fratures in either study. (Heppe , Medina-Gomez & Hofman, 2013) & (Tobias, Steer & Emmett, 2005).

On the other hand, a randomized controlled study of calcium supplementation was carried out in 256 mother-offspring pairs in Memphis, USA. Women were randomized to 2 g/day calcium or placebo from 22 weeks gestation until delivery. Maternal dietary intake was recorded and DXA measurements of the offspring's whole body and lumbar spine BMD were performed

before hospital discharge. No significant differences were found between the treatment groups overall (Koo, Walters & Esterlitz, 1999).

In a study which tested the effects of moderate maternal exercise during pregnancy on the bone and body composition of the offspring, the results suggest that moderate exercise during pregnancy can result in lasting changes to the musculoskeletal system and adiposity in offspring (Rosa, Blair & Vickers, 2013).

Neonatal bone abnormalities were attributable to maternal administration of magnesium sulphate. The gestational ages and the total doses of MgSO4 in pregnant women were the main factors related to the onset of neonatal bone abnormalities, but other factors also have a possible bearing on the condition. In addition, the cases with onset of bone abnormality seemed to be associated with symptoms attributable to hypermagnesemia of neonates (Matsuda, Maeda, Ito & Sakamoto, 1997).

It is now widely believed that habitual daily use of caffeine >500–600mg (four to seven cups of coffee or seven to nine cups of tea) represents a significant health risk and may therefore be regarded as ‘abuse’. Sustained abuse may in turn result in ‘caffeinism’, which refers to a syndrome characterized by a range of adverse reactions such as restlessness, anxiety, irritability, agitation, muscle tremor, insomnia, headache, diuresis, sensory disturbances (e.g. tinnitus), cardiovascular symptoms (e.g. tachycardia, arrhythmia) and gastrointestinal complaints (e.g. nausea, vomiting, diarrhoea) (James & Paull, 1988). In Canada, published values for the average daily intake of caffeine from all sources is about 2.4 mg/kg body weight for adults and 1.1 mg/kg for children 5–18 years old. Brown et al. (2001) reported daily caffeine intakes ranging from 288 to 426mg (equivalent to 4.5–6.5mg/ kg in a 65-kg person) in the adult population.

The potential adverse impact of caffeine consumption during pregnancy on fetal growth has been a concern for many years, as adenosine is involved in maintaining the balance between the availability and the use of tissue oxygen, blockage of its receptors could increase the susceptibility of the cell to hypoxia. Caffeine intake of 7025 women living in the Quebec City, Canada, was not related to low birth weight, but was associated with an increased risk of intrauterine growth retardation. For women whose average daily caffeine consumption was 0–10, 11–150, 151–300 or >300 mg, the adjusted ORs for delivering a newborn with growth retardation were 1.00, 1.28 (95% CI (1.04–1.59), 1.42 (1.07–1.87) and 1.57 (1.05–2.33), respectively (Fortier & Marcoux, 1993).

The most marked effects associated with heavy caffeine use (>300 mg/day) were reduced birth weight and small head circumference; the associations were still significant after adjustment for maternal nicotine use (Watkinson & Fried, 1985). However, in other study this effect was not detected with the same dose of caffeine intake (Godel, Pabst, and Hodges, 1992). It is difficult to establish the cause of the inconsistencies in the results of studies investigating the association between caffeine consumption and fetal growth, simply because of inadequate control for confounders or simply unknown study bias. 9 out of 11 studies showed that caffeine consumption at dose levels up to 530 mg/day was not an important risk factor for preterm delivery (Nawrot, Jordan, & Eastwood, 2003).

The fetus is exposed to caffeine ingested by the pregnant mother, since caffeine is rapidly absorbed from the gastrointestinal tract, readily crosses the placenta, and is distributed to all fetal tissues. In addition, exposure of the fetus to caffeine is enhanced because caffeine's half-life is markedly increased in the fetus and pregnant women in comparison with non-pregnant adults and older children. Because of the rapid growth that occurs during the late prenatal period, the

impact of chronic caffeine exposure may be far greater than at any other time of life. (Nawrot, Jordan, & Eastwood, 2003).

The umbilical cord length and diameter were measured as well as the newborn's tibial speed of sound (SOS). Infants with a short umbilical cord length have lower bone strength. Assuming that the fetus with a shortened umbilical cord may have low bone mineralization because of decreased movement or activity (Wright & Chan, 2009).

2.6. Vitamin D

Vitamin D is a fat-soluble vitamin where the dermal synthesis is its major natural source. This system is exceedingly efficient, and it is estimated that brief casual exposure of the arms and face is equivalent to ingestion of 200 international units per day (Haddad, 1992). However, the length of daily exposure required to obtain the sunlight equivalent of oral vitamin D supplementation is difficult to predict on an individual basis and varies with the skin type, latitude, season, and time of day. Prolonged exposure of the skin to sunlight does not produce toxic amounts of vitamin D3 because of photoconversion of previtamin D3 and vitamin D3 to inactive metabolites (lumisterol, tachysterol, 5,6-transvitamin D, and suprasterol 1 and 2). In addition, sunlight induces production of melanin, which reduces production of vitamin D3 in the skin (Terushkin, Bender, Psaty & Engelsen, 2010).

Dietary vitamin D is incorporated into micelles, absorbed by enterocytes, and then packaged into chylomicrons. Disorders associated with fat malabsorption, such as necrotizing enterocolitis, pancreatic insufficiency, cystic fibrosis, short gut syndrome, and cholestatic liver disease, are associated with low serum 25-hydroxyvitamin D (25[OH]D) levels. Infants, disabled persons, and older adults may have an inadequate sun exposure, while the skin of those older than 70 years of age also does not convert vitamin D effectively. In addition, at northern

latitudes, there is not enough radiation to convert to vitamin D, particularly during the winter. For these reasons, in the United States, milk, infant formula, breakfast cereals, and some other foods are fortified with synthetic vitamin D2 (ergocalciferol), which is derived from radiation of ergosterol found in plants, the mold ergot, and plankton, or with vitamin D3. In other parts of the world, cereals and bread products are often fortified with vitamin D.

Vitamin D from the diet or dermal synthesis is biologically inactive and requires enzymatic conversion to active metabolites. Vitamin D is converted enzymatically in the liver to 25-hydroxyvitamin D (25[OH]D), the major circulating form of vitamin D, and then in the kidney to 1,25-dihydroxyvitamin D, the active form of vitamin D. 25-hydroxyvitamin D2 has a lower affinity than 25-hydroxyvitamin D3 for vitamin D-binding protein. Thus, 25-hydroxyvitamin D2 has a shorter half-life than 25-hydroxyvitamin D3, and treatment with vitamin D2 may not increase serum total 25(OH)D levels as efficiently as vitamin D3. The plasma 1,25-dihydroxyvitamin D concentration is a function both of the availability of 25(OH)D and of the activities of the renal enzymes 1-alpha-hydroxylase and 24-alpha-hydroxylase. The renal 1-alpha-hydroxylase enzyme is primarily regulated by the following factors: PTH, Serum calcium and phosphate concentrations, and fibroblast growth factor 23 (FGF23) (Christakos, Ajibade & Dhawan, 2010). 25-hydroxyvitamin D (25[OH]D) is the major circulating form of vitamin D. It has a half-life of two to three weeks, compared with 24 hours for parent vitamin D. It has activity in the bones and intestines, however it is less than 1 percent as potent as 1,25-dihydroxyvitamin D, the most active form of vitamin D. The half-life of 1,25-dihydroxyvitamin D is approximately four to six hours. 1,25-dihydroxyvitamin D binds to intracellular receptors in target tissues and regulates gene transcription (Lowe, Maiyar & Norman, 1992). It appears to function through a single vitamin D receptor (VDR), which is nearly universally expressed in

nucleated cells. The receptor is a member of the class II steroid hormone receptor, and is closely related to the retinoic acid and thyroid hormone receptors (DeLuca, 2004).

The most important biological action is to promote enterocyte differentiation and the intestinal absorption of calcium. Other effects include a lesser stimulation of intestinal phosphate absorption, direct suppression of parathyroid hormone (PTH) release from the parathyroid gland, regulation of osteoblast function, and permissively allowing PTH-induced osteoclast activation and bone resorption. Both 1,25-dihydroxyvitamin D and 25(OH)D are degraded in part by hydroxylation by a 24-hydroxylase. The activity of the 24-hydroxylase gene is increased by 1,25-dihydroxyvitamin D, which therefore promotes its own inactivation, and decreased by PTH, thereby allowing more active hormone to be formed (Zierold , Darwish & DeLuca, 1994).

Various groups have been recognized to be at risk of low vitamin D levels, including dark skinned individuals, populations who for religious or cultural reasons extensively cover their skin, those who frequently use sunscreen, populations living at northerly latitudes, and overweight or obese individuals (Macdonald, Mavroeidi & Fraser, 2011). However, the incidence of vitamin D deficiency and insufficiency differs according to geographic location, ethnicity and skin pigmentation, clothing, cultural habits, exposure to sunlight, nutrition, and supplementation. The percentage of pregnant women with vitamin D insufficiency can vary from 96% in rural China, where there is a low exposure to sunlight and low vitamin D supplementation, to 1% close to the equator in Tanzanian women. Differences in 25(OH)D serum levels can be striking. Although mean serum levels of 25(OH)D in Tanzanian women are 138.5 nmol/l, they are 10 times lower (12.8 nmol/l) in Iranian women because of their lack of exposure to sunlight and low vitamin D intake (Christesen, Falkenberg, Lamont & Jorgensen, 2012). Vitamin D deficiency and insufficiency are highly prevalent. In a cohort of 198 pregnant

Caucasian women from Southampton, UK, 31 % had a circulating serum 25(OH)-vitamin D (25(OH)D) lower than 50 nmol/L, often considered “insufficient" and 18 % were vitamin D deficient (<25 nmol/L) (Dawodu & Tsang, 2012) . Subclinical vitamin D deficiency, as measured by low serum 25(OH)D, is very common. In the National Health and Nutrition Examination Survey (NHANES) in 2005 to 2006, 41.6 percent of adult participants (≥20 years) had 25(OH)D levels below 20 ng/mL (50 nmol/L) (Forrest & Stuhldreher, 2011).

2.7. **Neonatal Risk Factors**

Despite all challenges, survival has improved dramatically in the last few years. More than 60% of babies born at 24 weeks gestation regularly survive long term with improved nutrition, being one potential factor contributing to these improvements (Melton, 1995). Complications of prematurity are the underlying reasons for the high rate of infant mortality and morbidity in preterm infants compared with full-term infants. The risk of complications increases with increasing immaturity. Thus, infants who are extremely premature, born at or before 25 weeks of gestation, have the highest mortality rate (about 50 percent) and if they survive, are at the greatest risk for long-term morbidity. In preterm survivors, there is a high rate of long-term neurodevelopment impairment and chronic health problems. These chronic medical and neurodevelopmental complications often require additional health care and educational services, which add to the overall economic cost of caring for the premature infant.

More than 50 years ago, it was observed that premature infants, especially those with very low birth weight (<1500 g), are at increased risk for metabolic bone disease, including delayed longitudinal growth, osteopenia, and rickets (Greer & McCormick, 1986). In 1989, Nehra et al., found that the incidence of the condition increased and studies have reported poor

mineralization in up to 55% of infants <1000 g and 23% of infants <1500 g at birth (Nehra, Carlson & Fallon, 1989).

Osteopenia of prematurity is related to both low gestational age and prolonged need for intravenous nutrition. In 2009, a study reported the pathologic fractures in 30% of preterm infants with osteopenia (Vachharajani, Mathur & Rao, 2009).

A preterm baby faces many challenges. Feeding problems are almost inevitable in the very preterm group as a coordinated suck and swallow is not established until around 34 weeks corrected gestation. Extremely preterm infants and those who are unwell may require IV fluids or a period of total parenteral nutrition before full feeds can be established. The content of Calcium (Ca) and Phosphorus (P) in human milk is insufficient for VLBW infants to achieve intrauterine accretion rates or normal bone mineralization (Schanler& Oh, 1980) & (Schanler, 1991). In one study of 865 VLBW preterm infants, infants fed un-supplemented human milk had elevated serum alkaline phosphatase activity, indicating stimulation of bone resorption to normalize the serum Ca concentration (Brooke, Baker Lucas, Bishop, Morley, 1989). Those who have severe liver problems because of cholestasis, often a result of parenteral hyper-alimentation or biliary atresia are at greater risk for osteopenia (Done, 2012).

Mineral accretion is inadequate in VLBW infants who are treated with total parenteral nutrition (PN) for more than two weeks. This is related in part to the need to limit mineral concentrations because of their solubility in parenteral nutrition solutions. However, increased administration of Ca and P improves mineral retention. As an example, in a study of 24 VLBW infants receiving total parenteral nutrition, administration of 17 mmol/L (680 mg/L) Ca and 20 mmol/L (660 mg/L) P resulted in net retention of 70 to 80 mg/kg per day Ca and P respectively (Prestridge, Schanler, Shulman, Burns &Laine, 1993). The provision of minerals in PN is limited

by their solubility increasing the risk of reduced BMC further and this may be compounded by the need for fluid restriction; for example phosphate supplementation in PN is commonly linked to PN sodium supplementation. Aluminum contamination of PN has been reported and this may further affect bone formation and mineralization adversely (Naylor, Eastell& Shattuck, 1999).

Premature infants usually receive a lot of sedations that limit physical movement and hinder the muscle activity that increases the bone strength. However, passive motor physical therapy in premature newborns for 15 min per day, 5 times per week for 4 weeks permitted significantly greater weight gain, length, BMC, BMD which could contribute to the prevention of osteopenia in prematurity (Canani, Miura & Vignochi, 2008). Medications frequently used in the nursery, such as methylxanthines, steroids, and diuretics, can increase the risk of inadequate bone mineralization due to stimulation of osteoclasts, which resorb trabeculae to maintain adequate serum calcium levels (Weiler, Wang, Atkinson, &, 1995; Zanardo ,Dani&Trevisanuto, 1995; Venkataraman, Han & Tsang, 1983).

2.8. Medications

Medications used in NICUs can highly affect the bone turn over and development. These medications include caffeine, diuretics and steroids. All of them have negative impact of premature bone development.

2.8.1. Caffeine.

Caffeine is probably the most commonly consumed pharmacologically active compound in the world (Heaney, 2002). It is one of the most commonly prescribed drugs in the NICU, and has appropriately been described as a “silver bullet” in neonatology to treat apnea of prematurity (Aranda ,Natarajan& Davis, 2010). Caffeine is a trimethylxanthine that primarily exerts its effects by increases levels of 3'5' cyclic AMP by inhibiting phosphodiesterase: a central

stimulant which increases medullary respiratory center sensitivity to carbon dioxide, stimulates central inspiratory drive, and improves skeletal muscle contraction (diaphragmatic contractility). Prevention of apnea may occur by competitive inhibition of adenosine, blocking adenosine receptors, resulting in an increased respiratory drive. Adenosine receptors are present in the brain, heart, blood vessels, kidneys, gastrointestinal tract, and respiratory system (Fredholm, Battig, Holmen &Nehlig, 1999). Caffeine results in the release of norepinephrine, dopamine and serotonin in the brain and the increase of circulating catecholamines, consistent with a reversal of the inhibitory effect of adenosine (Benowitz 1990)

Inter-conversion between caffeine and theophylline has been reported in preterm neonates (caffeine levels are ~25% of measured theophylline after theophylline administration and ~3% to 8% of caffeine would be expected to be converted to theophylline). Caffeine has now largely replaced aminophylline and theophylline for routine treatment because of its wider therapeutic index and longer half-life that allows once-daily administration. The half-life in neonates is 72-96 hours (range: 40-230 hours) and the time to peak serum concentration after oral administration is ranging from 30 minutes to 2 hours, whereas 86% excreted unchanged in urine (Natarajan, Lulic-Botica & Aranda, 2007).

The liver is the primary site of caffeine metabolism (Arnaud, 1999). In adults, caffeine is virtually completely metabolized to 1- methylxanthine and 1-methyluric acid from the paraxanthine intermediate. Only 1–5% of ingested caffeine is recovered unchanged in the urine. Infants up to the age of 8–9 months have a greatly reduced ability to metabolize caffeine, excreting about 85% of the administered caffeine in the urine unchanged (Stavric & Gilbert 1990).

They found that caffeine half-life is increased in the neonatal period in both animals and

humans due to the immaturity of hepatic enzyme systems, namely cytochrome P-450 and some demethylation and acetylation pathways, ranging half- lives of 40-130 h are found in premature newborn infants

The enzymes responsible for caffeine metabolism mature progressively with increasing gestational age. Girls have a higher rate of caffeine metabolism than boys (Fredholm, Battig, Holmen &Nehlig, 1999). Clearance in infants born preterm is markedly lower and the volume of distribution is higher than at term-equivalent age and beyond. Elimination of caffeine is initially depressed in extremely premature infants and then increased nonlinearly to final assessment at 6 weeks postnatal age. The volume of distribution of caffeine is increased linearly with increasing weight (Charles, Townsend, Steer & Flenady, 2008).

Caffeine significantly increases calcium excretion and creatinine clearance; on the other hand, caffeine does not affect serum sodium, potassium, or phosphorus level (Natarajan, Lulic-Botica & Andrea, 2007).

Caffeine pharmacokinetic data are limited in VLBW infants. In preterm infants born at a mean gestational age of 29 weeks and receiving caffeine citrate at a dosage of 6 mg/kg per day intravenously, the 25th to 75th percentile range for the serum concentrations was 18 to 23 mg/L in the first 14 postnatal days. At this age, the serum caffeine concentrations are not dependent on PMA, weight, or postnatal age, and remained in a safe and therapeutic range over the ranges of renal and hepatic functions typically found in practice (Leon, Michienzi & Ma, 2007). In infants born at 24 to 29 weeks' gestation and birth weights of 570 to 1,570 g, oral caffeine is completely absorbed.

The routine dosing for caffeine citrate has been a loading dose of 20 mg/kg (10 mg/kg of caffeine base) followed by a daily maintenance dose of 5 mg/kg. Most US neonatal centers now

use maintenance doses of 5 to 8 mg/kg. Treatment is typically discontinued by 33 to 34 weeks PMA, following resolution of clinically apparent apnea of prematurity symptoms. Some centers continue caffeine beyond 34 weeks PMA, although there are no data beyond 33 to 34 weeks PMA to establish the maintenance caffeine dose necessary to achieve a therapeutic blood level. Due to clearance of caffeine progressively increases with increasing PMA, at the same maintenance dose per kilogram as at less than 34 weeks, caffeine levels at 36 weeks PMA would likely only be about half of levels achieved at 32 weeks PMA (Charles BG, Townsend & Steer, 2008).

Infants born at 28 to 33 weeks' gestation and treated with caffeine had increased oxygen consumption and energy expenditure over a 4-week period and the daily weight gain was significantly lower in comparison with a control group (Natarajan, Lulic-Botica & Aranda, 2007).

Caffeine toxicity in children is manifested by severe emesis, tachycardia, central nervous system agitation and diuresis. Chronic exposure to caffeine has been implicated in a range of dysfunctions involving the gastrointestinal system, liver, renal system and musculature (James 2004).

In a recent study, it was found that, in mice, low-concentration caffeine (0.005–0.1 mM) did not affect the bone marrow cell viability and alkaline phosphatase activity during osteoblast differentiation from bone marrow stromal cells, but it effectively enhanced the osteoclastogenesis from bone marrow hematopoietic cells and the bone resorption activity by pit formation assay. Moreover, caffeine effectively enhanced the receptor activator of NF-kB ligand (RANKL), but reduced the osteoprotegerin protein expressions in osteoblast MC3T3-E1 cells. Caffeine could also increase the cyclooxygenase-2 (COX-2) protein expression and

prostaglandin production in cultured neonatal mouse calvariae. In animal study, BMD in lumbar vertebra, femur, or tibia was significantly lowered in growing rats supplemented with 0.2% caffeine in diets for 20 weeks compared with the control group. Additionally, the calcium contents in tibia and femur of caffeine-treated rats were also lower, and the osteoclastogenesis of bone marrow cells isolated from caffeine-treated rats was markedly enhanced than that in the control group. Taken together, these results suggest that caffeine may reduce BMD in growing rats through the enhancement in osteoclastogenesis (Liu, Chen, Yang, Yen, Yang & Tsai, 2011). The effect of caffeine on bone metabolism is evident especially it has a prolonged half- life in premature infants. Caffeine caused a dose dependent increase of the spontaneous release of Calcium from neonatal mouse calvarial bones, as in another study (Leon & Ma, 2007).

Tolerance to the renal effects of caffeine does not develop in adults, as habitual coffee intake had no effect on the increase in calcium excretion associated with an acute caffeine dose (Bergman & Massey, 1990). On the other hand, increasing caffeine intakes were not associated with significant decreases in bone density in adolescent women (Lloyd, Rollings, Kieselhorst & Eggli, 1998). Sakamoto et al. (2001) found no effect of high-coffee diets on biochemical markers of bone metabolism or on cytokines implicated in bone loss in adult rats and coffee consumption did not lead to bone resorption. In another study on adult rats, comparing the high and low caffeine diet, there was no effect on the number of osteoclasts and levels of urinary deoxypyridinoline and serum osteocalcin, which reflect bone resorption and bone formation (Sakamoto, Nishihira & Fujie, 2001). Caffeine-containing beverage consumption has been reported to be associated with reduced bone mass and increased fracture risk. The biological significance of caffeine's negative effect on calcium balance continues to be the topic of scientific debate, as studies on both bone density and fracture risk have revealed conflicting

results (Barrett-Connor, Chang & Edelstein, 1994). To my knowledge, there is no previous research that looked at the effect of caffeine and its use as the most frequently used medication on bone of premature infants.

2.8.2. Steroids and Diuretics.

The significant role of inflammation in chronic lung disease pathogenesis has been well described. After the landmark publication by Cummings et al. in 1989, the administration of systemic corticosteroids to VLBW infants with CLD became widely adopted (Cummings, D'Eugenio & Gross, 1989).

Glucocorticoids increase bone resorption either directly by decrease estrogen and testosterone or indirectly by increase urinary calcium and decrease calcium absorption. Glucocorticoids also decrease intestinal calcium absorption, in part by opposing the action of vitamin D, and by decreasing the expression of calcium channels in the duodenum, which lead to increase PTH. (Huybers, Naber & Hoenderop, 2007).

The decline in bone formation is mediated by direct inhibition of osteoblast proliferation and differentiation and by an increase in the apoptosis rates of mature osteoblasts and osteocytes or by decreasing muscle strength (Canalis, Mazziotti & Giustina , 2007).

In addition, glucocorticoids alter parathyroid hormone (PTH) secretory dynamics (reduce tonic secretion and increase the amount released as pulses), antagonize the anabolic action of PTH, and inhibit production of insulin-like growth factor-I (IGF-I) and testosterone. The reduction in bone formation is associated with a decrease in the mineral apposition rate and in serum and urine biochemical markers of bone formation. The risk of bone loss is most pronounced in the first few months of use, followed by slower but steady loss of bone with continued use (Pereira, Carvalho & Canalis, 2010). In a study conducted by Subhedar et al.,

2001, they found that administration of dexamethasone was associated with a decrease in leg length growth velocity and decreased weight gain. However, a rapid recovery in growth was noted after drug discontinuation. Adverse effects on growth in head circumference have also been described (Subhedar, Ryan & Shaw, 2001). Several studies that follow up to 2 to 3 years of age have demonstrated possible long-term growth suppression (Cummings &D'Eugenio, 1989). As noted from the results, Glucocorticoid therapy increases the risk of fracture (Canalis , Mazziotti & Giustina, 2007).

Clark and colleagues reported that furosemide was the seventh most commonly reported medication in the NICU, with more than 8% of all NICU patients being exposed to the agent (Clark, Bloom & Spitzer, 2006). They act in the thick ascending loop of Henle to block the site of chloride in the Na+K+-2Cl− membrane carrier, thereby inhibiting sodium, chloride, and potassium reabsorption. Furosemide is primarily eliminated in the urine as unchanged drug. Plasma clearance is low, the volume of distribution greater, and the half-life prolonged in preterm. Mirochnick and colleagues reported that plasma half-life frequently exceeded 24 hours in infants less than 31 weeks' postconceptional age (Mirochnick , Miceli & Kramer, 1990). Due to significant amounts of divalent cations such as Ca2+ are reabsorbed in the loop of Henle by passive mechanisms that depend on the active transport of sodium chloride, administration of furosemide may result in hypercalciuria, bone demineralization, and renal calcifications. Exposure to furosemide more than 10 mg/kg body weight cumulative dose has recently been shown by multivariate analysis to be the strongest independent risk factor for nephrocalcinosis (Chang, Hsu & Tsai, 2011).

2.9. Diagnosis

2.9.1. Laboratory Diagnosis.

Currently, the diagnosis of metabolic bone disease is based mainly on biochemical markers and radiology. Biochemical markers (e.g., serum calcium, phosphorus and alkaline phosphatase) are not significantly correlated with bone mineral density (BMD). Furthermore, it is inconvenient to take blood samples from neonates, especially preterm infants (Catache & Leone, 2003).

Osteopenia typically develops in premature infants at 3 to 12 weeks of age. The condition is not clinically apparent and may not be detected by routine laboratory monitoring. The earliest indications of osteopenia are decreased serum phosphorus concentration, typically less than 3.5 to 4.0 mg/dL (1.1 to 1.3 mmol/L), and increased alkaline phosphatase activity. Alkaline phosphatase values >800 IU/L are worrisome, especially if combined with serum phosphorus values less than 4.0 mg/dL (1.3mmol/L) and are sensitive (100%) and specific (70%) for diagnosing low BMD.

However, distinguishing the normal rise in alkaline phosphatase activity associated with rapid bone mineralization from the pathologic increase related to early osteopenia often is difficult (Walters, Murphy, Henry & Gray, 1986). In a previous study, newborns with peak alkaline phosphatase activity >1200 IU/L had 1.6 cm less linear growth at 18 months and shorter stature at 9 to 12 years of age than those with lower levels (Fewtrell, Cole, Bishop & Lucas, 2000). Typical preterm NICU graduates have elevated alkaline phosphatase levels, with a range of 400 to 600 IU/L, and require vitamin D supplementation. The AAP recommends that all breastfed, partially breastfed, or formula-fed infants taking less than 1,000 mL of vitamin D–fortified milk per day should take 400 IU of vitamin D daily (Wagner & Greer, 2008).

2.9.2. Radiographic Diagnosis

Characteristic radiographic changes were seen in 55 percent of infants with birth weight <1000 g in 1996 (Backström, Kuusela&Mäki, 1996). The radiographic feature characteristic of osteopenia is decreased lucency of the cortical bone with or without epiphyseal changes. Although most infants with decreased bone mineralization do not have fractures even when osteopenia is severe, in some cases, a fracture can be the earliest sign. In a multicenter retrospective study of chest radiographs of infants born at <37 weeks gestation, rib fractures were identified in 26 of 1446 infants (1.8 percent) (Lucas-Herald, Butler, Mactier, McDevitt, Young & Ahmed, 2012). The radiographic appearance can be quite variable and depends on the amount of hypertrophic cartilage that has accumulated and to what extent it is partially mineralized, as the mineralization defect might not match the arrest of endochondral ossification (Glaser, 1949).

The most commonly used quantitative radiological methods to assess bone mass is dual energy x-ray absorptiometry (DXA) ((Nelson & Koo, 1999). DXA measures the calcium content in the bones, expressed as grams of hydroxyapatite per centimeter squared. The exposure to ionizing radiation is minimal (effective dose, 0.001 mSv; <0.1 mrem). The technique is precise and reproducible, and takes only 5 minutes. Although the trabecular bone is preferred, the lumbar region is generally used in neonates. Modern portable machines can analyze the forearm and the calcaneus. BMD measurement is the method of choice for children, because the results are independent of anthropometric variables and gestational age (Rigo, De Curtis & Pieltain, 2000). DXA is the “gold standard” for adult osteopenia; however, it has not been tested extensively in preterm infants because it is ionizing and cannot be performed at bedside (Godang, Qvigstad & Voldner, 2010).

Quantitative ultrasonography, using broadband ultrasonographic measurement, speed of sound (SOS), or bone transmission time, has been employed to assess bone density, but still a research tool. Quantitative computed tomography scan measures true volumetric bone density but involves exposure to high dose radiation.

2.10. Treatment

Providing adequate Calcium and Phosphorus for bone mineralization, through feeding fortified human milk or premature formula, treats osteopenia of prematurity. Infants who do not tolerate human milk fortifiers or premature formula because of lactose intolerance or cow's milk protein allergy should be given supplements of calcium and phosphorus. The maximum allowable parenteral mineral concentrations should be provided to infants not receiving enteral feeding. Vitamin D supplementation is rarely indicated for this condition. Total daily doses of 200 to 400 IU/day are adequate, although some (especially Europeans) prefer a higher dose of 800 to 1000 IU/day (Rigo, Pieltain, Salle &Senterre, 2007). The Canadian Pediatric Society advocates an increase of vitamin intake to 800 IU/day for northern Native communities during the winter months (First Nations, 2007).

Bone mineral content was similar when Calcium and Phosphorus were added to a multi-nutrient fortifier or given alone, although the multi-nutrient formulation resulted in better linear growth (Wauben, Atkinson, Grad, Shah &Paes, 1998). For VLBW infants, it is recommended to add elemental Ca (2 to 3 mmol/kg per day or 80 to 120 mg/kg per day) and P (1.5 to 2 mmol/kg per day or 45 to 60 mg/kg per day) to human milk, so that infants receive the recommended intake of 200 mg/day of calcium and 100 mg/day of phosphorus after enteral feeding is established. Current commercial human milk fortifiers provide stable suspensions of these minerals. Supplementation of human milk with Ca and P improves linear growth, increases bone

mineralization during hospitalization and after discharge, and normalizes serum calcium, phosphorus, and alkaline phosphatase activity and urinary excretion of calcium and phosphorus (Lucas, Brooke, Baker, Bishop & Morley, 1989). Net mineral retention and bone mineral content are improved with increased intake (Schanler& Abrams, 1995). Atypical elevations (alkaline phosphatase more than 650 IU/L) require the aforementioned supplementation plus oral calcium and phosphorus supplementation. Goals for calcium supplementation range from 60 to 90 mg/kg per day; however, most infants need 100 to 160 mg/kg to reach adequate bioavailability. The goal of phosphate supplementation is 60 to 90 mg/kg per day. These regimens are difficult in terms of preparation, in part because of precipitation of supplements when added directly to feedings. Assisting families with the schedule, preparation, and refilling of these prescriptions is important for compliance. Increased parenteral intakes of calcium and phosphorus resulted in greater retention of these minerals, during parenteral nutrition therapy and in greater bone mineral content after therapy (Prestridge, Schanler & Shulman, 1993).

Vitamin D may contribute little to calcium absorption in premature infants in the first weeks of life (Bronner, Salle, Putet, Rigo&Senterre, 1992). Vitamin D may need to be given as the active form, calcitriol (1,25 dihydroxy vitamin D), with appropriate monitoring of serum calcium to avoid hypercalcemia. Premature infants with fractures resulting from osteopenia are managed conservatively with supplemental minerals and vitamin D, but not with surgical intervention. Although vitamin D supplementation is effective in preventing vitamin D deficiency, the optimal vitamin D requirement in women remains unknown. Studies evaluating plasma vitamin D status have shown that vitamin D supplementation of <2000 IU/day is not effective in achieving sufficiency (Mallet, Gugi, Brunelle&Henocq, 1986). The standard recommended daily allowance for vitamin D supplementation in adults is 400 IU/day; the same

dose is recommended during pregnancy (Bischoff-Ferrari, Shao, Dawson-Hughes &Hathcock, 2010). Some sources recommend up to 1,000 IU for preterm infants. In addition, for non-breastfed infants, the use of a preterm formula provides additional calcium and phosphorus compared with standard formula.

Chapter 3. Objectives & Hypothesis

Objectives

1) The primary outcome is to study the effect of caffeine, as the most frequently used medication in NICU, on bone of premature infants. The effect on bone density is mainly based on radiological data, which were taken every two weeks during the hospital stay.
    a) To elucidate the effect of the duration of caffeine usage on bones of premature infants.
    b) To determine the effect of the cumulative dose of caffeine on osteopenia of prematurity.
2) As secondary outcome:
    a) To observe any effect of steroids and diuretics on bone metabolism, either cumulative dose or duration of usage.
    b) To determine the effect of Vitamin D intake, supplemented and included in the feeding, on the infants with osteopenia of prematurity evident in X rays.
    c) To observe any relation or causation between maternal parity and osteopenia of prematurity.

Hypothesis

Based on existing studies we hypothesize that caffeine usage and cumulative dose or duration of usage are associated with osteopenia of prematurity. Those with high dose of usage or longer duration of usage are more likely to develop OP. This association exists even when controlled for the effect of other neonatal or parental risk factors.

Chapter 4. Methods

Design

This is a quantitative descriptive cohort study was conducted at Health Sciences Centre (HSC) in Winnipeg from October 2007 to June 2012. The Neonatal Intensive Care Unit (NICU) at Health Science Centre, Winnipeg, Manitoba, is a referral center for infants (including preterm infants) transported from rural Manitoba, Nunavut, Northwestern Ontario and occasionally parts of Saskatchewan. This cohort study started from birth till 12 weeks of age. As a pilot study this research study included 109 infants. Cases were defined as premature infants less than 31 weeks gestational age and birth weight less than 1500 grams, with radiological evidence of osteopenia.

Variables considered in this study include

Demographic Data. The demographic data included: gestational age in weeks, gender, birth weight, average biweekly weight in grams, TPN days, and maternal parity level, which was recorded as categorical data; high if more five, moderate if three or four and low parity if one or two. Average biweekly vitamin D intake in units was included as longitudinal data.

Laboratory data. Laboratory data included serum phosphate levels were collected on biweekly basis plus or minus 1 week as found in Attachment Reflection Laboratory Data Retrieval System. The phosphate level was recorded as categorical data; high if more than 2.5 mmol/l, normal if ranged between 1.8 to 2.5 mmol/l , low if between 1.3 to 1.8 mmol/l and very low if less than 1.3 mmol/l . This was considered at any time point during the cohort study.

Radiological data: The chest radiographs were undertaken to investigate respiratory tract symptoms, to be part of an investigation of sepsis, to confirm the position of an endotracheal tube, nasogastric tube, or central line and to investigate abdominal symptoms.

The radiological data (X rays) were reviewed and interpreted by pediatric radiologist, who did not know about the patients' data, on a biweekly basis in the first 12 weeks of life at least, using Koo et al., criteria (Koo, et. al., 1982).

a) Grade 0: Normal density of bone cortex along shaft with normal dense white line at metaphysis and normal band of lucency, and thinning of cortex.
b) Grade 1: Loss of dense white line at the metaphysis, increased submetaphyseal lucency and thinning of cortex.
c) Grade 2: Changes in grade 1 plus irregularity and fraying of metaphysis, with splaying and cupping that is indicative of rickets.
d) Grade 3: Indications of rickets with evidence of fractures.

Identification of OP

Osteopenia of the newborn will be identified if the infant meets the radiological criteria of osteopenia. Any grade of osteopenia according to Koo et al. criteria was considered as disease.

4.1. Inclusion criteria

a) Premature infants less than 1500 gram or less than or equal to 31 weeks gestation age and admitted to NICU at HSC, Winnipeg from October 2007 to June 2012.
b) Infants who had at least 12 weeks of hospital stay.
c) Data include nutritional data, radiological data and laboratory data to interpret,

4.2. Exclusion criteria

a) Infants born with congenital anomalies including congenital heart disease.
b) Infants experiencing gut surgery affecting feeding.
c) Infants with non osteopenic fractures
d) Infants with insufficient data to analyze.

Chapter 5. Statistical Analysis

The descriptive statistics (means and standard deviations) were used to summarize the characteristics of the sample. As the grade level of bone of newborn babies were measured every fortnightly from birth to 12 weeks old, outcome variables (OP statuses) are longitudinal with up to seven time points. In analyzing repeated measures data, individual differences in changes over time, were typically captured by random effects using mixed modeling (the multilevel model for change). These random effects describe each person's trend across time, and explain the correlational structure of the longitudinal data. We should note that an advantage of using mixed model over the traditional repeated measures ANOVA is that it can use all available information even for those with missing data at some points. The ability to include subjects with incomplete data across time is important relative to procedures that require complete data across time because (a) by including all data, the analysis has increased statistical power, and (b) complete-case analysis may suffer from biases to the extent that subjects with complete data are not representative of the larger population of subjects. Whereas the traditional approaches estimate average change (across time) in a population, mixed model can also estimate change for each subject. These estimates of individual change across time can be particularly useful in longitudinal studies where a proportion of subjects exhibit change across time that deviates from the average trend.

The cumulative dose and duration of usage of caffeine were included in the generalized mixed model as covariates. Other covariates were added to the generalized mixed model include dose or duration of usage of steroids and diuretics, Vitamin D intake, and other demographic variables such as gestational age in weeks, gender. Vitamin D intake was treated as time-varying covariate.

The statistical analyses were carried out using SAS 9.3 (SAS Institute, Cary, NC). All p-values are two-sided, and significance was set at a value of 0.05.

Chapter 6. Ethics Approval

The proposed study was a quantitative chart review study; the collected data did not include any patients' identification. The collected data was stored in a personal computer with a password. I followed the Tri-Council Guidelines for Health Research involving data privacy throughout my research project.

1) The ethics approval was obtained from the Health Research Ethics Board (HREB) at University of Manitoba number# H2013: 231
2) The Health Sciences Center Research Impact Approval was obtained from the Health Science Center. Number# RI2013: 088

Chapter 7. Results

The initial cohort included 335 preterm infants, with a gestational age of less than 31 weeks and a birth weight less than 1500 grams, who were admitted to the neonatal intensive care unit between July 2007 and July 2012. Of these 335 infants, 35 infants died, and 5 infants were transferred to other facilities and 3 had surgical NEC ended with short bowel syndrome. Out of the remaining 292 infants, the final study group included 109 infants who had 12 weeks of hospital stay, available radiological data, and laboratory data to analyse.

Means and standard deviations were used to summarize the characteristics of the sample for most continuous variables (GA, birth weight, caffeine dose). For those variables, which were highly skewed, median and quantiles were reported (TPN duration, steroid dose and diuretic dose). The raw data were examined for any outliers and influential points before the start of the analysis. The results of GA, BW, sex, maternal parity and TPN duration are shown in Table 1, and average biweekly weight and vitamin D intake on Table 2.

Table 1. The cohort demographic data**:**

Variables	
Gestational Age (weeks) (mean±2SD)	27±1.6
Birth Weight (grams) Mean±2SD	665±229
Male/ female	54 male/55 female
Maternal Parity	
Low parity<2	85 (77.9%)
Moderate parity2-4	16 (14.6%)
High parity>4	8(7.5%
TPN days (Median)	21
Quantiles	11, 32

Table 2. The average biweekly weight and vitamin D intake of the study cohort:

	Week1-2	Week3-4	Week5-6	Week7-8	Week9-10	Week11-12
Average weight in grams	993±23	1108±2	1335±29	1660±4	1984±4	2348±5
Average Vitamin D in units	392±35	555±37	737±33	834±29	947±29	1034±32

The mean gestational age of this cohort was 27 weeks (27±1.6); the cohort includes 54 males and 55 females. The average birth weight of this cohort was 665±229 grams. Of the 109 infants, 85 were born to mothers of low maternal parity (77.9%), 16 (14.6%) of moderate maternal parity and 8 of high maternal parity (7.5%). The median duration of TPN were 21days with 1st and 3rd quantiles of 11 and 32 days respectively. The average biweekly weight is showed in Table 2.Vitamin D was manually calculated as average biweekly vitamin D intake including the dietary, parenteral and supplementary vitamin D source.

It was easier to include grade 1 and 2 of OP together, as the differentiation between the two grades is very subjective. For grade 3 OP, it was easy to distinguish, as callus formation was indicative of previous underlying spontaneous fracture. According to the frequency of the outcome variables (four grade levels), we dichotomized it by collapse of categories of grade levels. Grade 1, 2 and 3 were collapsed together as OP because we lack enough events in each of the three grades. In the same time we considered grade 0 as normal. So the mixed model is generalized mixed model for binary outcome.

There were eight infants with bone fractures (8%). The fractures involved the right and left lower ribs and none of them had spontaneous humerus fracture. The prevalence of OP based on Koo et al., in this cohort was 51.3%.

All of the infants received caffeine during their hospital stay, starting day one. The average dose of caffeine was 425.33±235.2 mg as a cumulative dose and the average duration of caffeine therapy was 60±45.8 days. The average dose of caffeine was 7.95 ±2.7mg per kg per day and the range of caffeine dose was (4.1-15.6 mg/kg/day) including the loading, the maintenance dose and the mini-load doses. The usual starting load was 10 mg/kg followed by maintenance of 5-7 mg/kg/day and the infant received mini-loads of caffeine in-between according to the severity of apnea of prematurity as long as the heart rate was less than 180 beat/min. The adjustment of the caffeine dose according to the current weight of the infants usually occurred according to the treating physician preference. During the study time, there was no systematic protocol to follow the serum caffeine level.

There were 79 infants who received diuretics (73%). The median diuretic dose was 5.9mg with 1st and 3rd quantiles of 1, 25.8 during the hospital stay. The steroids were calculated as dexamethasone dose or equivalent as 100 mg of hydrocortisone are equal to 20 mg of dexamethasone. In this cohort, the median steroid dose was 2 mg and the 1st and 3rd quantiles were 0, 42 during the hospital stay.

We first fitted generalized linear model to examine each individual variable associate with the probability of OP, including gestational age, average biweekly birth weight, maternal parity, TPN duration, vitamin D intake, and serum phosphate level, duration of caffeine treatment and the cumulative doses of caffeine, steroids, and diuretics. The results are reported in Table 3. Table 3 shows that lower gestational age and average biweekly weight are correlated with OP. Similarly, higher caffeine cumulative dose and longer caffeine duration of therapy showed a statistically significant correlation with OP ($p<0.05$). In the univariate model; steroids doses, TPN days and biweekly average intake of vitamin D display significant correlation with

OP. On the contrary, maternal parity, serum phosphate and diuretics were not associated with a significant association with OP ($p>0.05$). The cohort did not include enough high parity mothers to analyse. The maternal parity was analysed as low parity if less than 2 and moderate parity if more than 2. Similarly, serum phosphate was categorized as very low if less 1.3mmol/l and low if between 1.3 and 1.8 mmol/l and normal if more than 1.8, as there was not enough data of preterm infants with high phosphate >2.5mmol/l to analyze.

Table 3. Factors associated with OP: Results of Univariate analysis

Variables	Estimate	Standard Error	P value
Gestational age (weeks)	-0.645	0.147	<0.001
Average biweekly weight (grams)	0.0006	0.0002	0.006
Caffeine cumulative dose (mg)	0.005	0.001	<0.001
Caffeine duration (days)	0.051	0.013	<0.001
Steroids cumulative dose (mg)	0.09	0.046	0.038
TPN duration (days)	0.034	0.012	0.005
Vitamin D (units)	-1.863	0.36	<0.001
Diuretics cumulative dose (mg)	0.003	0.002	0.20
Serum phosphate (mmol/l) Phosphate<1.3 Phosphate(1.3-1.8) Phosphate>1.8 (ref)	 -0.09 0.11	 0.16 0.33	 0.57 0.74
Maternal Parity Low parity Moderate Parity (ref)	 -0.016	 0.42	 0.96

Then we fitted multivariable generalized mixed model with gestational age, average biweekly weight, cumulative dose of caffeine, cumulative steroids dose and vitamin D considering the clinical importance and statistical significance at univariate analysis. But because of the small sample size, we were only able to include 5 variables to fit in the mixed model. The results are showed in Table 4.

Table 4. Results from Multivariable Generalized Mixed Model

Effect	Estimate	Standard Error	P value
Intercept	5.63	5.59	0.321
Caffeine Cumulative Dose (mg)	0.39	0.05	0.007
Steroid Cumulative Dose (mg)	0.17	0.05	0.035
Vitamin D (units)	-1.64	0.47	0.006
Average Biweekly Weight (grams)	-0.01	0.0001	<0.0001
Gestational age (weeks)	-0.41	0.19	0.0408

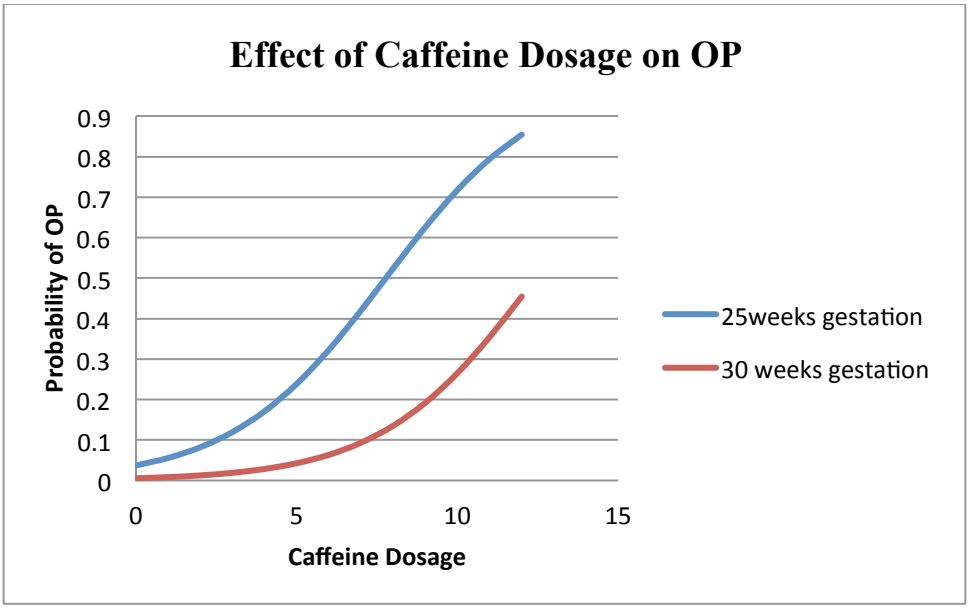
Table 4 indicates that higher cumulative dose of caffeine is associated with an increase in the probability of OP. The effect of caffeine was true even when we controlled the effect of other variables (average weight, the gestational age steroid and vitamin D). The odds of OP is 1.10 times (95%CI: 1.05-1.15) higher for every 5 mg/kg increase in caffeine dose when the effect of steroids, Vitamin, gestational age and average biweekly weight are controlled.

The steroid dosage has a statistically significant result in predicting OP with ($p<0.0001$) (estimated Odds ratio =1.1 and CI: 1.005-1.20). The average weight on biweekly basis showed statistically significant prediction of OP ($p=0.006$). The higher the average weight was, the less likely to develop OP.

The results showed that the average biweekly vitamin D intake, both included in the diet and supplemented, had a negative correlation with the OP ($p<0.0001$). The probability of OP is decreased by 0.4% when vitamin D increased from 400 to 800.

To illustrate the effect of caffeine dose on OP in 2 different groups of preterm infants at 25 and 30 weeks gestation, graph 1 was plotted using the previous model. Graph 1 shows the effect of increasing caffeine dosage on the probability of OP in different gestational age based on the above fitted generalized mixed models. The graph demonstrates the difference between the gestational ages, 25 and 30 weeks in regard of OP probability when both are exposed to the same dose of caffeine. The probability of OP is higher in lower gestational age (25 weeks) than the 30 weeks gestational age.

Graph 1: Probability of OP with increasing caffeine dosage in 25 weeks and in 30 weeks gestational age:



To examine whether the effect of caffeine dose on OP differs by duration of treatment, we fitted another generalized mixed model by including the interaction between caffeine dosage and duration of therapy, and other covariates, i.e., steroids and vitamin D, gestational age, average weight. The results are showed in table 5. This table shows that, the average caffeine dose, caffeine duration of therapy as well as the interaction between caffeine dose and duration

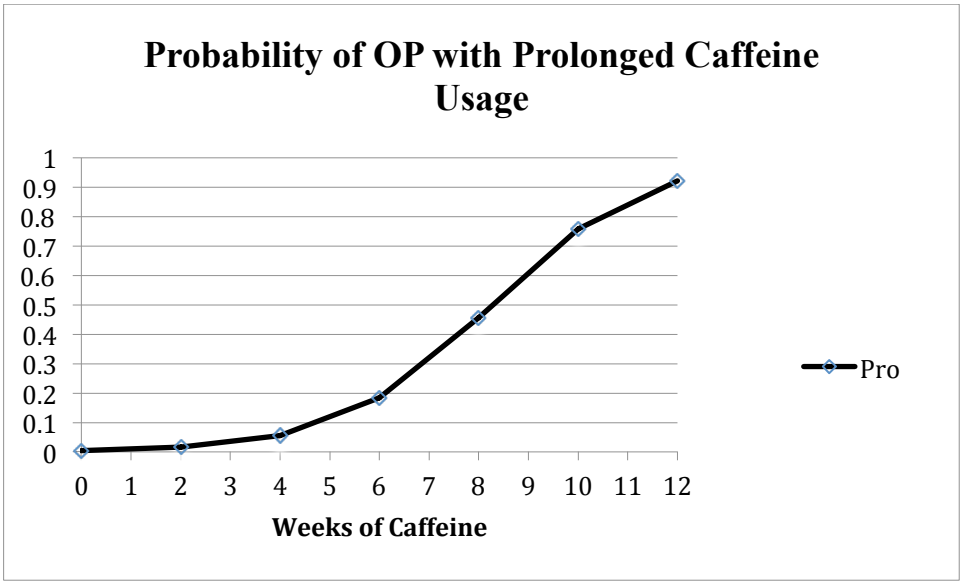
of caffeine treatment has a statistical significant correlation with OP even when controlling for the effects of gestational age, weight and vitamin D ($p<0.05$).

Table 5. Estimates with interaction of caffeine and duration of treatment

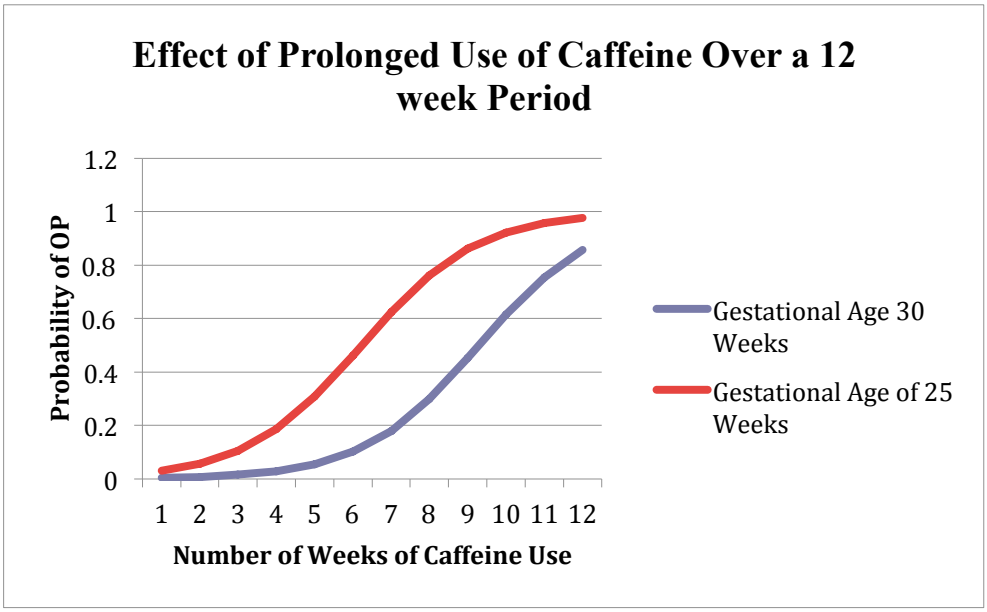
Effect	Estimate	Standard Error	P
Intercept	3.39	5.99	0.57
Average Caffeine dose (mg/kg/d)	0.24	0.09	0.029
Duration of caffeine treatment (days)	0.64	0.27	0.02
Caffeine dose* Duration of caffeine treatment (days)	0.07	0.04	0.05
Steroid cumulative dose (mg)	0.09	0.05	0.07
Vitamin D (units)	-1.86	0.36	0.04
Average biweekly Birth Weight (grams)	-0.06	0.02	0.001
Gestational age (weeks)	-0.64	0.15	0.001

Based on the model in Table 5, Graph 2 and 3 show the effect of duration of caffeine usage on the probability of OP. The probability of OP increased in 25 weeks preterm infants, is higher than the 30 weeks preterm infants. The graph exhibited that the lower the gestational age the higher the probability of osteopenia over prolonged caffeine use, even when controlling caffeine dose, steroid dose, birth weight, and vitamin D.

Graph 2**.** Probability of OP with prolonged caffeine use among the study cohort:



Graph 3. Probability of OP with same Caffeine dosage in 25 weeks and in 30 weeks gestational age over the weeks of treatment



Chapter 8. Discussion, Limitations and Conclusion

There is ample published information about the effect of caffeine on adult bone, but very little is available about the effect of caffeine dosages on premature bone. This cohort study was designed to examine the effect of caffeine on OP. The results of this study revealed a strong correlation between exposure to caffeine and the presence of OP. In this study the average caffeine dose was 7.9 mg/kg/day well beyond the dose used in the CAP study of 5 mg/kg/d (Schmidt, Roberts, Davis, et al., 2006).

The dose of caffeine the premature babies in this study received also was much greater than the average daily intake of caffeine in Canada from all sources which is ~ 2.4 mg per kg body weight for adults and 1.1 mg/ kg for children 5–18 years old (Chou, 1992).

Although the overall survival of extremely low birth weight infants has improved over the past 2 decades, these infants continue to have significant comorbidities. In this study of a recent cohort of extremely low birth weight (ELBW) infants who survived > 12 weeks, 50.1% had radiological evidence of OP during their NICU stay. About 8% of infants with MBD further developed spontaneous rib fractures secondary to OP. The prevalence of OP in our study is similar to that previously reported in the literature and suggests that MBD remains a significant comorbidity in ELBW infants and puts them at increased risk for spontaneous fractures during the NICU stay. Poor bone mineralization has been found in 30% of infants weighing less 1500 g at birth and in more than 50% of those weighing less than 1000 grams (Requirement, 1985). Later reports on the incidence of MBD in premature infants mostly range from 30% to 50%. (Koo et al., 1999). More recent reports by Mitchell et al., (2009) and Viswanathan et al., (2013) found that, the smaller the babies were, the higher was the incidence of MBD, ranging from 12% to 67% , and the incidence of osteopenic fractures ranged between 3% and 21 % (Mitchell,

Rogers, Hicks, Hawthorne, Parker, & Abrams, 2009) & (Viswanathan, Khasawneh, McNelis, Dykstra, Amstadt, Super & Groh-Wargo, 2013).

It is well studied that the frequency of MBD is inversely related to gestational age and birth weight (BW). (Vachharajani, Mathur, Rao, 2009). Our results are consistent with this concept, the younger and the smaller the babies were the higher was the incidence of OP. The OP in lower gestation and lower birth weight can be explained by the high turnover of bone matrix early in fetal life which decreases with advancing gestation and the mineral accretion rates in utero increase throughout gestation reaching a peak around term (Harrast &Kalkwarf, 1998). On the other hand, the premature delivery and the NICUs Stay are addional significant contributors to the high incidence of OP. The relation of bone turnover with gestational age and birth weight has been well described. (Vachharajani, Mathur, &Rao, 2009).

Apnea of prematurity is one of the most common respiratory problems in the neonatal intensive care unit. Advances in neonatal care in recent decades have resulted in an increased survival rate in low- birth-weight premature infants. Therefore, the number of newborns with this disorder is increasing (Erenberg & Allen, 2000).

In spite of the caffeine effect on treating apnea of prematurity with favorable long-term outcomes (Ofek-Shlomai, & Berger, 2014), our study results revealed a strong association between caffeine dose and duration of treatment and OP in longitudinal data analysis even when controlling for the effect of gestational age and weight gain and other medications. It is well studied that caffeine causes calciuria and creates negative calcium balance in preterm infants. A study done by Zandaro et al., (1995), investigating effect of caffeine on calciuria, found that caffeine (loading dose 10 mg/kg and maintenance dose 5 mg/kg/day) treatment caused a significant increase ($p < 0.05$) in calciuria (10 to 15 fold) compared to control healthy untreated

premature infants. (Zanardo, Dani, Trevisanuto, Meneghetti, Guglielmi, Zacchello & Cantarutti, 1995). Zanardo et al. assessed the caffeine effect after 5 days of treatment and in the current study the caffeine average duration of use was 60 days.

Caffeine creates a negative calcium balance by decreasing the renal threshold for calcium. Yeh et al. (1986) found that rats administered caffeine had increased urinary and fecal calcium excretion with a compensatory increase in intestinal calcium absorption in older rats but not in younger ones. In a separate study, the intestinal absorption coefficient of calcium declined gradually as observed in the young control group. (Yeh, Aloia, Semla, & Chen, 1986). In another study to support the effect of caffeine on bone metabolism, Yeh and Aloia (1986) found serum PTH and 1, 25(OH)2 D to increase 4 weeks after caffeine administration in young rats but not in older rats (Yeh, & Aloia, 1986). That study confirmed that the negative balance of calcium after caffeine administration, could lead to a compensatory increase in vitamin D and PTH to normalize serum calcium at the expense of bone.

Our study also indicates a strong correlation between the duration of caffeine treatment and OP. Caffeine causes direct stimulation of osteoclastogenesis, which leads to bone resorption. This is in agreement with the animal study done by Liu el al., (2011) who demonstrated that BMD in lumbar vertebra, femur, and tibia was significantly lowered in growing rats supplemented with caffeine in diets for 20 weeks compared with the control group. The calcium contents in tibia and femur of caffeine-treated rats were also lower than that in the control group. The osteoclastogenesis of bone marrow cells isolated from caffeine-treated rats was markedly enhanced as compared with the control group (Liu, Chen, Yang, Yen, Yang & Tsai, 2011). In a study investigating the effect of chronic caffeine administration on bone histomorphometry and serum markers of bone mineral metabolism, there was increase in serum PTH levels

demonstrated after 7 weeks, when the high-dose caffeine group showed a raised level ($p < 0.05$). Serum osteoclastin, which has been shown to be a useful marker of bone turnover, was significantly increased in high caffeine dose compared with control which indicate high bone turn over and positively support our study ($p < 0.001$) (Glajchen, Ismail, Epstein, Jowell, & Fallon, 1988).

In contrast to the current study results, in a retrospective study done by Viswanathan et al. (2013), there was no difference in duration of caffeine use between cases of OP and control group. Viswanathan et al. did not calculate caffeine dose, only caffeine duration was included between cases and controls. In contrast to our study, Viswanathan et al. included spontaneous rib fractures in the control group if there was no previous radiological evidence of MBD. In our study, the osteopenic fractures were included in the cohort data and identified as a severe grade of osteopenia. The average duration of caffeine treatment in both groups in the Viswanathan et al. study was about 40 days, while in our study, the average duration of caffeine treatment was 60 days (Viswanathan, Khasawneh, McNelis, Dykstra, Amstadt, Super & Groh-Wargo, 2013).

Although our current study results did not include the maternal caffeine intake during pregnancy and lactation time, it is worth mentioning that maternal caffeine intake negatively affects the bone formation and development especially in premature infants, which added to the risk of prematurity. In a study examining the effect of maternal caffeine intake on fetal bone, Nakamato et al (1999) found that, normal growth and development of the fetal bone are impaired as a result of maternal caffeine intake (Nakamoto, Grant & Yazdani, 1999). The early effects of caffeine in the maternal diet were lasting as noted by the lack of recovery of the offspring even after changing to a caffeine-free diet (Schneider, Miller & Nakamoto, 1990). These results may grant some backing to our study giving that caffeine in the form of coffee, tea and cola are

among the most consumable drinks in the world, and most of the study population was fed either expressed breast milk or donor breast milk.

The effect of caffeine on bone metabolism is evident especially as it has a prolonged half-life in premature infants. Natarajan et al, (2007) found that caffeine half-life is increased in the neonatal period in both animals and humans due to the immaturity of hepatic enzyme systems, namely cytochrome P-450 and some demethylation and acetylation pathways, ranging half- lives of 40-130 h are found in premature newborn infants (Natarajan, Lulic-Botica & Aranda, 2007)

In this study there was no gender difference between males and female infants regarding OP which is in agreement with other studies which compared female to male preterm infants using SOS (Littner, Mandel, Mimouni, & Dollberg, 2003), (Yiallourides, Savoia, May, Emmerson, & Mughal, 2004) and (Rubinacci, Moro, Boehm, De Terlizzi, Moro, & Cadossi, 2003). But our results differ from those found in a study by Teitelbaum (2006) who did find that male infants have higher bone density than females by comparing preterm and full term infants using SOS with average weight of 2500 grams, but when compared as preterm and full term groups with similar weights, this difference disappeared (Teitelbaum, Rodriguez, Ashmeade, Yaniv, Osuntokun, Hudome, & Fanaroff, 2006). Procollagen type-I carboxy-terminal propeptide concentration in cord blood, which was used as indicator of OP in the first day of life, was significantly higher in male versus female premature infants. Such an observation was explained as it may follow a recognizable trend for testosterone hormone in utero. Testosterone levels are increased in male fetuses during the period of sexual differentiation and throughout the second trimester of pregnancy. At term, this difference was no longer observable. The gender-difference in serum procollagen type-I carboxy-terminal propeptide may reflect differences in the production of the collagen degradation markers by skeletal and non-skeletal cells in premature

infants (Aly, Moustafa, Amer, Hassanein, Keeves, & Patel, 2005). But this difference in BMC was not observed in another study by Namgun, & Tsang, 2000 who stated that gender differences in BMC appear in early life between 1-18 month of age but probably not at birth (Namgung, & Tsang, 2000).

This study showed significant effect of TPN duration on the development of OP but this effect disappeared when we controlled for the effects of caffeine, GA, biweekly average weight and steroids. A study published in 1985 comparing the effect of different protein content in TPN on MBD, found a positive correlation between TPN duration and MBD in preterm infants less than 32 weeks. It is well documented that TPN infusion is associated with increased calcium excretion from the kidneys with different theories to explain this phenomenon that include that continuous infusion of glucose leads to increase insulin which increases calcium excretion as well as sulfated amino acids (Chu, Barkowski, & Buhac, 1990). Another study showed that prolonged TPN could cause metabolic bone disease, which can be gently ameliorated by changing Vitamin D intake (Sankaran, Berscheid, Verma, Zakhary, & Tan, 1985).

This study showed that TPN duration showed a statistical significant correlation with OP. TPN duration did not show similar significant effect when included with other determinants like GA, average biweekly weight and Caffeine. TPN duration did not show the significant effect but still with noticeable trend, the explanation for this may be because the effects of other risk factors contribute much to the OP more than the TPN duration alone and given that the TPN contains the maximum amount of calcium and phosphate according to the maximum solubility. In this study, TPN duration count included the null per os days and partial feeding days as well. Usually in clinical practice in this unit, TPN is provided till the infant can tolerate the full enteral feeding.

In agreement with another study by Rohana et al (2007) there was a correlation between TPN and OP (p=0.03) but in this latter study there was no inclusion of the content of phosphate and calcium in TPN and sample size was small, including 53 infants only (Rohana, Hasmawati, & Zulkifli, 2007).

In accordance to our study, (Pereira-da-Silva et al., (2011), found that, the bone speed of sound (SOS) was significantly higher in the TPN group with higher calcium contents rather than the duration of TPN, when they gave equivalent amounts of nutrients to both cases and control groups of premature infants (Pereira-da-Silva, Costa, Pereira, Filipe, Virella, Moreira, 2011).

Although Backström et al suggested that serum phosphate level lower than 1.8 mmol/L (5.5 mg/dl) may have a diagnostic sensitivity of 100% and specificity of 70% for MBD (Backström, Kouri, Kuusela, Sievänen, Koivisto, Ikonen, & Mäki, 2000) , In our study, serum phosphate on biweekly basis did not show a statistically significant correlation with OP but with a trend being low with OP. None of other studies examined serum phosphate as a longitudinal markerover the hospital stay. Yet serum phosphate is among the minerals that is regulated tightly and the average biweekly record may not representing the real situation of serum phosphate in infants on TPN for the first week at least and on partial feeding for another week. In the study hospital, serum phosphate is followed closely during TPN duration and the infant is usually given the maximum amount of phosphate allowed according to the calcium-phosphate solubility. On the other hand, the breast milk or the donor breast milk is fortified for all preterm infants. In agreement with our results, serum phosphate was not correlated with procollagen type-I carboxy-terminal propeptide as a predictor for OP in preterm infants by Aly et al., 2005 . In that study serum phosphate was recorded as single reading at birth. In another study serum phosphate and serum alkaline phosphatase were not correlated with OP at 3 weeks of age but were correlated

with OP at 3 and six months of age, which was explained by the other confounding factors that affect premature bone in early life in NICUs, which may include mechanical ventilation, nutrition, and medications used during the hospital stay. These factors abolish the ability of serum phosphate and alkaline phosphatase to correlate will with OP in NICUs, while the sensitivity was 100% at 3 months and 6 months of age. Serum phosphate was not specific indicator of OP as shown by the same study. Some of the infants in this study had normal serum phosphate but received caffeine, corticosteroids and diuretics during their hospital stay which indicate non specificity of serum phosphate as well as the negative effect of these medications on BMD (Backström, Kouri, Kuusela, Sievänen, Koivisto, Ikonen, Mäki, 2000). .

On the other hand, in a case series reported by Mutlu (2014), all babies had metabolic bone disease and low serum phosphate level, but all of them were about 42-45 weeks corrected gestational age, and were referred because of abnormal laboratory results but were asymptomatic. Those cases were discharged from the hospital and probably did not receive any milk fortifiers. Mutlu, G. (2014). Another study showed that serum phosphate is correlated with OP as a single reading at 21 days of age along with SOS reading from metacarpal bones (Betto, Gaio, & De Terlizzi, 2014). Hellstern et al., (2003), compared infants of 23–25 weeks GA to infants of 26-31 weeks GA; they found that the infants of 23-25 weeks are at risk for low renal phosphate threshold concentrations values, leading to urinary phosphate excretion even in the presence of low serum phosphate levels. This difference disappeared after 3-5 weeks post-natal age and with maturity. In that study, they recommend monitoring serum phosphate to keep the level greater than 2 mmol/l (Hellstern, Pöschl & Linderkamp, 2003).

While it is documented that the number of previous pregnancies of a healthy mother correlated negatively with BMD measurements, the effect of previous pregnancies did not show

the same effect on infants' bone formation.This supports the fact that an infant acquires the needed minerals and vitamin from the mother's body with active transport against the concentration gradient ignoring the mother's general status (Ghannam, Hammami, Bakheet, & Khan, 1999).

In this study there was no significant effect of maternal parity on OP in preterm infants. The preterm infants GA was 27 weeks in average and all of them missed the third trimester mineral accretion period. On the other hand this cohort study did not have enough of high parity mothers to reveal correlation, and thus further study is needed by including high parity mothers. The moderate maternal parity included mothers with more than 2 previous deliveries. To my knowledge, there are no published research studies on the effect of maternal parity as a predictor for OP less than 31 weeks. In agreement with our results, Godfrey (2001)observed that neonatal BMC and BMD were not related to maternal age, parity, or social class, while the birth weights of both parents and the height of the father were positively correlated with neonatal whole body BMC (Godfrey, Walker-Bone, Robinson, Taylor, Shore, Wheeler, & Cooper, 2001).

In a prospective cohort study from the UK Harvey et al. (2010) found that Neonatal whole body bone area adjusted for birth length (a measure of bone width) was predicted positively by maternal parity and late pregnancy triceps skinfold thickness and negatively by late pregnancy walking speed. These findings were similar in both genders. But that study included only full term infants and there were no defined parity groups, in fact, that study divided the mother to either primigravida or others and include other mothers with more than one pregnancy as a different group (Harvey, Javaid, Arden, Poole, Crozier, Robinson, & Cooper, 2010). On the other hand, in a study done by Aly et al., (2005) comparing maternal parity as a continuous variable to compare bone turnover in preterm and full term infants using procollagen type-I

carboxy-terminal propeptide as a predictor for OP, the maternal parity was negatively correlated with procollagen type-I carboxy-terminal propeptide($p=0.006$). In that study, the preterm infants gestational age was 32.17 ± 1.72 weeks and the full term infants GA were 38.75 ± 0.91 weeks and the preterm birth weight were 1.471 ± 0.167 kg (Aly, Moustafa, Amer, Hassanein, Keeves, & Patel, 2005).

Our study showed a statistically significant correlation between vitamin D and OP. Vitamin D calculations included supplementations and dietary content as a biweekly average for twelve weeks of hospital stay. Vitamin D supplementation did not show any change in the probability of OP with changing the dose of vitamin D from 400 to 800 units. In the study hospital, all infants are supplemented with vitamin D either in TPN or enterally. Enteral supplementation is started when the infants can tolerate half feeding. The infants are supplemented either with 400 unit or 800 units daily according to the type of milk.

Our study results are contradicting those in a study done by Kislal & Dilmen (2008). They compared three different doses of vitamin D, 200 IU, 400 IU and 800 IU, were administered to a total of 37 preterm infants between 15th day of birth until the 30th day of birth. There were no significant differences in levels of serum calcium and phosphate before and after vitamin D supplementation in all groups. Serum ALP levels were increased only in groups 1 and 3, suggesting that vitamin D in high doses can contribute to the bone turnover in neonates. In that study the study groups included a small sample size and they did not include the OP changes of the study group. According to our present results, there is no strong evidence for use of high-dose vitamin D. The available data suggest that vitamin D supplementation is beneficial in healthy term and preterm breastfed infants in addition to children with established malnutrition and rickets. There is also disagreement among professional organizations about vitamin D dose

in infants. The American Academy of Pediatrics recommends 400 IU (10 µg) per day, as does the Institute of Medicine with a goal 25-hydroxyvitamin D level >20 ng/mL. However, the Endocrine Society recommends that infants require at least 400 IU and that 1000 IU daily may be needed to obtain an optimal 25-hydroxyvitamin D level (>30 ng/mL) for non-skeletal health benefits (Nehra, Carlson, Fallon, Kalish, Potemkin, Gura, Puder, 2013). The Canadian Pediatric Society advocates an increase of vitamin intake to 800 IU/day for northern Native communities during the winter months (First Nations, I. A. M. H. C., 2007).

Although antenatal betamethasone exposure does not affect peak bone mass or femoral geometry in adulthood (Dalziel, Fenwick, Cundy, Parag, Beck, Rodgers, & Harding, 2006), systemic postnatal corticosteroid causes appreciable suppression of serum bone alkaline phosphatase and osteocalcin and, to a lesser extent, urinary deoxypyridinoline. The results suggest that the corticosteroid inhibits bone growth mainly by decreasing bone formation (Ng, Lam, Wong, & Lee, 2002).

Our study results show a statistical significant correlation between OP and steroid cumulative dose during the hospital stay. In accordance with another study done by Eelloo et al., (2008), dexamethasone treatment in the neonatal period appears to be associated with reduced total body and lumbar spine bone mineral density. Dexamethasone treatment results in increased excretion of calcium in stools and urine and of phosphate in urine (Eelloo, Roberts, Emmerson, Ward, Adams, & Mughal, 2008). In an agreement with an earlier study, dexamethasone treatment increased the daily excretion of phosphate in the urine and decreased the phosphate concentration in serum (Sonntag & Gaude, 1997).

Another prospective longitudinal study was to observe the effects of treatment with dexamethasone on somatic growth, mineral balance and bone mineralization in very low birth

weight preterm infants with chronic lung disease. The start of dexamethasone treatment was also associated with a significant fall in calcium absorption, calcium retention (60.8% to 40.6%) and phosphate retention (65% to 39.6%). Phosphate absorption was not significantly affected (Shrivastava, Lyon & Mcintosh 2000). It is proven that dexamethasone effect on bone catabolism is observed in neonates as well as adults.

Long-term use of diuretics especially furosemide in preterm infants suffering from chronic lung disease and congestive heart disease results in increased urinary excretion of calcium. Simultaneously, infants with chronic lung disease frequently suffer low calcium intake and fluid restriction (Rigo, 2000). These observations would lead us to expect an increase in the probability of OP. In a study done by Atkinson et al., (1988), twenty-four-hour urine specimens were collected from 30 patients who were treated with diuretics. They observed hypercalciuria in all treatment groups. Covariate analysis demonstrated a significant effect of diuretic treatment ($p<0.01$) and sodium excretion ($p<0.05$) on urinary calcium excretion. (Atkinson, Shah, McGee, & Steele, 1988). Treatment with any of the diuretics in neonates is associated with abnormal renal losses of calcium that creates negative calcium balance.

In our study, diuretics did show a positive trend in relation to OP. This correlation did not reach statistical significance. This result can be explained by the short duration of diuretics use and the relative small sample size. The use of high dose of caffeine that has a diuretic effect might explain the lower need for the diuretic use.

Limitations of the Study

This study has several limitations that may be affecting or skewing the results. This study was limited by the small sample size. It was a retrospective chart review. Some of the data was missing from the charts. The study was conducted at one center, and thus the results may not be generalizable on a wider scale. Manitoban mothers are known to have vitamin D deficiency and the effects of maternal vitamin D deficiency might be affecting the fetal outcome. In the future more preterm infants especially premature infants with high maternal parity should be studied to investigate the effect of high maternal parity on OP. Although the association seen between caffeine and OP was very dramatic, in the future, caffeine effect on OP can further be investigated as a case-control study in an animal model mimicking the human situation.

Conclusion

We conclude that caffeine has a strong association with OP. As limit of viability continues to decrease with 70% survival of infants between 24-26 weeks, OP will continue to increase and will results in significant morbidity in childhood and adulthood unless strategies to mitigate risk factors are developed. These might include further study of effective lower caffeine doses, different ventilation strategies, adequate vitamin D intake, passive movement as all these can provide protection against OP. Further studies are needed to evaluate which infants are at higher risk because of genetic susceptibilities and to possibly implement other modalities to treat apnea of prematurity particularly in genetically susceptible infants.

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