VITAMIN D STATUS AND BONE METABOLISM IN VERY PRETERM AND TERM INFANTS AND THEIR MOTHERS

BY

REBECCA J. VEITCH

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences
University of Manitoba
Winnipeg, Manitoba

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Vitamin D status and bone metabolism in very preterm and term infants and their mothers

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Vitamin D plays a role in bone growth and mineralization. It is therefore important to determine the vitamin D status of very preterm infants and its role in bone metabolism. Sixteen very preterm infants were recruited and followed throughout their hospital stay. At day 7, 21, 35 post-natal age (PNA) and hospital discharge, 25-hydroxyvitamin D (25(OH)D) and biochemical markers of bone metabolism (osteocalcin, urinary N-telopeptide (NTx) and urinary calcium) were measured. Bone mineral content (BMC) was measured using dual-energy x-ray absorptiometry at hospital discharge and 6 months corrected age. Thirty term infants (gestation age between 37 0/7 and 40 6/7) were also recruited to act as a reference group. In term infants, the above mentioned biochemical markers were measured in cord plasma and urine samples at birth. At birth and 6 months of age, BMC was also measured.

At birth, 31% of very preterm infants and 27% of term infants were deficient in 25(OH)D, defined as $\leq$ 27.5 nmol/L (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). Thirty one percent of the very preterm mothers and 50% of the term mothers were deficient in 25(OH)D at the time of delivery, defined as <40 nmol/L (Vieth et al., Eur J Clin Nutr 2001;55:1091). There was a significant positive relationship between term infants and their mother's 25(OH)D status at time of delivery. However, this was

not evident among the very preterm infants. Very preterm infants born between the months of April-September were found to have a significantly higher plasma 25(OH)D than infants born between October-March. Day 7 PNA 25(OH)D was significantly higher than day 35. At day 35 PNA, 6% of infants were deficient in plasma 25(OH)D. However, by hospital discharge, all infants had normal values. Day 7 PNA osteocalcin and urinary NTx and calcium concentrations were significantly lower than hospital discharge. At corrected term age, very preterm infants were found to have a higher level of all markers of bone metabolism in comparison to the reference group of term infants at birth. At corrected term age, very preterm infants were smaller than the reference group of term infants. As well, their lower lumbar spine and femur BMC remained lower, even after correction for weight and length. At 6 months corrected age, very preterm infants remained smaller than the reference group. As well, BMC continued to be lower in very preterm infants; however, after correcting for weight and length, the differences were no longer evident. There was a significant positive relationship between day 7 PNA 25(OH)D and lumbar spine BMC corrected to weight at hospital discharge within very preterm infants.

In conclusion, 25(OH)D deficiency exists in both very preterm and term infants in Manitoba, as well as their mothers. Throughout their hospital stay very preterm infants have increased concentrations of bone metabolism markers at hospital discharge compared to a reference group of term infants. By 6 months corrected age, very preterm infants have appropriately mineralized bone, but, their growth remains smaller.

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List of Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D
AGA	Appropriate for gestational age
ANOVA	Analysis of variance
BMC	Bone mineral content
BMD	Bone mineral density
BPD	Bronchopulmonary dysplasia
cAMP	Cyclic adenosine monophosphate
CA	Corrected age
CV%	Coefficient of variation
DEXA	Dual energy x-ray absorptiometry
DPA	Dual photon absorptiometry
DRI	Dietary Reference Intake
ELISA	Enzyme linked immunosorbent assay
GA	Gestational Age
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
NICU	Neonatal intensive care unit
NTx	N-telopeptide
PICP	Propeptide of type I collagen
PNA	Post-natal age
P-RNI	Premature Infant Recommended Nutrient Intake
PTH	Parathyroid Hormone
RIA	Radioimmunoassay
RNI	Recommended Nutrient Intake
SPA	Single photon absorptiometry
TPN	Total parenteral nutrition

1.0 Introduction

Preterm birth continues to challenge neonatal medicine and nutrition and is one of the most important perinatal health problems in industrialized countries (Health Canada, 1999). Preterm birth is defined as birth at or less than 37 completed weeks of gestation, while very preterm birth is defined as birth at or less than 32 completed weeks of gestation (Health Canada, 1999).

The rate of preterm and very preterm birth appears to be increasing in Canada, likely due to a reduction in mortality rate (Kramer et al., 1998). Several factors including improved respiratory support, total parenteral nutrition (TPN) and improved enteral nutrition have contributed to the reduced mortality rate (Berry et al., 1997). The increased survival rate justifies the need for improved medical and nutritional initiatives (Goldenberg & Rouse, 1998).

Preterm infants are prone to several health risks because they have not had the chance to fully develop in utero. Examples of health problems include immature sucking (Lau et al., 2000), hypocalcemia (Mayne & Kovar, 1991), neurological and respiratory diseases (Kramer et al., 1998), impaired growth (Gibson et al., 2000) and impaired bone growth and mineralization (Rigo et al., 2000). Very preterm infants are even more susceptible to the above mentioned health problems due to their early delivery. However, by optimizing the postnatal care available to preterm and very preterm infants, the health problems, developmental delays and impaired growth may be improved.

Nutritional management of very preterm infants can be complex. Most of the organs are immature and several of the health problems associated with very

preterm birth impact nutrition in some form. Fetal nutrient stores are established during the last trimester of pregnancy (28 weeks gestation), and therefore very preterm infants begin life with compromised nutrient stores (Namgung et al., 1998). Each individual infant has different needs (Greer & McCormick, 1988) making it difficult to administer the appropriate amount of nutrients in quantities similar to which the infant would have received in utero.

The Canadian Paediatric Society revised their recommendations on the feeding of preterm infants in 1995 (Nutrition Committee, Canadian Paediatric Society, 1995) and re-affirmed these recommendations in 2000 (Nutrition Committee, Canadian Paediatric Society, 1999-2003). This gives health care professionals a relatively up-to-date guideline, based on scientific research, for the feeding of very preterm and preterm infants according to their weight and gestational age. It is important to note however, that many of the nutrient guidelines for very preterm infants have been extrapolated from data on larger preterm infants. Very preterm infants may have different nutrient needs than preterm infants (Nutrition Committee, Canadian Paediatric Society, 1995).

The above guidelines are important because inadequate nutrition during early life can affect growth and development that will manifest in infancy (Cooke et al., 1998), childhood (Powls et al., 1996) and adulthood (Northway et al., 1990). In addition, inadequate infant nutrition may program the development of chronic diseases (Barker, 1994). Research has shown that infants born small and remaining small during childhood are at increased risk of developing

coronary heart disease, stroke, diabetes or hypertension during adult life (Barker, 1994).

Postmenopausal osteoporosis may also be predetermined based on infant and childhood nutritional practices. Cooper et al. (1995) showed that weight in infancy and childhood is strongly correlated with bone mineral content (BMC) at 21 years of age in women. As well, poor childhood growth has been shown to be linked to risk of fractures later in life (Javaid & Cooper, 2002). Inadequate nutrition will negatively impact growth, both weight and height, leading to negative consequences later in life in regard to bone mass. Very preterm infants are susceptible to low BMC and density that continues to young adulthood (Weiler et al., 2002). It has also been shown that days required to recover birth weight are linked to skeletal size in adulthood (Weiler et al., 2002). Since bone mass is a common prevailing sequelae of preterm birth, it is important to continue to conduct research to further understanding of the potential mechanisms. Risk factors which impact growth and bone mineralization, such as inadequate nutrition, must be identified and addressed in very preterm infants to decrease their risk for low bone mineral content and density throughout life.

Vitamin D is one such nutrient that very preterm infants likely have inadequate stores of at birth. Vitamin D plays a role in bone mass accretion, long bone growth and mineralization (Rigo et al., 2000). For humans, sunlight is the main source of vitamin D. Very preterm infants however spend the first several weeks in hospital without any exposure to sunlight. They are dependent upon the vitamin D stores developed in utero and supplemental oral vitamin D. It is

essential that the role vitamin D plays in infant growth and bone mineralization and the amounts required for vitamin D to fulfill its function adequately are determined.

The infant's vitamin D status is directly related to the mother's vitamin D status (Markestad et al., 1983), therefore, it is also important to consider the maternal vitamin D status during pregnancy. A deficiency of 25-hydroxyvitamin D (25(OH)D) in mothers during pregnancy has been shown to result in adverse effects on bone ossification (Specker et al., 1992) and congenital rickets (Moncrieff & Fadahunsi, 1974) in their infants. Knowledge of the maternal 25(OH)D concentration at time of delivery will allow us to interpret the vitamin D status and BMC of the infants.

This thesis research will examine the role of vitamin D in bone metabolism and bone mass in the very preterm infants. A secondary objective is to study vitamin D status at birth and bone mass in infants born at term age to serve as a comparison group from the same geographical region and using identical assessment techniques.

2.0 Literature Review

2.1 Endogenous and Dietary Vitamin D

Vitamin D is essential within the human body because of its involvement in calcium and phosphate homeostasis and maintenance of bone (Brown et al. 1999). Vitamin D will refer to both vitamin D_2 and vitamin D_3 in this thesis. Dietary vitamin D is measured in International Units (IU) or micrograms (μg). One microgram of vitamin D is equal to 40 IU. Plasma 25(OH)D is expressed as ng/mL or nmol/L. One ng/mL plasma 25(OH)D is equal to 2.496 nmol/L plasma 25(OH)D.

2.1.1 Vitamin D Sources

Humans obtain vitamin D through two sources; endogenous synthesis in the skin and through diet (Holick, 1994). When skin is exposed to ultraviolet beta radiation (wavelength 290-320 nm), 7-dehydrocholesterol, located in the epidermis, is converted to previtamin D_3. Previtamin D_3 is converted to vitamin D_3 that is transported to the dermal capillary bed where it enters circulation (Chen, 1999).

All layers of the epidermis have the capability to convert 7-dehydrocholesterol to vitamin D_3, however, the strata spinosum and strata basale, which are the inner layers of the epidermis, have the greatest capability (Norman, 1998). When skin is exposed to sunlight for an extended period of time, previtamin D_3 can also be converted to lumisterol and tachysterol, photoisomers of previtamin D_3 (Chen, 1999). The function of lumisterol and tachysterol are currently unknown (Chen, 1999), however, it is known that the

conversion is essential to prevent vitamin D intoxication (Holick, 1994). Several factors can affect the conversion of vitamin D in the skin such as skin pigmentation (Norman, 1998), latitude and season (Webb et al., 1988) and sunscreen use (Matsuoka et al., 1987).

The second source of vitamin D for humans is food products (Brown et al., 1999, Holick, 1994). The major, natural, food sources are fatty fish, such as salmon and fatty fish oils, such as cod liver oil (Holick, 1999). Neither is consumed in great amounts by the general population. Several foods, common in the North American diet are now fortified with vitamin D to allow for greater consumption, including milk and margarine. Fortification of fluid milk with vitamin D_2 was made mandatory in 1975 (Cheney, 2000). Table 2.1 identifies the vitamin D content of several foods, consumed by both infants and adults.

Presently, within North America, milk is considered to be the main dietary source of vitamin D, however fortification may be unreliable. The vitamin D content of milk samples was studied in several eastern states. Each quart of milk was labeled as containing 400 IU vitamin D, however, only 29% (12 samples) contained between 80-120% of the claimed amount of vitamin D. Sixty-two percent (26 samples) contained <80% of the claimed amount. Of these samples, 4 (15%) contained non-detectable amounts of vitamin D (Holick et al., 1992). In another study, 15 milk samples, labeled as containing 400 IU/liter, from British Columbia were studied. Only 27% (4 samples) contained between 80-120% of the amount claimed, while 73% contained either <80% or >120% of the vitamin D claimed. One sample had no detectable amount of vitamin D (Chen et al., 1993).

Unfortunately, there have been no recent studies published. The variability of vitamin D within fortified foods may pose problems for individuals who rely on these products as their source of vitamin D, due to limited sunlight exposure. This reinforces the fact that sunlight should be considered the main source of vitamin D.

2.1.2 Vitamin D Metabolism

Once vitamin D has either been synthesized in the skin and entered circulation or consumed through food or supplements and absorbed, vitamin D binds to vitamin D binding proteins, which transports it to the liver. Within the liver, vitamin D undergoes a hydroxylation reaction on carbon 25 to form 25(OH)D, the major circulating form of vitamin D (Holick, 1996; McCary & DeLuca, 1999). This reaction is poorly regulated, therefore, concentrations increase or decrease in proportion to oral intake of vitamin D or endogenous synthesis. Plasma concentrations of 25(OH)D are therefore indicative of vitamin D status and for this reason it is used as an indicator of vitamin D status in humans (Brown et al., 1999).

When required, the biologically inactive 25(OH)D enters the kidney where it undergoes a second hydroxylation reaction. Within the inner mitochondrial membrane of the renal cortex (Specker, 1994), 25(OH)D-1-α-hydroxylase mediates the conversion of 25(OH)D to $1,25(OH)_2D_3$. The hormone, $1,25(OH)_2D_3$, is the biologically active form of vitamin D (Brown et al., 1999). It is considered a hormone because it is produced at a site which differs from its target site and it is carried to target tissues via the blood (McCary & DeLuca,

1999). Within the kidney, 25(OH)D can undergo a second reaction using the enzyme 24-hydroxylase to form 24,25-dihydroxyvitamin D. In concert with $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ has recently been shown to play a role in the fracture-healing process (Kato et al., 1998). In target tissues, $1,25(OH)_2D_3$ can be hydroxylated by 24-hydroxylase to $1,24,25(OH)_3D_3$, a metabolite thought to be biologically inactive. The function of $1,24,25(OH)_3D_3$ is to prevent hypercalcemia and hyperphosphatemia (McCary & DeLuca, 1999).

In preterm infants, concentrations of $1,25(OH)_2D_3$ have been shown to be higher in arterial compared with umbilical venous blood, therefore suggesting that the fetus has the capability of converting 25(OH)D to the active form (Wieland et al., 1980, Fetter et al., 1985).

The conversion of 25(OH)D to $1,25(OH)_2D_3$ in the kidney is regulated by parathyroid hormone (PTH). In response to plasma calcium concentrations, PTH either stimulates or inhibits the enzyme, 1-α-hydroxylase. When hypocalcemia is present, PTH stimulates 1-α-hydroxylase, increasing the production of $1,25(OH)_2D_3$. The opposite occurs when hypercalcemia is present (Brown et al., 1999).

2.1.3 Vitamin D Functions

The major function of $1,25(OH)_2D_3$ is to keep the serum concentrations of calcium and phosphorus within their normal range allowing them to maintain cellular function and promote mineralization of bones (Holick, 1996). There are two ways in which $1,25(OH)_2D_3$ maintains serum calcium concentrations. The preferred way is to enhance the efficiency of the small intestine to absorb dietary

calcium. The active absorption or transport of calcium, which occurs in the small intestine, is enhanced by $1,25(OH)_2D_3$ (Halbert & Tsang, 1992). Calcium is transported from the small intestine into the enterocyte and across the basolateral membrane into circulation (Brown et al., 1999).

When dietary calcium is not present in adequate amounts, the second, least preferred route must be utilized. This process involves increasing the production of osteoclasts, which causes increased resorption of bone and mobilization of calcium stores (Holick, 1996). All ages, but specifically during fetal development and infancy, it is essential that adequate calcium is received so that the second route can be avoided and proper bone mineralization can occur.

A more detailed discussion of the effects $1,25(OH)_2D_3$ has on bone will follow in section 2.2.4.1.

During reproductive years, women should have adequate vitamin D status and calcium intakes to allow for the mineralization of their own and their developing infants' bones. The fetus only has access to nutrients, including vitamin D, by way of placental transfer from the mother (Paulson & Deluca, 1986). An infant's vitamin D status is therefore directly related to their mother's (Markestad, 1983), and a woman who enters pregnancy with a deficiency of vitamin D is putting both herself and her infant at risk for reduced bone mass. If the mother is vitamin D deficient, the fetus will be unable to build vitamin D stores for use after birth, when sunlight exposure is still limited. Very preterm infants, who typically spend the early part of life in hospital, depend upon their stores of

vitamin D accumulated during development in utero plus nutrient intake to assist them in maintaining a rapid rate of bone formation. Very preterm infants miss the rapid bone growth and mineralization that occurs during the third trimester (Fewtrell et al., 1999). Therefore, optimum nutrition must be provided to maintain adequate vitamin D status to allow very preterm infants to grow and mineralize bone at a rate similar to what they would have in utero.

2.1.4 Description of Vitamin D Deficiency

Vitamin D deficiency recommendations exist for both infants and adults. The primary indicator of vitamin D status is circulating plasma 25(OH)D (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). Concentrations of plasma 25(OH)D $\leq$27.5 nmol/L ($\leq$11 ng/mL) are considered deficient in infants (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). In adults, plasma 25(OH)D concentrations $\leq$40 nmol/L ($\leq$16 ng/mL) are considered deficient (Vieth et al., 2001). Plasma 25(OH)D $\leq$73nmol/L ($\leq$29 ng/mL) are considered the low end of normal (Vieth et al., 2001). There is little information available regarding the level of 25(OH)D required by young and middle-aged adults to maintain normal calcium metabolism (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). However, a normal range of 25 to 137.5 nmol/L 25(OH)D is suggested (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997).

2.1.5 Prevalence of Vitamin D Deficiency

Recently there has a been an increase in reports of the incidence of 25(OH)D deficiency in both infants and adults. Specker et al. (1992) studied term infants (>37 weeks gestation) in China and found that 57% had cord serum 25(OH)D concentrations considered deficient (≤27.5 nmol/L). At 6 months of age, 19% continued to have deficient 25(OH)D status. Lebrun et al. (1993) studied infants and mothers in the Island Lake Community (Northern Manitoba). Forty-three percent of the children (n=76) aged 3-24 months and 76% of the mothers had 25(OH)D concentrations less than 25 nmol/L. Dawodu et al. (2003) studied Arab and South Asian term infants (between 4 and 16 weeks of age) in the United Arab Emirate. Eighty-two percent of the infants were found to have deficient 25(OH)D status (≤10 nmol/L) (Dawodu et al., 2003).

More recently, reports of 25(OH)D deficiency in adults have come from several areas within Canada. In the North West Territories, the calculated risk for vitamin D deficiency (defined as 25(OH)D ≤30 nmol/L) for a group of both aboriginal and non-aboriginal women was found to be 80.8%. For aboriginal mothers taking a vitamin D supplement, 48.4% were found to have a vitamin D deficiency (Waiters et al., 1998). Vieth et al. (2001) studied women, aged 19-35 in Toronto, Ontario. During the winter months (November to April), 22.5% of the women were found to have a vitamin D deficiency (<40 nmol/L). In Calgary, 188 men and women, aged 27-89 were followed for a year. Thirty-four percent were found to have a 25(OH)D deficiency (≤40 nmol/L) at least once during the year (Rucker et al., 2002).

2.1.6 Vitamin D Deficiency Rickets

A vitamin D deficiency during active bone growth manifests itself as rickets. Rickets is one of the oldest diseases. In 1919, Mellanby first reported rickets as a nutritional disease, however, rickets had been described several centuries earlier (Haworth, 1995). Rickets may present with hypocalcemic seizures or bowing of the tibias, and can have long term effects on growth and ability to walk (Moffat, 1995). Many consider vitamin D deficiency rickets a disease of the past, however, it continues to be a nutritional disease (Joiner, 2000).

In Manitoba, between 1989 and 1994, 21 patients between 6 weeks and 2 and a half years of age presented at Children's Hospital, Winnipeg with rickets caused by vitamin D deficiency (Haworth, 1995). All but one of these patients was Native. Prior to that, between 1972 and 1984, 48 cases of vitamin D deficient rickets were reported at the same hospital (Haworth & Dilling, 1986).

Data from the Canadian Institute of Health Information (CIHI) show that between 1995 and 2000 in Canada, 6.19 per 100 000 males and 4.14 per 100 000 females between the ages of 0-19 had active rickets. However, this data likely underestimates the prevalence as it reports the diagnosis in patients hospitalized for various reasons and does not include all provinces (Ward, L, Personal communication).

Unfortunately at this time, recent data from Canada, or more specifically Manitoba, does not exist.

2.1.7 At-risk Populations for Vitamin D Deficiency

Several groups of individuals are at an increased risk for vitamin D deficiency. Factors that play a role in determining availability of vitamin D include sunlight exposure, season and latitude, and skin pigmentation. Sunlight exposure is typically an individual's most abundant source of vitamin D. Vitamin D synthesis depends on both the quantity or intensity and the quality of the ultraviolet irradiation (Norman, 1998). Geographic location is an important determinant in the above two requirements. In northern geographic locations, especially during winter months, the zenith angle of the sun is increased, which decreases the amount of ultraviolet irradiation that is filtered (Norman, 1998). The less ultraviolet irradiation filtered, the less able the skin is to synthesize vitamin D. Colder temperatures, more clothing worn and less time spent outdoors (Norman, 1998) also contribute to decreased synthesis of vitamin D in the skin. Using an in vitro model, Webb et al. (1988), showed that during the months of November and February in Boston (latitude 42°N), no vitamin D synthesis occurred in the skin. In Edmonton (latitude 52°N), vitamin D synthesis ceased during the months of October to April (Webb et al., 1988). In Norway (latitude 70° N), Markestad et al. (1984) studied the breast milk of mothers with infants born during autumn or winter to determine if adequate vitamin D could be supplied to their term infants. During autumn and winter, days are very short and the sun stays below the horizon for 2 ½ months at this latitude. The breast milk of all mothers, including those who took a supplement (~400 IU/day), did not supply adequate amounts of vitamin D for their infants, suggesting these women

were not able to adequately synthesize enough vitamin D to meet both their needs and their infants (Markestad et al., 1984).

Winnipeg, Manitoba has a latitude of 49°N, similar to Edmonton, and therefore it can be anticipated that vitamin D synthesis is not adequate to promote vitamin D synthesis during the winter months (October to April). Although Winnipeg is further south than Norway, it is likely that the breast milk of mothers during winter months in Manitoba may not provide adequate vitamin D for their infants.

Although humans are able to synthesize vitamin D during the summer months, increasing concerns of skin cancer are leading to an increased use of sunscreen. A sunscreen of SPF 8 reduces vitamin D production by 97.5%, while SPF 15 reduces vitamin D production by 99% (Centers for Disease Control and Prevention, 2001). The American Academy of Pediatrics recently released new guidelines which suggest that infants younger than 6 months of age should be kept out of direct sunlight, and protective clothing should be worn and sunscreen should be used (American Academy of Pediatrics, 2003). These guidelines, if followed, would minimize sunlight exposure and negatively impact vitamin D synthesis in infants.

Individuals with dark skin are also at increased risk for 25(OH)D deficiency. The skin of these individuals contains an increased amount of melanin. Melanin absorbs ultraviolet light and functions as a light filter, therefore competing with 7-dehydrocholesterol for the ultraviolet photons (Norman, 1998). Harris and Dawson-Hughes (1998) studied both Black and White women in

Boston to assess seasonal changes in 25(OH)D. Black women were found to have substantially lower plasma 25(OH)D than White women all year long suggesting a reduced capability for vitamin D synthesis (Harris & Dawson-Hughes, 1998).

2.1.8 Maternal Vitamin D Deficiency and the Implications on the Infant

A woman entering pregnancy with a vitamin D deficiency or developing a deficiency during pregnancy due to inadequate vitamin D intake or sunlight exposure may experience several negative effects in both herself and her infant. The mother will have a decreased concentration of 25(OH)D and calcium which may negatively impact her bone mineralization (Specker, 1994). The consequences for the infants tend to be more serious since they depend on their mother's vitamin D stores. The outcomes could include delayed fetal growth, delayed bone development (Specker, 1994) and a disturbance in calcium regulation (Specker, 1994; Salle, 1988). Moncrieff and Fadahunsi (1974) studied a case report of a term infant born with congenital rickets and determined the cause to be the severe malnourished state and extreme vitamin D deficiency of the mother. Mawer et al. (1986) studied vitamin D status in very preterm infants (25-32 weeks gestation) at birth in England. Initial plasma 25(OH)D concentrations indicated that one third of the infants were vitamin D deficient and another third had plasma 25(OH)D concentrations in the lower region of the adult normal range. The cause of the vitamin D deficiencies was determined to be inadequate maternal sunlight exposure during pregnancy. Subsequently, it was determined that a majority of the infants would have been vitamin D deficient by

corrected term age if not given a supplement (1000 or 3000 IU per day) when oral feeds commenced (Mawer et al., 1986). The above studies illustrate the importance of adequate vitamin D intake and stores in mothers during pregnancy.

Inadequate maternal vitamin D intake and stores impact fetal bone growth and calcium homeostasis. Namgung et al. (1992) studied seasonal differences in term newborn BMC and found it to be lower in summer-born infants compared to winter-born infants. The authors speculated that maternal vitamin D status was inadequate during the winter due to lack of sunshine, which influenced fetal bone mineralization and became evident at birth (Namgung et al., 1992). Maternal vitamin D status may also play a role in the infant's ability to regulate calcium homeostasis. Cockburn et al. (1980) conducted a supplementation trial (400 IU/day vs. placebo) on women from 12 weeks gestation throughout pregnancy to determine if vitamin D supplementation during pregnancy is beneficial to both mothers and infants. Mothers who received the placebo had low serum 25(OH)D and their infants exhibited an increased incidence of hypocalcemia (13% compared to 6% in the vitamin D supplemented group). Delvin et al. (1986) studied term infants of mothers who were supplemented with either vitamin D (1000 IU / day during 3rd trimester) or a placebo during pregnancy. Infants born to supplemented mothers had a smaller decrease in serum total calcium, a significant increase in serum PTH and inorganic phosphate between birth and day 4. In the infants of supplemented mothers, serum 25(OH)D concentrations were significantly higher at birth and decreased

between birth and day 4. The researchers suggested that providing a vitamin D supplement during pregnancy allowed the fetus to store a larger amount of 25(OH)D which was available after birth for rapid hydroxylation to $1,25(OH)_2D_3$ leading to increased calcium absorption (Delvin et al.,1986). This outcome would have positive effects on bone mineralization in infants. Research continues to provide evidence of the importance of adequate vitamin D during pregnancy on the developing infant.

2.1.9 Fetal and Infant Vitamin D Metabolism

Fetal and infant vitamin D status is dependent on maternal stores. Currently, the most accepted method for studying fetal physiology is through analysis of cord blood samples (Salle et al., 1987). Within cord blood, 25(OH)D has been shown to be significantly correlated with maternal concentration, implying that it is capable of diffusing across the placenta (Delvin et al., 1982). Within cord blood, $1,25(OH)_2D_3$ is not correlated with maternal concentration implying that it is not capable of crossing the placenta (Delvin et al., 1982; Steichen et al., 1980). Umbilical cord concentrations of $1,25(OH)_2D_3$ are low since it is thought that the developing fetus does not have a need for intestinal calcium absorption until birth (Steichen et al., 1980). As well, the fetus is capable of synthesizing $1,25(OH)_2D_3$ on their own, and can meet their demands in utero through this mechanism (Wieland et al., 1980).

2.1.10 Neonatal Hypocalcemia

Within the first 24 hours after birth, serum $1,25(OH)_2D_3$ concentrations rise coinciding with the need for intestinal absorption of calcium and phosphorus

(Steichen et al., 1980). Irrespective of this, hypocalcemia is still often present in preterm infants due to both the immaturity of the vitamin D activation pathway and the inefficiency of calcium absorption (Salle et al., 1987). As well, intravenous fluids, which very preterm infants receive initially, do not typically contain calcium, which may negatively impact bone mineralization (Williams, 2000). The adaptation of the very preterm infant from maternal transplancental nutrition supply to an external nutrition source is a traumatic event for very preterm infants and this is also likely involved in the hypocalcemia (Salle et al., 1987, Saggese et al., 1991). Inadequate vitamin D stores at birth, as evidenced in Cockburn et al. (1980) and Delvin et al. (1986) may also be a cause of hypocalcemia in infants. Prevention of hypocalcemia in very preterm infants is important because hypocalcemia will lead to resorption of calcium from bone stores, decreasing the mineral content of the bone.

2.1.11 Vitamin D Supplementation in Infancy

Supplementing infants with vitamin D has been shown to be beneficial (Mawer et al., 1986). Infants supplemented with vitamin D immediately after birth were found to have improved serum 25(OH)D status (Salle et al., 2000). Foregoing a vitamin D supplement following birth can lead to vitamin D deficiency (Mawer et al., 1986). Mawer et al. (1986) determined that if vitamin D supplementation was not administered daily to their study preterm infants, the majority would have had a vitamin D deficiency upon reaching corrected term age. It remains difficult to define the appropriate amount of vitamin D for very preterm infants since inadequate amounts may lead to inadequate calcium

absorption (Mawer et al., 1986) while excess can lead to hypercalcemia, hypercalciuria, polyuria and dehydration (Backstrom et al., 1999).

Several studies have looked at vitamin D supplementation, but varying amounts have been used. Backstrom et al. (1999) studied vitamin D supplementation and very preterm infants (<33 weeks gestational age) in Finland (63°N). Infants were divided according to 300 grams birth weight ranges and then randomly assigned to receive a vitamin D supplement of either 200 IU/kg of body weight/day up to a maximum of 400 IU/day or 960 IU/day which is the current amount recommended in Europe (Backstrom et al., 1999). Receiving a dose of vitamin D up to 400 IU/day maintained normal vitamin D status and bone mineral accretion rates similar to those receiving the higher amounts (Backstrom et al., 1999). In Finland (latitude between 60°N and 70°N), Greer et al. (1981) compared bone mineral content and 25(OH)D in term infants (between 38 to 40 weeks gestation) fed either unsupplemented breast milk or supplemented breast milk (400 IU/day vitamin D). Infants receiving the supplementation had a significantly higher BMC than the unsupplemented infants by 12 weeks of age (Greer et al., 1981). The above research indicates that in Northern latitudes, supplementation of 400 IU/day may be satisfactory in maintaining adequate vitamin D status and bone mineral content in preterm and term infants. Canada's geographic location ranges from 83°N in Nunavut to 41°N in Southern Ontario, which encompasses Finland's geographic location. This indicates that for some areas of Canada, 400 IU/day may be adequate.

The area of vitamin D supplementation requires further research, specifically for very preterm infants. Supplementation is required to maintain adequate 25(OH)D stores (Mawer et al., 1986, Backstrom et al., 1999), however the appropriate amount still remains unknown. Although 400 IU/day may seem adequate, several factors influence vitamin D stores and metabolism including maternal stores of 25(OH)D (Markestad et al., 1983), geographic location (Markestad et al., 1984) and season of birth (Mawer et al., 1986). Therefore, when determining the amount of vitamin D necessary for very preterm infants, all of the above variables must be considered. This further challenges the health care profession in determining an adequate amount for all very preterm infants. Rather than determining a general amount used by all countries, an amount may need to be determined specific for each geographic location.

2.1.12 Vitamin D Recommendations for Very Preterm and Preterm Infants

Based on a review of the applicable research, the Canadian Pediatric Society has set recommendations regarding the necessary amount of vitamin D required by preterm infants (Nutrition Committee, Canadian Paediatric Society, 1995). During the transition period, infants with a birth weight of <1000 grams should receive 40-120 IU/day and infants with a birth weight of >1000 grams should receive 40-260 IU /day. During the stable-growing and post-discharge periods, 400 IU/day is the recommended amount. For infants that are Black or of Asian descent, or have a low plasma concentration of 25(OH)D at birth (25-50 nmol/L), it is recommended they receive 800 IU/day (Nutrition Committee, Canadian Paediatric Society, 1995).

In order to reach these amounts, it is clear that supplementation of vitamin D is required (Nutrition Committee, Canadian Paediatric Society, 1995). D-Vi-SOl® is a commonly used infant vitamin D supplement and contains 400 IU/dropper (1 mL). Preterm formula also contains vitamin D in amounts ranging from 182-219 IU/100 mL (Mead Johnson, 1999), while human milk fortifier contains 150 IU/100 mL (Mead Johnson, 2001).

2.1.13 Vitamin D Recommendations for Term Infants

Several recommendations exist for vitamin D requirements in term infants. Nutrition for Healthy Term Infants (1998) suggests 400 IU/day (10 µg/day) for all full-term breast-fed infants. For those infants living in Northern communities, supplementation of 800 IU/day (20 µg/day) is recommended. The dietary reference intakes (DRI) have established an adequate intake of 200 IU/day (5 µg/day) vitamin D for infant's ages 0-12 months. This recommendation assumes no exogenous source of vitamin D from sunlight exposure (Standing Committee on the DRI, 1998). The Manual of Clinical Dietetics (2001) states that vitamin D supplementation of 400 IU/day (10 µg/day) is recommended for breast fed infants in both Canada and the United States. Several reasons exist for this recommendation including human milk being a poor source of vitamin D, vitamin D deficiency can lead to serious acute and long-term consequences that are preventable, it is not possible to identify all infants at risk of a vitamin D deficiency and no harm is associated with supplementation at the recommended level (American Dietetic Association/Dietitians of Canada, 2001). Lastly, the American Academy of Pediatrics released a Clinical Report in 2003 reaffirming

the adequate intake of 200 IU/day (5 µg/day). They suggest a 200 IU/day supplement to all breastfed infants unless they are weaned to at least 500 mL per day of vitamin D fortified formula or milk, and to all non-breastfed infants who are ingesting less than 500 mL per day of vitamin D-fortified formula or milk (American Academy of Pediatrics, 2003). Term infant formula contains 410 IU / liter.

2.1.14 Vitamin D Recommendations during Pregnancy

Currently, the Dietary Reference Intake (DRI) for pregnancy suggests there is no need to increase vitamin D intake above the requirement for women aged 19-50. The Adequate Intake of 200 IU / day is still recommended, however, this is based on individuals obtaining adequate sunshine. The DRI further mentions that an intake of 400 IU / day would not be excessive (Standing Committee on Dietary Reference Intakes,1997). Cockburn et al. (1980) have illustrated that even 400 IU/day may not be enough for some women, as this level of supplementation did not completely abolish hypocalcemia in infants. As illustrated earlier, several factors can interfere with vitamin D synthesis. Many women are not able to obtain adequate sun exposure during pregnancy and may require a higher dietary intake of vitamin D. It is recommended that pregnant women take a prenatal multi-vitamin during pregnancy as a precautionary measure. Materna® is a common brand in Canada and this brand contains 250 IU/capsule. During summer months, when sunlight exposure is adequate, the supplemental vitamin D, plus sunlight and diet contributions will likely support the

mother with adequate vitamin D. During winter months, when sunshine exposure is inadequate, diet plus supplemental vitamin D may or may not be adequate.

2.1.15 Seasonal Variation in Vitamin D Status

Many studies have been done that have looked at the seasonal variation of vitamin D status in term infants. Only one study has looked at vitamin D status and seasonal variation in very preterm infants (Mawer et al., 1986). Because preterm infants miss the last trimester in utero when nutrient stores are being rapidly built, their seasonal variation of vitamin D status may be different from term infants. Table 2.2 summarizes the research that currently exists looking at seasonal variation of vitamin D status. Currently there have been no studies done on seasonal variation of vitamin D in infants at a similar latitude and climate as Manitoba.

Research has found that infants born in the summer tend to have higher plasma 25(OH)D than infants born in the winter (Kuroda et al., 1981; Mawer et al., 1986; Namgung et al., 1998). Namgung et al. (1998) attribute their results to the cultural habits of Koreans. A similar study was conducted in Ohio which found no seasonal variation of plasma 25(OH)D among infants (Namgung et al., 1994). Korean women typically do not have much sunlight exposure during pregnancy due to cultural beliefs, and as well, mothers in America are more likely to ingest prenatal vitamins during pregnancy (Namgung, 1998). Infant 25(OH)D status at birth is directly related to their mothers (Markestad, 1983), therefore, a infant born to a mother obtaining adequate vitamin D will have normal 25(OH)D status at birth.

Two studies have also looked at the BMC of the infants in relation to 25(OH)D status and their results are conflicting. Namgung et al. (1998) studied Korean term infants and found winter-born infants had a lower BMC than summer-born infants. Namgung et al. (1994) studied infants born in Ohio and found that winter-born infants had higher BMC than summer-born infants. Based on the results of the research done in Ohio, the researchers speculated that if maternal vitamin D status influences fetal bone mineralization, the effect occurs early in pregnancy with the result being evident at birth (Namgung et al., 1994).

Because of the relationship between maternal vitamin D status and infants' vitamin D stores (Markestad, 1983), several studies have also looked at seasonal variation of maternal vitamin D status at birth. These results are summarized in Table 2.3. The general consensus is that mothers giving birth in summer or autumn had higher plasma 25(OH)D than mothers giving birth in winter or spring (Kuroda et al., 1981; Namgung et al., 1998; Nehama et al., 1987; Verity et al., 1981).

Table 2.1 Vitamin D content of foods and infant formulas

	Vitamin D Content
Pasteurized cow's milk, 1 litre[1]	400 IU
Salmon, baked, 3 ½ oz[1]	300 IU
Sardines, canned in oil, drained, 3 ½ oz[1]	270 IU
Cod Liver Oil, 1 tbsp[1]	1360 IU
Expressed breast milk[2]	33-68 IU / litre
Enfalac with Iron[3]	410 IU / litre

1. National Institute of Health, 2002.
2. Hollis et al., 1986.
3. Mead Johnson, 1999.

Table 2.2: 25-hydroxyvitamin D status and bone mineral content in infants at birth according to location and season

Authors	Year	Location	Term/ Preterm	Birth wt (mean)	Vitamin D Results[1,2]	BMC Results
Verity et al	1981	Britain	Term (appropriate for gestational age (AGA))	N/A	- Spring-born infants had lower 25(OH)D than autumn born (26.5 ± 9.7 vs. 57.2 ± 17.7 / 41.7 ± 13.2)[3]	
Kuroda et al	1981	Japan	Term	>2.5kg	- Summer-born infants had higher 25(OH)D than winter-born (47.2 ± 21.0 vs. 22.0 ± 8.5)[4]	
Mawer et al	1986	England	Preterm (25-32 weeks gestation)	1.36 kg	- Summer-born infants had higher 25(OH)D than winter-born (54.9 ± 23.4 vs. 15.0 ± 7.0)[5]	
Nehama et al	1987	Israel	Term	N/A	- Spring-born infants had lower 25(OH)D than autumn-born (28.2 ± 2.5 vs. 45.2 ± 3.2)[6]	
Namgung et al	1994	Ohio	Term (AGA)	3.3 kg	- There was no difference in 25(OH)D levels between seasons (60.4 ± 3.2 (summer) vs. 54.0 ± 2.7 (winter))[4]	- Summer-born infants had lower BMC than winter-born (82.7 ± 1.4 vs. 89.9 ± 2.3 mg/cm)[1]
Namgung et al	1998	Korea	Term (AGA)	3.3 kg	- Summer-born infants had higher 25(OH)D than winter-born (30.0 ± 15.0 vs. 10.7 ± 7.5)[4]	- Summer-born infants had higher BMC than winter-born (93.9 ± 7.8 vs. 86.7 ± 7.7 g)[1]

1. Values are mean ± SD except Nehama et al (1987) and Namgung et al (1994) which are mean ± SE.
2. Values measured in nmol/L.
3. Values are from infants from vitamin D supplemented mothers / unsupplemented mothers.
4. Samples taken were cord blood.
5. Samples taken between 0-21 days after birth.
6. Samples taken within 24 hours after birth.

Table 2.3: Maternal plasma 25-hydroxyvitamin D at time of delivery according to location and season

Author	Year	Location	Results[1,2]
Verity et al.	1981	Britain	- Mothers giving birth in spring had lower 25(OH)D than mothers giving birth in autumn (55.9 ± 10.5 / 41.7 ± 11.7 vs. 82.4 ± 31.4 / 62.6 ± 17.5)[3]
Kuroda et al.	1981	Japan	- Mother giving birth in summer had higher 25(OH)D than mothers giving birth in winter (84.6 ± 31.2 vs. 39.4 ± 16.5)
Nehama et al.	1987	Israel	- Mothers giving birth in spring had lower 25(OH)D than mothers giving birth in autumn
Namgung et al.	1998	Korea	- Mothers giving birth in summer had higher 25(OH)D than mothers giving birth in winter (42.9 ± 18.2 vs. 24.0 ± 13.2)

1. Value are mean ± SD
2. Measurements are nmol/L
3. Values are from vitamin D supplemented / unsupplemented mother

2.2 Bone

2.2.1 Bone Structure

The human skeletal system is composed of three types of bone; flat bones (ie., skull, scapula), irregular bones (ie., vertebrae, metacarples) and long bones (ie., femur, tibia). Flat and long bones are derived from two different types of development, intramembranous ossification and endochondral ossification, respectively (Baron, 1999), which will both be discussed in a subsequent section.

Mature bone is comprised of cortical and trabecular bone. Cortical bone is the outer layer, which is a thick, dense, highly calcified matrix. In adults, 80-90% of this bone is calcified (Baron, 1999). In infants, this proportion is likely much lower because the initial structure of bone is woven. During woven bone formation, calcification is delayed. Regardless of age, the function of cortical bone is to provide support and protect the internal organs and bone marrow (Baron, 1999). Trabecular bone, found in the interior of bone, has a structure resembling sponge. In adults, 15-20% of trabecular bone is calcified with the remainder being occupied by bone marrow, blood vessels and connective tissue. Regardless of age, the metabolic function of trabecular bone is to act as a reservoir for the mineral ions, calcium and phosphorus (Baron, 1999).

2.2.2 Bone Metabolism

Bone metabolism refers to the components and processes that occur in both bone resorption and formation. It is composed of a cyclical sequence of events that include activation, resorption and formation. During modeling, or rapid bone growth, this sequence is typically not present because activation is

not required and resorption does not necessarily precede formation (Parfitt, 1990).

2.2.2.1 Modeling vs. Remodeling

Modeling occurs during infant and childhood growth when bone formation occurs at a higher rate than bone resorption (Martin et al., 1989). Modeling is continuous, does not require activation, occurs on approximately 90% of the bone surface and results in a net gain of bone (Parfitt, 1990). During periods of slow growth or during adulthood, remodeling occurs, in which bone formation and bone resorption occur in balance (Martin et al., 1989).

2.2.2.2 Cells involved in Bone Metabolism

Several cells are involved in bone formation and resorption and include osteoblasts, osteocytes, osteoclasts, chondroblasts and chondrocytes (de Bernard, 1992). Osteoblasts are derived from mesenchymal cells and are responsible for the production of the matrix constituents (Baron, 1999). The plasma membrane of the osteoblast is rich in alkaline phosphatase and expresses receptors for PTH and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Baron, 1999).

After osteoblasts have finished synthesizing the bone matrix, they become osteocytes. Osteocytes are mature osteoblasts that are found immersed in the matrix of the bone (de Bernard, 1992). Osteocytes are able to synthesize new bone matrix at the surface of the osteocytic lacunae, which can then be calcified.

Osteoclasts are the cells responsible for bone resorption and are involved in preparing the bone surface for new bone growth (de Bernard, 1992).

Osteoclasts typically work alone or in pairs at the resorption site, although up to 4 or 5 may work together (Baron, 1999). During osteoclast bone resorption, osteocytes are phagocytized and digested along with the other components of bone (Baron, 1999).

Chondroblasts are the cells involved in the growth of long bones during the cartilaginous phase where they secrete the cartilaginous matrix (Baron, 1999). Eventually chrondroblasts become embedded in their own matrix and are re-termed chondrocytes. Chondrocytes proliferate for some time, however, they do eventually enlarge, become hypertrophic and die (Olsen, 1999).

2.2.3 Bone Development and Growth

As mentioned earlier, two processes are involved in bone development. Intramembranous ossification is involved in the development of flat and irregular bones, while endochondral ossification is involved in the development of long bones. During bone growth, resorption and formation do not occur in a cyclical sequence.

2.2.3.1 Intramembranous Ossification

Intramembranous ossification begins with a layer of mesenchymal cells which become highly vascularized. The mesenchymal cells proliferate and differentiate into preosteoblasts and subsequently osteoblasts (Baron, 1999; Javaid & Cooper, 2002). The osteoblasts synthesize a bone matrix of woven bone, characterized by irregular bundles of collagen fibers, large osteocytes and delayed calcification which do not proceed in an orderly fashion. At the periphery, mesenchymal cell differentiation and woven bone formation continue

to occur. Eventually, the woven bone will be remodeled and replaced by mature bone (Baron, 1999). Different from endochondral ossification, which will be discussed in the next section, there is no cartilage model preceding ossification in this type of bone development (Javaid & Cooper, 2002).

2.2.3.2 Endochondral Ossification

Three stages occur in endochondral ossification. A cartilage model is formed, followed by longitudinal growth and vascular invasion and finally growth in diameter of the bone. In the first stage, mesenchymal cells proliferate and differentiate into prechondroblasts and then chondroblasts. Chondroblasts secrete the cartilaginous matrix, in which they eventually become embedded and become chondrocytes. However, different from osteocytes, chondrocytes continue to proliferate and differentiate and this type of growth is called appositional growth. Growth in the cartilage, due to synthesis of new matrix between the chondrocytes, is called interstitial growth. Eventually the chondrocytes will become hypertrophic and die (Baron, 1999). The development of the cartilage model can be seen by 5 weeks gestation (Javaid & Cooper, 2002).

During development, a ring of woven bone is formed by intramembranous ossification and following calcification of this woven bone, blood vessels penetrate the cartilage. The blood vessels will provide the blood supply that will form the bone marrow. In the growth plate, chondroblasts are actively dividing and synthesizing matrix in the proliferative zone. At the epiphyseal plate which is directly above the growth plate, the matrix calcifies and following, the cartilage

matrix is partially resorbed and blood vessels appear. After resorption, osteoblasts differentiate and form a layer of woven bone on top of the cartilaginous remnants. Growth in diameter occurs as new membranous bone is deposited beneath the periosteum and this growth continues throughout life (Baron, 1999).

2.2.3.3 Bone Resorption

Bone resorption is dependent upon the activity of osteoclasts (Martin et al., 1989). The osteoclast identifies the area of bone to be resorbed and attaches itself through the clear zone and ruffled border. The ruffled border, which contains finger-like projections of the plasma membrane, allows for the concentration of resorption activities (Chambers, 1991), creating a microenvironment in which osteoclasts can work. The clear zone lies between the ruffled border and the bone surface and acts to anchor the osteoclast to the bone giving stability to the motile ruffled border (Holtrop, 1991). An acidic environment is created in the resorption area through the action of a proton pump located within the ruffled border (Price & Thompson, 1995; Baron, 1999). The low pH of the area dissolves the crystals on the bone surface and exposes the matrix (Baron, 1999). Lysosomal enzymes, secreted through the ruffled border, reach a significantly high concentration because the resorption area is sealed off. These enzymes, which are at optimal pH in the acidic environment, degrade the matrix components and cause the release of calcium into the extracellular fluid (Chambers, 1991). The end result is an open area for bone to be formed.

The regulatory mechanism that inhibits osteoclast activity is poorly understood. It has been suggested that the osteoclast has the ability to monitor a change in calcium concentration by means of a "calcium receptor" on its surface and then couple this to the inhibition of resorption (Zaidi et al., 1991).

In the process of modeling, resorption is not coupled to formation. Parathyroid hormone stimulates the differentiation of progenitors to fuse and form osteoclasts, as well as activates pre-formed osteoclasts to resorb bone (Mundy, 1999). Interleukin 1, 3, 6 and 11 also play a role in stimulating the development of osteoclasts (Roodman, 1999).

2.2.3.4 Bone Formation

Once osteoclasts are finished their work, osteoblasts enter the area. Osteoblasts never appear or work individually, but rather present themselves in a group (Heersche & Aubin, 1990; Baron, 1999). They function to produce and secrete the matrix constituents of bone, including type I collagen and specialized bone-matrix proteins (Lian et al., 1999a). A lag time of approximately 10 days is present before calcification occurs. The lag time is essential because the collagen fibers must mature before they can support mineral deposition. During this lag time, the matrix is termed osteoid tissue. After bone formation is complete, osteoblasts lose their synthetic capacity and become trapped within the matrix and form osteocytes (Lian et al., 1999a).

During modeling, parathyroid hormone related protein (PTHrP) stimulates the proliferation of fetal growth plate chondrocytes and inhibits the differentiation

of these cells into hypertrophic chondrocytes, thereby stimulating bone formation leading to bone elongation and growth (Rigo, 2000).

2.2.4 Hormones and Growth Factors Involved in Bone Metabolism

Several hormones and growth factors play a role in bone metabolism. These include $1,25(OH)_2D_3$, PTH, calcitonin, insulin and insulin-like growth factor I & II (IGF-I and IGF-II).

2.2.4.1 1,25-dihydroxyvitamin D

Both bone formation and resorption are effected by $1,25(OH)_2D_3$. Using a system of fetal mouse metatarsals, Tao and Minken (1994) found $1,25(OH)_2D_3$ to be a stimulator of osteoclast formation. As well, $1,25(OH)_2D_3$ regulates calcium metabolism (Holick, 1999). When plasma calcium concentrations are low, $1,25(OH)_2D_3$ causes resorption of bone leading to demineralization of bone and calcium homeostasis within the body (Holick, 1999).

Adequate vitamin D, either by diet or sunshine exposure, is essential in maintaining and promoting adequate mineralization of bone. A deficiency of vitamin D leads to a decreased mineral deposition in the skeleton. Histological sections of bone from vitamin D deficient patients show an increase in unmineralized matrix (Lian et al., 1999b). When the body senses a vitamin D deficiency, intestinal absorption of calcium decreases, which results in a decrease in the plasma calcium concentrations. Low plasma calcium signals the calcium sensor in the parathyroid gland to increase synthesis of parathyroid hormone. In an attempt to conserve calcium, PTH acts by increasing renal tubular reabsorption of calcium, but it also causes formation of osteoclasts from

stem cells, leading to the resorption of bone and mobilization of calcium. The end result is abnormal bone formation (Holick, 1999). Osteoblast differentiation is also influenced by $1,25(OH)_2D_3$ at several stages and plays a role in the coupling of bone resorption to formation (Lian et al., 1999b).

It is through a vitamin D receptor which $1,25(OH)_2D_3$ works. Previously, it had been demonstrated that the vitamin D receptor was present only in osteoblasts and monocytes (Merke et al., 1986). Therefore, it had been understood that $1,25(OH)_2D_3$ exerted its actions on osteoclasts indirectly. Langub et al. (2000) have recently disputed this theory. Using immunohistochemical evidence, they demonstrated that the vitamin D receptor is also present in osteoclasts, suggesting $1,25(OH)_2D_3$ may also exert a direct response. The exact mechanism however requires further research.

The vitamin D receptor has been found to be present at an early gestational age. Vitamin D receptors have been found in tooth germs of fetus as early as 8 weeks gestation (Bailleul-Forestier et al., 1996), and in kidney and fetal skin at 17 weeks gestation (Weisman et al., 1990). In proximal small intestine, $1,25(OH)_2D_3$ binding activity has been shown at 13 weeks gestation (Delvin et al., 1990).

2.2.4.2 Parathyroid Hormone

The main function of PTH is to stimulate osteoclastic bone resorption (Dempster et al., 1993) by causing the release of calcium and phosphorus from bone (Holick, 1999). However, PTH receptors have been identified on both osteoclasts and osteoblasts, suggesting a dual regulatory mechanism for PTH

action on bone (Dempster et al., 1993). Several functions have been identified including stimulating release of calcium and phosphorus from bone and stimulating the growth of osteoprogenitor cells (cells that contribute to maintaining the osteoblast population) (Holick, 1999).

When calcium concentrations are low, PTH functions to stimulate osteoclast bone resorption. The calcium is reabsorbed from the kidney and the activity of 1-α-hydroxylase is increased via stimulation from PTH. This leads to the enhanced synthesis of $1,25(OH)_2D_3$, which then acts on the intestine to increase absorption of calcium and acts on the bone to cause resorption and demineralization. These actions of PTH lead to accelerated bone resorption and decreased bone formation (Heersche & Aubin, 1990).

Bone formation is stimulated by PTH through the increased synthesis of bone type 1 collagen. It has been hypothesized that IGF-I mediates the stimulatory effect, since both IGF-I and PTH have similar effects (Canalis et al., 1989).

In preterm infants, concentrations of PTH were found to be suppressed in umbilical cord samples (Rubin et al., 1991, Saggese et al., 1991). Rubin et al. (1991) found that between birth and 24-48 postnatal hours, PTH concentrations increased significantly and by 7-10 days PNA, PTH had declined. Saggese et al. (1991) found that at 24 hours of age, mean calcium concentrations declined and reached hypocalcemic levels. PTH concentrations rose significantly during this time and then progressively declined between days 2-5 PNA corresponding to a normalization of calcium values (Saggese et al., 1991). The rise and decline of

PTH evident in both of these studies coincides with the observed decline and subsequent rise in calcium. As well, the rapid PTH response to the decline in calcium concentrations suggests that during the first hours of life, parathyroid glands display a normal response to the early hypocalcemia (Saggese et al., 1991).

The main regulatory determinant of the secretory rate of PTH from the parathyroid gland is the extracellular calcium concentration. Low plasma calcium concentrations stimulate the parathyroid gland to synthesize and secrete PTH, whereas high calcium concentrations inhibit the parathyroid gland (Jupper et al., 1999).

2.2.4.3 Calcitonin

Calcitonin is a polypeptide hormone that inhibits the bone resorbing activity of osteoclasts (Bronner, 1992) by causing shrinkage of mature osteoclasts decreasing their resorptive properties (Deftos et al., 1999). Calcitonin secretion is regulated by blood calcium and has a parallel response. In other words, as blood calcium increases, calcitonin secretion increases, inhibiting the release of calcium from bone through resorption (Deftos et al., 1999). Calcitonin works through its surface receptor, present on osteoclasts, by activating the cyclic adenosine monophosphate (cAMP) secondary messenger pathway. In infants, serum calcitonin concentrations have been shown to increase significantly between birth and 24 hours of age. Very preterm infants have higher concentrations at birth compared with term infants (Venkataramen et

al., 1987). The exact role of calcitonin in calcium homeostasis and skeletal metabolism in human has not yet been established (Deftos et al., 1999).

2.2.4.4 Insulin

Insulin is associated with the stimulation of osteoblastic activity both in vivo and in vitro (Heersche & Aubin, 1990). Insulin is necessary for normal bone mineralization and at physiological concentrations can stimulate collagen synthesis and matrix production, but does not alter bone cell replication (Hock et al., 1988). Insulin receptors have been found in fetal tissues, including lung and liver (Styne, 1998).

2.2.4.5 Insulin-Like Growth Factors –I and –II

The liver is where IGF-I is mainly synthesized, after which it is released into the blood (Straus, 1994), however, IGF-I can be synthesized by a variety of other cells, including those of the bone and cartilage (McCarthy et al., 1989). Within the body, IGF-I and -II have similar biological activities, although in bone, IGF-I is more potent (Lian et al., 1999a). The functions of IGF-I include stimulation of preosteoblast cell replication and increasing bone collagen synthesis (Hock et al., 1988). Bone matrix synthesis is stimulated by IGF-I by enhancing bone collagen production and causing an increase in osteoprogenitor cell replication, leading to a larger number of functional osteoblasts (Hock et al., 1988).

Bone resorption is stimulated through IGF-I by enhancing osteoclast recruitment and activation. Using bone cells from mouse femora and tibiae, Mochizuki et al. (1992) showed that IGF-I increased dose dependently the area

of pits formed by osteoclasts, suggesting that IGF-I has a stimulatory effect on osteoclasts.

Rosen et al. (1995) studied the effects of IGF-I on bone in growing rats. They found that IGF-I stimulated bone growth and mineral deposition, however, growth was stimulated more than deposition. Therefore, in growing rats, IGF-I caused an increase in BMC and bone size and a decrease in bone mineral density (BMD).

In fetal development, both IGF-I and IGF-II are expressed in most tissues (Jones & Clemmons, 1995), although it has been suggested that IGF-II may play more of a role in fetal growth, due its high concentrations in embryonic and fetal tissues (Straus, 1994). Research on mice lacking IGF-II found that at birth, they had a dwarf phenotype, providing evidence that IGF-II is required for normal growth of the fetus (DeChiara et al., 1990). In very preterm infants, it has been suggested that IGF-I may regulate fetal growth in part by influencing type 1 collagen turnover (Kajantie et al., 2001).

2.2.5 Markers of Bone Metabolism

Biochemical markers of bone metabolism are useful because they reflect the elements that contribute to bone formation and bone resorption (Price & Thompson, 1995). Several markers are available for both components of bone growth.

2.2.5.1 Bone Resorption Markers

The markers for bone resorption include urinary NTx and the pyridinolines.

2.2.5.1.1 Urinary N-telopeptide

Urinary NTx is a sensitive marker and is generated directly by osteoclast proteolysis at the resorbing site (Apone et al., 1997). A validation study for urinary NTx, carried out on adult men, found that NTx correlated well with other biochemical markers such as pyridinoline and hydroxyproline validating its use (Rosen et al., 1994). Through further research, it was determined that NTx may be more specific that pyridinoline and hydroxyproline. Treatment with bisphosphonate reduced urinary NTx by 85% suggesting that when bone resorption is suppressed, very little NTx is excreted from other sources (Rosen et al., 1994). Hanson et al. (1992) found that the interactions forming pyridinolines at the NTx site on bone collagen were predominantly α_1(I) to α_2(I) and α_2(I) to α_2(I). This is a distinguishing feature of bone type 1 collagen and further validates the specificity of NTx.

In preterm infants (mean ($\pm$ SD) gestational age 34.4 $\pm$ 1.4 weeks), urinary NTx concentrations have been shown to be higher than in term infants (mean ($\pm$ SD) gestational age 39.5 $\pm$ 1.2 weeks) (Mora et al., 1997). As well, infant concentrations are several hundred times higher than in healthy young women (mean ($\pm$ SD) age 27.3 $\pm$ 6.1 years) (Mora et al., 1997).

Urinary NTx follows a circadian rhythm, meaning it has a characteristic 24-hour pattern. Concentrations are characterized by a high level in the early morning and a low level in the afternoon (Bollen et al., 1995). This is an important characteristic to consider when taking spot samples to reflect osteoclast activity.

Along with NTx, C-telopeptide and urinary hydroxyproline are two other potential markers of bone resorption. C-telopeptide is less commonly used because it lacks specificity. The structure is common to all tissues in which type 1 collagen is cross-linked with pyridinolines (Hanson et al., 1992). Urinary hydroxyproline, similar to C-telopeptide, also lacks specificity and is not a useful marker for bone resorption (Greer et al., 1991). Bone turnover is not the only source of hydroxyproline (Hanson et al., 1992), as excretion may be influenced by alteration in renal and hepatic function as well as the diet (Price & Thompson, 1995).

2.2.5.2 Bone Formation Markers

Osteocalcin, alkaline phosphatase and propeptide of type 1 collagen constitute the markers for bone formation.

2.2.5.2.1 Osteocalcin

Osteocalcin, also known as bone Gla-protein, is a bone specific marker (Wong, 1990) and the most abundant noncollagenous protein found in bone (Naylor et al., 1999). Osteocalcin is synthesized in bone upon stimulation by $1,25(OH)_2D_3$ (Koshla & Kleerekoper, 1999). When secreted, osteocalcin is incorporated into the matrix and released into circulation during bone resorption. Therefore, osteocalcin may be a marker of bone turnover, rather than a specific marker of bone formation. In preterm infants, concentrations of osteocalcin have been shown to be low in cord blood and rise significantly within the first week, after which it remains constant (Bhandari et al., 1999). At four weeks of age, preterm infants (mean ($\pm$ SEM) gestational age 33.1 $\pm$ 0.4 weeks) have been

found to have a significantly lower osteocalcin level than term infants (mean (± SEM) gestational age 39.6 ± 0.3 weeks) (Mora et al., 1994).

In adults, serum and urinary osteocalcin concentrations exhibit a circadian rhythm with a high level during early morning (4-5 am) and a low in the afternoon (Gundberg et al., 1985). In research with older children, this circadian rhythm has also been reported (Saggese et al., 1994), however, there has been no data available in infants.

2.2.5.2.2 Alkaline Phosphatase

Serum total alkaline phosphatase activity is an established marker of bone formation. It is very commonly used, especially in the clinical setting, yet it lacks sensitivity and specificity (Delmas et al., 1987). Alkaline phosphatase lacks specificity because bone alkaline phosphatase is similar to that found in the kidney, liver and placenta (Price & Thompson, 1995). Currently, there are immunoassays available to measure bone-specific alkaline phosphatase. In infants, measuring total alkaline phosphatase may be appropriate because the majority of circulating alkaline phosphatase is of bone origin (Koo, 1996). Faerk et al. (2002) in 108 very preterm infants (mean GA 29 ± 2 weeks) measured alkaline phosphatase weekly until term age, and performed a dual energy x-ray absorptiometry (DEXA) scan at term age to measure BMC. Bone mineral content was found not to be associated with mean serum alkaline phosphatase or peak serum alkaline phosphatase suggesting routine measurements of serum alkaline phosphatase is of no use in predicting bone mineralization outcome in very preterm infants (Faerk et al., 2002).

2.2.5.2.3 Propeptide of Type I Collagen (PICP)

Propeptide of type 1 collagen (PICP) is released from the collagen precursor, procollagen, during type 1 collagen synthesis. Therefore, measurement of PICP can be used as a marker of osteoblast function or bone formation (Namgung et al., 1996). Very preterm infants appear to have concentrations higher than adults, indicative of their rate of bone growth (Crofton et al., 1999). In infants, PICP also has a strong positive correlation with weight gain. Bhandari et al. (1999) found that in very preterm infants, PICP paralleled the weight lost during the first week of life with a decrease in concentration. As weight began to rise, PICP concentrations also rose (Bhandari et al., 1999). It has also been shown that the younger the infants, the higher the level of PICP. Infants born at 24-29 weeks gestation were found to have higher concentrations compared with infants born at 30-34 weeks gestation (Seibold-Weiger et al., 2000). As well, significantly higher concentrations have been shown in very preterm male infants cord plasma compared with female infants. This gender difference was not evident in term infants (Seibold-Weiger et al., 2000). Similar to other biochemical markers, PICP also exhibits a circadian rhythm in adults (Bollen et al., 1995). However, current immunoassays for PICP are not linear preventing use in a population with wide variation in plasma concentration (Weiler & Fitzpatrick-Wong, unpublished data).

2.2.6 Bone Metabolism In Utero

As stated earlier, bone formation occurs via intramembranous ossification and endochondral ossification. At 5 weeks gestation the development of the

cartilaginous model can be seen (Javaid & Cooper, 2002). By 19 weeks gestation, bone is in the early stages of mineralization and the matrix organization exhibits a degree of linearity (Glorieux et al., 1991). As early as 20 weeks gestation, the materno-fetal gradient for calcium transfer emerges (Javaid & Cooper, 2002). During the third trimester, the majority of calcium is accumulated (Javaid & Cooper, 2002) allowing for bone mineralization to continuously increase until term (40 weeks gestation) (Cole & Hanley, 1991). It is during the third trimester as well, when the majority of fetal bone is gained (Javaid & Cooper, 2002) and skeletal growth occurs at the greatest rate in life (Glorieux et al., 1991). Femoral shaft length has been measured to be 95.5 mm at term age, compared with 34 mm at 16 weeks gestation (Salle et al., 2002). Very preterm infants, who miss the third trimester in utero, miss much of the bone growth and development.

2.2.7 Bone Metabolism and Bone Mass in Infancy

Preterm infants, specifically very preterm infants, miss the opportunity to rapidly increase bone mineralization in utero and tend to reach corrected term age with sub-optimal bone mass (Fewtrell et al., 1999). Gestational age has been found to be a major determinant in the rate of bone turnover. Mora et al. (1997) found that bone turnover is elevated in preterm infants (32 weeks gestation) and slowed in proximity to corrected term age in line with a reduced rate of growth. In this study, urine samples were collected immediately after birth and between 24 and 48 hours after birth to measure urinary NTx, a biochemical marker of bone resorption. Although these results illustrate the activity of bone

resorption, they do not consider bone formation and mineral content of bone. It is important to consider all aspects of bone metabolism when doing research to obtain a clear understanding of the physiology behind outcomes of bone mass. Likewise, bone resorption for the purpose of modeling becomes elevated as early as 7 days postnatally in term infants (Pratico et al., 2002), Therefore, whether bone resorption becomes slower with maturing age in very preterm infants is not clear.

Beyers et al. (1994) provide a more complete study of bone metabolism in preterm infants. All aspects of bone metabolism were studied at birth and corrected term age in preterm infants and at birth in term infants for a control group. Bone mineral density (BMD) was measured using bone measurements of the humoral bone. When BMD was expressed as cortical index (combined cortical thickness/total shaft width), which negates the influence of body size, similar results were found between preterm and term infants. However, at corrected term age in preterm infants, a significantly lower cortical index was found, suggesting that BMC is significantly less by term age than what it would have been had they remained in utero to term (Beyers et al., 1994).

Based on their radiological and biochemical results, Beyers et al. (1994) suggested that high turnover osteopenia, rather than low bone formation may characterize the pathogenesis of neonatal osteopenia. Neonatal osteopenia is a common metabolic disorder seen in preterm infants, which refers to a decreased amount of bone tissue (Rauch & Scheonau, 2001). The causes of the high bone turnover could not be determined; however, they suggested that alteration in

parathyroid function may play a role, as secondary hyperparathyroidism was suggested in the preterm infants due to their high urinary excretion of cyclic adenosine monophosphate (cAMP) (Beyers et al., 1994).

Koo et al. (1999) considered maternal diet in relation to infant bone metabolism. Mothers were recruited prior to 22 weeks gestation to receive either 2 grams elemental calcium or placebo. As well, 24-hour food recalls were done at randomization and 32-33 weeks gestation to assess calcium intake. Infants were born at term age. Mothers with inadequate calcium intake during pregnancy (less than approximately 600 mg/day) delivered infants with lower bone mineral content, suggesting that maternal diet may play a role in developing neonatal osteopenia.

Schanler et al. (1992) completed a follow-up study, although only for two years. They found that the significant differences in bone mineralization between preterm and term infants at one year were no longer present at 2 years suggesting that catch-up bone mineralization was attained by the second year. These results may be debated because it is possible that the term children were experiencing a pause in their growth while the preterm children were experiencing a growth spurt. It is important that this research be continued to 3 or 5 years to determine bone mineral status and determine if catch-up growth is actually attained and maintained. Fewtrell et al. (1999), in an 8-12 year follow-up study of children born prematurely, found these children to have lower bone mass than their term born peers. It is important to note that their bone mass was appropriate for their size. The researchers concluded that attempts to improve

bone mass should be directed at increasing size as small individuals are more prone to develop osteoporosis (Fewtrell et al., 1999).

Bronchopulmonary dysplasia (BPD) is a common cause of chronic respiratory disease and remains a major cause of long term morbidity (Vaucher, 2002). Dexamethasone, a corticosteroid, is routinely provided to infants with BPD to decrease pulmonary edema. However, bone loss and impaired growth are adverse effects (Weiler et al., 1995; Vaucher, 2002).

Weiler et al. (1995) studied piglets receiving 15 days of either dexamethasone or placebo. After 15 days of treatment, dexamethasone piglets showed significantly lower weight and length gain, as well as significantly lower whole body, lumbar and femur BMD (Weiler et al., 1995).

Brunton et al. (1998) studied 60 very preterm infants with BPD (mean GA 26 $\pm$ 1.5 wks) fed either standard or enriched formula (high-energy, high-protein, high-mineral formula) starting at corrected term age. At 3 months corrected age, infants fed enriched formula had attained significantly greater length, radial BMC and lean mass. Therefore, it was suggested that infants with BPD attain faster catch-up growth when fed higher amounts of protein and minerals, specifically calcium, phosphorus and zinc (Brunton et al., 1998).

Ziegler et al. (1976) provided a reference guide for representative body composition of fetuses. Infants with a gestational age of 32 weeks should have a body weight of 1830 g with a calcium content of 640 mg/100 g fat-free weight and phosphorus content of 406 mg/100 g fat-free weight (Ziegler et al., 1976). During the last trimester, approximately two-thirds of the calcium and phosphorus is

deposited in the fetus (Javaid & Cooper, 2002). Therefore, by 37 weeks gestation, their body weight should be 2940 g, with 758 g of calcium/100 g fat-free mass and 479 g phosphorus/100 g fat-free mass (Ziegler et al., 1976). Venkataraman (1995) in a review article, suggested that calcium and phosphorus intake in preterm infants are below the intra-uterine accretion rate. Inadequate amounts of these two minerals within the body of very preterm fetuses may suggest the infants have inadequate bone mineralization since these are the two main minerals found within bone.

2.2.8 Measurement of bone mass

Bone mass, or BMC, can be measured using DEXA. Bone mass is dependent upon both the size and density of skeletal bone (Molgaard et al., 1997). In recent years, DEXA has been validated as a safe, accurate and reproducible technique for measuring whole body composition and bone mineral content in the infant (Brunton et al., 1993; Brunton et al., 1997; Koo, 1996; Rigo et al., 1998). There is a very low level of irradiation with DEXA scans (1.0 mrad for whole body scan, QDR4500A), and therefore is a useful tool in longitudinal pediatric studies (Koo & Hockman, 2000). There is a very short scan time that also makes it feasible to use in infant research studies (Salle et al., 1992). A whole body scan of an adult takes less than 3 minutes, while an infant whole body scan will be shorter using a QDR4500A. Research and validation studies have shown that artifacts, such as diapers and blankets do not result in significant differences in DEXA BMC values (Koo et al., 1995). However, they do interfere with measurement of soft tissue (Brunton et al., 1997). It should be

noted that all studies validating the use of DEXA within infants, have been done looking at whole body, rather than lumbar spine or femur.

There are several other options available for measuring whole body composition and BMC, although DEXA seems to have several advantages. Single photon absorptiometry (SPA) is unable to measure trabecular bone (Salle et al., 1992) and therefore predominantly measures cortical bone (Hori et al., 1995). This restricts the areas in which bone scans can be performed. The whole body and lumbar vertebrae are unable to be scanned with SPA (Salle et al., 1992), leaving the forearm as the only bone that can be scanned (Barden & Mazess, 1988). Dual photon absorptiometry (DPA) is another option available, however there is a long scan time with DPA and therefore decreased precision compared to DEXA (Barden & Mazess, 1988; Salle et al., 1992). Other methods such as computed tomography are unreasonable to use due to the high level of radiation the infant would be exposed to (Brunton et al., 1997).

2.2.9 Measurement of Growth

Growth is assessed three ways in infants: weight, length and head circumference. In children greater than 2 years of age and adults, growth is assessed using height and weight. Each of these growth measurements is obtained using simple non-invasive techniques and provides precise, accurate measurements provided standardized techniques are utilized. However, errors can also occur typically due to examiner error resulting from inadequate training and instrumental error (Gibson, 1990a).

Standardized techniques exist for all growth measurements. Infant weight is measured using a pediatric weigh scale. The infant should be naked and placed on the scale with weight distributed equally about the center (Gibson, 1990b). Adult weight is measured using a standing weigh scale. Weight should be measured after the bladder is emptied and before a meal. Subjects should stand in the center of the platform, looking straight ahead and standing relaxed, while wearing light underclothing (Gibson, 1990b).

Length is measured for infants less than 2 years of age. Two individuals are required to correctly position the infant and ensure a reliable and accurate measurement. The infant is placed face upward with the head at the fixed end of the length board. One individual ensures the crown of the head is in contact with the fixed headboard. The second individual holds the infants feet and brings the moveable foot board to rest against the heels, while keeping the toes upward and knees straight. The reading is taken to the nearest millimeter (Gibson, 1990b).

Height is measured for children greater than 2 years of age and adults. Proper technique involves having the individual stand tall with the head and knees straight and heels, buttocks and shoulder blades in contact with the wall surface. The arms should hang loosely at the sides with the palms facing thighs. The individual is asked to take a deep breath and stand tall with shoulders relaxed while the headboard is lowered until it reaches the crown of the head. The height is also recorded to the nearest millimeter (Gibson, 1990b).

Head circumference is an important measurement in infants because it is closely related to brain size. A narrow, flexible measuring tape should be used.

The tape is placed just above the supra-orbital ridges covering the most prominent part of the frontal bulge. The measurer should ensure the tape is pulled tightly and that it is at the same level on each side of the head. The measurement is made to the nearest millimeter (Gibson, 1990b).

2.3 Infant Feeding Practices

Adequate nutrition is critical to growth and development of infants. Very preterm infants are at a higher nutritional risk due to immaturity of gastrointestinal tract and lack of nutrient stores at birth (Nutrition Committee, Canadian Pediatric Society, 1995). The goal of feeding very preterm infants is to mimic in utero growth. The gastrointestinal tract does not reach maturity until 38 weeks gestation (Grand et al., 1976), which can make this difficult to achieve. As well, the majority of nutrient stores are established during the third trimester (Vitamin D Panel Expert Meeting, 1999) which very preterm infants miss. The Canadian Pediatric Society has divided their feeding recommendations of feeding preterm infants into three time periods. Initially, during the transition period (birth to day 7), the goal is provision of sufficient nutrients to prevent nutrient deficiencies and substrate catabolism (Nutrition Committee, Canadian Paediatric Society, 1995). Nutrition can be provided either through parenteral or enteral feeds. During the stable-growing period (day 8 to hospital discharge) the nutritional goal is growth and nutrient retention rates similar to those that would have been achieved in utero (Nutrition Committee, Canadian Paediatric Society, 1995).

Total parenteral nutrition is typically the first route of feeding. However, several nutrients are not provided adequately in TPN, including calcium and

phosphorus (Williams, 2000) which likely contributes to hypocalcemia often seen in very preterm infants after birth. Controversy still exists as to when enteral feeds should be initiated. Enteral feeding is thought to provide the substrate for bacterial growth within the intestinal mucosa which leads to necrotizing enterocolitis (Ostertag et al., 1986). However, several studies have suggested that initiating enteral feeds earlier may be beneficial to the infant. Ostertag et al. (1986) studied preterm infants who were started on enteral feeds either on day 1 or day 7 of life. There was no significant difference noted between groups regarding development of necrotizing enterocolitis. As well, infants who started enteral feeds early were found to have significantly higher total energy and protein intakes during the second week of life (Ostertag et al., 1986) which may have beneficial effects on growth of the infant. Berseth and Nordyke (1993) studied 32 preterm infants who were fed either formula or water in conjunction with parenteral nutrition for 10 days. Those infants who received formula established enteral feedings and full nipple feedings sooner and were discharged sooner than water fed infants (Berseth & Nordyke, 1993).

Health professionals and parents have two types of feeds to choose from when enteral feeds start breast milk or formula. Although for term infants breast milk is considered the gold standard, it is not the reference standard for preterm infants. Unless fortification of breast milk occurs, there is risk of inadequate growth of the infant and nutrient deficiencies. However, due to the psychological benefits breast feeding provides to the mother and the anti-infective benefits for the infants, it is still the recommended source of nutrition (Nutrition Committee,

Canadian Paediatric Society, 1995). The energy density and protein content of preterm milk have both been found to be greater than term milk, however, it likely is still not adequate to promote growth of the infant (Anderson, 1981). Calcium and phosphorus content of preterm milk is also inadequate, requiring fortification. Providing preterm milk without fortification may not allow for adequate mineral intake to warrant bone mineralization and skeletal growth rates similar to in utero rates (Mayne and Kovar, 1991) further increasing the risk of metabolic bone disease (Faerk et al., 2000).

The vitamin D content of human milk is typically the infants' only source unless supplementation is offered. Vitamin D content of mothers milk is low. Hollis and colleagues (1986) found the vitamin D content to be between 33-68 IU/liter. They also found that there was a significant relationship between mothers vitamin D status and their milk vitamin D concentration (Hollis et al., 1986). This level of vitamin D is inadequate to support infant's requirements (Weisman et al., 1982).

As stated above, fortification of several nutrients, including vitamin D, is required to allow for growth rates similar to in utero rates. Human milk fortifiers contain protein, minerals and vitamins, including vitamin D (150 IU/100 mL) to ensure the infant's nutrient intake meet their established needs (Nutrition Committee, Canadian Pediatric Society, 1995). Several studies have shown that infants given fortified milk have faster rates of growth than those receiving unfortified preterm milk (Nutrition Committee, Canadian Pediatric Society, 1995). Moyer-Mileur and colleages (1992) studied preterm infants fed either fortified or

unfortified human milk and concluded that consumption of unfortified human milk with inadequate levels of calcium and phosphorus may predispose the preterm infants to osteopenic changes, rickets and fractures.

2.3.1 Measurement of nutrient intake

Several methods exist for measuring nutrient intake of both infants and adults. Three-day food records are appropriate for both infants and adults, while the 24-hour food recall is appropriate for adults. When completing a 3-day food record, the participant is asked to record the time of consumption, all foods and beverages consumed, including brand names and cooking method, and amount of food. For complex foods, such as a casserole, participants are asked to provide the recipe. Food portion size is estimated via measuring cups and spoons, food counts and measurements (Gibson, 1990c).

For infants who are breast-fed, the test weigh method is utilized to obtain a 3-day food record. Mothers are provided with an electronic weigh scale and asked to weigh infants prior to feeding and immediately after feeding. They are advised not to change anything in between, such as a diaper, clothing or blanket. The difference in grams is the amount in milliliters of breast milk the infant consumed (Scanlon et al., 2002). There are several disadvantages to test-weighing which include disruption of the mother-infant interaction, difficulty weighing an active infant and measuring night feedings (Scanlon et al., 2002).

Twenty-four hour food recalls are a quick, relatively inexpensive way to obtain nutrition intake from adults. The participant is asked to recall their exact food intake for the preceding day. A detailed description of all food and

beverages, including brand names and cooking methods is obtained. The success of the 24-hour food recall depends on the participant's memory, ability of the participant to convey accurate estimates of portion sizes, degree of motivation of the participant and the persistence of the interviewer (Gibson, 1990c). Twenty-four hour food recalls can be performed via the telephone or in person. Results from telephone 24-hour food have been found to be similar to those obtained with in-person interviews (Tran et al., 2000).

3. HYPOTHESES: Very preterm infants born with low vitamin D status will have lower bone mass compared with very preterm infants born with adequate vitamin D stores and term infants. Very preterm infants born during spring and early summer are at an increased risk for vitamin D deficiency.

The **global objective** of the study is to:

1. Characterize bone metabolism and bone mineral content measured at term in relation to gestational age, vitamin D and bone metabolism in infants born prematurely.

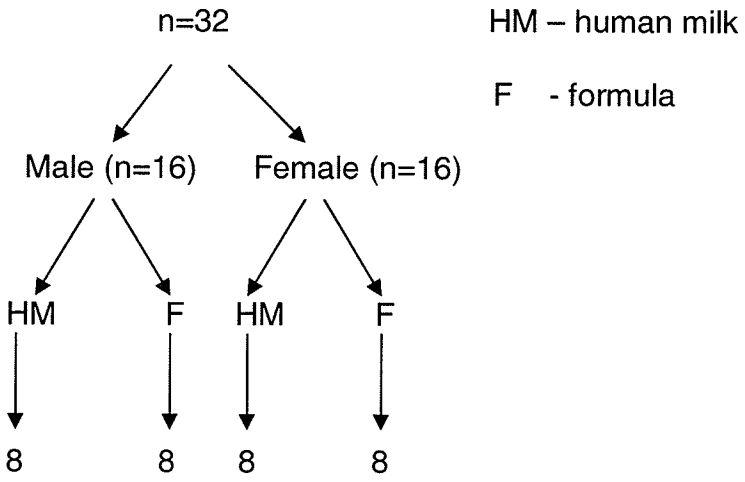
The **specific objectives** of the study are to:

1. Determine if mother's vitamin D status at birth has an impact on the infants vitamin D status at birth,
2. Compare seasonal variation of vitamin D status in very preterm and term infants born in Manitoba, as well as their mothers,
3. Determine if vitamin D status affects bone mineral content at birth and 6 months corrected age in very preterm and term infants.

4.0 Methods: Very Preterm Infants and their Mothers

4.1 Population: The original goal was 32 infants based on seasonal 25(OH)D research within term infants (Namgung et al., 1998), in which a difference of 19.3 nmol/L between groups was seen with a SD of 15. The alpha is 0.05 and the power is 0.80, which suggests a population of n=11 for each group. In this research, we had an original goal of n=16 per group. We increased the group population to n=16 to account for drop-out of infants and incomplete data sets. As well, this allowed us to separate groups between breast-fed and formula fed infants. Figure 4.1 illustrates the justification of the original goal. This original goal of 32 infants was unattainable due to time frame. Our final population was 16 very preterm singleton infants recruited to the study.

Figure 4.1: Justification of original sample size



4.2 Ethical Approval: Ethical approval was granted through the University of Manitoba Ethics Committee. Informed consent was obtained from mothers prior

to being included in the study. Appendix A provides contains a copy of the very preterm infant/mother consent form.

4.3 Recruitment: Very preterm infants and mothers were recruited over an eleven month period (May 2001 to April 2002) from Women's Hospital and St. Boniface General Hospital High Risk Labor and Delivery. Both centers are located in Winnipeg, Manitoba, Canada. All very preterm infant-mother pairs born were invited to participate, provided they met the inclusion criteria. Recruitment rate for very preterm infants was 57% during the eleven month time period.

4.4 Inclusion Criteria: The inclusion criteria for very preterm infants was birth $\leq$ 32 weeks gestation, birth weight <1500 grams, appropriate for gestational age as defined by 2 of 3 growth charts (Usher & McLean, 1969, Arbuckle et al., 1993, Kramer et al., 2001) and no known congenital abnormalities. Mothers were eligible to participate provided they did not have any form of diabetes (Type 1, Type II or gestational diabetes), did not consume alcohol or use recreational drugs throughout the pregnancy, and did not use medications known to affect bone metabolism.

Diabetes has been shown to alter vitamin D metabolites and minerals in pregnancy (Kuoppala et al., 1988), and infants born to diabetic mothers have been found to have lower BMC at birth (Demarini et al., 1995). Substance abuse during pregnancy has been shown to be associated with neonatal abnormalities, such as major anomalies, hearing loss, and fetal alcohol syndrome-like facial features (Scheeres & Chudley, 2002). It has been suggested that steroid

medications ingested during pregnancy may negatively impact bone metabolism of the fetus and infant. However, research confirming this has not been forthcoming.

4.5 6 months Corrected Age Follow-up: Fourteen of the sixteen infants were followed until 6 months corrected age. Two infants were not followed until 6 months corrected age due to death and lost contact, respectively. Infants were followed for growth measurements, BMC and nutrition.

4.6 Growth Measurements: Growth measurements were collected weekly until discharge. The measurements included length, weight and head circumference. Length was measured using crown-heel length (to the nearest 0.1 cm) using an infant length board. Lengths were obtained by one of the investigators with the assistance of the bedside nurse. Head circumference (measured to the nearest 0.1 cm) was also obtained by one of the investigators. Weight (measured to the nearest 1 gram) was taken from the infant's chart.

Intra-variability measurements between researchers (n=4) for length and head circumference were obtained on two infants. Each member obtained both measurements on the same infant and the Coefficient of Variation (CV%) was obtained. The values for length and head circumference were 0.49% and 1.04%, respectively.

4.7 Nutrition: In Hospital - Nutrient intake was obtained from the infant's medical chart. Intake was recorded daily until the infant reached full feeds, defined as 150 mL/kg. The amount, type of feed (total parenteral nutrition, intravenous solution, formula, or expressed breast milk), and residuals were

collected daily until full feeds were met, and twice weekly thereafter until discharge. Supplements, such as Human Milk Fortifier, Microlipid and vitamins were also recorded. Nutrient intake of very preterm infants in hospital was divided into the following periods for analysis; birth to day 7 PNA, day 8 to day 21 PNA, day 22 to 35 PNA and day 36 PNA to hospital discharge. These periods were chosen as they coincided with the blood and urine sampling time points. Average calories, macronutrients (protein, fat and carbohydrate), calcium, magnesium, phosphorus and vitamin D were calculated during each time period. Post Discharge – Infants fed formula at discharge, or those receiving supplemental formula feeds were provided with infant formula (Enfalac 20 with Iron, Mead Johnson, Ottawa, Ontario) for 6 months. Tri-Vi-Sol (Mead Johnson, Ottawa, ON) was provided to all breastfed infants for 6 months.

At 6 months of age, mothers were asked to complete a 3-day food record for the infant. Appendix B -1 contains a copy of the 3-day food record instructions and forms mothers were provided with. Mothers were asked to record the amount of breast milk and/or formula and solids the infant consumed. If infants were still being breast-fed, mothers were provided with a weigh scale and asked to weigh infants pre- and post-feed, and record the weights. The difference in weight is the amount of milk consumed by the infant. Mothers were also asked to record supplement use, such as Tri-Vi-Sol.

The food records were analyzed by one of two investigators using Nutrient Program (E. Warwick, PEI, Canada). Food records were analyzed for energy, macronutrients (protein, fat and carbohydrate), calcium, phosphorus, magnesium

and vitamin D. Each investigator performed and analyzed a 24-hour food recall on the same three individuals to determine percent agreement between investigators. Percent agreement measurements between investigators for calories and macro-nutrients were as follows: calories 92.3%; protein 92.98%; fat 88.73%; and carbohydrate 93.85%.

4.8 Medical Management: Medical management information, including current health issues, medications, as well as respiratory/ventilatory support was also recorded from the infant's medical chart.

4.9 Blood and Urine Sampling: Venous blood samples were to be collected on day 7, 21 and 35 PNA. Actual mean ($\pm$ SD) sampling days for the above three time points were day 7.4 $\pm$ 1.3, day 21.4 $\pm$ 1.1, and day 35.5 $\pm$ 1.6, respectively. Hospital Lab Technicians obtained the sample of approximately 0.5 mL via the heel prick method. Blood was sampled between 0600 and 0900 to control for diurnal variation of markers of bone metabolism. The blood was collected in a microtainer containing heparin. The blood was separated into plasma and red blood cell fractions within 2-3 hours of sampling and subsequently stored at -80°C.

Urine samples were to be obtained within 48 hours of birth, day 7, 21 and 35 PNA. Actual mean sampling times for the above four time points were day 1.8 $\pm$ 0.7, day 7.5 $\pm$ 1.3, day 21.7 $\pm$ 1.3 and day 36.4 $\pm$ 1.2, respectively. Urine samples were requested to be collected between 0200 and 0900 to control for diurnal variation however, samples were obtained anytime between 0000 and 1200. A urine sample of 1-5 mL was collected using either urine bags or plastic

bags by the Nursing Staff in the Neonatal Intensive Care Unit (NICU). Samples were stored within 12 hours at -80°C.

Blood and urine were also obtained prior to discharge, unless discharge was similar to the day 35 PNA sampling time point.

4.10 Maternal Information: Maternal information was obtained to assist in interpreting the outcomes of the infants. Demographic information was recorded from the medical chart and was further verified with the mother at the 6 month follow-up appointment. Prior to hospital discharge, a morning (0800-1000) venous blood sample (5-10 mL) was obtained by Hospital Lab Technicians using a vacutainer containing heparin. The sample was separated into plasma and red blood cell fractions within 2-3 hours of sampling and stored at -80°C, until required.

Mothers were asked to complete a 3-day food record within 6-8 weeks of delivery and mail it back to the investigators in a self-addressed stamped envelope. Appendix B-2 contains the 3-day food record instructions and form provided to mothers. Instructions were provided outlining the procedure for completing the 3 day food records. Food records were reviewed by one of two investigators and any information that was unclear was obtained by telephone.

Mothers were also asked to complete a 24-hour food recall performed by one of two investigators, either in person or by telephone. Results from 24-hour food recalls in person are similar to results obtained from 24-hour food recalls done via telephone (Tran et al., 2000). Appendix B-3 contains a copy of the 24-

hour food recall form. At the time of their DEXA scan, mothers were again asked to complete a 3 day food record and 24 hour food recall.

One of two investigators analyzed all the food records using Nutrient Analysis Program (E. Warwick, PEI, Canada). Food records and 24-hour food recalls were analyzed for average energy intake, macronutrients (protein, fat and carbohydrate), calcium, phosphorus, magnesium and vitamin D.

4.11 25-hydroxyvitamin D: 25-hydroxyvitamin D was measured in plasma using an ^{125}iodine (^{125}I) radioimmunoassay (RIA) kit (DiaSorin Inc., Stillwater, MN) in very preterm infants (day 7, 21, 35 PNA and discharge) and maternal samples. The assay is a two-step procedure. The first procedure involves extraction of 25(OH)D, after which the treated sample is then assayed using an RIA procedure.

Extraction Procedure: For infant samples, 525 µL of acetonitrile was added to each borosilicate glass tube, followed by the addition of 25 µL of standard, control or sample. Each tube was vortexed for approximately 10 seconds, and centrifuged for 10 minutes at room temperature at a speed of 1200 x g. In duplicate, 50 µL of supernatant was pipetted into tubes. For maternal plasma, standards and controls, 500 µL of acetonitrile was added to each tube, followed by the addition of 50 µL of sample, standard or control. Each tube was vortexed for approximately 10 seconds and centrifuged for 10 minutes at room temperature at a speed of 1200 x g. In duplicate, 25 µL of supernatant and 25 µL of acetonitrile were added to tubes for the assay procedure. The addition of

the acetonitrile allows for the rapid extraction of the 25(OH)D and other hydroxylated metabolites.

Assay Procedure: Following addition of the supernatant to the tubes, 50 µL of ^{125}I-25(OH)D and 1.0 mL of goat anti-25(OH)D serum was added to the tubes. Each tube was then vortexed, followed by a 90 minute incubation period at room temperature. After incubation, 500 µL of donkey anti-goat precipitating complex was added to each tube, followed by a second incubation period of 20-25 minutes, to permit time phase separation. After the second incubation period, 500 µL of 25(OH)D NSB/addition buffer was added to each tube. The tubes were then vortexed for 30 minutes at room temperature at a speed of 1800 x g. The supernatant was decanted and the tubes were counted in the gamma scintillation counter (1282 Universal Gamma Counter, Wallac Oy) for 300 seconds per tube. Six standards at concentrations of 2.5, 5, 12, 20, 50, 100 ng/mL were assayed as well, and used to generate a standard curve. The standard curve was generated using Graph Pad Prism Software (San Diego, CA) and the concentration of 25(OH)D was interpolated from the curve. Values obtained were multiplied by 0.289 to covert ng/mL to nmol/L.

Two assays had low control 2 values, however all samples done on these assays were within the control 1 range, which was appropriate. Therefore, it was felt that the results from the assays were appropriate.

Lot#109063
25(OH)D Control1 and 2: 9.8-20.6 ng/mL (mean – 15.2 ng/mL) and 33.5-71.5 ng/mL (mean – 52.5 ng/mL)
Measured: 14.4 ng/mL and 74.04 ng/mL
Recovery/Accuracy: 94.7% and 141%

Lot#109662
25(OH)D Control1 and 2:10.2-21.4 ng/mL (mean – 15.8 ng/mL) and 30.1-63.7 ng/mL (mean – 46.9 ng/mL)
Measured:16.8 ng/mL and 45.8 ng/mL
Recovery/Accuracy:106% and 98%

Lot#109789
25(OH)D Control1 and 2: 10.2-21.4 ng/mL (mean – 15.8 ng/mL) and 30.1-63.7 ng/mL (mean – 46.9 ng/mL)
Measured: 13.7 ng/mL and 22.1 ng/mL
Recovery/Accuracy: 87% and 47%

Lot#109789
25(OH)D Control1 and 2: 10.2-21.4 ng/mL (mean – 15.8 ng/mL) and 30.1-63.7 ng/mL (mean – 46.9 ng/mL)
Measured: 12.1 ng/mL and 23.3 ng/mL
Recovery/Accuracy: 77% and 50%

Lot#110047
25(OH)D Control1 and 2: 10.2-21.4 ng/mL (mean – 15.8 ng/mL) and 30.1-63.7 ng/mL (mean – 46.9 ng/mL)
Measured: 16.95 ng/mL and 37.43 ng/mL
Recovery/Accuracy: 107% and 79.8%

Lot#110314
25(OH)D Control1 and 2: 10.2-21.4 ng/mL (mean – 15.8 ng/mL) and 30.1-63.7 ng/mL (mean – 46.9 ng/mL)
Measured: 13.3 ng/mL and 51.0 ng/mL
Recovery/Accuracy: 84.0% and 108.7%

4.12 Osteocalcin: Osteocalcin was measured in infant (day 7, 21, 35 PNA and discharge) plasma samples using an ^{125}I radioimmunoassay kit (DiaSorin Inc., Stillwater, MN). Day 7 PNA infant samples were measured undiluted. Day 21, 35 PNA and discharge infant plasma samples were diluted 2-5 fold with Standard 0. All samples were measured in duplicate. Fifty µL of standard, controls and samples were mixed with 200 µL of rabbit anti-osteocalcin serum and 200 µL of ^{125}I osteocalcin by vortexing and then incubated for approximately 16-20 hours at 4˚C. Following incubation, 500 µL of precipitating complex was added, and the

samples were vortexed and incubated for 2 hours at 4°C. Following incubation, the samples were centrifuged for 30 minutes at 760 x g at room temperature. The supernatant was decanted and the precipitate was counted in a gamma scintillation counter (1282 Universal Gamma Counter, Wallac Oy) for 300 seconds per tube. Concentrations of plasma osteocalcin were expressed as ng/mL. Serial dilutions were made to generate the standard curve (21.60,10.80, 5.40, 2.70, 1.35, and 0.86 ng/mL). The standard curve was generated using Graph Pad Prism Software (San Diego, CA) and the concentration of osteocalcin was interpolated from the curve. Values obtained were multiplied by a conversion factor of 0.171 to convert ng/mL to nmol/L.

Lot# 109815
Osteocalcin Control1: 3.8-6.2 ng/mL (mean – 5.0 ng/mL)
Measured: 4.3 ng/mL
Recovery/Accuracy: 86.0%

Lot# 109815
Osteocalcin Control1: 3.8-6.2 ng/mL (mean –5.0 ng/mL)
Measured: 4.6 ng/mL
Recovery/Accuracy: 92.0%

Lot# 109815
Osteocalcin Control1:3.8-6.2 ng/mL (mean – 5.0 ng/mL)
Measured: 5.2 ng/mL
Recovery/Accuracy: 104.0%

Lot# 110285
Osteocalcin Control1: 3.7-5.9 ng/mL (mean – 4.8 ng/mL)
Measured: 4.6 ng/mL
Recovery/Accuracy: 95.8%

Lot# 110511
Osteocalcin Control1: 3.7-5.9 ng/mL (mean – 4.8 ng/mL)
Measured: 4.7 ng/mL
Recovery/Accuracy: 97.9%

4.13 Cross linked N-telopeptide: Cross linked N-telopeptide (NTx), a sensitive marker of bone resorption, (Apone et al., 1997), was measured in duplicate in infant urine samples (birth, day 7, 21, 35 PNA and discharge) using a competitive-inhibition enzyme-linked immunosorbent assay (ELISA) (Ostex, Seattle, US). Infant urine samples were diluted 10 fold with distilled water. Twenty-five microliters of sample was added to micro-wells pre-coated with NTx, followed by the addition of 200 µL of conjugate solution (purified murine monoclonal antibody directed against NTx and conjugated to horseradish peroxidase). The plate was then allowed to incubate for 90 minutes at room temperature. During the incubation period, NTx in the sample competes with NTx on the micro-wells for binding sites of the monoclonal antibodies, therefore the amount of antibody bound to micro-wells is inversely proportional to the amount of NTx in the specimen. Following incubation, the plate was washed 5 times with working strength wash solution and 200 µL of chromogen/buffered substrate (buffered hydrogen peroxide) was added to act as a substrate for horseradish peroxidase. This was followed by an incubation period of 15 minutes. The reaction was stopped with the addition of 100 µL sulfuric acid to each well. The absorbance of the plate was read after a 5 minute incubation period at 450 nm and 630 nm using a spectrophotometer (Microplate Scanning Spectrophotometer; Bio-Tek Instruments). The value obtained from 630 nm was subtracted from 450 nm value to correct for imperfections on the plate. Urinary NTx values were corrected to urinary creatinine and expressed as molar ratio NTx:Creatinine.

Lot# 043L03
NTx Control Level 1 and Level 2: 392 nM BCE and 1385 nM BCE
Measured: 954 nM BCE and 2189 nM BCE
Recovery/Accuracy: 243.4% and 158.1%

Lot # 043L03
NTx Level 1 and Level 2 Control: 392 nM BCE and 1385 nM BCE
Measured: 342 nM BCE AND 1045 nM BCE
Recovery/Accuracy: 87.2% and 75.5%

Lot # 043L03
NTx Level 1 and Level 2 Control: 392 nM BCE and 1385 nM BCE
Measured: 372 nM BCE and 1279 nM BCE
Recovery/Accuracy: 94.9% and 92.3%

Lot # 043L03
NTx Level 1 and Level 2 Control: 392 nM BCE and 1385 nM BCE
Measured: 464 nM BCE and 1132 nM BCE
Recovery/Accuracy: 118.4% and 81.7%

Lot# 220L09
NTx Level 1 and Level 2 Control: 440 nM BCE and 1468 nM BCE
Measured: 454 nM BCE and 1333 nM BCE
Recovery/Accuracy: 103.2% and 90.8%

Lot# 220L09
NTx Level 1 and Level 2 Control: 440 nM BCE and 1468 nM BCE
Measured: 335 nM BCE and 1181 nM BCE
Recovery/Accuracy:76.1% and 80.4%

Lot# 233L05
NTx Level 1 and Level 2 Control: 440 nM BCE and 1468 nM BCE
Measured: 447 nM BCE and 1489 nM BCE
Recovery/Accuracy: 101.6% and 101.4%

Lot# 267L40
NTx Level 1 and Level 2 Control: 350 nM BCE and 1324 nM BCE
Measured: 339 nM BCE and 1175 nM BCE
Recovery/Accuracy: 96.9% and 88.7%

4.14 Calcium and Phosphorus: Urine samples (birth, day 7, 21, 35 PNA and discharge) were assayed for calcium and phosphorus as outlined below. In a glass tube, 0.25 mL or 0.75 mL urine was mixed with 0.5 mL or 1 mL nitric acid.

The tubes were covered and allowed to sit for 12-18 hours at room temperature in the fumehood. Once contents were clear in color, 9.5 mL or 19 mL of deionized water was added, making a 5% nitric acid solution and the mixture was transferred to 20 mL scintillation vials for measurement. Total calcium and phosphorus were measured via emission spectroscopy (Varian Liberty 200, Varian Canada, Mississauga, ON). The average CV% was 6.4% and 3.4% for calcium and phosphorus, respectively.

4.15 Creatinine: Creatinine was measured in infant urine samples (birth, day 7, 21, 35 PNA and discharge) using colorimetric determination (Procedure number 555-A, Sigma Diagnostics Inc., St. Louis, MO). Urine samples were diluted 2-5 fold with deionized water and the mixture was vortexed. Twenty microliters of standard or sample was pipetted into the appropriate well on the microplate, followed by 200 µL of alkaline picrate solution (5:1 picric acid and sodium hydroxide). The microplate was incubated at room temperature for 8-12 minutes. Following incubation, absorbance was read at 500 nm on a spectrophotometer (Microplate Scanning Spectrophotometer, Bio-Tek Instruments). An acid reagent was then added to each well to destroy the color derived from creatinine. The microplate was then re-incubated for 5 minutes, following which the absorbance was read again at 500 nm. A standard curve was developed using 4 concentrations of standards as follows: 2.5, 5, 7.5, 10 mg/dL. The creatinine concentration was then calculated as follows:

$$\text{Creatinine (mg/dL)} = \frac{\text{initial absorbance} - \text{final absorbance}}{\text{Initial absorbance std} - \text{final absorbance std}} \times \text{Std. Conc}$$

Values were converted to mmol/L by first multiplying by the dilution factor followed by 88.4. The average CV% for all samples measured in duplicate was <10%.

4.16 Dual Energy X-ray Absorptiometry Scan: Infant bone mineral content measurements were obtained using DEXA (QDR4500A Elite X-ray Bone Densitometer, Hologic Inc., Bedford, MA, USA) at corrected term age and 6 months corrected age. Infant femur and spine (L1-4) were measured and analyzed using the Hologic QDR Software for Windows, Version 11.2.

Mothers were advised prior to the scan to ensure the infant was not wearing metal. If the infant's clothing had metal, a hospital gown was provided. In most cases, infants were wrapped in a blanket. Research and validation studies have shown that artifacts, such as diapers and blankets do not result in significant differences in bone mineral content values (Koo et al., 1995). Scans took place when the infant was quiet, and in most cases, asleep. No sedation was used. Infants were positioned on the scan bed, on their back and were scanned once.

Quality control scans were performed three times per week on a manufacturer-supplied spine phantom. Coefficient of variation for BMC was 3.078% for 306 scans. Coefficient of variation for BMD was 0.408% for 306 scans.

Mothers – Dual energy x-ray absorptiometry measurements of both the femur and lumbar spine (L1-4) were also obtained from the mothers. Scans were done either at 6 months post-partum, if not breastfeeding or 6 months after

finishing breastfeeding. Pregnancy and lactation have both been shown to alter bone metabolism and it takes approximately 6 months to normalize (Yasumizu et al., 1998, Kalkwarf & Specker, 1995).

Mothers wore a hospital gown and ensured all jewelry was removed. Mothers were asked to lie as still as possible for the scans.

Height and weight of mothers was also obtained at the time of the DEXA scan. Height was measured to the nearest 0.1 cm, using a stadiometer (Model 242, SECA Weighing and Measuring Systems, Hanover, MD). Weight, was measured in kilograms using an electronic weight scale (Model 551K/552KL/591KL/592KL/595KL, Healthometer, Bridgeview, IL).

Intravariability measurements between researchers (n=4) for height were obtained on 4 adults. Each member obtained the measurement on the same adult and CV% was calculated. The CV% for height was 0.09%.

4.17 Statistical Analysis: Statistical analysis was done using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA) and SAS (SAS Institute Inc,, Cary, NC). Repeated measures ANOVA was performed to determine differences between biochemical sampling point and Tukey's post-hoc test was used to determine differences between means. Student's t-test was performed to determine differences between 25(OH)D status according to season of birth. Correlation analysis was performed to detect relationships between infant and mother's vitamin D status and between infant's vitamin D status and bone metabolism markers. Outliers were identified as being >3 SD from the mean. For all analyses, differences were considered significant with a $p<0.05$.

5.0 Methods: Term Infants and their Mothers

5.1 Population: 30 term infant-mother pairs were recruited to the study. This population served as a reference group to the very preterm infant population.

5.2 Ethical Approval: Ethical approval was granted through the University of Manitoba Ethics Committee. Informed consent was obtained from mothers prior to being included in the study. Appendix C provides a copy of the term consent form.

5.3 Recruitment: Term infant and mother pairs were recruited from Women's Hospital High Risk Labor and Delivery and Labor; Delivery; Recovery; Postpartum (LDRP) Units. All infant and mother pairs who met inclusion criteria and had a cord blood sample obtained at delivery were invited to participate. Recruitment rate for the term infants was 26%.

5.4 Inclusion Criteria: Inclusion criteria for term infants was birth between 37 0/7 and 40 6/7 weeks gestation, appropriate for gestational age according to 2 of 3 growth charts (Usher & McLean, 1969, Arbuckle et al., 1993, Kramer et al., 2001), and healthy. The gestational age range was chosen as it matches the typical discharge age of very pre-term infants from the NICU.

Mothers - The eligibility criteria for the mothers of term infants is the same as that outlined for the preterm mothers in Chapter 4.4.

5.5 6 month Follow-up: Infants were followed until 6 months of age, for growth measurements, nutrition and bone mineral content.

5.6 Growth Measurements: Growth measurements from birth were collected from the hospital chart and included length, weight and head circumference.

Growth measurements were also collected at the birth and 6-month follow-up visit. Length was measured using crown-heel length (to the nearest 0.1 cm) using an infant length board. Lengths were obtained by two of the research team members. Weight (measured to the nearest 1 gram) was measured using an electronic weigh scale. Head circumference (measured to the nearest 0.1 cm) was measured using a disposable measuring tape. Growth measurements were obtained by one of four members of the research team. Intra-variability measurements among the researchers are provided in Chapter 4.6.

5.7 Nutrition: Mothers were asked to complete a 3-day food record of infant's intake at both birth and 6 months of age (Appendix B-1). For infants who were fed formula, the amount and type of formula consumed was recorded. For infants who were breast fed, the test-weigh method was utilized. Mothers were provided with a weigh scale and asked to weigh infants pre- and post-feed, and record the weights. The difference in weight is the amount of milk consumed by the infant. Mothers were also asked to record supplement use, such as Di-Vi-Sol.

Formula (Enfalac 20 with Iron, Mead Johnson, Ottawa, Ontario) was provided for the first 6 months to formula fed infants. All breast-fed infants received Di-Vi-Sol (Mead Johnson, Ottawa, Ontario) supplements for the first 6 months of age.

All records were analyzed by one of two investigators using Nutrient Analysis Program (E. Warwick, PEI, Canada). Percent agreement between investigators is outlined in Chapter 4.7. Records were analyzed for calories,

macronutrients (protein, fat and carbohydrate), calcium, phosphorus, magnesium and vitamin D.

5.8 Blood and Urine Sampling: At the time of delivery, a cord blood sample was obtained using a vacutainer containing heparin. The sample was separated into plasma and red blood cell fractions within 18 hours of sampling and subsequently stored at -80°C.

A urine sample was collected within 48 hours of birth between 0200 and 0900 to control for diurnal variation. The sample was collected using either urine bags or plastic bags placed in the diaper. The sample was stored at -80°C within 12 hours of collection.

5.9 Maternal Information: Maternal information was obtained to assist in interpreting the outcomes of the infants. Demographic information was recorded from the medical chart. The type and amount of vitamin and mineral supplements used during pregnancy was also recorded. Verification of the information occurred at the 6 month follow-up appointment. A venous blood sample (5-10 mL) was obtained prior to discharge from hospital. Hospital Lab Technicians obtained the blood sample between 0800 and 1000. The sample was separated into plasma and red blood cell fractions within 2-3 hours of sampling and stored at -80°C, until required.

Mothers were asked to complete a 3-day food record (Appendix B-2) within 4-6 weeks of delivery and return via mail in a self-addressed stamped envelope which was provided. Instructions were provided to mothers outlining

the procedure for filling out the 3 day food records. Any information which was unclear was obtained by telephone.

A 24-hour food recall (Appendix B-3), was completed with all mothers and was performed by one of two investigators, either in person or via telephone.

At the time of their DEXA scan, mothers were again asked to complete both a 3-day food record and a 24-hour food recall.

5.10 25-hydroxyvitamin D: 25-hydroxyvitamin D was measured in infants cord plasma and maternal plasma. The method used for measuring 25(OH)D is outlined in Chapter 4.11. Deficient levels are similar to those obtained in the aforementioned chapter.

5.11 Osteocalcin: Osteocalcin was measured in infant plasma, using the same method outlined in Chapter 4.12.

5.12 Cross linked N-telopeptide: Cross linked N-telopeptide was measured in infant urine. The method used to measure NTx is outlined in Chapter 4.13.

5.13 Calcium and Phosphorus: Calcium and Phosphorus were measured in infants urine using via emission absorptiometry. The method used is outlined in Chapter 4.14.

5.14 Creatinine: Creatinine was measured in infants urine using colorimetric determination. The method used is outlined in Chapter 4.15.

5.15 Dual Energy X-ray Absorptiometry Scan: Infant BMC measurements were obtained using DEXA (QDR4500A, Elite X-ray Bone Densitometer, Hologic Inc., Bedford, MA, USA) within 14 days of birth and 6 months of age. Infant femur and lumbar spine (L1-4) were measured and analyzed using the Hologic

QDR software for Windows, Version 11.2. DEXA scans of mothers were also obtained at 6 months post-partum, if not breastfeeding or 6 months after finishing breastfeeding. The method used to obtain scans is outlined in Chapter 4.16.

5.16 Statistical Analysis: Statistical analysis was done using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). Student's t-test was performed to determine differences between 25(OH)D status according to season of birth. Correlation analysis was performed to detect relationships between infant and mother's vitamin D status and between infant's vitamin D status and bone metabolism markers. For all analyses, differences were considered significant with a $p<0.05$.

6.0 Results

The hypothesis and objectives both discuss vitamin D status at birth as the baseline measurement. However, we were unable to obtain a cord plasma sample at birth from our very preterm infants; therefore we used the day 7 PNA sample as our baseline.

6.1 Descriptive Characteristics – Infants and Mothers

Sixteen very preterm infants were recruited for the study, as well as 30 term infants which acted as a reference group. The gestational age of the very preterm infants at birth ranged from 23.9 to 29.6 weeks (mean 27.2 $\pm$ 1.8) (Table 6.1). Ten (62.5%) were male and 6 (37.5%) were female. Thirteen (81%) were Caucasian, and 3 (19%) were non-caucasian. Mean birth weight was 1011 $\pm$ 251 g and birth weights of all infants were within the 3rd and 97th percentile on infant growth charts (Usher & McLean, 1969, Arbuckle et al., 1993, Kramer et al.,2001). As well, length and head circumference were within the 3rd and 97th percentiles on infant growth charts (Center for Disease Control and Prevention, 2000). Very preterm infants spent on average 84 $\pm$ 28 days in hospital and had a mean discharge age of 39.5 $\pm$ 2.5 weeks gestation (Table 6.1).

Four (25%) of the very preterm infants developed Bronchopulmonary Dysplasia (BPD), defined as oxygen dependence at 36 weeks post-conceptual age (Vaucher, 2002) (Table 6.1). Three of the four infants with BPD received Dexamethasone for an average of 11 $\pm$ 9 days. Three of the infants with BPD received Lasix (Furosemide) as a diuretic therapy for an average of 22 $\pm$ 16 days.

Fourteen of the very preterm infants were followed until 6 months corrected age. Contact was lost with one infant-mother pair and one infant passed away.

Table 6.1 also outlines the descriptive characteristics of the term infant group (n=30). The mean gestational age was 38.8 $\pm$ 1.2 weeks. There were 16 (53%) male and 14 (47%) female infants. Twenty-one (70%) of the term infants were Caucasian. All infants were appropriate for gestational age at birth, with birth weight, length and head circumference between the 3rd and 97th percentile on infant growth charts (Center for Disease Control and Prevention, 2000) (Table 6.1).

The descriptive characteristics of the mothers are outlined in Table 6.2. The mean age of the mothers at time of delivery was 26 $\pm$ 6 years for the very preterm mothers and 27 $\pm$ 7 years for the term mothers. Thirteen (81%) of the very preterm infants and 27 (90%) of the term infants were delivered vaginally. Nine (56%) of the very preterm mothers and 19 (63%) of the term mothers did not smoke during their pregnancy. Ten (63%) of the very preterm mothers and 23 (77%) of the term mothers reported using a prenatal supplement (Table 6.2).

6.2 Nutrient Intake of infants:

The nutrient intake of the very preterm infants was separated into 4 time periods for analysis. The first period, birth to day 7 PNA, corresponded with the transition requirements of the Premature Infant Recommended Nutrient Intakes (P-RNI). The last three periods (day 8-21 PNA, day 22-35 PNA and day 36 PNA-hospital discharge) corresponded with the Stable-Discharge requirements of the

P- RNI's (Nutrition Committee, Canadian Paediatric Society, 1995) and also represented the intervals between blood sampling (Table 6.3). On average, infants did not meet the energy requirement of 70-80 kcal/kg during the transition period. As well, the average intakes of calcium, phosphorus and magnesium were not adequate to meet the transition period P-RNI. However, average intake of protein, fat and carbohydrate were within the recommended amount. Average vitamin D intake was also within the recommended range of 40-120 IU/day (Table 6.3).

The P-RNI for energy during the stable-growing period is 105-135 kcal/kg. On average, this requirement was not met until day 22-35 PNA in the very preterm infants. The protein P-RNI was met during day 22-35 PNA, however average protein intake was not maintained between day 36 PNA and hospital discharge and dropped below the P-RNI. Very preterm infants did not meet the stable-growing period P-RNI for calcium or phosphorus, however, it was met for magnesium. Intake of vitamin D continued to increase throughout the hospital stay, however intake did not meet the stable-growing period P-RNI of 400 IU/day until day 36 PNA-hospital discharge time period (Table 6.3). Supplementation of vitamin D (Tri-Vi-Sol®, Mead Johnson) was initiated in 12 infants within hospital. Supplementation is typically initiated once tolerance to full enteral feeds is established. Full enteral feeds were established, on average, at 19.7 $\pm$ 7.6 days PNA (range 4-37 days PNA), however, average Tri-Vi-Sol initiation was not until 27 $\pm$ 8.1 days PNA (range 19-43 days PNA). One infant did not receive Tri-Vi-Sol supplementation until after hospital discharge (day 44 PNA), while 3 infants

did not receive Tri-Vi-Sol. One of the three infants was being fed expressed breast milk, while the other two were receiving formula. The two infants who received formula, had adequate oral intake of vitamin D and did not require supplementation. The infant who was receiving expressed breast milk was not receiving adequate vitamin D orally prior to hospital discharge.

Nutrient intake of term infants was measured via a 3-day food record completed by their mother. There was a 67% response rate. At birth, 17 infants were breast-fed and this continued for an average of 4.7 $\pm$ 3.9 months. Thirteen infants were fed infant formula (Enfalac with Iron, Mead Johnson). Fourteen infants received Di-Vi-Sol. Average nutrient intake was compared to the Recommended Nutrient Intake (RNI) and Dietary Reference Intake (DRI), when applicable (Table 6.4). Ninety percent of the infants did not meet the energy requirement of 600 kcals/day, while 95% did not meet the protein requirement of 12 g/day (DRI is 13 g/day). On average, infants met the RNI for all other nutrients measured. The DRI values differ slightly and therefore infants did not meet the recommended intake of magnesium. The RNI for vitamin D is 10 µg/day, while the DRI has been decreased to 5 µg/day. On average, infant's vitamin D intake was 6.8 $\pm$ 4.6 µg/day (Table 6.4). Twenty-five percent of the infants had a vitamin D intake less than 5 µg/day.

6.3 Nutrient Intake of Infants at 6 month Follow-up:

Nutrition intake of infants at 6 months of age was assessed using 3-day food records completed by their mothers. The response rate was 71% (n=10) for very preterm infants and 74% (n=22) for term infants.

Four very preterm infants continued receiving breast milk at 6 months corrected age, while 10 were receiving formula. All infants had been introduced to solids. Their intake was compared to the post-discharge period P-RNI's (Nutrition Committee, Canadian Paediatric Society, 1995) (Table 6.5). Infants, on average, met the P-RNIs for energy, macronutrients, calcium, phosphorus and magnesium. As well, infant's intake met the P-RNI of 400 IU/day vitamin D (Table 6.5).

By 6 months of age, all term infants had been introduced to solid foods. Twenty seven percent of infants were still receiving breast milk, while 73% of infants were receiving formula. Two infants receiving formula were still receiving 1 feed per day of breast milk.

Average intake of term infants was compared to both RNI and DRI values (Table 6.5). Infants, on average, did not meet the RNI for energy of 900 kcals/day. However, average intake did meet the RNI for protein, calcium, phosphorus, magnesium and vitamin D. The DRI for protein has been increased to 14 g/day, therefore, infants average intake did not meet this requirement. Calcium, phosphorus, magnesium and vitamin D average intakes met the DRI's (Table 6.5).

6.4 Nutrient Intake of Mothers at Time of Delivery:

Very preterm and term mothers were asked to complete a 3-day food record, as well as a 24-hour food recall. Very preterm mothers had a 53% response rate for 3-day food records, while term mothers had a 70% response rate. Twenty-four-hour food recalls were done on 14 (87.5%) of the very preterm

mothers. Contact was lost with one mother and the 24-hour food recall for the second mother was misplaced. All term mothers had a 24-hour food recall performed and analyzed. Twenty-four hour food recalls were done on average 77 ± 28 days post-delivery in the very preterm mothers and 58 ± 29 days post-delivery in the term mothers. Due to the poor response rate for the 3-day food records, only 24-hour food recalls are reported (Table 6.6).

Average energy intake of the very preterm mothers was 2181.3 ± 655.4 kcals. Sixteen percent of calories were from protein, 36% were from fat and 49% were from carbohydrate. On average, calcium, phosphorus and magnesium intakes met the RNI. Average intake of calcium and phosphorus met the DRI, however, average magnesium intake did not. The average intake of vitamin D was 5.8 ± 4.9 µg/day, which met both the RNI and DRI (Table 6.6).

Term mothers had an average calorie intake of 1783.9 ± 567.6 kcals. Seventeen percent of calories were from protein, 29% from fat and 54% from carbohydrate. Term mothers met the RNI for calcium, phosphorus, magnesium and vitamin D. The DRI values are different and therefore average calcium and phosphorus intakes were below the recommended value. Average vitamin D intake was 3.1 ± 3.0 µg/day which did not meet the DRI of 5 µg/day (Table 6.6).

6.5 Nutrient Intake of Mothers at DEXA Scan.

Mothers were again asked to fill out a 3-day food record at their DEXA scan. The response was 50% and 65.5%, respectively, for very preterm and term mothers. One hundred percent of very preterm mothers and 83% of term mothers had a 24-hour food recall performed, therefore, only 24-hour food recalls

are reported (Table 6.7) Very preterm mothers received 16% of calories from protein, 32% from fat and 52% from carbohydrate. Term mothers received 15% of calories from protein, 31% from fat and 54% from carbohydrate. Neither group received adequate calcium. Average vitamin D intake was 3.1 $\pm$ 3.6 µg/day and 3.4 $\pm$ 2.7 µg/day for very preterm and term mothers, respectively, which did not meet the DRI of 5 µg/day (Table 6.7).

6.6 Vitamin D Status

Thirty-one percent of very preterm infants were deficient in plasma 25(OH)D at day 7 PNA with a value <27.5 nmol/L (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997) (Figure 6.1). All infants with deficient plasma 25(OH)D status were born between the months of October and March. It has been shown that at a similar latitude as Winnipeg, vitamin D synthesis does not occur during these months (Webb et al., 1988). A significant relationship was not evident between very preterm infants plasma 25(OH)D at day 7 PNA and their mother's plasma 25(OH)D status at time of delivery ($r=0.457$, $p=0.075$).

Very preterm infants were divided into two groups according to month of birth and plasma 25(OH)D status to consider seasonal variation. Group one included those infants born between April and September and group two included those infants born between October and March. The difference between groups was not significant (43.79 $\pm$ 8.33 nmol/L and 36.37 $\pm$ 23.30 nmol/L, $p=0.4385$) (Figure 6.2a). However, group two was noted to have an outlier. After the removal of the outlier, group two was found to have a

significantly lower plasma 25(OH)D level at birth compared to group one (29.67 ± 12.56 nmol/L and 43.79 ± 8.33 nmol/L, p=0.0254) (Figure 6.2b).

We obtained plasma 25(OH)D concentrations throughout infants hospital stay at day 7, 21 and 35 PNA and hospital discharge (Figure 6.3). At day 7 PNA, the mean plasma 25(OH)D of very preterm infants was 39.62 ± 18.21 nmol/L. Thirty-one percent of infants had deficient values. Day 7 PNA plasma 25(OH)D was significantly lower than day 35 PNA plasma 25(OH)D (p=0.0045)(Figure 6.3). At day 35 PNA, 6% of infants continued to have deficient plasma 25(OH)D values. By hospital discharge, all infants had plasma 25(OH)D values within normal range. Twelve of the infants received at least one blood transfusion during their hospital stay, which may have influenced their plasma 25(OH)D status over time. 25-hydroxyvitamin D was measured in cord plasma samples of term infants (Figure 6.4). Thirty-seven percent of term infants had plasma 25(OH)D considered deficient (<27.5 nmol/L). Five of the eleven term infants with deficient concentrations were born between October and March. A significant relationship existed between term infants cord plasma 25(OH)D and their mothers plasma 25(OH)D at birth (r=0.8006, p<0.0001) (Figure 6.5).

Term infants were also divided into 2 groups according to month of birth and plasma 25(OH)D status to consider seasonal variation. Similar to very preterm infants, group one included those infants born between October and March and Group two included those infants born with April and September. There were no significant differences between groups (28.94 ± 12.13 nmol/L (n=14) and 37.93 ± 16.25 nmol/L (n=16), p=0.1010).

Very preterm infants had a similar discharge age as the term reference group (39.5 $\pm$ 2.5 weeks and 38.8 $\pm$ 1.2 weeks). At hospital discharge, very preterm infants had a similar plasma 25(OH)D level as term infants at birth (39.62 $\pm$ 18.21 nmol/L vs. 36.64 $\pm$ 20.83 nmol/L).

Five (31%) of the very preterm mothers had deficient concentrations (<40.0 nmol/L) (Vieth et al., 2001) of plasma 25(OH)D at the time of delivery (Figure 6.6). Four of the 5 mothers with deficient concentrations had infants with deficient plasma 25(OH)D values at day 7 PNA. Twelve mothers (75%) had plasma 25(OH)D values <73 nmol/L, which is considered the low end of normal for adults (Vieth et al., 2001). One hundred percent (n=4) of the very preterm mothers with normal plasma 25(OH)D value took a prenatal supplement, containing vitamin D, during their pregnancy. Sixty percent of the mothers with a deficient plasma 25(OH)D did not take a prenatal supplement during pregnancy.

Fifty percent (n=15) of the term mothers had deficient concentrations of plasma 25(OH)D at the time of delivery (Figure 6.7). Ninety-seven percent of the term mothers had plasma 25(OH)D below the low end of normal (<73 nmol/L) (Figure 6.7). Eleven (73%) of the plasma 25(OH)D deficient mothers took a prenatal supplement during pregnancy. The one term mother with a normal plasma 25(OH)D value did not take a prenatal supplement during pregnancy.

Seasonal variation of both very preterm and term mothers was also considered at their time of delivery. Both populations were divided into two seasons, October through March and April through September. There were no significant differences in plasma 25(OH)D status between seasons in very

preterm mothers (51.82 ± 23.51 (n=9) vs. 54.82 ± 20.92 (n=7), p=0.7942), nor term mothers (34.35 + 13.99 (n=14) vs. 44.93 + 16.78 (n=16), p=0.0735).

In very preterm infants, correlation analysis was performed to determine if there were any relationships between day 7 PNA plasma 25(OH)D with lumbar spine and femur BMC at hospital discharge. There were no relationships noted between day 7 PNA plasma 25(OH)D and hospital discharge lumbar spine and femur BMC (Table 6.8). However, with day 7 PNA plasma 25(OH)D and lumbar spine BMC corrected to weight, there was noted to be an outlier (Figure 6.8a). There was a significant positive relationship noted after the removal of the outlier (Figure 6.8b).

Correlation analysis was also performed with hospital discharge plasma 25(OH)D and hospital discharge lumbar spine and femur BMC (Table 6.8). There were no relationships noted, even after correcting both lumbar spine and femur with weight and length (Table 6.8).

There were no significant relationships noted between plasma 25(OH)D at day 7 PNA and 6 month corrected age lumbar spine and femur BMC in very preterm infants (Table 6.9). However, after correcting femur BMC to weight, there was a significant negative relationship (Figure 6.9). Hospital discharge plasma 25(OH)D also did not have any significant relationships with lumbar spine and femur BMC at 6 months corrected age (Table 6.9).

In term infants, cord plasma 25(OH)D concentrations were found to have no relationship with BMC of lumbar spine and femur at hospital discharge. Even after correcting lumbar spine and femur to weight and length, the relationships

remained non-significant (Table 6.10). As well, there were no significant relationships noted between cord plasma 25(OH)D and 6 month lumbar spine and femur BMC (Table 6.11).

6.7 Measurements of Bone Markers

Plasma osteocalcin, a marker of bone formation, was measured in very preterm infants at day 7, 21 and 35 PNA and hospital discharge, as well as in term infants cord plasma. In very preterm infants, osteocalcin concentrations were similar between day 7 PNA and day 21 PNA. However, at hospital discharge, plasma osteocalcin was significantly higher than both days 7 and 21 PNA concentrations (Figure 6.10). At hospital discharge, plasma osteocalcin in very preterm infants was higher than term infant's osteocalcin in cord plasma (2.09 ± 1.13 nmol/L vs. 0.54 ± 0.49 nmol/L) (Figure 6.11).

Urinary NTx is a measure of bone resorption. Urinary NTx was measured at birth, day 7, 21, 35 and hospital discharge. In very preterm infants, birth and day 7 PNA NTx was significantly lower than hospital discharge (Figure 6.12). A urine sample was also collected in term infants at birth. Birth urinary NTx concentration in the reference group of term infants were lower than very preterm infants at hospital discharge (1634 ± 812 molar ratio to creatinine vs. 4537 ± 3080 molar ratio to creatinine) (Figure 6.13).

Urinary calcium and phosphorus were also measured at birth, day 7, 21 35 PNA and hospital discharge. Urinary calcium at birth was significantly lower than day 35 PNA and hospital discharge. As well, day 7 PNA was significantly lower than hospital discharge urinary NTx (Figure 6.14). There was no pattern

noted among phosphorus concentrations throughout the infant's hospital stay and there were no significant differences noted between any of the time points (Figure 6.15).

Very preterm infant's urinary calcium concentrations were higher at hospital discharge than the reference group of term infants at birth (4.59 $\pm$ 5.08 molar ratio to creatinine vs. 0.16 $\pm$ 0.17 molar ratio to creatinine) (Figure 6.16). Urinary phosphorus concentrations were similar between both very preterm infants at hospital discharge and term infants at birth (data not shown).

6.8 DEXA Measurements at Hospital Discharge and 6 months corrected age in Very Preterm Infants and Birth and 6 months in Term Infants:

The gestational age of very preterm infants was within the range of the reference group of term infant's at the time of the initial DEXA scan (term 38.8 $\pm$ 2.2 weeks vs very preterm 40.0 $\pm$ 1.7 weeks) (Appendix D). For very preterm infants, the scan coincided with hospital discharge and for the reference group the scan occurred on average 9.5 $\pm$ 6.1 days after birth (range 2-23 days). Weight, length and head circumference were lower in very preterm infants at the initial DEXA scan at hospital discharge compared to the reference group of term infants. At hospital discharge, lumbar spine and femur BMC were lower in the very preterm infants than in the reference group of term infants (Figures 6.17 and 6.18). When corrected for body weight, both lumbar spine and femur continued to be lower in very preterm infants when compared to the reference group of term infants (0.41 $\pm$ 0.21 grams vs 0.61 $\pm$ 0.11 grams and 0.45 $\pm$ 0.21 grams vs 0.71 $\pm$ 0.22 grams, respectively) (Figures 6.19 and 6.20). As well, after correcting

lumbar spine and femur BMC to body length, very preterm infants BMC continued to be lower (Figures 6.21 and 6.22).

At 6 months corrected age, very preterm infants continued to be smaller in weight and length compared to the reference group of term infants (Appendix E). Lumbar spine and femur BMC also remained lower than the reference group (Figures 6.176 and 6.18). However, after corrected for weight and length, both lumbar spine and femur BMC values were similar (Figures 6.19, 6.20, 6.21, 6.22).

6.9 DEXA Measurements of Mothers:

Dual energy x-ray absorptiometry was performed on mothers 6 months post-delivery or 6 months after finishing breast-feeding. Mothers had DEXA scans performed to assist in interpreting the bone mass data of the infants in multivariate regression analysis. However, due to our small sample size, we were unable to perform multivariate regression analysis. The mother's data will still be included and is presented below.

Preterm mothers had an average body mass index (BMI) of 26 $\pm$ 5.7 kg/m^2, while term mothers had an average BMI of 28 $\pm$ 5.7 kg/m^2. Average lumbar spine (L1-4) BMC of the very preterm mothers was 52.52 $\pm$ 6.51 grams, while term mothers had an average lumbar spine (L1-4) BMC of 58.09 $\pm$ 6.8 grams. Total hip BMC in very preterm mothers was 25.67 $\pm$ 4.43 grams, while it was 29.41 $\pm$ 6.02 grams in term mothers (Table 6.12).

T-scores and z-scores were also obtained on the mothers. The T-score is the number of standard deviations that the individuals BMD is above or below the

mean reference value for young adults (Khan et al., 2002). A T-score of > -1.0 is suggestive of normal BMD, -1.0 to -2.5 is suggestive of osteopenia and below -2.5 suggests osteoporosis. On average, both very preterm and term mothers had T-scores of >-1.0 for both lumbar spine and total hip suggestive of normal BMD (Table 6.14). However, 7 very preterm mothers and 12 term mothers had t-scores for spine within the osteopenic range. Three of the 7 very preterm mothers and all of the term mothers had plasma 25(OH)D concentrations less than 73 nmol/L. Three very preterm mothers and 10 term mothers had total hip t-scores within the osteopenic range. All of the very preterm mothers and 5 of the term mothers had 25(OH)D concentrations less than 73 nmol/L.

The z-score is the number of standard deviations an individual's BMD is above or below the mean reference value for people of the same age (Khan et al., 2002). For the spine, very preterm mothers had on average -0.68 $\pm$ 0.81, while term mothers had -0.19 $\pm$ 0.92. Very preterm mothers had a z-score of the femur of -0.28 $\pm$ 1.01, while term mothers had a z-score of -0.08 $\pm$ 0.93.

Very preterm mothers with below normal 25(OH)D (<73 nmol/L), on average, had similar lumbar spine and total hip BMC compared with those mothers who had adequate 25(OH)D concentrations (52.81 $\pm$ 6.97 grams vs. 51.80 $\pm$ 6.06 grams and 25.95 $\pm$ 5.02 grams vs. 24.96 $\pm$ 2.90 grams, respectively). Term mothers with deficient 25(OH)D (<40 nmol/L) had similar lumbar spine and total hip BMC compared with those mothers with below normal 25(OH)D (58.30 $\pm$ 7.18 grams vs. 57.44 $\pm$ 6.72 grams and 28.98 $\pm$ 6.15 grams vs. 30.24 $\pm$ 6.05 grams, respectively).

Table 6.1: Descriptive characteristics of very preterm and term infants.

		Very Preterm Infants	Term Infants
		(n=16)	(n=30)
Mean Gestational Age at birth (weeks)		27.2 ± 1.8	38.8 ± 1.2
Gender	male	10 (62.5%)	16 (53%)
	female	6 (37.5%)	14 (47%)
Ethnicity	Caucasian	13 (81%)	21 (70%)
	Non-caucasian	3 (19%)	9 (30%)
APGAR Score	1 minute	5 ± 3	8 ± 1
	5 minute	7 ± 2	9 ± 1
Anthropometry			
Birth			
Mean weight (g)		1011 ± 251	3546 ± 556
Mean length (cm)		35.8 ± 3.0	51.7 ± 2.3
Mean head circumference (cm)		25.2 ± 1.9	35.4 ± 1.2
Mean Discharge Gestational Age (weeks)		39.5 ± 2.5	
Average number of days in hospital		84 ± 28	
Average number of days on oxygen		53 ± 31	
Average number of days on ventilator		19 ± 18	
# of infants on oxygen at 36 weeks gestational age		4	

Data shown as mean ± SD except for gender and ethinicity data which is shown as number(%).
Term infant anthropometry was done at birth DEXA visit.

Table 6.2: Descriptive characteristics of very preterm mothers and term mothers.

	Very Preterm Mothers	Term Mothers
	(n=16)	(n=30)
Age	26 ± 6	27 ± 7
Gravida	2.8 ± 2.1	2.8 ± 1.6
Parity	1.0 ± 1.4	1.3 ± 1.1
Delivery Type		
Vaginal	13 (81%)	27 (90%)
Cesarean	3 (19%)	3 (10%)
Length of Labor (hours)	6.9 ± 7.1	7.0 ± 7.8
Smoker		
Yes	7 (44%)	11 (37%)
No	9 (56%)	19 (63%)
Race		
Caucasian	13 (81%)	20 (67%)
Non-caucasian	3 (19%)	10 (33%)
Prenatal Supplement		
Yes	10 (62.5%)	23 (77%)
No	6 (37.5%)	7 (23%)

Data shown are mean ± SD, or as number (percent).

Table 6.3: Nutrient intake of very preterm infants during hospital stay separated into 4 time points compared to the premature infant recommended nutrient intakes[1].

	Transition Period		Stable-Growing Period			
Nutrients	**Birth-Day 7**	**P-RNI**	**Day 8-21**	**Day 22-35**	**Day 36-d/c**	**P-RNI**
Energy (kcal/kg)	66.5 ± 11.9 (7)	**70-80**	96.2 ± 9.8 (3)	104.9 ± 15.6 (6)	118.3 ± 11.5 (14)	**105-135**
Protein (g/kg)	1.8 ± 0.4 (16)	**1-3**	3.1 ± 0.6 (4)	3.7 ± 0.8 (9)	2.7 ± 0.5 (1)	**3.5-4**
Fat (g/kg)	2.1 ± 0.8 (15)	**0.5-3.6**	4.2 ± 0.9 (6)	4.9 ± 0.9 (11)	6.7 ± 0.6 (16)	**4.5-6.8**
Carbohydrate (g/kg)	11.8 ± 1.9 (16)	**5-20**	13.4 ± 1.7 (16)	12.0 ± 1.1 (16)	11.7 ± 1.1 (16)	**7.5-15.5**
Calcium (mg/kg)	24.9 ± 12.3 (0)	**60.1 – 80.2**	50.7 ± 28.5 (0)	112.9 ± 42.6 (2)	140.5 ± 39.2 (5)	**160.3 – 240.5**
Phosphorus (mg/kg)	18.7 ± 8.7 (1)	**31.0 – 46.5**	33.7 ± 13.6 (0)	59.4 ± 19.3 (3)	67.8 ± 21.2 (5)	**77.4 – 117.7**
Magnesium (mg/kg)	3.4 ± 2.0 (4)	**4.9 – 6.1**	5.1 ± 3.1 (7)	5.7 ± 1.6 (10)	7.3 ± 2.1 (16)	**4.9 – 9.7**
Vitamin D (IU/day)	101.9 ± 30.3 (16)	**40-120**	117.0 ± 38.5 (0)	388.1 ± 157.0 (9)	504.0 ± 206.3 (14)	**400**

Data shown as mean ± SD, (number meeting P-RNI), n=16

[1]. Nutrition Committee, Canadian Paediatric Society. Nutrient needs and feeding of premature infants. Can Med Assoc J 1995;152:1765-1785.

Table 6.4: Nutrient intake of term infants at birth compared to both the Recommended Nutrient Intakes (RNI)[1] and the Dietary Reference Intakes (DRI)[2].

	Nutrient Intake	RNI	DRI
Energy (kcal)	396.7 ± 97.0	600	
Protein (g)	7.3 ± 2.4	12	13
Fat (g)	22.3 ± 4.1		
Carbohydrate (g)	43.5 ± 15.5		
Calcium (mg)	259.7 ± 101.8	250	210
Phosphorus (mg)	159.5 ± 86.7	150	100
Magnesium (mg)	26.8 ± 10.4	20	30
Vitamin D (µg)	10.0 + 12.2	10	5

Data Shown are mean ± SD, n=20.

[1]. Minister of National Health & Welfare, Health Canada. Nutrition Recommendations. The report of the scientific review committee. Canadian Government Publishing Center, Ottawa, Ontario, Canada. 1990.

[2]. American Dietetic Association / Dietitians of Canada. Manual of Clinical Dietetics, 6th Edition. 2000.

Table 6.5: Nutrient intake of very preterm infants at 6 month corrected age compared to Premature Infant Recommended Nutrient Intakes[1] and term infants at 6 months of age compared to both Recommended Nutrient Intakes[2] and Dietary Reference Intakes[3].

	Nutrient Intake	P-RNI	
Preterm Infants (n=10)			
Energy (kcal/kg)	109.6 ± 35.3	100-120	
Protein (g/kg)	2.2 ± 0.5	2.2	
Fat (g/kg)	5.2 ± 2.7	4.4-7.3	
Carbohydrate (g/kg)	13.9 ± 3.7	7.5-15.5	
Calcium (mg/day)	616.4 ± 272.7	252.0 (breast-fed) 376.0 (formula-fed)	
Phosphorus (mg/day)	569.9 ± 192.2	105.4 (breast-fed) 272.8 (formula-fed)	
Magnesium (mg/kg)	17.8 ± 16.2	4.9 – 14.6	
Vitamin D (IU)	500.0 ± 487.5	400	
Term Infants (n=22)		**RNI**	**DRI**
Energy (kcal)	599.4 ± 198.3	900	
Protein (g)	12.1 ± 5.6	12	14
Fat (g)	29.5 ± 10.7		
Carbohydrate (g)	74.4 ± 27.8		
Calcium (mg)	564.1 ± 299.6	400	210
Phosphorus (mg)	341.5 ± 197.8	200	100
Magnesium (mg)	56.1 ± 22.5	32	30
Vitamin D (µg)	11.2 + 9.9	10	5

Data shown as mean ± SD.

[1]. Nutrition Committee, Canadian Paediatric Society. Nutrient needs and feeding of premature infants. Can Med Assoc J 1995;152:1765-1785.

[2]. Minister of National Health & Welfare, Health Canada. Nutrition Recommendations. The report of the scientific review committee. Canadian Government Publishing Center, Ottawa, Ontario, Canada. 1990.

[3]. American Dietetic Association / Dietitians of Canada. Manual of Clinical Dietetics, 6th Edition. 2000.

Table 6.6: Nutrient intake of very preterm and term mothers at time of delivery as determined by 24-hour food recalls compared to both the Recommended Nutrient Intakes (RNI)[1] and Dietary Reference Intakes (DRI)[2]

	Nutrient Intake	RNI	DRI
Very Preterm Mothers (n=14)			
Energy (kcal)	2181.3 ± 655.4		
Protein (g)	90.7 ± 38.2 (16%)		
Fat (g)	88.4 ± 33.3 (36%)		
Carbohydrate (g)	263.6 ± 78.1 (49%)		
Calcium (mg)	1149.4 ± 626.0	700	1000
Phosphorus (mg)	1418.8 ± 730.6	850	700
Magnesium (mg)	283.6 ± 141.5	200	310-320
Vitamin D (µg)	5.8 ± 4.9	2.5	5
Term Mothers (n=30)			
Energy (kcal)	1783.9 ± 567.6		
Protein (g)	74.6 ± 33.5 (17%)		
Fat (g)	60.90 ± 30.9 (29%)		
Carbohydrate (g)	236.7 ± 80.4 (54%)		
Calcium (mg)	730.7 ± 462.4	700	1000
Phosphorus (mg)	1089.9 ± 488.4	850	700
Magnesium (mg)	239.1 ± 138.2	200	310-320
Vitamin D (µg)	3.1 ± 3.0	2.5	5

Data shown are mean ± SD, except for protein, fat and carbohydrate which are shown as mean ± SD (percentage of calories).
RNI values shown are for women aged 16-49.
DRI values shown are for women aged 19-50.

[1]. Minister of National Health & Welfare, Health Canada. Nutrition Recommendations. The report of the scientific review committee. Canadian Government Publishing Center, Ottawa, Ontario, Canada. 1990.
[2]. American Dietetic Association / Dietitians of Canada. Manual of Clinical Dietetics, 6th Edition. 2000.

Table 6.7: Nutrient intake of very preterm and term mothers at time of dual energy x-ray absorptiometry scan as determined by 24-hour food recalls compared to both the Recommended Nutrient Intakes (RNI) and Dietary Reference Intakes (DRI)

	Nutrient Intake	RNI[1]	DRI[2]
Very Preterm Mothers (n=14)			
Energy (kcal)	1735.7 ± 654.8		
Protein (g)	71.1 ± 39.4 (16%)		
Fat (g)	65.0 ± 42.4 (32%)		
Carbohydrate (g)	222.6 ± 65.2 (52%)		
Calcium (mg)	663.6 ± 366.7	700	1000
Phosphorus (mg)	944.7 ± 461.4	850	700
Magnesium (mg)	215.6 ± 113.0	200	310
Vitamin D (µg)	3.1 ± 3.6	2.5	5
Term Mothers (n=24)			
Energy (kcal)	1640.5 ± 512.0		
Protein (g)	60.4 ± 26.5 (15%)		
Fat (g)	56.4 ± 22.7 (31%)		
Carbohydrate (g)	223.7 ± 87.8 (54%)		
Calcium (mg)	605.3 ± 311.7	700	1000
Phosphorus (mg)	855.1 ± 373.3	850	700
Magnesium (mg)	167.3 ± 70.5	200	310
Vitamin D (µg)	3.4 ± 2.7	2.5	5

Data shown are mean ± SD, except for protein, fat and carbohydrate which are shown as mean ± SD (percentage of calories).

[1]. Minister of National Health & Welfare, Health Canada. Nutrition Recommendations. The report of the scientific review committee. Canadian Government Publishing Center, Ottawa, Ontario, Canada. 1990.

[2]. American Dietetic Association / Dietitians of Canada. Manual of Clinical Dietetics, 6th Edition. 2000.

Figure 6.1: Individual plasma 25-hydroxyvitamin D of very preterm infants at day 7 PNA. Deficient (<27.5 mmol/L) indicators and season of birth shown (n=16)

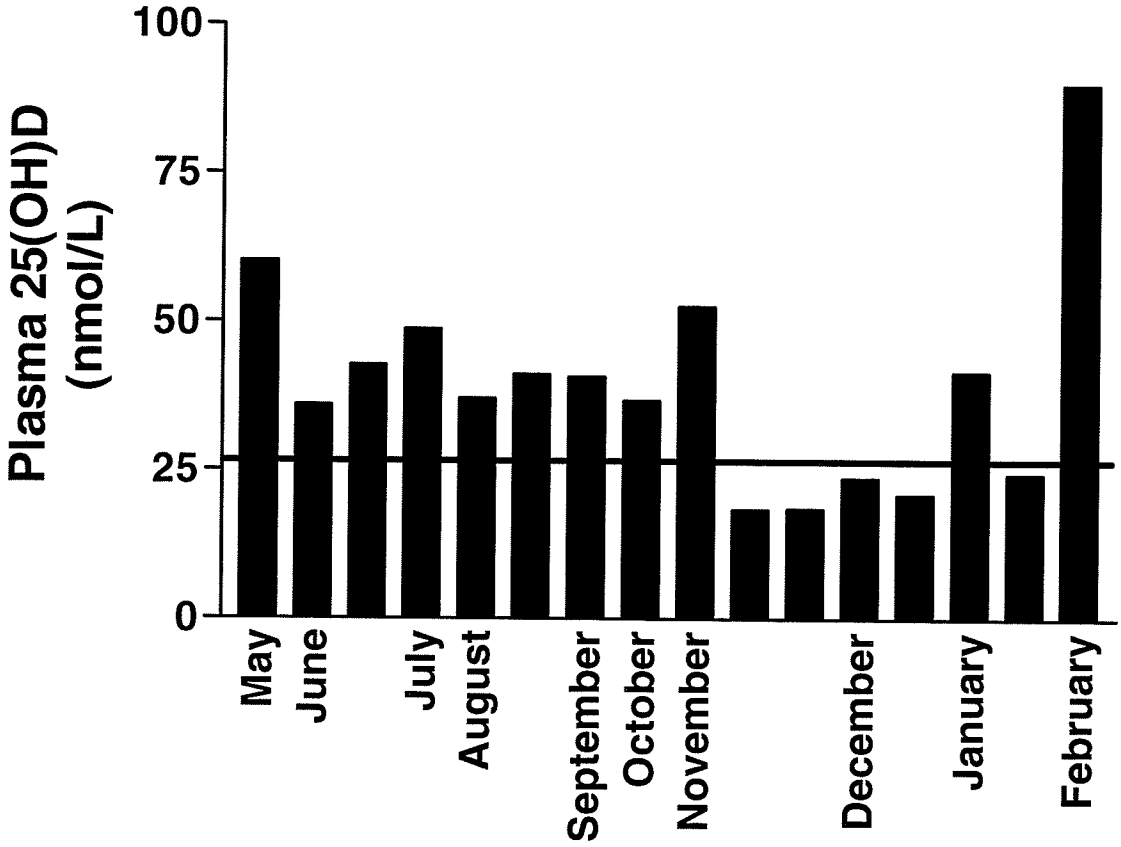
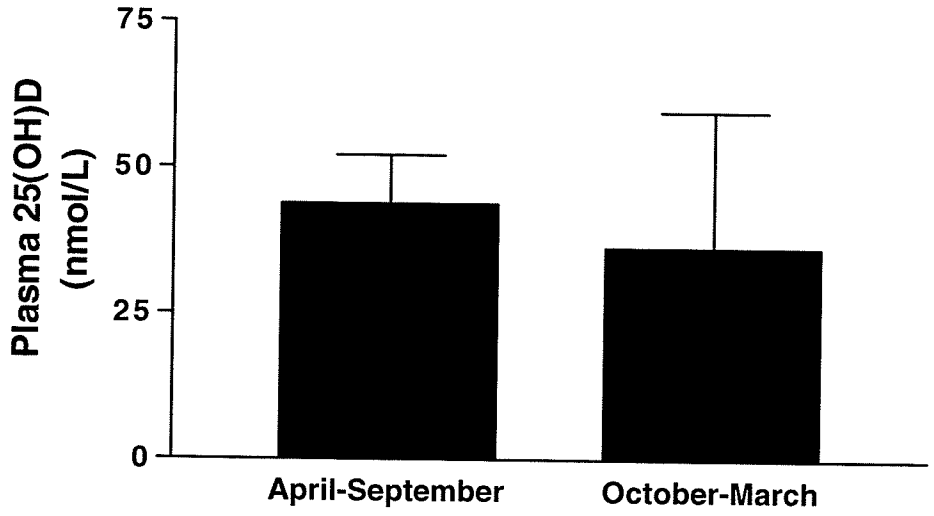
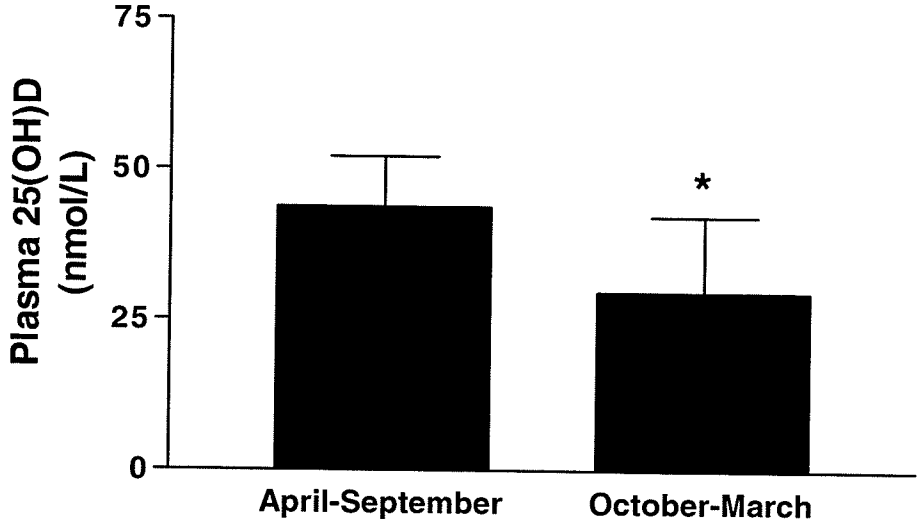


Figure 6.2a. Seasonal variation of plasma 25-hydroxyvitamin D status of very preterm infants at Day 7 PNA.



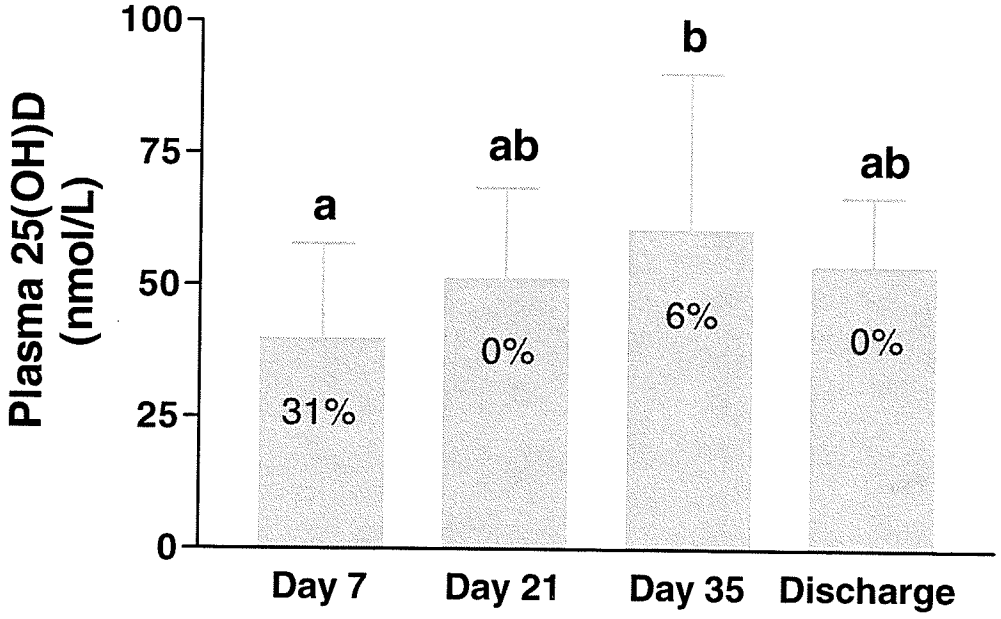
Data shown as mean + SD.
n=7 for April - September and n=9 for October - March.
No significant difference as analyzed by Student's t-test.

Figure 6.2b. Seasonal variation of plasma 25-hydroxyvitamin D status of very preterm infants at Day 7 PNA after removal of outlier.



Data shown as mean ± SD.
n=7 for April - September and n= 8 for October - March.
* Significant difference as analyzed by Student's t-test (p=0.0254).

Figure 6.3: Plasma 25-hydroxyvitamin D at day 7, 21, 35 PNA and hospital discharge in very preterm infants.



Data shown as mean ± SD, n=16.
Percentages indicate the percent of infants with deficient level of 25(OH)D at that time point.
Significant differences as analyzed by Repeated Measures ANOVA and Tukey's Post-Hoc Test (p=0.0045).

Figure 6.4: Individual 25-hydroxyvitamin D of term infants measured in cord plasma. Deficient (≤27.5 nmol/L) indicator and season of birth shown. (n=30)

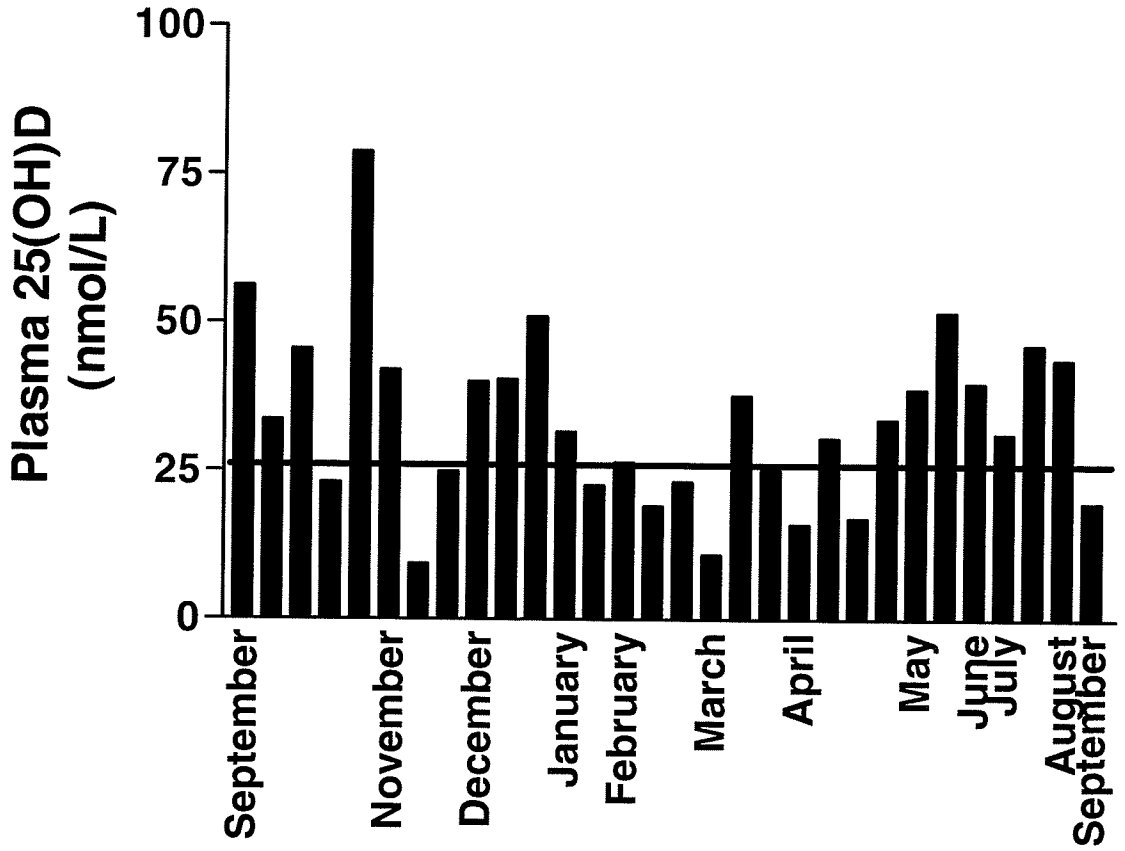


Figure 6.5. Relationship between term infants cord plasma and their mother's plasma 25-hydroxyvitamin D at time of delivery.

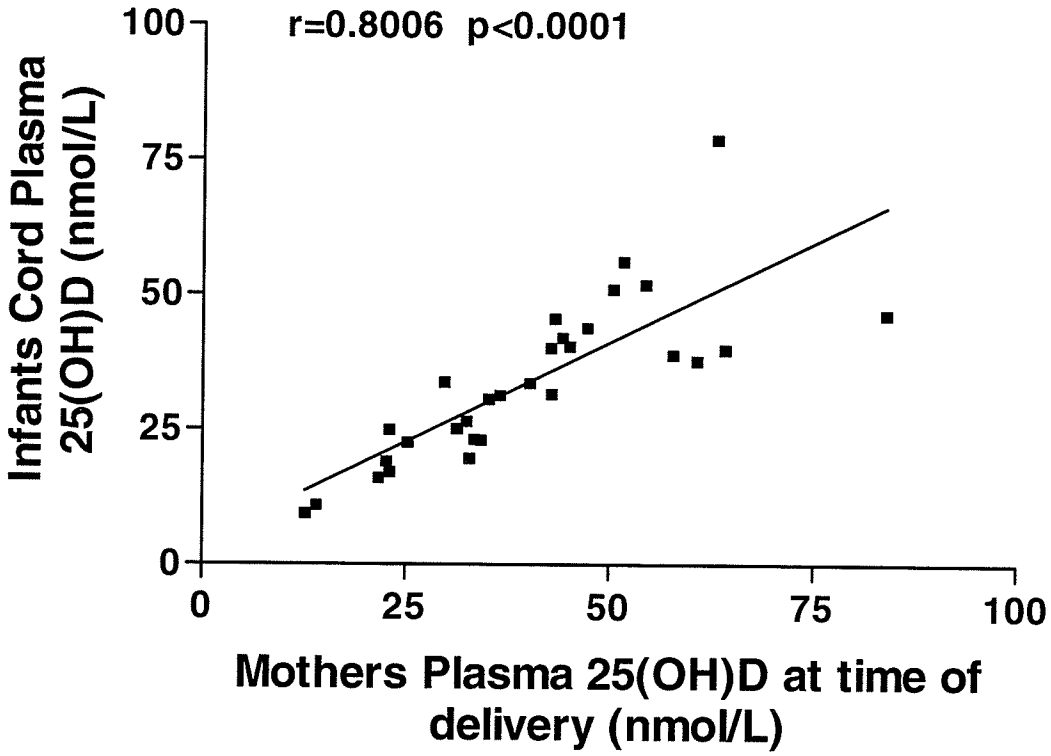


Figure 6.6: Individual plasma 25-hydroxyvitamin D of very preterm mothers measured at time of delivery. Deficient (<40.0 nmol/L) and low end of normal (<73 nmol/L) indicators and season of delivery shown (n=16)

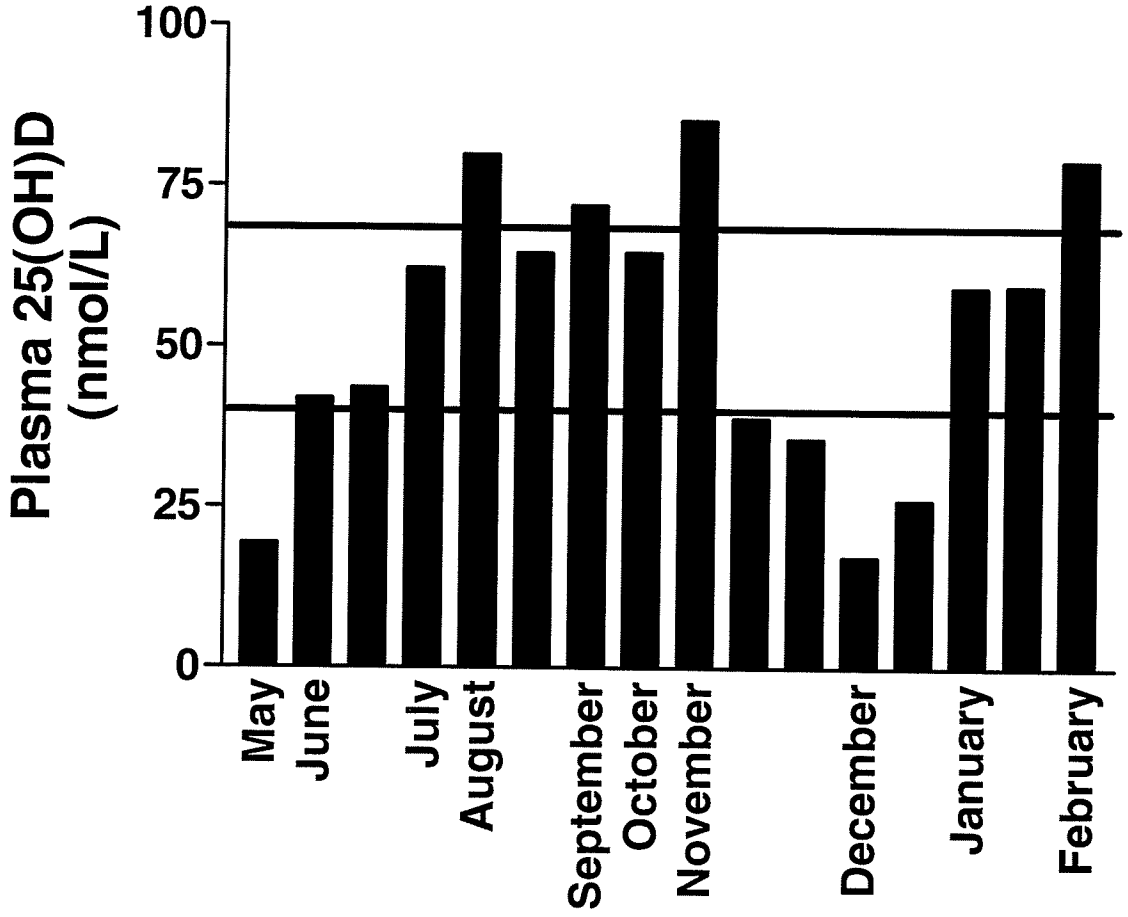


Figure 6.7: Individual plasma 25-hydroxyvitamin D of term mothers measured at time of delivery. Deficient (<40.0 nmol/L) and low end of normal (<73 nmol/L) indicators and season of delivery shown (n=30)

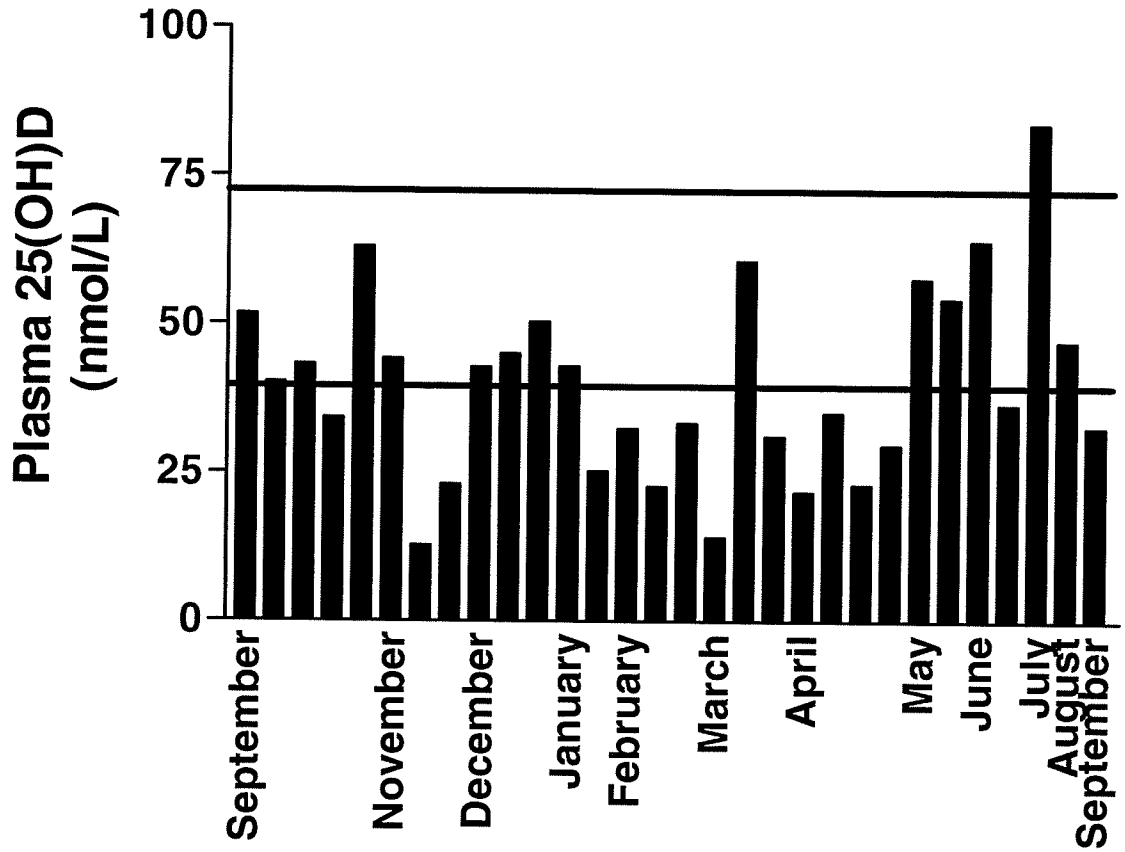
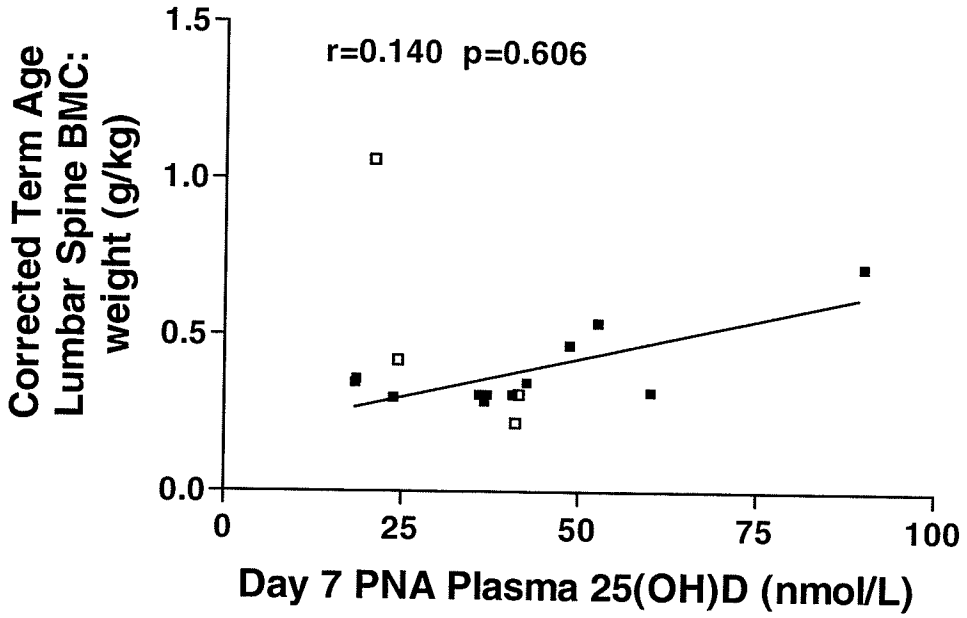


Table 6.8: Relation of day 7 PNA plasma 25-hydroxyvitamin D and hospital discharge plasma 25-hydroxyvitamin D of very preterm infants to their hospital discharge lumbar spine and femur bone mineral content.

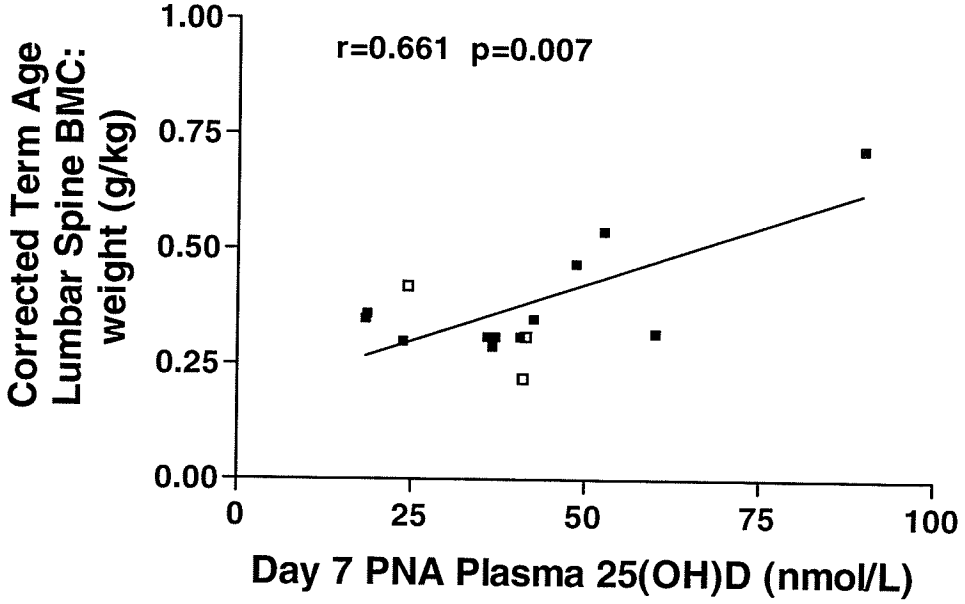
	Variable	r value	p value
Plasma 25(OH)D Day 7 PNA	Lumbar Spine BMC (g)	0.2617	0.3275
	Lumbar Spine BMC: length (g/cm)	0.3861	0.1552
	Femur BMC (g)	0.3544	0.2138
	Femur BMC: weight (g/kg)	0.3159	0.2713
	Femur BMC: length (g/cm)	0.3332	0.2444
Plasma 25(OH)D Hospital Discharge	Lumbar Spine BMC (g)	-0.01598	0.9532
	Lumbar Spine BMC: weight (g/kg)	-0.02403	0.3699
	Lumbar Spine BMC: length (g/cm)	0.01087	0.9681
	Femur BMC (g)	0.4978	0.0701
	Femur BMC: weight (g/kg)	0.1165	0.6916
	Femur BMC: length (g/cm)	0.3722	0.1900

Figure 6.8a: Relationship between plasma 25-hydroxyvitamin D status at day 7 PNA and lumbar spine bone mineral content corrected to weight (g/kg) at corrected term age (n=16).



Non-colored squares represent infants with BPD

Figure 6.8b: Relationship between plasma 25-hydroxyvitamin D status at day 7 PNA and lumbar spine bone mineral content corrected to weight (g/kg) at corrected term age after removal of outlier (n=15).

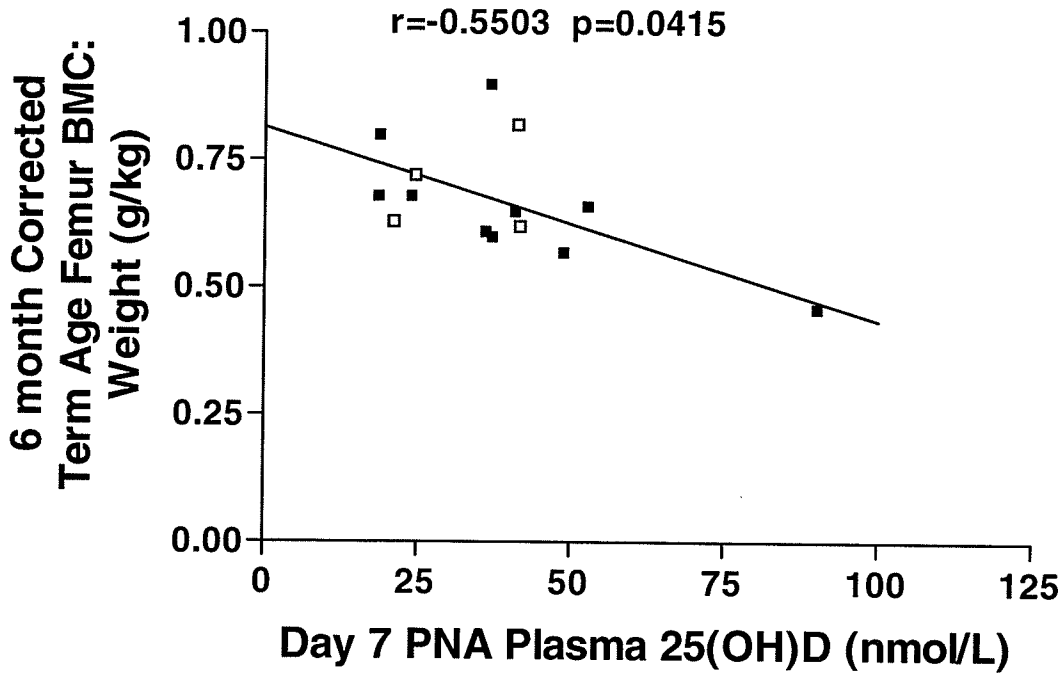


Non-colored squares represent very preterm infants with BPD

Table 6.9: Relation of day 7 PNA plasma 25-hydroxyvitamin D and hospital discharge plasma 25-hydroxyvitamin D of very preterm infants to their 6 month corrected age lumbar spine and femur BMC.

	Variable	r value	p value
Plasma 25(OH)D Day 7 PNA	Lumbar Spine BMC (g)	-0.2039	0.5041
	Lumbar Spine BMC: weight (g/kg)	-0.1931	0.5273
	Lumbar Spine BMC: length (g/cm)	-0.3012	0.3174
	Femur BMC (g)	-0.4626	0.0958
	Femur BMC: length (g/cm)	-0.5121	0.0612
Plasma 25(OH)D Hospital Discharge	Lumbar Spine BMC (g)	-0.0116	0.9701
	Lumbar Spine BMC: weight (g/kg)	0.1457	0.6348
	Lumbar Spine BMC: length (g/cm)	0.0473	0.8897
	Femur BMC (g)	-0.3558	0.2119
	Femur BMC: weight (g/kg)	-0.2284	0.4322
	Femur BMC: length (g/cm)	-0.3385	0.2364

Figure 6.9: Relationship between plasma 25-hydroxyvitamin D status at day 7 PNA and femur bone mineral content corrected to weight (g/kg) at 6 months corrected age (n=14).



Non-coloured squares represent infants with BPD.

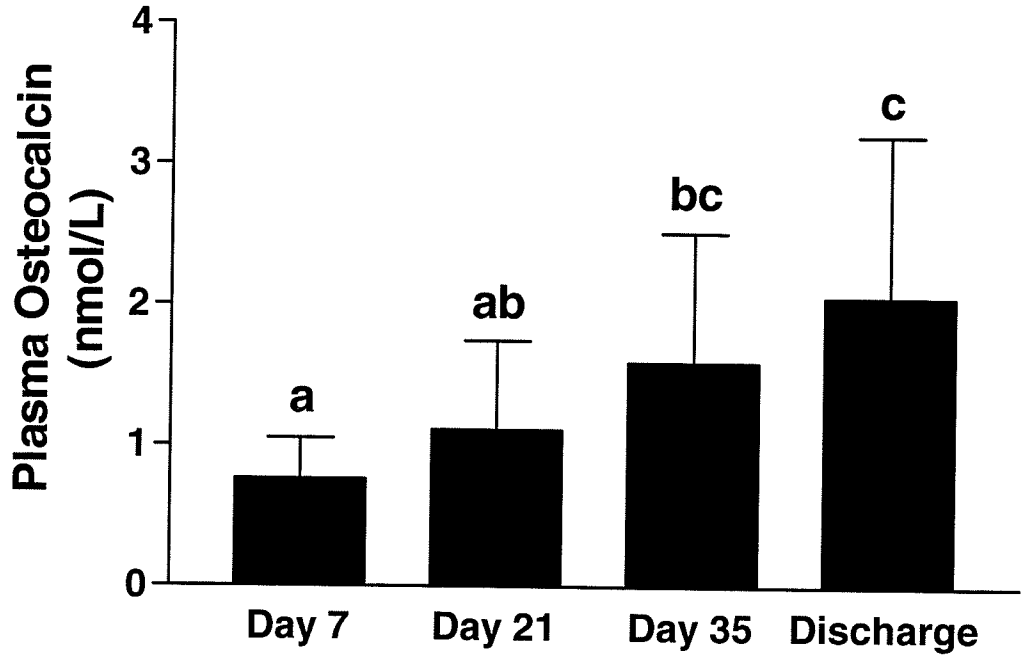
Table 6.10: Relation of cord plasma 25-hydroxyvitamin D of term infants to their birth lumbar spine and femur bone mineral content.

	Variable	r value	p value
25(OH)D Cord Plasma	Lumbar Spine BMC (g)	-0.0778	0.6827
	Lumbar Spine BMC:weight (g/kg)	0.1622	0.3917
	Lumbar Spine BMC: length (g/cm)	0.07579	0.6906
	Femur BMC (g)	-0.0059	0.9758
	Femur BMC: weight (g/kg)	0.2539	0.1758
	Femur BMC: length (g/cm)	0.1561	0.4100

Table 6.11: Relation of cord plasma 25-hydroxyvitamin D of term infants to their 6 month lumbar spine and femur bone mineral content.

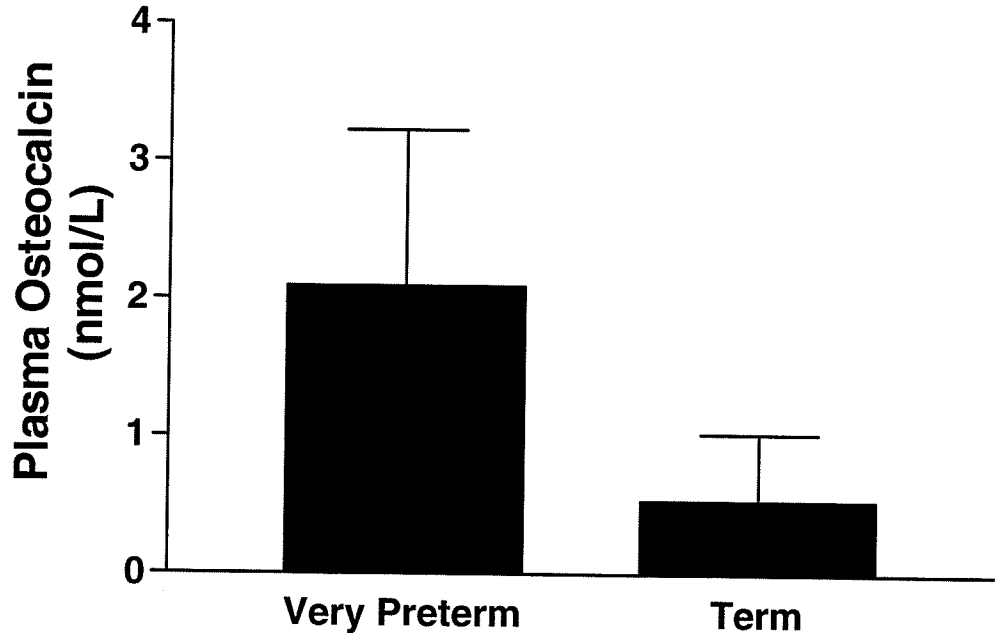
	Variable	r value	p value
25(OH)D Cord Plasma	Lumbar Spine BMC (g)	-0.1290	0.4969
	Lumbar Spine BMC:weight (g/kg)	-0.08869	0.6412
	Lumbar Spine BMC: length (g/cm)	-0.0344	0.8569
	Femur BMC (g)	0.1451	0.4442
	Femur BMC: weight (g/kg)	0.1470	0.4382
	Femur BMC: length (g/cm)	0.1989	0.2920

Figure 6.10: Plasma osteocalcin at day 7, 21, 35 PNA and hospital discharge in very preterm infants.



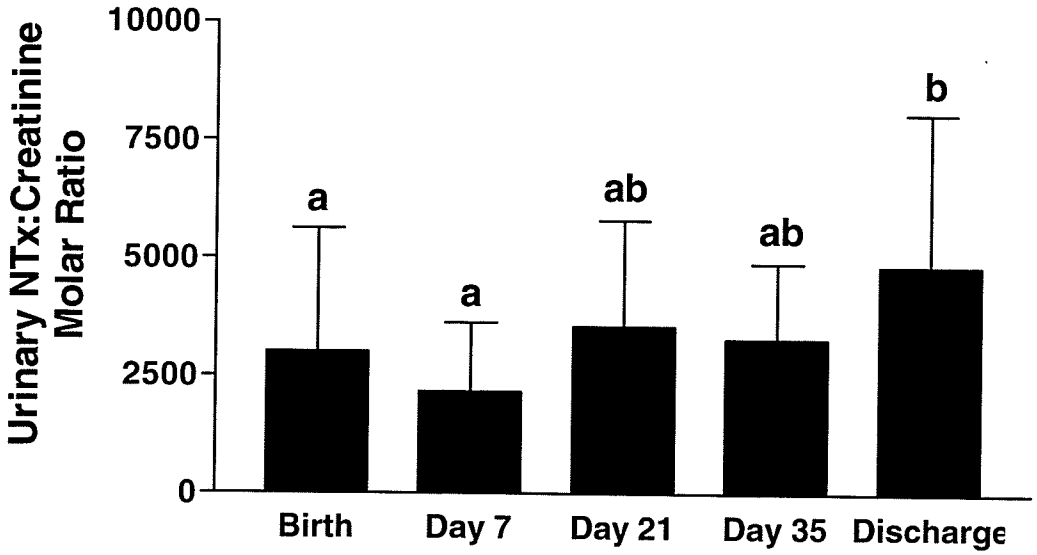
Data shown as mean ± SD, n=16, unless otherwise noted.
Discharge (n=14): 2 infants hospital discharge coincided with day 35 measurement.
Significant differences as analyzed by Repeated Measures ANOVA and Tukey's Post-hoc Text ($p \leq 0.003$).

Figure 6.11: Plasma osteocalcin at hospital discharge in very preterm infants and cord plasma in term infants.



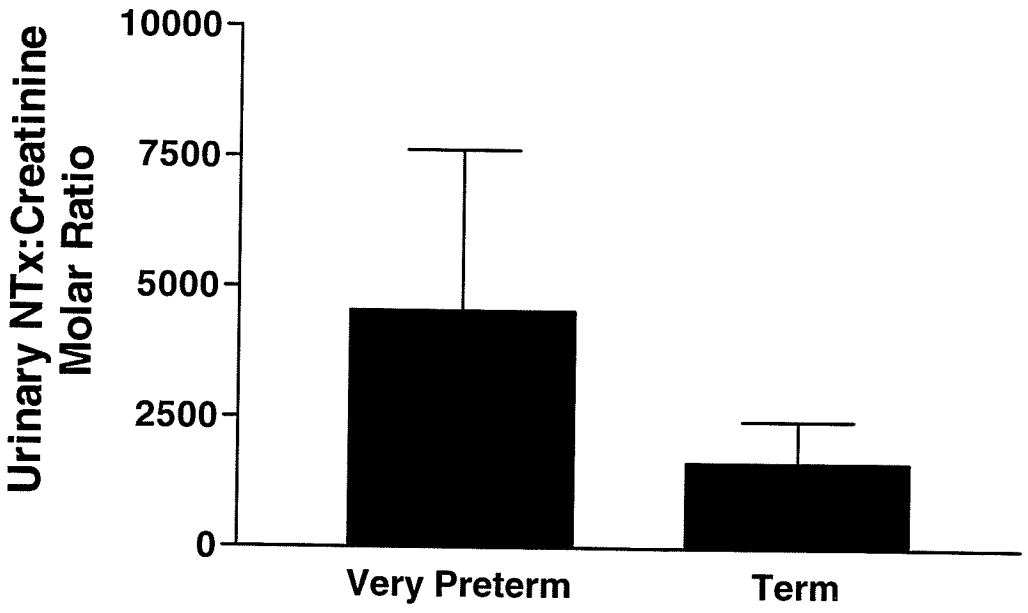
Data shown as mean ± SD.

Figure 6.12: Urinary NTx at birth, day 7, 21, 35 PNA and hospital discharge in very preterm infants.



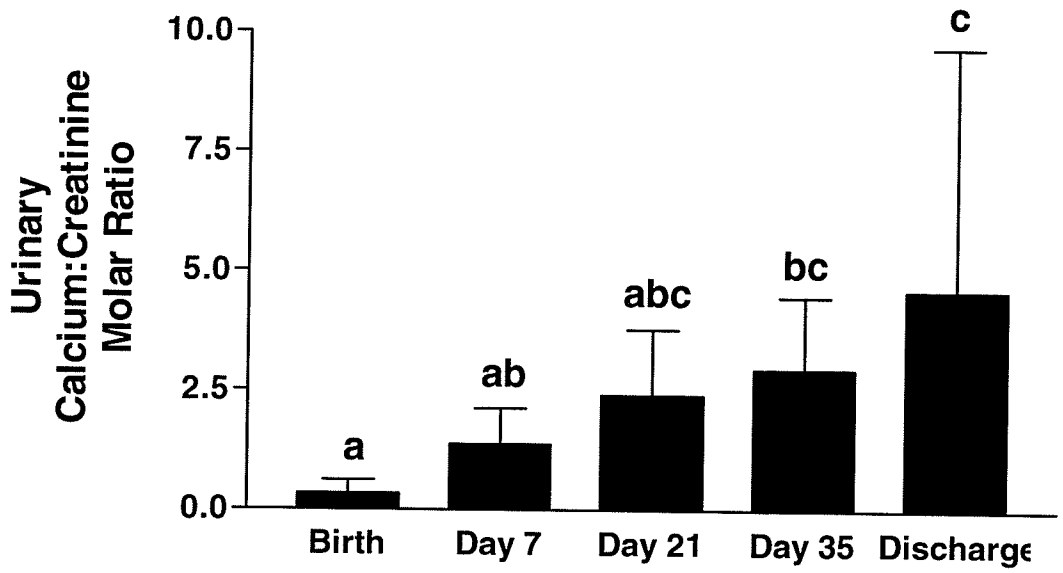
Data shown as mean ± SD.
n=16 unless otherwise noted.
Birth (n=15): unable to obtain urine sample from one infant.
Discharge (n=12): two infants hospital discharge coincided with day 35 measurement and unable to obtain a urine sample on the other two infants.
Significant differences as analyzed by Repeated Measures ANOVA and Tukey's Post-hoc Test ($p \leq 0.03$).

Figure 6.13: Urinary NTx at hospital discharge in very preterm infants and birth in term infants.



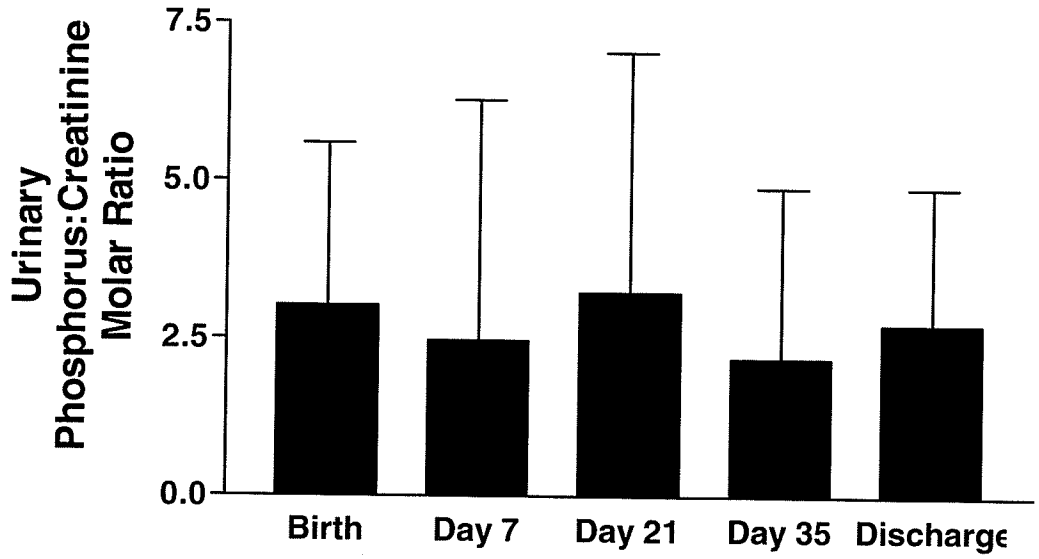
Data shown as mean ± SD.
n=16 for very preterm infants.
n=25 for term infants as unable to obtain urine sample from 5 infants.

Figure 6.14: Urinary calcium at birth, day 7, 21, 35 PNA and hospital discharge in very preterm infants.



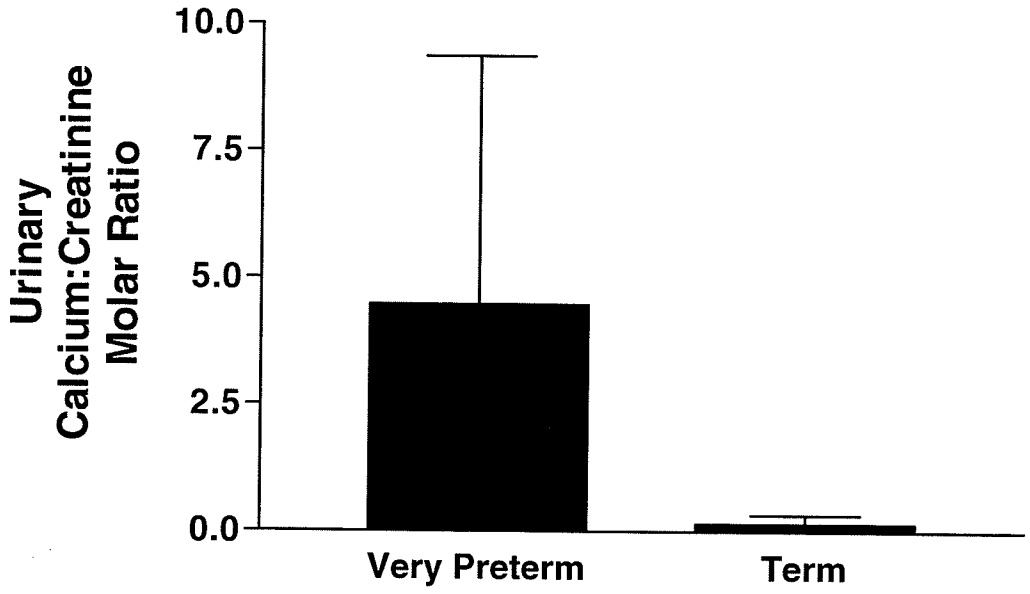
Data shown as mean ± SD.
n=16 unless otherwise noted.
Birth (n=15): unable to obtain urine sample from one infant.
Day 21 (n=15): one urine sample was non-detectable at < 0.05 ppm.
Day 35 (n=15): one urine sample was non-detectable at < 0.05 ppm.
Discharge (n=12): two infants hospital discharge coincided with week 5 and unable to obtain urine sample from two infants.
Significant differences as analyzed by Repeated Measures ANOVA.
Bars with different superscripts are significantly different ($p \leq 0.02$)

Figure 6.15: Urinary phosphorus at birth, day 7, 21, 35 PNA and hospital discharge in very preterm infants.



Data shown as mean ± SD.
Birth (n=15): unable to obtain sample from one infant.
Day 7 (n=10): 6 urine samples had non-detectable concentrations (< 0.10 ppm).
Day 21 (n=15): 1 urine sample had non-detectable concentrations (< 0.10 ppm).
Day 35 (n=14): 2 urine samples had non-detectable concentrations (< 0.10 ppm).
Discharge (n=11): two infants hospital discharge coincided with week 5 measurement, unable to obtain urine sample from two infants and one infant with non-detectable concentrations (< 0.10 ppm).
No significant differences as analyzed by Repeated Measures ANOVA and Tukey's post-hoc Test.

Figure 6.16: Urinary calcium at hospital discharge in very preterm infants and birth in term infants.



Data shown as mean ± SD.

Figure 6.17: Lumbar spine bone mineral content of very preterm infants at hospital discharge and 6 months corrected age. Reference group indicated.

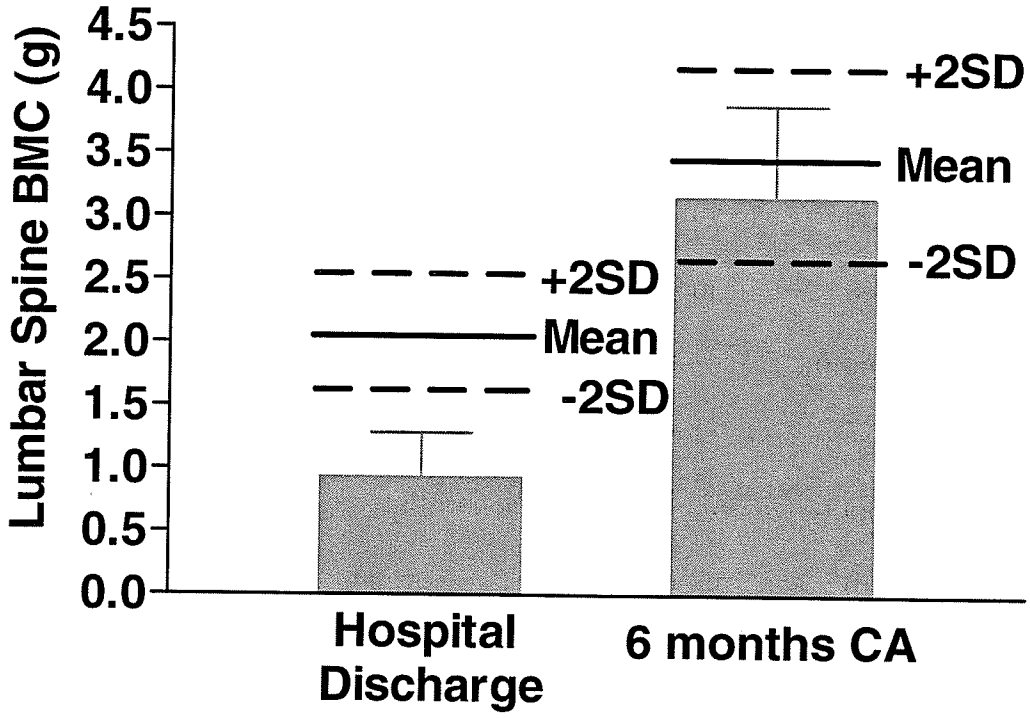


Figure 6.18: Femur bone mineral content of very preterm infants at hospital discharge and 6 months corrected age. Reference group range indicated.

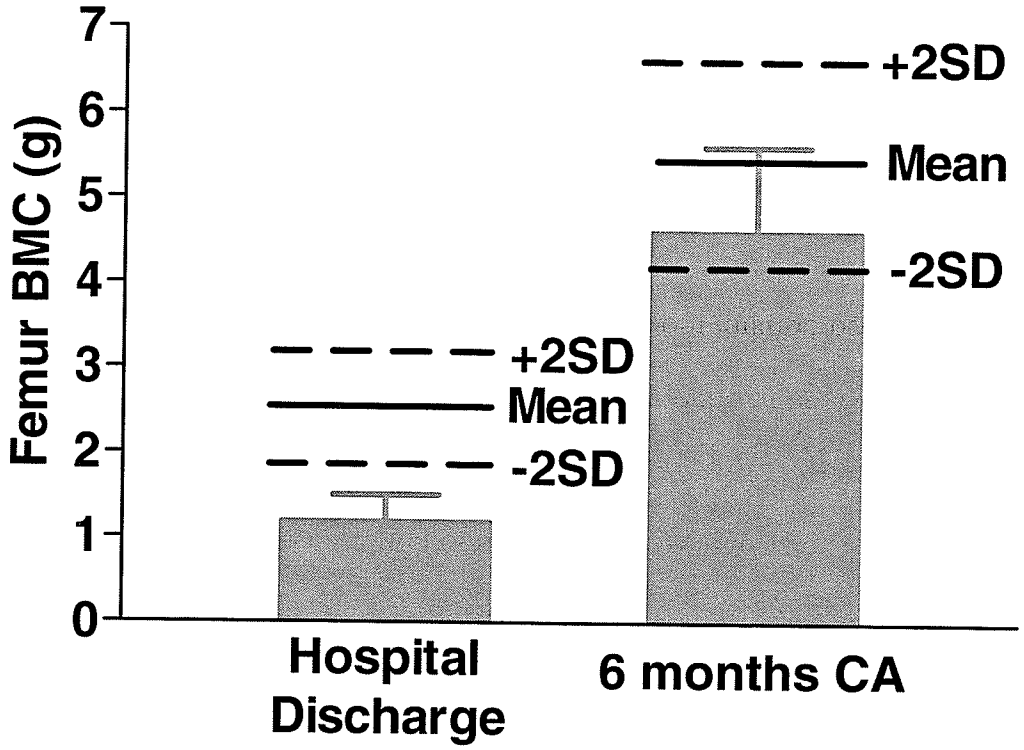


Figure 6.19: Lumbar spine bone mineral content of very preterm infants corrected to weight at hospital discharge and 6 months corrected age. Reference range indicated.

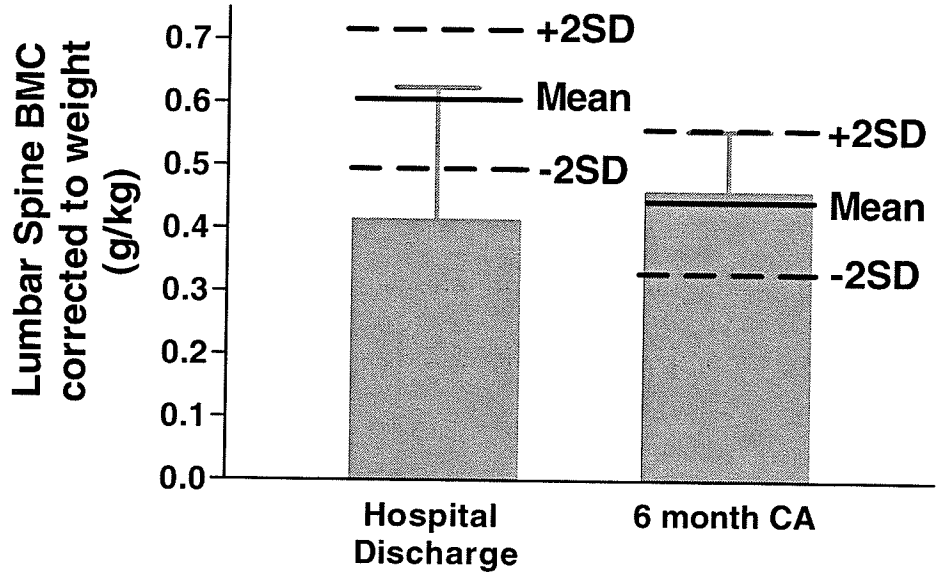


Figure 6.20: Lumbar spine bone mineral content of very preterm infants corrected to length at hospital discharge and 6 months corrected age. Reference range indicated.

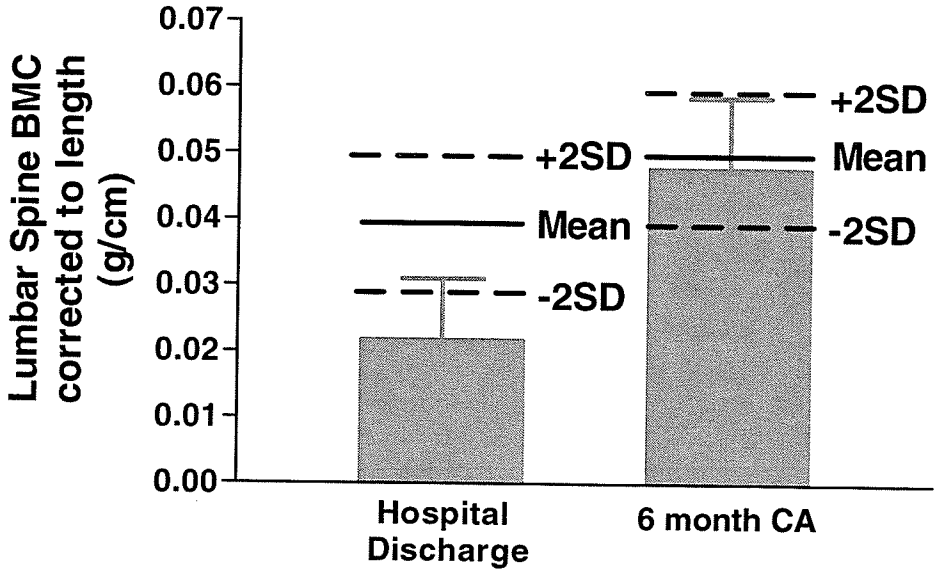


Figure 6.21: Femur bone mineral content of very preterm infants corrected to weight at hospital discharge and 6 months corrected age. Reference range indicated.

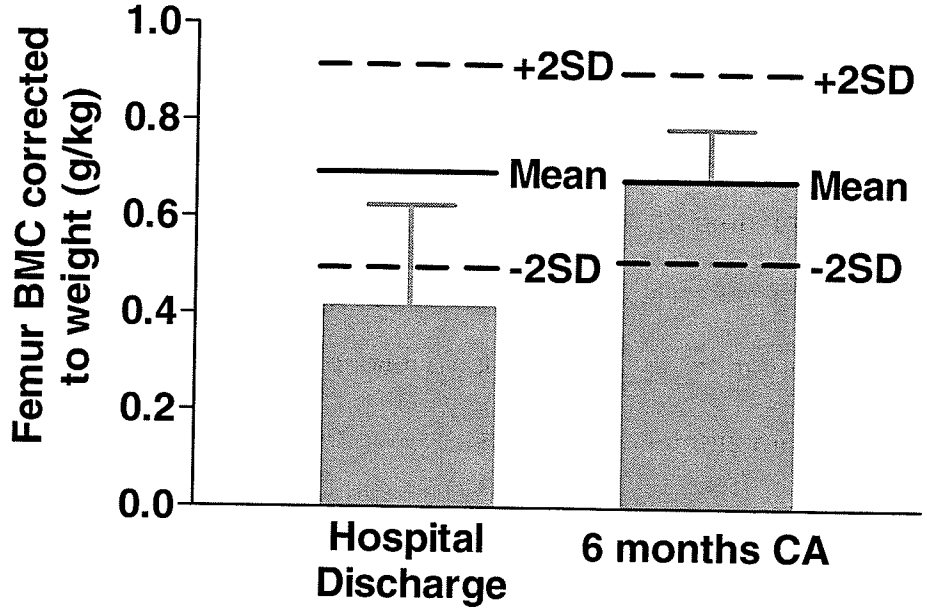


Figure 6.22: Femur bone mineral content of very preterm infants corrected to length at hospital discharge and 6 months corrected age. Reference range indicated.

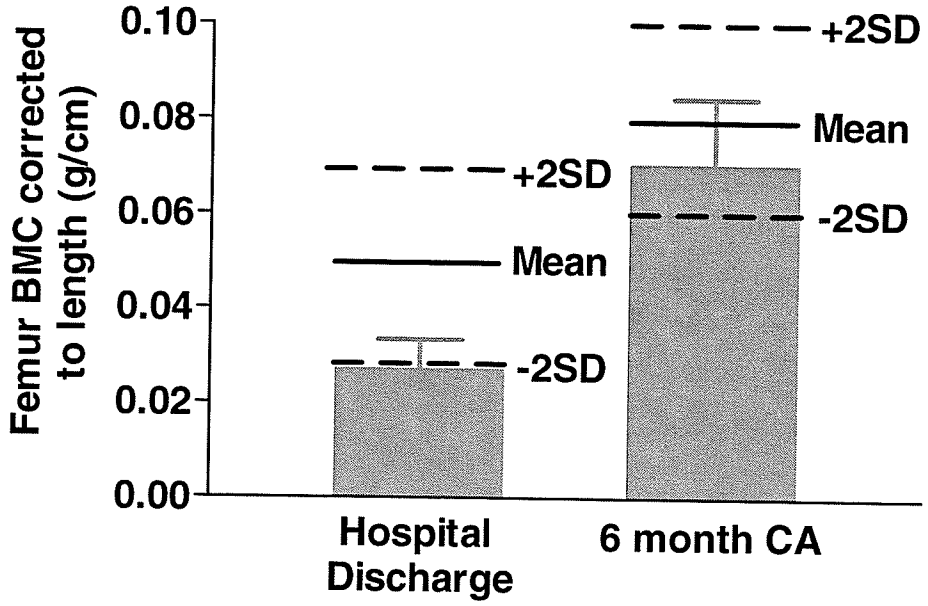


Table 6.12: Anthropometry and bone mineral content and bone mineral density values as measured by dual energy x-ray absorptiometry of very preterm and term mothers.

	Very Preterm Mothers	Term Mothers
	(n=14)	(n=29)
Weight (kg)	66.6 ± 14.8	73.6 ± 15.6
Height (cm)	159.3 ± 6.4	161.6 ± 7.8
BMI (kg / m^2)	26 ± 6	28 ± 6
Lumbar Spine (L1-4)		
BMC (g)	52.52 ± 6.51	58.09 ± 6.80
BMD	0.95 ± 0.10	1.011 ± 0.11
T-Score	-0.80 ± 0.85	-0.32 ± 0.89
Z-Score	-0.68 ± 0.81	-0.19 ± 0.92
Total hip		
BMC (g)	25.67 ± 4.43	29.41 ± 6.02
BMD	0.89 ± 0.11	0.92 ± 0.11
T-Score	-0.43 ± 0.96	-0.19 ± 0.90
Z-Score	-0.28 ± 1.01	-0.08 ± 0.93

Data shown as mean + SD.
Scans were performed on mothers 6 months after delivery or 6 months after finishing breast-feeding.
Weight and height were measured at the time of the DEXA scan.

7.0 Interpretation of Results and Future Research

7.1 Discussion

Several populations in different geographical regions show evidence of plasma 25(OH)D deficiency in recent years including term infants in China (Specker et al., 1992), children in Northern Manitoba (Lebrun et al., 1993), women of child-bearing age in Northern Manitoba (Lebrun et al., 1993), in North West Territories (Waiters et al., 1998), and in Toronto (Vieth et al., 2001) and men and women throughout the age span in Calgary (Rucker et al., 2002). This research also shows prevalence of plasma 25(OH)D deficiency in term infants and women of child-bearing age as well as very preterm infants within Manitoba (latitude 49°N). Thirty-one percent of very preterm infants and 27% of term infants were deficient at birth. Seventy-three percent of very preterm mothers and 97% of term mothers had plasma 25(OH)D concentrations below the low-end of normal, defined as $\leq$ 73 nmol/L (Vieth et al., 2001). Term infants cord plasma 25(OH)D status has been shown to be significantly related to maternal plasma 25(OH)D at delivery (Markestad, 1983), which was also evident in our term infant-mother population. A significant relationship was not seen with very preterm infants and their mothers. However, we did not have a cord plasma 25(OH)D sample, but rather were looking at the relationship between day 7 PNA and mothers plasma 25(OH)D. By day 7, infants had received vitamin D through their feeds, therefore, their plasma 25(OH)D status was likely different and may not have been truly reflective of their cord plasma 25(OH)D status. Therefore, a significant relationship may have been evident if cord blood 25(OH)D was known.

During pregnancy, over half of all mothers took a prenatal supplement, which contained vitamin D. However, this was still not enough vitamin D, along with diet and sun exposure to maintain adequate 25(OH)D. Indeed, 50% of the term mothers who took a prenatal supplement during pregnancy had deficient concentrations of 25(OH)D. Nutrient intake during pregnancy was unknown; however, post-delivery, average intake of vitamin D was not adequate in term mothers to meet the DRI. When the DRIs were developed, there were difficulties in setting the adequate intake (AI) due to the lack of research and confounding factors, including sunlight exposure, sunscreen use, location and season (Standing Committee on Dietary Reference Intakes, 1998). Our findings suggest that at our latitude and geographical location, the recommended amount for women of child-bearing age may be inadequate to promote adequate plasma 25(OH)D stores throughout pregnancy.

Although term infants can be at risk of 25(OH)D deficiency, very preterm infants are at further risk. It is during the third trimester, which very preterm infants miss, when the majority of nutrient transfer occurs (Paulson & Deluca, 1986). As mentioned earlier, 31% of very preterm infants and 27% of term infants had deficient concentrations of plasma 25(OH)D at birth. A deficiency of plasma 25(OH)D in infants with growing bones may lead to rickets. Rickets, by many, is considered a disease of the past. However, with the recent evidence of plasma 25(OH)D deficiency in infant and child populations (Specker et al., 1992, Lebrun et al., 1993, Namgung et al., 1998, Park et al., 1998), it can be assumed

this disease likely continues to exist. Indeed, in Manitoba, rickets has still been shown to be evident in recent years (Haworth & Dilling, 1986, Haworth, 1995).

We followed the plasma 25(OH)D status of very preterm infants through their hospital stay. At day 7 PNA, approximately one third of the infants had deficient plasma 25(OH)D. At day 21, all infants had normal plasma 25(OH)D, however, by day 35, 6% had deficient plasma 25(OH)D. In the hospitalized very preterm infant, synthesis of vitamin D can not occur regardless of time of year; therefore they must initially rely solely on their vitamin D stores and oral vitamin D to reach their required intake. For the first several days of life, infants are receiving parenteral feeds, during which time they receive adequate vitamin D to meet their requirements. However, it is during the transition from parenteral feeds to enteral feeds where vitamin D intake is compromised. On average, vitamin D supplementation was not initiated in our infants until day 26 $\pm$ 6.6 PNA. During the transition period, infants received adequate vitamin D, which likely assisted in normalizing their plasma 25(OH)D stores. However, infants were compromised by not receiving adequate vitamin D orally between day 8-35 PNA leading to 6% of infants having a deficient concentration again at day 35 PNA. This illustrates the importance of ensuring adequate oral vitamin D to very preterm infants consistently through their first weeks of life.

In order to meet the requirements for vitamin D, supplementation is required in infants. We did not influence the supplementation of our infants, however, on average, infants were provided with 200-400 IU/day. Supplementation may need to be initiated sooner in very preterm infants in order

to help maintain/achieve plasma 25(OH)D. Vitamin D supplementation is typically not initiated until full feeds are achieved and tolerated, which was the case with our study infants, which may be too late. There is currently no research that considers if it would be safe to initiate supplementation earlier to eliminate the difficulty in achieving adequate concentrations during the transition between parenteral and enteral feeds.

Synthesis of 25(OH)D does not occur at our latitude between April and September (Webb et al., 1988). Studies have looked at seasonal variation of plasma 25(OH)D among term infants (Verity et al., 1981, Kuroda et al., 1981, Nehama et al., 1987, Namgung et al., 1994, Namgung et al., 1998), and very preterm infants (Mawer et al., 1986). Mawer et al., 1986 found that very preterm infants born during summer months (July to September) had higher concentrations of plasma 25(OH)D than those born during winter months (January to March). Due to the small population size of our study, we divided the year into two seasons, spring/summer and fall/winter. We also found that very preterm infants born during spring/summer had significantly higher plasma 25(OH)D concentrations than infants born during fall/winter. If the very preterm infants born during spring/summer would have continued until term, they likely would have been born with adequate plasma 25(OH)D stores. This is based on two assumptions: these infants had increased 25(OH)D stores at 28-32 weeks gestation and the duration of pregnancy would be during sun synthesizing months, therefore the infants would likely have maintained their stores. Those very preterm infants born during fall/winter, if continued to term, would have been

born during winter/spring, likely with inadequate 25(OH)D stores. This would agree with the term infant research on seasonal variation of plasma 25(OH)D. Term infants born during fall/winter have been shown to have lower 25(OH)D than those infants born during spring/summer (Kuroda et al., 1981, Nehama et al., 1987). Interestingly, our term infants did not show a seasonal difference in plasma 25(OH)D. Namgung et al. (1994) looked at term infants in Ohio, USA and also found no seasonal variation. They attributed this to an increase in the number of mothers taking a prenatal supplement towards the end of pregnancy. This may also explain our results as 93% of the mothers delivering between October and March took a prenatal supplement.

We did not observe a seasonal variation among our mothers at time of delivery. This is contrary to several other studies which found that mothers delivering in spring/summer had higher plasma 25(OH)D than those mothers delivering during autumn/winter (Kuroda et al., 1981, Nehama et al., 1987, Namgung et al., 1998). However, in these studies, mothers either did not take a vitamin D supplement during pregnancy (Nehama et al., 1987) or very few mothers took a supplement (Namgung et al., 1998). In our study, 71% of very preterm mothers delivering between April and September and 44% of mothers between October and March took a prenatal supplement containing vitamin D. For term mothers, 62.5% of those delivering between April and September and 93% of those delivering between October and March took a prenatal supplement. The higher rate of prenatal supplement use in our study may explain why we did not observe a difference in plasma 25(OH)D status among seasons.

Several studies have looked at bone metabolism markers in very preterm infants at birth and corrected term age, however, few have measured these markers at time points throughout the infant's hospital stay. As well, it is important to look at markers of both bone resorption and formation to obtain the full picture of bone metabolism. Urinary NTx and calcium are both indicative of bone resorption. We found that both of these markers increased throughout hospital stay until discharge. As well, urinary NTx and calcium were both higher at corrected term age/hospital discharge than term infants at birth. Mora et al. (1997) also looked at urinary NTx excretion in infants ranging in age from 32 to 42 weeks gestation. In contradiction to our results, they suggested urinary NTx concentrations slow in proximity to term age. However, they looked only at birth samples of the infants at different gestational ages and did not consider the pattern over time. Indeed, if we looked at the birth samples of our very preterm infants and term infants we could make the same conclusion. However, this is not indicative of the pattern of NTx over the first few weeks of life in very preterm infants. Similar to our results, Beyers et al. (1986), found that preterm infants at corrected term age had significantly higher concentrations of hydroxy-proline, another marker of bone resorption, than term infants at birth. They also found higher concentrations of urinary calcium excretion in preterm infants than term infants. Their conclusion was an increased level of bone resorption during first weeks of life in preterm infants, which would agree with our findings.

Plasma osteocalcin is used to measure bone formation. We found that osteocalcin concentrations continuously increased throughout very preterm

infant's hospital stay. Compared with term infants, very preterm infants had a higher level at corrected term age than term infants at birth. Shiff et al. (2001) also found that osteocalcin concentrations continuously increased throughout the first 10 weeks of life in very preterm infants. Bhandari et al. (1999) also looked at osteocalcin for the first 6 weeks of life in very preterm infants and found that concentrations increased significantly between birth and week 1 and then reached a plateau. All of these studies, and ours, point to an increased rate of bone formation occurring in very preterm infants.

Lumbar spine (L1-4) and femur BMC was measured in very preterm infants at corrected term age and term infants at birth using DEXA. Very preterm infants had lower lumbar spine (L1-4) and femur BMC compared to term infants. Very preterm infants continued to have lower lumbar and femur BMC even after correction for weight and length. Beyers et al. (1986) also found that very preterm infants had significantly lower cortical index (a measure of BMC which negates the influence of body size) in very preterm infants at corrected term age compared with term infants. Minton et al. (1979) found that the post-natal rate of increase in BMC in preterm infants was significantly less than that expected in utero. For infants born term, BMC had increased by 46% between birth and 12 weeks of age. In preterm infants (31-32 week gestation age), BMC increased 12% during this similar time period (Minton et al., 1979). Therefore, at corrected term age, very preterm infants are compromised in both size and mineralization of bone.

At the follow-up at 6 months corrected age, very preterm infants remained smaller in weight and length. Lumbar spine and femur DEXA scans were again performed. Lumbar spine and femur BMC continued to be lower in very preterm infants compared to term infants. However, after correcting for weight and length, the differences in BMC were no longer evident. This observation suggests that infants are able to mineralize their bones, but their growth is smaller. This observation continues to be evident in very preterm infants into their early teen years. Fewtrell et al. (1999) also found that preterm infants at 12 years of age remained shorter and lighter, but had BMC appropriate for size.

To look at the effects 25(OH)D status had on BMC, correlation analysis was performed. A significant positive relationship was shown between day 7 PNA 25(OH)D and lumbar spine BMC corrected to weight at hospital discharge. This suggests that higher 25(OH)D status close to birth leads to higher lumbar spine BMC at corrected term age.

This relationship could be an effect of programming. Early diet has been shown to affect BMC in childhood. At 5 years of age, BMC adjusted for size was higher in preterm infants who were fed human milk vs. those fed formula (Lucas, 1998). Within our study, we may be seeing signs of both maternal and transition period nutrition affecting BMC of the infants later in life. Infants born with inadequate 25(OH)D have lower BMC of lumbar spine at hospital discharge. As well, our infants on average, throughout their hospital stay received inadequate amounts of both calcium and phosphorus in their diet. Inadequate calcium intake leads to decreased plasma calcium concentrations, which activates PTH and

eventually leads to resorption of bone. If programming was evident, one might expect to see other bones, including femur affected. However, there was no relationship seen with femur BMC, and as well, by 6 months corrected age, the significant relationship between day 7 PNA 25(OH)D and lumbar spine corrected to weight was no longer evident.

At 6 months corrected age, a negative relationship is noted between day 7 PNA 25(OH)D and femur BMC corrected to weight in very preterm infants. Several variables affect bone growth and mineralization, including, but not limited to genetics (Rubin et al., 1999), and nutrition (Rigo et al., 2000). Due to our sample size, we were limited to doing correlation analysis, and therefore were unable to perform multiple regression analysis, which would have eliminated confounding factors, such as those listed above. Therefore, it is possible that the results we are seeing are a false correlation and simply an effect of our sample size.

7.2 Strengths and Limitations

The strength of this research is that all aspects of bone metabolism, including resorption, formation and bone mass were studied in very preterm infants. This allows for a better understanding of the mechanisms at play. As with most research, there were several limitations. The sample size of very preterm infants was small in this study due to lesser amounts of infants being born that met our criteria during our recruitment period, therefore, regression analysis was unable to be performed. When looking at the effect 25(OH)D has on bone, we were unable to account for the confounding factors. A second

limitation was that infants were only followed until 6 months of age. The consequences of the low 25(OH)D on bone may not be evident until later in life. Infants may be developing rickets, however, the diagnosis may not be evident until after 6 months of age. Another limitation of the study is that we were unable to obtain biochemical data from the infants at their 6 month follow-up appointment due to ethical issues. Six month biochemical data would have allowed us to look again at all aspects of bone metabolism. As well, 25(OH)D could again be obtained to determine if infants were able to maintain a normal level post-discharge.

7.3 Future Research

On the basis that vitamin D deficiency is still evident, future research is required in terms of both mothers and infants.

In regard to the mothers, research should focus on amount of knowledge regarding vitamin D and appropriate concentrations of vitamin D intake, taking into consideration season and latitude. Determining the level of knowledge, in women of child-bearing age to the importance of nutrition during pregnancy and how it impacts their impending fetus would be of benefit. Secondly, it would be beneficial to determine when mothers are informed about vitamin D during the prenatal period and if intervention and increased awareness are required. Research focusing on the appropriate level of intake of vitamin D during pregnancy to maintain plasma 25(OH)D in both mothers and fetus is also important.

Research on infants should focus on prevalence of rickets, vitamin D supplementation and growth of the infants. Rickets occurs when a vitamin D deficiency is present; therefore determining the prevalence, both provincially and nationally, would be helpful in understanding the extent of the problem. Vitamin D supplementation research could focus on 2 areas. First, looking at safety of initiating vitamin D supplementation earlier than tolerance to full enteral feeds would be of benefit. If vitamin D supplementation was safe to start when enteral feeds are initiated, this may help maintain 25(OH)D stores from birth onward. Secondly, several factors including sunlight exposure, oral intake, latitude and season affect vitamin D requirements. Determining requirements for specific geographical locations, seasons and ages may also help lower the rate of 25(OH)D deficiency.

By 6 months of age, we are seeing compromised growth of very preterm infants, although they are adequately mineralizing their bones. Individuals with petite stature are at an increased risk of fractures later in life (Javaid & Cooper, 2002), therefore focusing research on optimizing growth through nutrition would be of benefit. Following these infants throughout their life would also be value to see if catch-up growth occurs and also to see if the deficient 25(OH)D concentrations at birth have an impact later in life on their bone.

7.4 Conclusions

In conclusion, we continue to see high rates of 25(OH)D deficiency among very preterm and term infants and women of childbearing age. Very preterm infants have high rates of both bone resorption and formation by corrected term

age, as well as compromised growth and bone mineral content. However, by 6 months corrected age, although their growth remains smaller than term infants, they are able to adequately mineralize their bones.

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Appendix A

THE UNIVERSITY OF MANITOBA

FACULTY OF HUMAN ECOLOGY

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RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Growth and bone mineralization in premature infants

Protocol Number: B2000:204

Principal Investigator: Dr. H. Weiler, RD, PhD
Co-Investigator: Dr. M.M. Seshia, MBChB; Dr. C.K. Yuen, MD, MBA;
Sponsor: University of Manitoba, Winnipeg, Manitoba, R3T 2N2

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

This study is being conducted to study nutritional status and growth of infants. You are being asked to take part in this study because you are soon to or have just delivered a newborn infant. A total of 64 mother infant pairs will participate in this study.
The purpose is to study the growth patterns and bone development of babies that are born early (prematurely) and to examine if specific nutrients are linked to infant growth and bone development. The growth and bone development of these babies will also be compared with that of babies born at the expected due date (full-term). This research is being done because research suggests that growth in height, weight, and head size and the amount of bone mineral are different in babies born prematurely compared to those born at the expected term date. The nutrient of interest is arachidonic acid. This nutrient is normally made by the liver and is essential for growth. Thus this study will help to determine if prematurely born babies with greater amounts of arachidonic acid, as indicated in a small blood sample, experience improved growth and bone mineral content

and will help to advance nutritional practices. In addition, the influence of growth over the first year of life on body size at one year of age will also be studied.

Study Procedures

Phase One: Since your baby was born early, you and your baby are being asked to participate in this research study as part of the preterm group of the study. The study will begin at the time of your baby's birth and will continue until he/she is discharged from the hospital or reaches the age he/she would have been had they been delivered at expected due date. Your infant will also be followed in Phase Two – see below.

In order to participate in this study, you must not have a personal history of diabetes before or during your pregnancy and your doctor will determine whether or not any medications you are currently taking will interfere with the proposed measurements. Your baby will be examined at birth as part of routine clinical care for any medical conditions that may exclude him/her from participating in this study.

Phase Two: at discharge from hospital, 6 and 12 months thereafter, we ask that you and your infant visit our research facility to enable measurement of growth and bone as well as collect information on what you and your infant are eating. We would also like to measure your bones at the 12-month visit.

If you take part in this study, you will have the following tests and procedures:

Your Infant's Involvement

We request your permission to obtain the following samples from your premature baby: a sample of blood from the placenta (afterbirth) taken routinely at birth; measurement of your baby's length, weight, and head size once a week starting from one week of age; a urine sample from your baby at birth, one, three and five weeks of age and at term. At one, three and five weeks of age and at term, a blood sample (approximately 0.5 ml or 1/10 a teaspoonful) from your baby will be collected at the same time as for routine monitoring.

We also ask your permission to scan your baby's whole body, lower (lumbar) spine and thigh bone (femur) using a low dose x-ray technique called dual energy x-ray absorptiometry at the research facility immediately after hospital discharge. For the second part of the study we wish to measure your baby's weight, length and head size as well as the same measurements on bone at 6 and 12 months. This will help us learn about the relationships among birth size, nutrition, growth and growth by one year after discharge from hospital.

Your Own Involvement

We request your permission to take a blood sample (approximately 10 ml or 2 teaspoonfuls) from you prior to your discharge from hospital. To help us learn about mother's diet and breast milk, you will be asked to keep a three-day record of food intake after you have been discharged. This will be reviewed with you approximately one week

later at a convenient time, for example, while you are visiting your baby in hospital or over the telephone when you are at home. This should take no more than half an hour.

You will decide on your own whether you wish to breast-feed or to feed your baby infant formula. If breast-feeding your baby, you will be asked to provide a sample of your breast milk on two days of each week, to be collected at 9:00 a.m. and 9:00 p.m. by manual expression or with a breast pump, for nutrient analysis. The amount required is small (approximately 5 ml or 1 teaspoonful) and should not interfere with the normal feeding of your infant.

At the follow-up visits, at your infant's discharge from hospital, 6 and 12 months, we will ask you about the amounts and types of food that you and your infant consume to help us learn about nutrition and growth. In addition, we ask that at the 12 month visit that we measure your height, weight and bones using the same approach as for your infant. This will help us learn if maternal size and bone are also related to your infant's growth over the first year.

Participation in the study will be from the time you join the study until your infant reaches one year of age or one year corrected age.

The researcher may decide to take you off this study if you or your infant develop illnesses that affect growth and bone.

Risks and Discomforts

There are no risks associated with measurements of growth or collection of the urine, and breast milk samples. There is low risk associated with sampling of blood such as bruising but we will try to take blood when samples are taken for monitoring to limit the number of times blood is sampled. The sampling of blood from you and the placenta pose minimal or no risk with bruising a potential with sampling from you. The physical risks associated with the scan of you and your infant's whole body, lumbar spine and femur are considered to be low. The device uses an x-ray source to measure bone density. However, the amount of radiation is much less than a regular chest x-ray and equivalent to the radiation accumulated during one normal day.

Benefits

There are no monetary benefits to you or others in this study. You will be provided a breast pump kit if you choose to breastfeed. The knowledge gained through this study will help us to improve nutritional care for infants who are born prematurely.

Costs

All laboratory tests which will be performed as part of this study are provided at no cost to you and your infant. There will be no cost for the study measurements that you participate in.

Payment for Participation

There will be no financial remuneration for participating in this study with exception of coverage of parking at the follow-up visits at 6 and 12 months after discharge.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and your infant's name will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the University of Manitoba Research Ethics Board.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not effect your other medical care at this site.

Questions

You are free to ask any questions that you may have about your rights as a research participant. If any questions come up during or after the study, contact the study doctor and the study staff:

Dr. H. Weiler	Dr. M. Seshia	Dr. K. Yuen
975-7716	787-1827	788-5710

For questions about your rights as a research participant, you may contact The University of Manitoba Faculty of Medicine Research Ethics Board at (204) 789-3389.

Do not sign this consent unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with either of Drs. Weiler, Seshia, Yuen and/or their study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my, and my infant's, medical records by the University of Manitoba and the University of Manitoba Research Ethics Board.
By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature:_____Date:_____

Participant printed name:_____

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name:_____Date:_____

Signature:_____

Role in the study:_____

Appendix B-1-

Dear participant and parent(s): Date:_____

Re: Study: Growth and bone mineralization in premature infants

Thank you for your continued participation in this project. Prior to your return to the research facility for a six month and one year visit, we would like you to complete the enclosed three day food record and return them to us at your next visit or by using the enclosed addressed and stamped envelope. It would be best if you completed the record two weeks prior to your scheduled visit to make it easier to also record what your infant eats. For your infant please record food intake the week just prior to visiting for the research measurements.

If you have any questions or concerns, please do not hesitate to call one of the investigators listed below. To make the information useful to us, the accuracy of the recorded information we receive from you is essential. In order to complete the food record properly, please follow the instructions outlined below.

1) Choose 2 week days and 1 weekend day to keep records of everything you or your infant eat or drink on those days. Please try to choose non-consecutive days (e.g. Tuesday, Thursday and Saturday would be good choices). Do not use a day when you or your infant are feeling sick.
2) For reasons of confidentiality, we have written your subject number on each form, therefore, please **do not** write your name on any of the forms. Your forms have "mother" written on the top and your infant's are indicated by "infant".
3) Record all foods/liquids consumed each day starting when you or your infant wake-up, making sure to write down the time when any food /liquid is consumed. Start a new line for each food/liquid recorded. Try to list foods/liquids immediately after eating/drinking.
4) Indicate the food/liquid type in detail, including brand names if appropriate and any toppings or spreads. If you are eating a mixed dish such as a stir fry, or use home make infant foods, please write the recipe on the back of the food record.
5) If you run out of space please write on the back or attach another piece of paper.
6) Indicate the amount of food/liquid consumed using standard household measuring cups/spoons or the number of slices if a product such as bread. For items such as meat or cheese, approximate dimensions would be helpful (example is 2x2x2 inches).
7) **If your infant is breastfed**, we will provide a weigh scale to estimate how much milk is consumed at each feed.
8) If you or your infant take any vitamins/supplements/medications on a regular basis, please write them down at the bottom of the food record and indicate the amount taken.

Thank you for your continued support.
If you have any questions, please call one of the following investigators:
Dr. Hope Weiler
(204) 474-6798

EXAMPLE Food Record - Day 1- Infant

Subject: P-I-6 **Date and day of week:** Tuesday, Mar 8, 2000

Time	Food	Brand	Amount
If Breast Fed			
1:30 AM	MILK	MOTHER'S	Weight before – 4.100 kg Weight after – 4.175 kg
5:30 AM	MILK	MOTHER'S	Weight before – 4.110 kg Weight after – 4.165 kg
7:30 AM	CEREAL	RICE - Gerber	1 TBSP dry made with expressed milk
If Formula Fed			
1:30 AM	MILK	Enfalac 20 Kcal/OZ SKIM	2 OZ consumed of 3
5:30 AM	MILK	Enfalac 20 Kcal/OZ SKIM	1 OZ.consumed of 3
7:30 AM	CEREAL	RICE - Gerber	1 TBSP dry made with formula

Vitamins/Supplements/Medications: D-Vi-Sol 1 mL

Food Record - Day 1 - Infant

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Food Record - Day 2 - Infant

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Food Record - Day 3 - Infant

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Appendix B-2

Dear participant and parent(s): Date:_____

Re: Study: Growth and bone mineralization in premature infants

Thank you for your continued participation in this project. Prior to your return to the research facility for a six month and one year visit, we would like you to complete the enclosed three day food record and return them to us at your next visit or by using the enclosed addressed and stamped envelope. It would be best if you completed the record two weeks prior to your scheduled visit to make it easier to also record what your infant eats. For your infant please record food intake the week just prior to visiting for the research measurements.

If you have any questions or concerns, please do not hesitate to call one of the investigators listed below. To make the information useful to us, the accuracy of the recorded information we receive from you is essential. In order to complete the food record properly, please follow the instructions outlined below.

9) Choose 2 week days and 1 weekend day to keep records of everything you or your infant eat or drink on those days. Please try to choose non-consecutive days (e.g. Tuesday, Thursday and Saturday would be good choices). Do not use a day when you or your infant are feeling sick.
10) For reasons of confidentiality, we have written your subject number on each form, therefore, please **do not** write your name on any of the forms. Your forms have "mother" written on the top and your infant's are indicated by "infant".
11) Record all foods/liquids consumed each day starting when you or your infant wake-up, making sure to write down the time when any food /liquid is consumed. Start a new line for each food/liquid recorded. Try to list foods/liquids immediately after eating/drinking.
12) Indicate the food/liquid type in detail, including brand names if appropriate and any toppings or spreads. If you are eating a mixed dish such as a stir fry, or use home make infant foods, please write the recipe on the back of the food record.
13) If you run out of space please write on the back or attach another piece of paper.
14) Indicate the amount of food/liquid consumed using standard household measuring cups/spoons or the number of slices if a product such as bread. For items such as meat or cheese, approximate dimensions would be helpful (example is 2x2x2 inches).
15) **If your infant is breastfed**, we will provide a weigh scale to estimate how much milk is consumed at each feed.
16) If you or your infant take any vitamins/supplements/medications on a regular basis, please write them down at the bottom of the food record and indicate the amount taken.

Thank you for your continued support.
If you have any questions, please call one of the following investigators:
Dr. Hope Weiler
(204) 474-6798

EXAMPLE Food Record - Day 1- Mother

Subject: P-M-6 Date and day of week: Tuesday, Mar 1, 2000

Time	Food	Brand	Amount
7:30 AM	TOAST	COUNTRY HARVEST CRACKED OAT	2 SLICES
	MARGARINE	REGULAR BECEL	2 TSP.
	JAM	DIET RASPBERRY	1 TBSP.
	PEANUT BUTTER	KRAFT LIGHT SMOOTH	2 TBSP.
	BANANA		1 SMALL
	MILK	SKIM	2 CUP
10:30 AM	CHEESE	KRAFT 27% M.F. CHEDDAR	2 OZ.
	CRACKERS	SODA	4
12:00	SOUP	CAMPBELL'S CREAM OF MUSHROOM - HALF THE FAT	1 CUP - MADE WITH SKIM MILK EQUAL PARTS
	2 HAM SANDWICH		
	BREAD	SOUR DOUGH	1 LARGE SLICE (cut in half)
	HAM	DELI SHAVED	1 OZ
	MUSTARD	YELLOW	1 TSP
	MAYONNAISE	KRAFT LIGHT MIRACLE WHIP	1 TSP
	APPLE		1 MED.
4:00 PM	BANANA BREAD	HOMEMADE	One slice is about 3 inches by 4 inches and ½ inch thick.

Vitamins/Supplements/Medications: Centrum multivitamin/mineral – 250 mg tablet

Food Record - Day 1 - Mother

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Food Record - Day 2 - Mother

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Food Record - Day 3 - Mother

Subject:_____ **Date and day of week:** _____

Time	Food	Brand	Amount

Vitamins/Supplements/Medications:_____

Appendix B-3

24 Hour Food Recall

Subject: **Date and Day of Week:**

Time	Food	Brand	Amount

Vitamins / Supplements / Medications: _____

Appendix C

THE UNIVERSITY OF MANITOBA

FACULTY OF HUMAN ECOLOGY

Department of Foods and Nutrition

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RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Growth and bone mineralization in premature infants

Protocol Number: B2000:204

Principal Investigator: Dr. H. Weiler, RD, PhD
Co-Investigator: Dr. M.M. Seshia, MBChB; Dr. C.K. Yuen, MD, MBA;
Sponsor: University of Manitoba, Winnipeg, Manitoba, R3T 2N2

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

This study is being conducted to study nutritional status and growth of infants. You are being asked to take part in this study because you are soon to or have just delivered a newborn infant. A total of 64 mother infant pairs will participate in this study.
The purpose is to study the growth patterns and bone development of babies that are born early (prematurely) and to examine if specific nutrients are linked to infant growth and bone development. The growth and bone development of these babies will also be compared with that of babies born at the expected due date (full-term). This research is being done because research suggests that growth in height, weight, and head size and the amount of bone mineral are different in babies born prematurely compared to those born at the expected term date. The nutrient of interest is arachidonic acid. This nutrient is normally made by the liver and is essential for growth. Thus this study will help to determine if prematurely born babies with greater amounts of arachidonic acid, as indicated in a small blood sample, experience improved growth and bone mineral content

and will help to advance nutritional practices. In addition, the influence of growth over the first year of life on body size at one year of age will also be studied.

Study Procedures

Phase One: Since your baby was born at term, you and your baby are being asked to participate in the term phase of the study. You and your baby will be followed in this phase of study from the time of birth to the time of discharge from hospital and also in Phase Two – see below.

In order to participate in this study, you must not have a personal history of diabetes before or during your pregnancy and your doctor will determine whether or not any medications you are currently taking will interfere with the proposed measurements. Your baby will be examined at birth as part of routine clinical care for any medical conditions that may exclude him/her from participating in this study.

Phase Two: at discharge from hospital, 6 and 12 months thereafter, we ask that you and your infant visit our research facility to enable measurement of growth and bone as well as collect information on what you and your infant are eating. We would also like to measure your bones at the 12-month visit.

If you take part in this study, you will have the following tests and procedures:

Your Infant's Involvement

We request your permission to obtain the following samples from your baby: a sample of blood from the placenta (afterbirth) taken routinely at birth; measurement of your baby's length, weight, and head size and a urine sample before discharge from hospital.

We also ask your permission to scan your baby's whole body, lower (lumbar) spine and thigh bone (femur) using a low dose x-ray technique called dual energy x-ray absorptiometry at the research facility immediately after hospital discharge. For the second part of the study we wish to measure your baby's weight, length and head size as well as the same measurements on bone at 6 and 12 months. This will help us learn about the relationships among birth size, nutrition, growth and growth by one year after discharge from hospital.

Your Own Involvement

We request your permission to take a blood sample (approximately 10 ml or 2 teaspoonfuls) from you prior to your discharge from hospital. If breastfeeding your baby, you will be asked to keep a three-day record of food intake after you have been discharged. This will be reviewed with you approximately one week later at a convenient time over the telephone when you are at home. This should take no more than half an hour.

You will decide on your own whether you wish to breast-feed or to feed your baby infant formula. If you choose to breastfeed your infant, we ask for a sample of milk at the end of

the first, second, third and fourth weeks after delivery with collection at 9:00 am and 9:00 pm (approximately 5 ml or 1 teaspoonful).

At the follow-up visits, at your infant's discharge from hospital, 6 and 12 months, we will ask you about the amounts and types of food that you and your infant consume to help us learn about nutrition and growth. In addition, we ask that at the 12 month visit that we measure your height, weight and bones using the same approach as for your infant. This will help us learn if maternal size and bone are also related to your infant's growth over the first year.

Participation in the study will be from the time you join the study until your infant reaches one year of age or one year corrected age.

The researcher may decide to take you off this study if you or your infant develop illnesses that affect growth and bone.

Risks and Discomforts

There are no risks associated with measurements of growth or collection of the urine, and breast milk samples. The sampling of blood from you and the placenta pose minimal or no risk with bruising a potential with sampling from you. The physical risks associated with the scan of you and your infant's whole body, lumbar spine and femur are considered to be low. The device uses an x-ray source to measure bone density. However, the amount of radiation is much less than a regular chest x-ray and equivalent to the radiation accumulated during one normal day.

Benefits

There are no monetary benefits to you or others in this study. You will be provided a breast pump kit if you choose to breastfeed. The knowledge gained through this study will help us to improve nutritional care for infants who are born prematurely.

Costs

All laboratory tests which will be performed as part of this study are provided at no cost to you and your infant. There will be no cost for the study measurements that you participate in.

Payment for Participation

There will be no financial remuneration for participating in this study with exception of coverage of parking at the follow-up visits at 6 and 12 months after discharge.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and your infant's name will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the University of Manitoba Research Ethics Board.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not effect your other medical care at this site.

Questions

You are free to ask any questions that you may have about your rights as a research participant. If any questions come up during or after the study, contact the study doctor and the study staff:

Dr. H. Weiler	Dr. M. Seshia	Dr. K. Yuen
975-7716	787-1827	788-5710

For questions about your rights as a research participant, you may contact The University of Manitoba Faculty of Medicine Research Ethics Board at (204) 789-3389.

Do not sign this consent unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with either of Drs. Weiler, Seshia, Yuen and/or their study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my, and my infant's, medical records by the University of Manitoba and the University of Manitoba Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Participant signature:_____Date:_____

Participant printed name:_____

I, the undersigned, attest that the information in the Participant Information and Consent Form was accurately explained to and apparently understood by the participant or the participant's legally acceptable representative and that consent to participate in this study was freely given by the participant or the participant's legally acceptable representative.

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent.

Printed Name:_____Date:_____

Signature:_____

Role in the study:_____

Appendix D: Anthropometry and bone mineral content values, as measured by dual energy x-ray absorptiometry, of very preterm infants at hospital discharge and term infants at birth.

	Very Preterm Infants	Term Infants
	(n=16)	(n=30)
Gestational Age (weeks)	38.8 ± 2.2	40.0 ± 1.7
Weight (g)	2338 ± 257	3546 ± 556
Length (cm)	45.1 ± 2.0	51.7 ± 2.3
Head Circumference (cm)	33.1 ± 1.8	35.4 ± 1.2
Lumbar Spine (L1-4) BMC (g)	0.93 ± 0.35	2.14 ± 0.49
Lumbar Spine (L1-4) BMC (g/kg)	0.41 ± 0.21	0.61 ± 0.11
Lumbar Spine (L1-4) BMC (g/cm)	0.02 ± 0.01	0.04 ± 0.01
Femur BMC (g)	1.20 ± 0.30	2.62 ± 0.64
Femur BMC (g/kg)	0.45 ± 0.21	0.71 ± 0.22
Femur BMC (g/cm)	0.03 ± 0.01	0.05 ± 0.02

Data shown as mean ± SD.
Body weight, length and head circumference at time of DEXA visit.

Appendix E: Anthropometry and bone mineral content values, as measured by dual energy x-ray absorptiometry, of very preterm infants at 6 months corrected age and term infants at 6 months of age

	Preterm Infants	Term Infants
	(n=14)	(n=30)
Weight (g)	6859 ± 721	8011 ± 1556
Length (cm)	65.5 ± 2.3	68.6 ± 4.0
Head Circumference (cm)	43.1 ± 1.5	44.2 ± 1.8
Lumbar Spine (L1-4) BMC (g)	3.15 ± 0.73	3.51 ± 0.76
Lumbar Spine (L1-4) BMC (g / kg)	0.46 ± 0.10	0.45 ± 0.11
Lumbar Spine (L1-4) BMC (g / cm)	0.05 ± 0.01	0.05 ± 0.01
Femur BMC (g)	4.62 ± 0.99	5.35 ± 1.30
Femur BMC (g/kg)	0.67 ± 0.11	0.68 ± 0.17
Femur BMC (g / cm)	0.07 ± 0.01	0.08 ± 0.02

Data shown as mean ± SD.
Body weight, length and head circumference at time of 6 month DEXA visit.