University of Alberta

Bone Health and Growth in Children post Liver Transplant

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Abstract

The study aim was to compare the impact of corticosteroid-free and corticosteroid-containing immunosuppressive regimes on parameters of bone health and growth in children post-liver transplant (LTX). One-hundred fifteen charts were screened, thirty-nine charts meeting study inclusion were reviewed. The prevalence of decreased lumbar and whole-body bone mineral density (BMD z-score <-2) was 15% and 8% respectively. Children on corticosteroid-free immunosuppressive regimens had greater height z-scores (0.23± 0.22 versus -0.71± 0.13, *p*=0.002) and whole-body bone mineral content (944± 88 versus 889 ± 57 g p<0.001) than those prescribed corticosteroids. A trend to lower absolute whole-body BMD (g/cm^2) was observed in children on corticosteroid therapy for more than 365 days (p=0.055). While suboptimal vitamin K and calcium intakes may have contributed to poor bone health, vitamin D intakes (with supplementation) appeared to meet recommended levels. Low-dose corticosteroid therapy is associated with reduced BMC and linear growth in children post-LTX.

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Table of Contents

List of Tables

List of Figures

Abbreviations List

$1,25(OH)_2D$: 1,25 Dihydroxycholecalciferol- Vitamin D

24-hr recall: 24-hour Recall

25(OH)D: 25 Hydroxycholecalciferol-Vitamin D

AAAs: Aromatic Amino Acids

AH: Autoimmune Hepatitis

AHS: Alberta Health Services

AI: Adequate Intake

ALP: Alkaline Phosphatase

ALT: Alanine Aminotransferase

AMDR: Acceptable Macronutrient Distribution Range

AS: Alagille Syndrome

AST: Aspartate Aminotransferase

BA: Biliary Atresia

BASM: Biliary Atresia Splenic Malformation

BCAA: Branched-chain amino acid

BMC: Bone Mineral Content

BMD: Bone Mineral Density

BMD-z: Bone Mineral Density z-score

BMI: Body Mass Index

BMI-z: Body Mass Index z-score

Ca: Calcium

CHO: Carbohydrate

DBD: Vitamin D Binding Protein

DXA: Dual-X-ray Absorptiometry

DRI: Dietary Reference Intakes

EER: Estimated Energy Requirement

ESLD: End-Stage Liver Disease

ESPGHAN: European Society for Pediatric Gastroenterology, Hepatology, and Nutrition

FFQ: Food Frequency Questionnaire

FTT: Failure to Thrive

GGT: Gamma-glutamyltransferase

GH: Growth Hormone

GI: Gastrointestinal

ht-z: Height-for-age z-score

IGF: Insulin-like Growth Factor

KP: Kasai Portoenterostomy

LCFAs: Long Chain Fatty Acids

LTX: Liver Transplant

MCT: Medium Chain Triglyceride

MELD: Model for End-stage Liver Disease

Mg: Magnesium

NRV: Nutrient Reference Values

OPTN: Organ Procurement and Transplantation Network

P: Phosphorus

PBM: Peck Bone Mass

PELD: Pediatric End-stage Liver Disease

PEM: Protein Energy Malnutrition

PIVKA-II: protein induced by vitamin K absence/antagonist-II

PSC: Primary Sclerosing Cholangitis

PTH: Parathyroid Hormone

PUFAs: Polyunsaturated Fatty Acids

QCT: Quantitative Computed Tomography

RDA: Recommended Dietary Allowance

REE: Resting Energy Expenditure

RQ: Respiratory Quotients

SPLIT: Studies of Pediatric Liver Transplantation

unCO: Undercarboxylated Osteocalcin

wt-z: Weight-for-age z-score

Zn: Zinc

Chapter1: Literature Review

1.1 Introduction

Neonatal cholestasis (1 in 2,500 live births) is the main indication of liver failure and liver transplantation (LTX) in infants and children (1). The Pediatric LTX Program at the Stollery Children's Hospital started in the early 1990's with over 150 children undergoing a total of 178 LTXs. The main cause of end-stage liver disease (ESLD) in children within this program was Biliary Atresia (BA); affecting approximately 55% of the children needing LTX (2). Infants and children with BA experience significant growth delay and poor bone health related to malnutrition in the pre-transplant period. Post-LTX, infants and children typically experience improved growth and bone health; but this may be prolonged by the use of immunosuppressive therapy that includes the use of corticosteroid therapy (3,4) and inadequate intakes of calcium, vitamin D and K (5). Because of these potential effects, The Pediatric LTX Program at the Stollery Children's Hospital transitioned to a steroid-free immunosuppressive protocol in late 2003. Poor bone health can lead to an increased risk for bone fracture which can significantly impair overall quality of life in transplant recipients (6). Given that LTX is a life saving procedure with excellent long-term outcomes for children with end stage liver failure, it is imperative to understand all the potential factors influencing quality of life and overall health status in the pediatric LTX recipient. The purpose of this literature review is to review the medical (e.g immunosuppressive) and lifestyle (e.g diet and physical activity) factors influencing bone health in children who undergo LTX.

1.2 Chronic Liver Diseases and Complications of Liver Failure

1.2.1 Cholestasis

Neonatal cholestasis (1 in 2,500 live births) (1) is the main etiology of chronic liver disease in early childhood. Cholestasis is an impairment of bile formation and/or flow which is the main reason of jaundice (1,7). There are two types of cholestasis: intrahepatic (functional) and extrahepatic (obstruction) (7). Intrahepatic cholestasis is characterized by decrease in bile flow without obstruction in the bile duct (7). In contrast, extrahepatic cholestasis is characterized by decreases in bile flow with obstruction in the bile duct (7). The etiology of cholestasis could be due to viral, bacterial or autoimmune resulting in biliary stasis and damage (7).

Neonatal cholestasis is defined as having jaundice (related to hyperbilirubinaemia) beyond two weeks of life (1). If the conjugated bilirubin exceeds 1 mg/dl and total serum bilirubin is at or below 5 mg/dl with a direct bilirubin fraction of more than 20% of the total bilirubin, this is considered abnormal (1). In addition, the predominant disturbance in alkaline phosphatase (ALP) and Gamma-glutamyltransferase (GGT) suggest biliary obstruction (1), while abnormalities of serum aminotransferases (alanine aminotransferase (ALT), and aspartate aminotransferase (AST)) imply hepatocellular disease (1,8).

Cholestasis is associated with steatorrhoea and fat-soluble vitamin (A, D, E & K) deficiency due to bile acid deficiency (7,9,10). When ESLD occurs, intrahepatic damage causes hepatocellular inflammation and fibrosis

and portal hypertension can be the result (9). Other complications will be discussed later within this chapter include: protein energy malnutrition (PEM) and failure to thrive (FTT).

1.2.2 Liver Disease Severity

Liver disease severity is typically defined in childhood by two validated scoring systems (11,12). The two scoring systems are the Pediatric End-stage Liver Disease (PELD) for children younger than 12 years of age; and the Model for End-Stage Liver Disease (MELD) for children over 12 years of age (11,12) (**Table 1-1)**. These scoring systems are typically used to assess the three month risk of death and/or admission to the intensive care unit in patients who are on the LTX waiting list (11-13). PELD/MELD score above 25 is related to severe cases and prioritizes the patient to the top of the LTX waiting list (13). Based on the OPTN (Organ Procurement and Transplantation Network), the scores were categorized by defining cut off points: above 25, 24 to >18, 18 to 11, and below 10 (13).

Table 1-1: PELD and MELD Formulas (11,12)

Formula	Comments
PELD score = 0.480 x Loge (bilirubin mg/dL) + 1.857 x Loge(INR) - 0.687x Loge(albuming/dL) + 0.436 if the patient is less than 1 year old* + 0.667**	*Scores for patients listed for LTX before the patient's first birthday continue to include the value assigned for age (< 1 year) until the patient reached the age of 24 months ** If the patient has growth failure <-2 Standard deviation
MELD score = 0.957 x Loge (creatinine mg/dL) + 0.378 x Loge(bilirubin mg/dL) + 1.120 x Loge(INR) + 0.6431	For candidates on dialysis, defined as having 2 or more dialysis treatments within the prior week; or candidates who have received 24 hours of continuous veno-venous hemodialysis (CVVHD) within the prior week, will have their serum creatinine level automatically set to 4.0 mg/dl
Multiply the score by 10 and round to the nearest whole number Laboratory values less than 1.0 are set to 1.0 for the purposes of the PELD/MELD score calculation	

1.2.3 Chronic Liver Diseases and Liver Transplantation

1.2.3.1 Biliary Atresia (BA)

BA is a liver disease that results is an accumulation of bile in the liver as a result of obstruction in the bile ducts (1,9). This obstruction can result in the build up of toxic bile acids within the liver which can lead to ESLD. Most children with BA require a LTX within the first two years of life. The incidence of this disease is rare (1 in 10,000 to 15,000 live births) (1,14,15). The highest incidence of BA is in East Asian countries, i.e. Taiwan (1 in 5000) (16) and among females rather than males (16,17). The causes of BA are still unknown with four different theories of the etiology of BA. These include 1) perinatal infection with a virus in an immature immune system, 2) abnormal or toxic bile components, and 3) an error in development of the hepatic arterial supply or 4) defect bile duct (9).

Some researchers reported two forms of BA: perinatal (acquired) and embryonic form (congenital or fetal). Perinatal (acquired) form is as consequence of obstruction of the biliary tree during the gestation and is the majority of cases (9). This form usually has no jaundice at birth and appears after birth (17). The embryonic form (congenital or fetal) is a result of a defect of stem cells and is associated with other congenital abnormalities of the intestine and cardiac system (9,16). This is not a common form, accounting for 10% to 20% of infants with a diagnosis of BA (9,17). BA splenic malformation (BASM) is an example of the embryonic form of BA with associated splenic abnormalities (17). The diagnosis of BASM significantly

decreases transplant free survival rate (17).

BA is classified based on the place of obstruction to three types **(Figure 1-1)**. Type 1 BA is a stricture in the common bile duct; Type 2 BA is a blockage in common hepatic duct; and Type 3 BA obstructs the entire exptrahepatic biliary (16,18,19). More than 90% of the cases are the third type of BA (16,18,19).

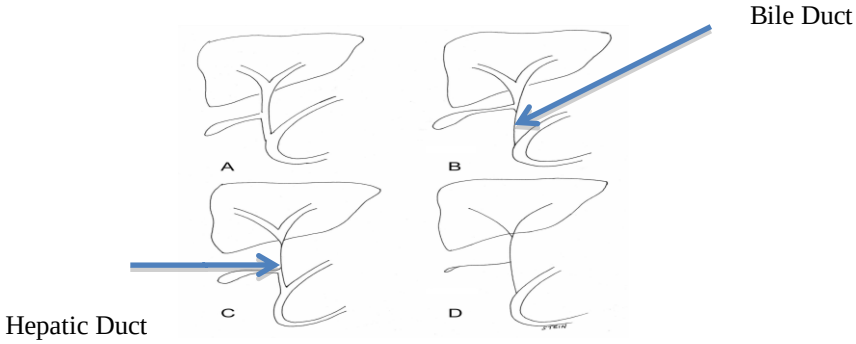


Figure 1-1: Classification of Biliary Atresia (place of obstruction): A) Normal, B) Type 1; atresia of the bile duct, C) Type 2; atresia of the hepatic duct, D) Type 3; atresia of the entire extrahepatic biliary tree Adapted from Ling, 2007 (19); Khalil, 2010 (16); and Hartley, 2009 (18)

The prompt management of BA is crucial for the survival of the native liver; children with BA treated prior to 60 days of life have a 10 year native liver survival of 73% compared to 11% past 90 days (20). In 1950, the Kasai Portoenterostomy (KP) was the first operation described for BA patients (16,17). KP is an attempt to drain the intrahepatic biliary tree by replacing the damaged extrahepatic biliary tree with a length of intestine (16,18). Approximately, two thirds of patients will clear their jaundice after a KP procedure, and about one third will retain their livers after the first decade of life (16); Portal hypertension and infections are complications of KP; resulting in intermittent admissions to hospital for ascending cholangitis (infection of

the biliary tree) (21). The average life span of patients with inoperative KP is 19 months (9). In Canada, 60% of BA children will need a LTX within the first two years of life (21,22); 37% will have a LTX before 1 year of age (21). Most children with BA prior to LTX experience significant PEM and poor bone health secondary to anorexia, malabsorption of fat and fat-soluble vitamins and altered nutrient utilization (5,18).

1.2.3.2 Alagille Syndrome (AS)

AS is as an autosomal dominant syndrome that affects the liver, heart and other organs (9). The mutation is in the *Jagged 1* gene (10). AS is characterized by intrahepatic cholestasis (96%) with biliary ductopenia, congenital heart disease (97%), skeletal abnormalities (51-80%), posterior embryotoxin of the eye (78%), dysmorphic facies (96%) and renal anomalies (15%) (9,23). Fifty-one to seventy- five percent of patients with AS have a 10-20 year survival rate (9,10). Approximately, 20% will develop ESLD within this time (9) and 60% of them will require LTX (9).

Poor bone health and growth failure are common in children with AS (23,24). Poor growth is common in children with AS because of liver disease (FTT, malabsorption and malnutrition) (23). Furthermore, hypothyroidism and Growth Hormone (GH) resistance are other factors contributing to delayed growth (23). A study reported that the weight-for-age z-score and height-for-age z-score were lower in children with AS (-1.70 ± 1.65; -2.1 ± 1.36); respectively (24). Skeletal abnormalities or "butterfly vertebrae" is a deformity in the spine that leads to high fracture rate up to 28% (23). It has

been shown that children with AS had low bone mass and size (24).

1.2.3.3 Autoimmune Hepatitis (AH)

AH is seen in both children and adults. It is a progressive inflammatory condition of the liver as a result of dysregulation of the immune system (25,26). It is more common in females (25). The etiology of AH is multifactorial: viral infection, inherited or acquired metabolic abnormalities or drug-associated liver injury. Therefore, immunosuppressive medications (corticosteroids and/or azathioprine) are prescribed to suppress the disease (25). There is not a well-established protocol of AH treatment, but it is similar to adults: 60 mg/day in the first week, 40 mg/day in the second week, and 30 mg/day in the third and fourth weeks (25). Then, it will be tapered to 20 mg/day for maintenance or to 10 mg/day with combination of azathioprine (25). Approximately, 80% of the patients respond to treatment; although, approximately 75 -90 % of patients experience at disease relapses (25). As a result many children with AH are often on long-term corticosteroid treatment pre-and-post LTX, resulting in a higher risk for poor bone health.

There are two types of AH: type 1, with positive anti-smooth muscle antibodies and type 2, with anti-liver/kidney microsomal antibodies (26,27). Type 2 AH is more common in children (26,27). Ten to twenty percent of children with AH need LTX and around 20% of them could re-develop the disease post LTX (26). This often means that children with AH are on long-term corticosteroid therapy, even after LTX.

1.2.3.4 Primary Sclerosing Cholangitis (PSC)

PSC is an autoimmune liver disease which leads to inflammation, fibrosis, and destruction of the bile ducts affecting both the intra and extrahepatic biliary tree (28,29). It appears to occur predominantly in males. The etiology of PSC has not yet been determined. Because there is no cure for PSC, liver failure will develop and LTX is required (29). The typical length of time between diagnosis of PSC and LTX is between 3–5 years (28). Around 10% of PSC patients have overlapping features of AH (29). Inflammatory bowel disease is commonly associated with PSC (50 to 70%) that could be treated by immunosuppressive medication (28,29)

1.2.4 Nutritional Complications of Cholestatic Liver Diseases

Children who are awaiting LTX develop various co-morbidities, the majority secondary to their ESLD. The nutrition and growth complications can be summarized as: anorexia, FTT, PEM, fat-soluble vitamins deficiency and poor bone health (5,30). As a result of the above complications, 60 % of children with ESLD awaiting for LTX have delayed growth (5) consequent to malabsorption, malnutrition (20,31,32) anorexia (17,31,32), and GH resistance (30). The prevalence of FTT, weight and/or height <2 standard deviations below the mean, or < 3rd percentile, is observed in approximately 60% of children with ESLD (30). PEM occurs in 20% of patients with mild or chronic liver disease without cirrhosis, 65% to 90% of patients with ESLD, and almost 100% of LTX candidates (33). Anorexia, malabsorption and hypermetabolism are the major factors that contribute to an increased risk for

PEM in children with cholestatic liver diseases (33).

Low intake typically occurs in infants and children with ESLD as a result of early satiety, loss of appetite, nausea and vomiting. One factor of low intake is ascites which causes abdominal distention and early satiety (20,32). Nausea and vomiting related to altered gut motility and severity of liver disease is also another major factor known to contribute to anorexia (20). Other potential reasons for low intake is due to zinc and/or magnesium deficiency which can contribute to changes in taste perception; thereby contributing to suboptimal food intake (32).

Malabsorption of fat and fat-soluble vitamins as a result of bile salt deficiency is one of the main nutritional complications in ESLD. Furthermore, high nutrient and energy requirements is related to malabsorption and changes in nutrient utilization (20,32). Pancreatic insufficiency is a confounding factor for fat malabsorption in specific liver disorders such as Alagille Syndrome (32).

Some children with ESLD (particularly BA) have increased energy expenditure and altered branched chain amino acid metabolism leading to a significantly higher risk for PEM (34-36). Hypermetabolism can occur as a result of several factors such as inflammation and complications of the disease (ascites, portal hypertension and variceal hemorrhage) (32) and altered nutrient utilization (20,32). Infants and children with cholestatic liver diseases typically have much lower fasting respiratory quotients (RQ) than

healthy infants and children. Fasting RQ's may range between 0.7 to 0.8, which indicates that children with cholestatic liver diseases mobilize fat and lean body stores as a major energy substrate in the fasted state. In contrast healthy children tend to have fasting RQ's in the range of 0.8 to 0.85, (34,35). All of these factors lead to a reduction in hepatic and muscle glycogen storage which leads to increased fat and protein utilization for energy homeostasis in the fasted state (31).

1.2.5 Bone Health in End-stage Liver Disease

Hepatic osteodystrophy is a long-term complication in children with ESLD. Children with liver diseases waiting for LTX are at high risk of bone fractures (6.6%–28%) (5,37,38). Most fractures are vertebral which are associated with low spinal BMD (15,30,37). The high rate of fracture is due to decreased osteoblast proliferation and activity leading to decreased wall thickness and defective bone matrix synthesis (37). This may be due to a variety of factors including suboptimal vitamin K/D and calcium status, altered parathyroid hormone status (PTH), presence of chronic inflammation and anorexia (30,37). Once 25-hydroxycholecalciferol (25(OH)D) levels fall below 50nmol/L, many children experience significant increases in serum levels of PTH which promotes bone resorption (30). Besides PTH, low production of hepatic proteins in liver disease is associated with reductions in vitamin D binding protein (DBP) and albumin leading to artificially elevated serum concentrations of 25(OH)D concentrations (30). The extent to which reduced DBP synthesis in liver disease contributes to changes in overall

vitamin D utilization is still unclear (30). Other factors such as vitamin K deficiency (known to be highly prevalent in children with cholestatic liver diseases), and altered weight bearing activity in the chronically ill child are also factors that likely contribute to poor bone health in this population (39). These factors are all discussed in **Section 1.4.4**.

1.3 Liver Transplantation

The survival rate of children post-LTX is 93% (1 year), 87-97% (5 years) and 81% (10 years) (4,11). The 10 years survival rate for children who have had LTX at age 2 years and younger is 75% and 2-15 years old is 61% (40). The main indication for LTX in childhood in North America is BA (55%) (2); approximately 60% of children with BA need LTX before the age of 2 years (21,41). The mortality rate in the first year post-LTX typically ranges between 4-10% in most pediatric LTX centers (42). Among patients with BA, the 1-year post-LTX patient survival is 92% and the 1-year post-LTX graft survival rate is 84%. Among patients transplanted for BA, the 5-year patient survival rate has been reported to range between 85–98% (43). Medical treatment (e.g. immunosuppressive) and health care help to improve long-term survival rates above 80% (30).

1.3.1 Post-Liver Transplant Complications

Even though, LTX is a life saving procedure for patients who suffer from different liver failure complications, some of the complications are recurrent (such as rejection) even after LTX and new complications appear (such as lymphoproliferative disease). Rejection episodes, increased risk of

developing lymphoproliferative disease, steroid induced growth failure/osteoporosis and delayed intellectual development, are among the main concerns that infants and children post-LTX can experience (4,11,44).

1.3.1.1 Acute and Chronic Organ Rejection

Rejection is a fundamental issue that children post-LTX can experience. There are two main types of rejections that are different histologically and clinically: acute and chronic rejection. Acute cellular rejection is "inflammation of the liver graft as a consequence of immunological changes between the donor and recipient immune systems" (45). The risk for acute rejection is highest in the in the early period following the LTX and may persist for days to weeks (45). Some children with specific underlying liver diseases are at greater risk of acute cellular rejection post-LTX: AH, PSC and Autoimmune Cholangiopathy (45). Based on Studies of Pediatric Liver Transplantation (SPLIT) reports, 80% of LTX patients experienced acute rejection in the first 6 months post-LTX (2) and 60% in the first 5 years post-LTX. The primary reason of acute rejection is immunological factors (i.e. T cells activation) that result can result in necrosis of the graft organ if left untreated (46). Acute and marked increases in serum aminotransferases (ALT, AST), and total bilirubin are indicators of acute rejection. Acute rejection is typically treated with high dose intravenous bolus of corticosteroids and/or tacrolimus (45,46). Clinically the severity and assessment of acute rejection is assessed using a clinical scoring system following liver biopsy. The Banff Rejection Activity Index (RAI) is a tool that

measures the severity of acute rejection. The RAI is composed of two major parts: the global assessment and the grading score (47,48). The global assessment classifies the rejection to mild, moderate and severe (47,48). The grading assessment is divided to three criteria: portal inflammation, bile duct inflammation damage and venous endothelial inflammation; each criteria is scored from 0-3 with maximal score of 9 (47,48) **(Appendix A: Rejection Activity Index (RAI)).**

Acute rejection, infection and history of AH and Primary Biliary Cirrhosis (PBC) are the main risk factors for chronic rejection in individuals who have undergone LTX (45). Individuals with PBC and AH are at particularly high risk, as these are autoimmune diseases that predispose the individual to changes in the immune response, even when a new 'graft organ' is in situ. Chronic rejection is a histological process characterized as bile duct loss (45). It occurs in 3 to 4% of LTX patients and is more typically observed after the first three months of LTX (45). Chronic rejection can extend to durations of years; and if not resolved can lead to graft failure and the need for re-LTX (45). Treatment typically results in the need for higher dose immunosuppressive therapy which can place the child at high risk for several complications: including increased risk for renal failure, lymphoproliferative disease and suboptimal bone health (4,45). Liver enzymes, alkaline phosphate and bilirubin are frequently disturbed during chronic rejection diagnosis (45).

1.3.1.2 Growth

One of the major post-LTX complications in children is delay in linear

growth. This occurs in up to 20-30% of infants and children who undergo LTX (30). This delay in linear growth appears to be multi-factorial; related to pre-transplant growth, age at growth and the use of immunosuppressive therapy. Most children appear to experience accelerated rates of growth (typically referred to as catch up growth) within the first 18 months post-LTX (5,30). Furthermore, age at LTX seems to have an influence on growth post-LTX (49). It has been noticed that younger children pre-LTX seem to have faster catch up growth post-LTX (30). LTX before or at the age of 2 years is associated with more improved growth than transplantation at older ages (30). In 39-95 % of children post-LTX, the growth acceleration rate can occur at any time between 6 months to 7 years post-LTX (49). The growth pattern typically is returned to normal after 2-3 years post-LTX (30). Based on results from SPLIT group in 2012, height-for-age z-score was -0.51 ± 1.11; 23% of children with height below the 10^{th} percentile correlated to corticosteroid use (2).

1.3.1.3 Bone Health

Poor bone health is a long-term complication in children post-LTX. This could be as a result of chronic and/or high doses of corticoesteroids which is called " steroid induced osteoporosis" (50,51). See **Section 1.4.4.7** for further discussion.

1.3.1.4 Other Complications

Other complications following LTX are delayed intellectual development, reduced quality of life and increased risk for renal and

lymphoproliferative disease (8,41). It has been observed that children with cholestasis have delayed language and gross motor skills (40). Around 25% of children post- LTX had learning problems and 33-40% of them are receiving special education services (40,52). Risk for renal disease is related to the use of immunosuppressive therapy (tacrolimus, cyclosporin) and may contribute to long standing issues with renal insufficiency (4). High dose immunosuppressive therapy (for treatment of acute and chronic rejection) and/or ineffective treatment for Epstein-Barr infection can also result in an increased risk for lymphoproliferative disease (4).

1.3.2 Post Liver Transplantation: Other Immunosuppressive Therapy

Children who have undergone LTX are on medications to help graft and patients survival (45,53). There are different immunosuppressive protocols that are used post-LTX: calcineurin inhibitors (cyclosporine and tacrolimus), anti-metabolites (mycophenolate mofetil and mycophenolate sodium), mTOR inhibitors (sirolimus and everolimus) and corticosteroids (45,53). Calcineurin inhibitors (e.g tacrolimus), the most common immunosuppressive used currently after discharge (95%), is the backbone maintenance immunosuppressive therapy post LTX (53). Calcineurin inhibitors decrease the incidence of rejection and graft loss (45,53); however, 20% of LTX patients experience chronic renal failure within 5 years post-LTX (53). This leads to decreased t production of $1,25(OH)_2D$ and increase bone resorption (6). Antibody-based therapy or induction therapy helps to reduce immunosuppressant dose and rejection rate (54,55). Immunosuppressants can

cause different side effects: calcineurin inhibitors cause nephrotoxicity, anti-metabolites cause gastrointestinal side effects, and corticosteroids could cause hypertension, osteoporosis and growth retardation (45,55-57). All are associated with an increased risk of malignancy. It has been reported that a calcineurin inhibitors free protocol (sirolimus and mycophenolate mofetil) leads to 70% graft and patient survival rate, better renal function and low incidence of cancer (56).

Other medications could be prescribed to improve growth and nutrition status. Growth hormone (GH) therapy could be prescribed to improve growth in children who have had LTX and fail to experience catch-up growth (30). Vitamin D supplementation is given to prevent deficiency or treat deficiency (58). High doses of vitamin D can be given orally or intramuscularly (60000 to 150000 IU), when clinically warranted for treatment of deficiency (30). Bisphosphonate could be recommended for patients with high fracture rate or risk (30) to prevent bone mass loss (59) for patients with low bone mineral density (BMD) (51,58). Also, weight-bearing exercise is recommended to improve bone health and prevent fracture (51,59).

1.4 Bone Health

1.4.1 Bone Assessment

There are many variables important in determining bone health. One of these variables is bone age (different than biological age). Bone age represents the physiological growth and maturation of a bone and is compared to population norms of age-and-gender matched children (60). Bone age is

determined after x-ray of the right hand and wrist by two methods: Greulich and Pyle and the Tanner-Whitehouse method (14,60). Variables that influence delays in bone development (bone age) include: delayed growth, GH deficiency, hypothyroidism, malnutrition and the presence of chronic illness (14,60). A bone age that is determined to be within one year of actual chronological age is within normal ranges (61). Any child with a bone age more than 1 year younger than chronological age is assessed to have delayed bone growth/bone maturation.

BMD is an indicator of overall bone health and marker of osteoporosis, osteopenia and fracture risk (61). BMD (g/cm^2) is the amount of matter per area of bones and mathematically the ratio of Bone Mineral Content (BMC) to bone area (cm^2) (62). BMC (g) is the amount of minerals per square centimeter of bone (62). Osteoporosis is defined as decreased bone mass resulting in a high risk for fracture (60). BMD z-score (BMD-z) < -2 is referred to osteoporosis; however, The International Society of Clinical Densitometry classified BMD-z < -2 as "low bone density for chronological age" (62).

Peak Bone Mass (PBM) is typically achieved between the ages of 25-30 (63). However, the maximal rates of bone accrual typically occur between the ages of 12-17 years; with slower rates of accrual after this age (63,64). Bone mass declines after 40-50 years of age in women (post menopausal) and in the early 50's for men (65). There are modifiable and un-modifiable factors that affect PBM. Gender and hormonal factors are the un-modifiable factors;

nutrition and physical activity are the modifiable factors (65). One of the factors that has shown an association with increased bone mass is body weight; with those experiencing FTT having a high risk for decreased bone mass (62).

1.4.2 Bone Histology and Physiology

Bone protects internal organs and supports motion. The structure of bones can be divided into two main parts: minerals and organic. It is composed of 50 to 70% mineral, 20 to 40% organic matrix and 5 to 10% water (66) (**Figure 1-2)**.

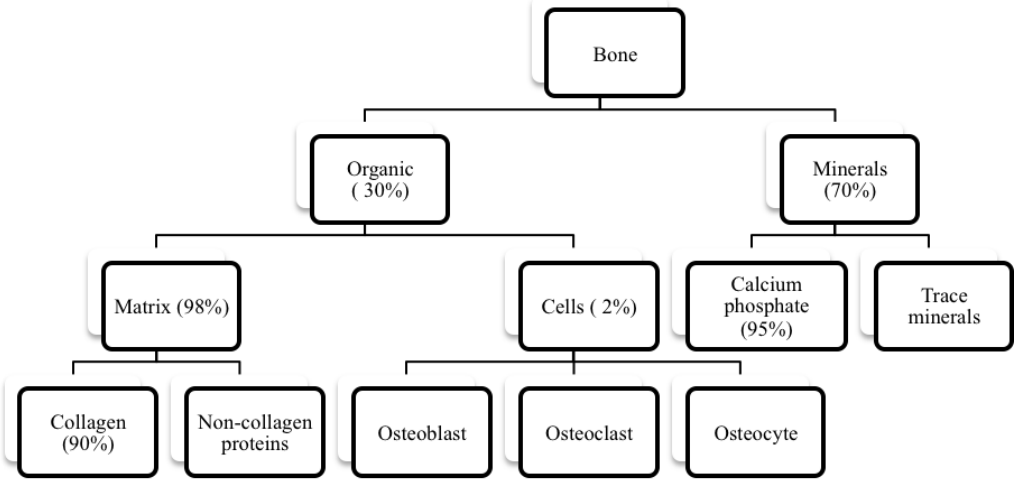


Figure 1-2: Bone Composition: Adapted from Dogan & Posaci, 2002 (57)

There are three different types of cells in skeletal bone and each one has a special function: osteoblast, osteocyte and osteoclast (**Figure 1-2**). Osteoblasts located on the surface of bone tissue originate from stem cells and are involved in bone formation (67). There are many factors affecting osteoblast activity, which are: osteoblast stimulating factor, PTH, GH and insulin like growth factor (IGF-1) (68-70) and micronutrient status (68,70)

(such as calcium, vitamin D and K). Osteocytes are found in the matrix and play a major role in producing cannaliculi (68). Cannaliculi has a role in bone resorption involving calcium and phosphorus exchange between blood and bones (67,68). Osteoclasts are multinuclear cells that originate from the macrophage-monocyte system in bone marrow and locate within the calcified matrix (68-70). When osteoclast activity is increased, bone resorption increases and this causes different bone breakdown and leads to decreased BMD and osteoporosis (68).

The cycle of bone growth is based on bone modeling and remodeling, bone formation and resorption. Bone formation (osteogenesis) is the process when osteoblasts play the main role in bone formation by depositing collagen to produce osteoid (68,70). Bone formation is stimulated by IGF-1 (57). After osteoid is produced, the mineralization rate increases to deposit calcium and phosphorus in the bones thus mineralization is complete (70). Bone resorption is a process involving the breakdown of bone by osteoclasts (68). Resorption is a multistep process initiated by the proliferation of immature osteoclast precursors, the commitment of these cells to the osteoclast phenotype, and finally, degradation of the organic and inorganic phases of bone by the mature resorptive cells (69). PTH stimulates bone resorption (57,70). Bone formation is greater than bone resorption until adolescence when bone formation may be more equal to bone resorption (64). During growth, bone accrual (bone acquisition) mainly leads to increase bone size (64,71) and bone mass (71,72).

Bone modeling is the process when the bone is formed from another existing bone in order to increase the bone's length and diameter (66). Bone formation and resorption are not tightly coupled and occur in different surfaces; bone formation occurs on periosteal surface, while bone resorption occurs on endosteal surface (66). Hypoparathyroidism, renal osteodystrophy, and anabolic agent medications (corticosteroids) are factors that increase bone modeling (66).

Bone re-modeling is a process when bone is resorbed and a new bone is formed. All cells in the bone play a role in bone re-modeling (66,69). Bone re-modeling begins before birth and continues until death (69) whereby bone formation and resorption are tightly coupled on the same surface (66). Bone re-modeling is a process consisting of four phases (3,67): activation phase, resorption phase, reversal phase and formation phase. Activation phase stimulates the bone cell by cytokines and growth factors for resorption (3,67). After resorption ends (reversal phase), bone formation starts. The formation phase is the final phase which needs 4-6 months to complete to generate a new bone (3,66). Resorption of the surface of trabecular bone is important for supplying needed calcium and phosphorus during mineral deficiency and prevents the accumulation of weak old bone (57) which is seen in post-menopause women and elderly men (66).

Both bone modeling and re-modeling have a fundamental function in bone growth. Modeling leads to an increase in the bone diameter and cortical thickness (64), while bone re-modeling leads to an increase in cortical

volumetric bone mineral density (vBMD). High re-modeling rates may lead to lower cortical BMD that may have an influence on increased fracture risk for this type of bone (57).

The rate of bone modeling is high in childhood for growth (66). Most of the gains in bone mineral content (BMC) during growth are due to increases in bone size rather than density (73). Bone growth in childhood contains longitudinal and radial growth. Longitudinal growth occurs at the growth plates before mineralization (66). On the other hand, bone re-modeling rate is high with aging and leads to increase bone size (66). Consequently, bone formation is higher than bone resorption in growth during childhood (68). The factors that affect bone modeling and remodeling are hormones (GH, PTH, calcitonin) and nutrition (vitamin D, vitamin K and calcium) (64,68). Other factors with an influence on bone health are physical activity and sunlight exposure (32,68,69) (**Figure 1-3**).

There are two types of bone: cortical or compact bone and cancellous or trabecular bone (66,74). The ratio of cortical bone to trabecular bone is 80:20 (66,74). Cortical bones are dense, ordered structure and found in long bones (such as the leg) and the surfaces of flat bones (66,68,74). Trabecular bones are lighter, less compact with an irregular structure and forms the ends of long bones and the inner parts of flat bones. Trabecular bones are found in bones with high stress loads such as vertebra (66,68,74). Bones are composed of both cortical and trabecular bone but in different ratios (68). For example, vertebra is composed of a ratio of 25:75 (cortical: trabecular), femoral head in

a ratio of 50:50 and radial diaphysis in a ratio of 95:5 (cortical: trabecular) (66). The rate of bone turnover is higher in trabecular than cortical bone (particularly in the elderly) to maintain the strength and mineral metabolism (66,68).

1.4.3 Methods to Assess Bone Health

There are different methods (images) to measure bone variables: dual-emission X-ray absorptiometry (DXA), quantitative computed tomography (QCT), quantitative ultrasound and X-rays and radiographic absorptiometry.

1.4.3.1 Dual-emission X-ray Absorptiometry (DXA)

Dual-emission X-ray absorptiometry (DXA) was first used in postmenopausal women in 1980 (62,75). In 1990, DXA software was available for the pediatrics population (76). It is basically two beams of x-ray; each with a different energy level (75,76). Difference in how the beams of X-ray interact with the bone during the actual DXA scan reflect bone mineral density (BMD); enabling estimations of BMD to be determined. DXA is used to measure bone densitometry and body composition with minimal radiation exposure, low cost and with 1-2% precision (75,77). DXA has its disadvantage including an inability to separate cortical and trabecular bone. It measures total BMD (cortical and trabecular bone). It also measures bone mineral content (BMC) which reflects bone size (longitudinal) (62,75,78).

DXA measures BMD, BMD-z, BMD t-score and BMC. Z-score and t-score are used to help better interpret bone health data (61). BMD t-score is the score of a healthy young adult (thirty years old) of the same gender and

ethnicity (61). In contrast, BMD-z is the score of a healthy person of the same age, gender and ethnicity. BMD t-score is used as predictor of bone health in adult (61,75). On the other hand, BMD-z is used as predictor of bone health in pediatric populations (62,79).

DXA is able to measure aBMD (area) and BMC at different parts of the body such as the spine, hip and/ or whole-body (75). Measuring BMD at different sites indicates the type of the bone examined., Spinal DXA is predominantly trabecular bone (75). In contrast, whole-body DXA is a combination of cortical and trabecular bone (62,75).

Siminoski in 2011 (61) has reported a new protocol and recommendation for DXA scans in pediatrics. The new protocol includes BMC z-score and adjusts the z score to bone age which gives a clearer picture and better understanding of bone health especially in a pediatrics who are exposed to corticosteroids. More data about BMC z-score and adjusted z-score to height and bone age will help the researcher to study the effect of corticosteroids on both BMC and BMD.

1.4.3.2 Other Methods

In 1976, quantitative computed tomography (QCT) was developed which is a type of computed image used to measure volumetric BMD (vBMD) (g/cm^3) in peripheral skeletal bone (62). QCT can measure vBMD in trabecular bone separate from cortical bone and bone size (62). It has several disadvantages: QCT image leads to high radiation exposure, cortical vBMD is an underestimation if the bone is less than 2 mm, movement causes error in

values and repeated measurements at the same bone site in pediatric is difficult due to longitudinal bone growth (62).

1.4.4 Factors Influencing Bone Health in Children: Pre and Post Liver Transplantation

The rate of bone modeling is high in childhood because of growth (66). Most of the gains in bone mineral during growth are due to increases in bone size rather than density (73). Bone growth in childhood contains longitudinal and radial growth. Longitudinal growth occurs at the growth plates before mineralization (66). On the other hand, when bone re-modeling rate is high, (with aging) increases in bone width occur (66). The factors that affect bone modeling and re-modeling are hormones (such as GH, PTH, calcitonin) and nutrition (vitamin D, vitamin K, calcium and phosphorus) (64,68), weight bearing physical activity (stimulation of osteoblast activity) and other factors such as sunlight exposure (via vitamin D synthesis) and the presence of chronic disease (32,68,69).

There are many factors that suppress or enhance bone mass in children post-LTX. These can be divided into potentially modifiable (e.g. dietary intake, weight bearing activity) and to factors that are not readily modifiable (e.g. hormonal status, pubertal status). These variables have the ability to influence bone growth and PBM in childhood; an important factor in terms of long-term risk for osteoporosis (80) (**Figure 1-3**).

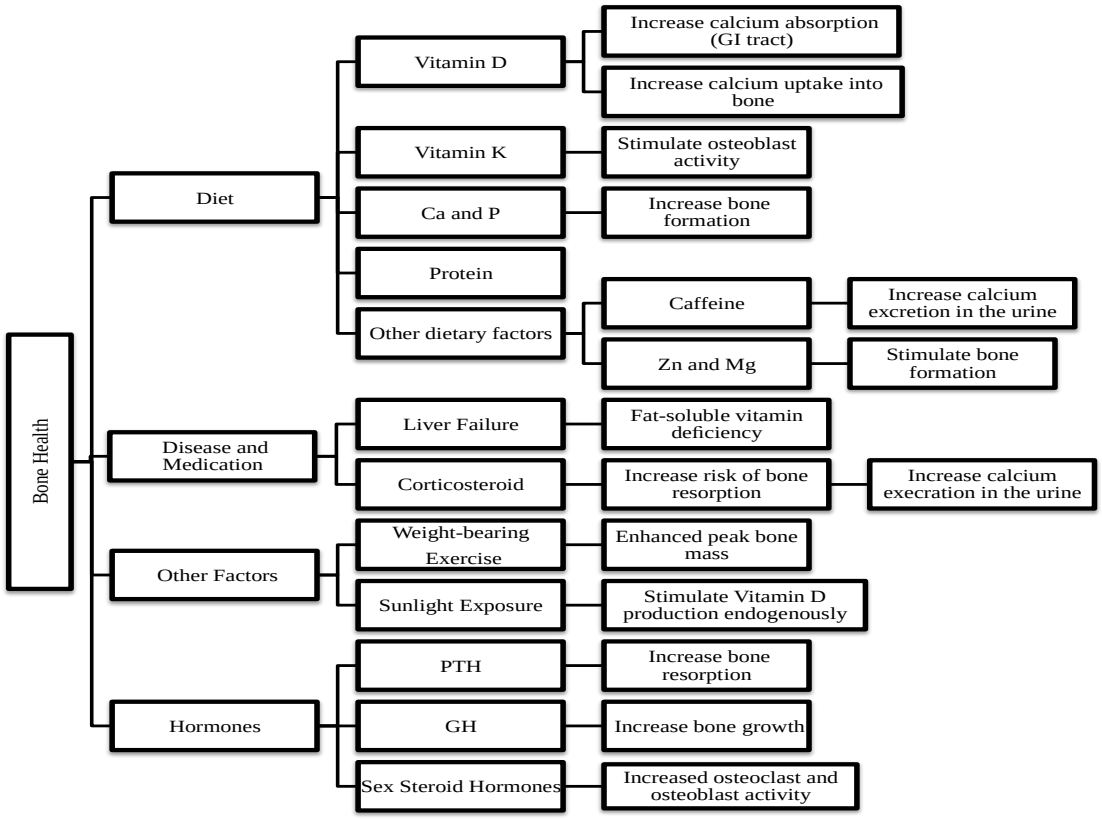


Figure 1-3: Factors Influencing Bone Health in Children post Liver Transplant

1.4.4.1 Hormones Influencing Bone Health

There are several hormones that affect bone growth and bone development: GH, PTH and sex steroid hormones. GH is released from the pituitary gland in the brain, stimulated by GH-releasing hormone and inhibited by Somatostatin (72); GH release increases the production of IGF-1 in the liver (71). GH has a major role in bone turnover by stimulating osteoblast proliferation and activity, and by stimulating the activity of osteoclasts through IGF-1 (57,64,71,72).

PTH regulates calcium-phosphate metabolism. PTH is stimulated by low calcium levels in blood resulting in increases in bone resorption (80). PTH enhances osteoblast proliferation and differentiation, as well as suppressing osteoblast apoptosis (38,81). This causes bone loss and is primarily observed in cortical bone (38). In contrast, calcitonin increases bone formation (57) in trabecular bone and increases BMD (80) **(Figure 1-4).**

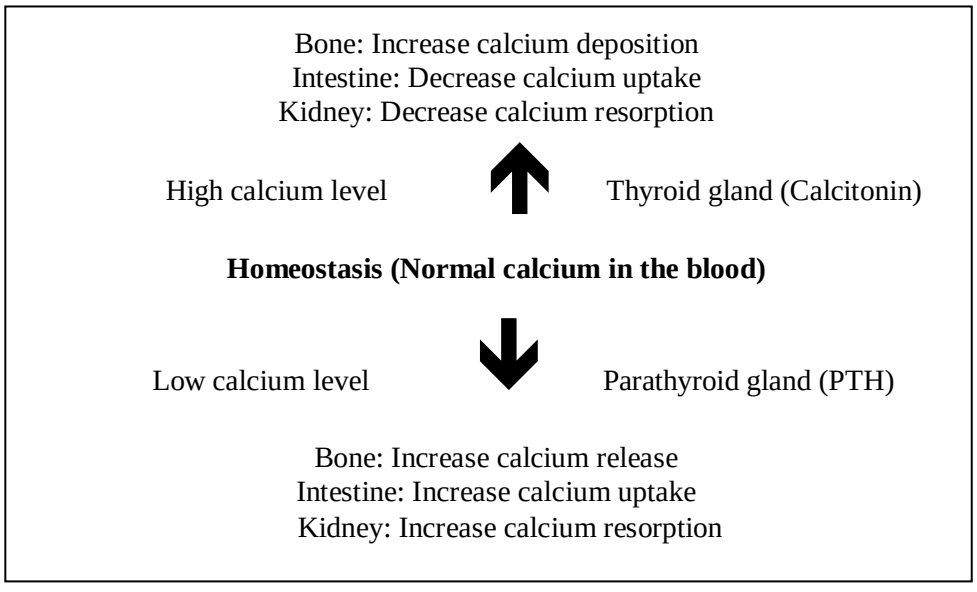


Figure 1-4: Calcium Homeostasis

Sex steroid hormones (Estrogen and Androgens) have a role in bone development and maintenance (63). Androgen deficiency is associated with low BMD by decreasing osteoblast proliferation and inhibiting osteoblast apoptosis (82,83). Moreover, low Estrogen results in increased bone turnover by increasing osteoclast and osteoblast activity (82,83). Estrogens increase osteoblast proliferation, decrease osteoblast and osteocyte apoptosis and induce osteoclast apoptosis (82,83).

1.4.4.2 Vitamin D

Vitamin D or calciferol (fat-soluble vitamin) is a steroid derived from cholesterol. It is present in food as vitamin D_2 (Ergocalciferol) and vitamin D_3 (Cholecalciferol) (32). Ergocalciferol is found in plants and Cholecalciferol is found in animal sources. Cholecalciferol is produced endogenously from 7-dehydrocholestrol when it is activated by ultraviolet light on the skin (32,84). Those forms are the inactive forms of vitamin D; the liver and kidney are the organs which are responsible to convert calciferol to the active form which is 1,25(OH)D (84). The activation process is divided to two steps, each being a hydroxylation of the compound as shown in **Figure 1-5**. 25(OH) D, the first hydroxylation step occurring in the liver, is the biochemical marker of vitamin D status determined from blood (9,84). Hence, children with liver and renal disease are at risk for suboptimal vitamin D status due to impaired conversion of vitamin D to its active forms.

The prevalence of vitamin D deficiency rickets (serum vitamin D less than 12.5 nmol/L) in children living in Northern Canada is 2.9 per 100,000 children (85). In order to maintain bone health, the optimal serum level of 25(OH)D is 75 nmol/L; serum 25(OH)D levels lower than 50 nmol/L is considered deficient in terms of promoting optimal bone health; serum 25(OH)D between 50 –75nmol/L is considered suboptimal vitamin D (86). Vitamin D induced rickets, is typically not seen in children until serum 25(OH) vitamin D levels drop below 25 nmol/L; a true sign that a full blown vitamin D deficiency has developed (87).

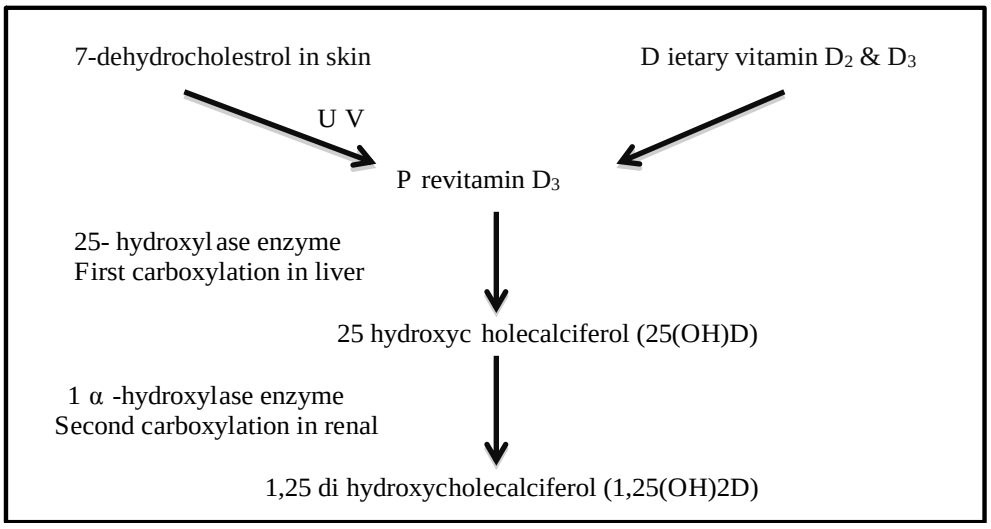


Figure 1-5: Vitamin D Metabolism in the Body

Vitamin D has a major role in skeletal health by maintaining plasma calcium and phosphate levels for bone formation and promoting bone resorption by optimizing rates of ostooclast formation (64,72,84). Thirty to forty percent of calcium and 80% of phosphorus from intestinal absorption is influenced by overall vitamin D status (88). Vitamin D deficiency increases PTH level, which increases bone turnover. Vitamin D level of 25(OH)D< 80 mmol/L was found in 67% of children with cholestatic liver diseases (89).

There are few food sources of vitamin D. These foods include fish, liver, egg yolk and vitamin D-fortified foods such as milk, some orange juices and margarine (88). In Canada, milk is fortified with 100 IU of vitamin D per cup (88). Nevertheless, some children still experience low vitamin D level as a result of deficient sunlight exposure; with estimates of suboptimal vitamin D status ranging from 37.4 to 97% during winter, and especially in northerly communities such as Alberta (90-94). The average intake of vitamin D in Canadian children is 157-165 IU/ day (dietary with supplementation). Approximately, 4-20 % of children are on vitamin D supplementation (94,95). The main dietary source of vitamin D is milk (35%) with an average milk intake of 0-1.5 cup/day (94,95). In 2009, Health Canada reported the median intake of vitamin D in children age 1-3 years was 157 IU/day and 4-8 years was 140 IU/day (96).

Health Canada recommends vitamin D supplementation for infants who are exclusively breastfeeding since birth, and two cups of milk for all Canadians over two years old (97). The Dietary Reference Intakes (DRI) for vitamin D has been changed based on Health Canada recommendations to 400 IUs for infant and 600 IUs for children and adolescents (recommended daily allowance or RDA)_ (97). The RDA for vitamin D was based on promoting 25(OH) vitamin D levels to 50 mmol/L; a level that is associated with vitamin sufficiency. However, there is substantial evidence, that 25(OH) vitamin D levels in the blood associated with optimal bone health should exceed more than 75 nmol/L and that at least 1000 IU/day of vitamin D in adults may need to be consumed in order to achieve blood levels of this magnitude (88,98). Exceeding the tolerable upper intake (UL) is

not recommended without medical supervision (**Table 1-2**) (88).

Nutrition intervention is fundamental to prevent and/or treat complications and improve health. Vitamin D malabsorption is the main concern in children with cholestatic liver disease. Therefore, high dose vitamin D (ergocalciferol or cholecalciferol), 3-10 times the RDA is recommended to treat vitamin D deficiency (30). Some cases may need more aggressive treatment of vitamin D; monthly intramuscular injections of 60000 to 150000 IU is an alternative (30). One publication recommended 2000 IU/day or 50000IU/ week for 6 weeks of vitamin D; then followed 400- 1000 IU/day of vitamin D for infants and 600-1000IU/day for children and adolescent (88).

Table 1-2: Recommendations of Vitamin D Intake (IU) (88)

	DRI[1]			Recommendations for Patients at Risk [2] Requirement/day
	AI	**EAR**	**RDA**	
0-6 months	400	-	-	400-1000
6-12 months	400	-	-	400-1000
1-3 years	-	400	600	600-1000
4-8 years	-	400	600	600-1000
9-18 years	-	400	600	600-1000

AI: Adequate Intake; DRI: Dietary Reference Intakes; EAR: Estimated Average Requirement; RDA: Recommended Dietary Allowance (IU)
[1] by Institute of Medicine
[2] by Endocrine Practice Guidelines Committee

1.4.4.2.1 Sun light exposure

The amount of vitamin D produced in the body is affected by different factors: ultraviolet spectrum, latitude of the city, cloud coverage, amount of skin exposed, age, obesity, ethnicity and the use of sun screen (99). Even though ultraviolet radiation has a role in vitamin D synthesis inside the body, it was been reported the high ultraviolet exposure is associated with an increase risk for skin cancer and therefore recommendations regarding the amount and duration of

sunlight exposure to promote vitamin D sufficiency are not available (99). Whereas cities at high latitude and cloudy weather can result in less exposure to sunlight, the use of sunscreen to prevent skin cancer also influences vitamin D production by decreasing ultraviolet light exposure (99). Moreover, dark skin color produces less vitamin D as a result of melanin, while obese individuals and older age also result in lower production of vitamin D (99). Therefore, the recommendation of sunlight exposure to promote cutaneous vitamin D production is difficult to standardize and as a result the prevalence of vitamin D deficiency is high in Northern cities such as Edmonton and Quebec City (92). Up to 97% of Canadians do not receive adequate levels of vitamin D in winter (90). It has been reported that individuals living in Northern Alberta have lower amounts of vitamin D synthesis between October and March in (52°N) (90).

1.4.4.3 Calcium and Phosphorus

Calcium and phosphorus are the minerals that make up 95% of bone (57). It is important to maintain the normal level of calcium and phosphorus in the blood to prevent bone resorption (100,101). PTH has a fundamental role in maintaining calcium and phosphate homeostasis in blood (100,101) (**Figure 1-3)**. An increased phosphorus level in the blood could be a sign of bone resorption (101).

Calcium is important to achieve maximal PBM and increase BMC (77). A study conducted in Indian toddlers supplemented with calcium and vitamin D with an Indian dessert (laddoo) lead to increases in whole-body BMC. The children were divided to two groups. One group was on 405 mg of calcium given

5 times a week and 30000 IU of Cholecalciferol given monthly for a year; the another group was on 156 mg of calcium given 5 times a week and 30000 IU of Cholecalciferol given monthly for a year, respectively (102). The study found that one-year calcium supplementation along with monthly vitamin D supplementation lead to increased whole-body BMC by 35% in the first group versus 28% in the second group (102). Another study conducted in Canadian children (between 9-18 years) concluded that calcium intake improves whole-body BMC, but with varying efficacy between boys and girls (102). The study recommended 1100 mg/day of calcium for boys and girls from ages 9 -13 years, 1200 mg/day of calcium for boys ages between 14 -18 years and 1000 mg/day of calcium for girls ages between 18 -18 years.

Health Canada in 2009 reported the median calcium intake of children 1-3 years is 1041 mg/day and 1003 mg/day for children 4-8 years (96). The major food source of calcium is dairy products (100). Health Canada recommendation is summarized in **Table 1-3**. Likewise, animal food is the food source of phosphorus such as diary products, poultry and meat (101). Health Canada did not reported any recommendation of phosphorus intake. This could be as a result of naturally high intake of phosphorus (1000-15000 mg/day) (101) (**Table 1-3**).

Table 1-3: Recommendations of Calcium and Phosphorus Intake (mg)

	DRI of Calcium		DRI of Phosphorus [1]		
	RDA[2]	**EAR[3]**	**AI**	**EAR**	**RDA**
0-6 months	200	-	100	-	-
6-12 months	260	-	275	-	-
1-3 years	700	500	-	380	460
4-8 years	1000	800	-	405	500
9-18 years	1300	1100	-	1055	1250

AI: Adequate Intake; DRI: Dietary Reference Intakes; EAR: Estimated Average Requirement; RDA: Recommended Dietary Allowance (mg)

[1] (103)

[2] (97)

[3] (104)

1.3.4.4 Vitamin K

Vitamin K is a fat-soluble vitamin with roles in bone metabolism and blood coagulation (105). There are two forms of vitamin K: phylloquinone (vitamin K1) and menaquinones (vitamin K2). K1 is found in leafy green vegetables (106-108). K2 is found in low dose in meat and egg yolk and endogenously produced by normal flora (106-108). There are seven forms of K2; classified by the n-unsaturated isoprenyl groups (MK-n) (106-108). The number of unsaturated isoprenyl group starts from MK-4 to MK-10 (106-108). Menadione (vitamin K3) is a synthetic water-soluble form of vitamin K and biologically active found in the blood (108). Both K1 and K3 could be converted to MK-4 (108).

Vitamin K1 is an important co-factor in the gamma-carboxylation of several important proteins needed for coagulation (prothrombin), bone formation (osteocalcin), sphingolipid biosynthesis and neuronal function (70,109). Without vitamin K1; these proteins remain in an inactive form (undercarboxylated) and serious problems related to coagulation and bone health can occur (70,109). Vitamin K status can be assessed by measurement of the levels of these

undercarboxylated proteins. These are typically referred to as proteins induced in vitamin K absence (PIVKA) (39). PIVKA-II represents the amount of decarboxylated factor II (prothrombin) that is synthesized in the liver; undercarboxylated osteocalcin reflects vitamin K status related to bone health. Vitamin K plays a vital role in bone re-modeling (108) and a healthy bone matrix (106). It helps produce osteocalcin which is a vitamin K dependent protein produced by osteoblasts in a carboxylated form (70,109). Osteocalcin is critical in terms of supporting bone strength (107) by increasing BMC (110). Osteocalcin deficiency could develop in liver diseases because of vitamin K deficiency (39). Vitamin K deficiency was noted in 67% of children with cholestatic liver disease and 56% in BA children (34,89). The extent to which vitamin K deficiency contributes to poor bone health in children with ESLD pre-and-post LTX remains largely unknown and requires further investigation. This could be investigated by measurement of surrogate markers of vitamin K status: PIVKA II and undercarboxylated osteocalcin (ucOC) (39).

The two forms of vitamin K are taken orally from a variety of food. In the intestine, triacylglycerol-rich lipoprotein transports both K1 and K2 to the liver (106). Some of K1 is execreted and the rest together with K2 is transported by low-density lipoprotein to the tissue (84). The median intake of vitamin K observed in 3-16 years old US children was 45 μg/day (111). The AI of vitamin K is summarized in **Table 1-4** (103).

Table 1-4: Recommendations of Vitamin K Intake (µg)

	AI
0-6 months	2
6-12 months	2.5
1-3 years	30
4-8 years	55
9-13 years	60
14-18 years	75

AI: Adequate Intake (µg)

A study conducted in healthy adults showed that vitamin K intake was 3.5±1.1µg/body weight/day; serum PIVKA-II concentration consistent with deficiency was noted in 14 % but undercarboxylated osteocalcin (unCO) was deficient by 43% (112). The explanation of this is that the amount of vitamin K intake exceeded the sufficient amount in adult (1 µg/body weight/day) and that was more sufficient in the carboxylation in the liver than the bone (112).

1.4.4.4 Other Dietary Factors

The average caffeine consumption among children (5-18 years) in Canada is 1.1 mg/kg/day (113). There are controversial opinions regarding the negative impact of caffeine on bone health (114,115). It has been reported that the negative effect of caffeine occurs by increasing calcium excretion and interfering with bone re-modeling (113,115). Magnesium and zinc are other nutrients that affect bone health in children with liver failure (32).

1.4.4.5 Lifestyle: Physical Activity

Physical activity especially weight-bearing exercise helps to enhance PBM to reach its maximum potential during childhood (8-17 years of age) (116,117) Physical activity has been shown to improve BMD by up to 17% (118) A study conducted with female Tennis players found that weight-bearing exercise is

associated with significant increases in trabecular BMD (by 10.6%) (119). In addition, physical activity increases bone mineralization and longitudinal growth in childhood with consequent long-term benefits (119).

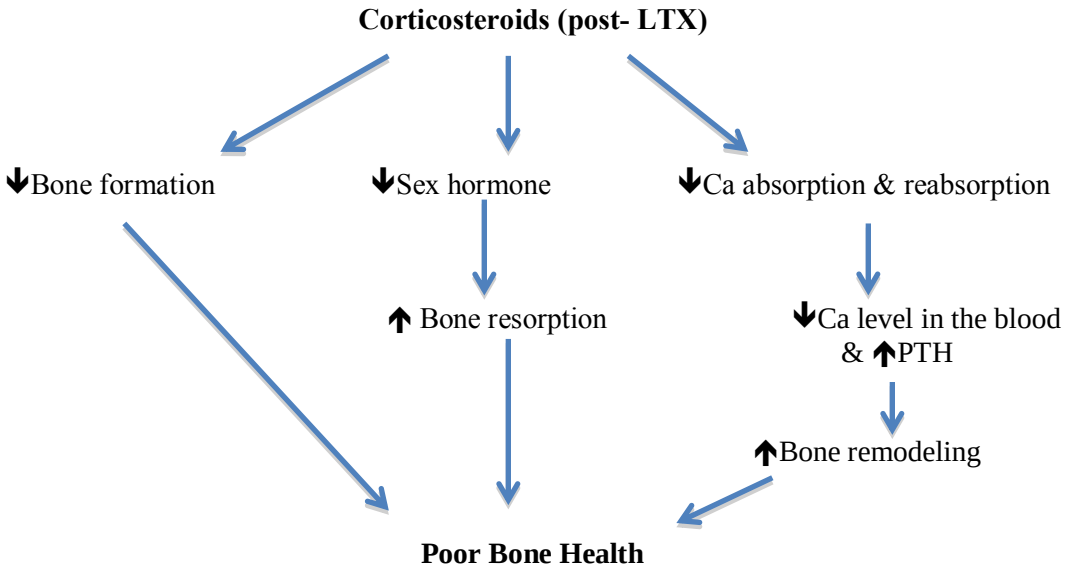
Another study conducted with young boys (10.6 years), found that there were no differences in lumbar, hip and lower limbs BMC and BMD between control and tennis players who play 5 and 3 days a week (120). Additionally, the dominant arm was greater in BMC (32%), bone area (11%) and BMD (15%) than non-dominant arm (119,120). This shows that the volume and intensity of exercise improves BMC, bone area and BMD (120). While the data in children does show that weight bearing activity is associated with overall improvements in BMC and BMD, the data regarding the impact on bone fracture risk in children is less clear (90,118). Some of this controversy may be related to the fact that children and adolescents often experience higher rates of 'stress fractures' related to higher risk physical activities (such as gymnastics) (121). Very little is known the extent to which these types of activities contribute to a higher fracture risk in children with chronic liver disease because few children participate in these types of activities (122).

The American College of Sports Medicine recommends 10 to 20 minutes of moderate to vigorous intensity activity for at least three days a week for children and adolescents to enhance bone health (123). Canadian physical activity guidelines (2011) recommend 60 min of moderate to vigorous intensity physical activity daily for children (5-11years) and adolescents (12-17 years): vigorous intensity activities at least 3 days per week and weight bearing exercise

at least 3 days per week (124).

1.4.4.6 Corticosteroids

Corticosteroid (prednisone and its derivatives) is a steroid hormone that is produced in the body and pharmaceutically for treatment purposes. Corticosteroids have a negative impact on bone health and are an immunosuppressant. Corticosteroids influences bone health by: increasing bone re-modeling, suppressing bone formation and increasing bone resorption (38,125,126). Corticosteroids induce osteoblast formation by inhibiting the release of cellular growth factors (125). Corticosteroids also decrease the absorption of calcium in the intestine, promote calcium excretion in the urine leading to an increased rate of bone resorption. These changes also lead to increase PTH secretion and bone resorption to maintain serum calcium concentrations, and the formation of new bone with lower calcium contents (126). While corticosteroids affect both cortical and trabecular bone; the greatest effects are observed with trabecular bones. This is likely due to the fact that trabecular bone has faster rates of turnover, when compared to cortical bone and hence are more susceptible to acute effects in calcium homeostasis (38,51,73). Therefore, bone loss from long-term corticosteroid use frequently appears first in the lumbar bones before other body parts are affected (38). The fracture rate is higher among patients receiving corticosteroid therapy by 1.3- to 2.6-fold in comparison to no corticosteroids therapy (125). **Figure 1-6** summarizes the negative impact of corticosteroids on bone health (73,126).

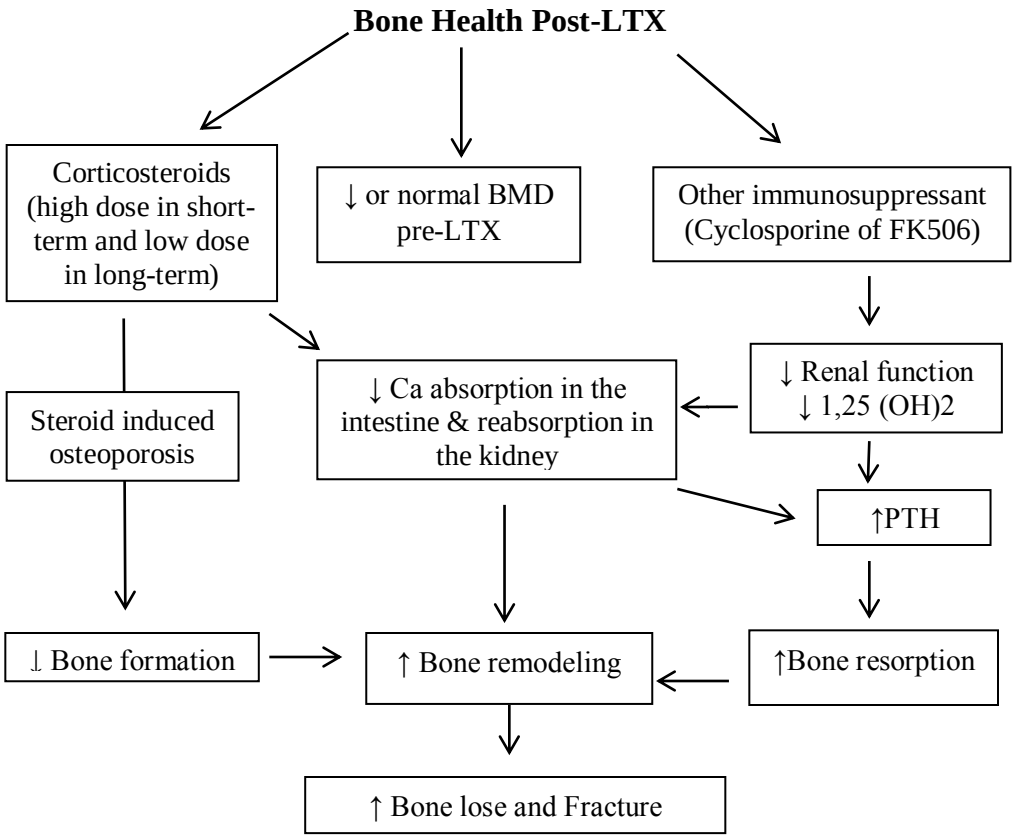


Ca: Calcium; LTX: Liver Transplant; PTH: Parathyroid Hormone

Figure 1-6: Corticosteroids Induced Poor Bone Health; Adapted from: Allen, 2002; Leonard & Bachrach, 2001 (73,126)

Both the duration of corticosteroid usage and the dose of corticosteroids have negative effects in LTX recipients (50,51). Reductions in bone mass; particularly lumbar BMD have been reported to range between 5-24% in the first year post-LTX (6,50); particularly when corticosteroid doses have been high (> 2.5 mg/day) (50,51). Other studies have shown even higher rates of poor bone health (as defined by BMD-z < -2) for as long as 6-7 years post-LTX in children (30). Consistent features of the effects of corticosteroids on bone health in the post-LTX period in children, suggest that maximal bone loss occurs around 6 months post-LTX, with some recovery of bone loss in the first 2 years post-LTX (6,127). The potential results of these changes in bone health are an increased risk for bone fracture; a significant co-morbidity in children who have undergone a life saving surgery. Fracture rates in children post-LTX have been reported to

range between 10-40% (30), with the highest rates of fracture (25-65%) in the spine in the first 6-12 months post-LTX (6,37). The fracture risk increases after 3 to 6 months with the addition of corticosteroid administration (50,51) with an average 12-38 % in the first year of LTX (30,37). Even after tapering off of corticosteroid therapy, the fracture rate in children 5 years post organ transplant has been reported to be six times higher that the fractures rates reported with a control population, particularly vertebral fractures (160 times higher) (128). Body mass index pre-LTX, type cholestatic liver disease and older age at LTX are also other factors associated with the high risk for fractures in this population (51,128).



Ca: Calcium; LTX: Liver Transplant; PTH: Parathyroid Hormone

Figure 1-7: Mechanisms of Poor Bone Health post Liver Transplant in Short and Long-term Period; **Adapted form: Kulak et al, 2010** (127)

1.5 Dietary Assessment Methods

There are three main methods to assess dietary intake: 24-hour recalls, use of 3 days food intake records and food frequency questionnaire (FFQ). The twenty-four-hour recall is a recall of what has been consumed in the previous day which could be done on more than one occasion (129). A Dietary record is the prospective record of what is been consumed in the next 1-7 days (130). A FFQ is one tool that contains a list of food items and estimates the frequency (per day, week or month) and the usual serving size (130). Each method has its weakness such as the lack of precision and the alteration of food behavior reporting to become socially acceptable (131). **Table 1-5** summarizes the advantages and disadvantages of these three dietary assessment methods.

Table 1-5: The Advantages and Disadvantages of Dietary Assessment Methods (129,130,132,133)

	Advantage	Disadvantage
24- hour Recall	• Quick tool to compare dietary intake between groups for large scale survey • Low respondent burden • Able to be administered by phone	• Accuracy decreases with few days applied • Memory dependent • Estimation of portion size
Dietary Record	• Widely used method • Precise method • Serving size measurement could be household or weighed • Recording could be done by participant or external person	• Expensive • High respondent burden
Food Frequency Questionnaire	• Practical and cost effective • Extends long period of time • Quick tool to assess the epidemiological deficiency • Low respondent burden • Posted or administered by phone • Self completed	• Low precision • Time consuming in validation of the tool • Possible for over or under estimation of specific food

The differences in the results of nutrient intake assessment by FFQ compared to other dietary methods (24-hour recall) is the lower precision that is experienced with the use of FFQ (132,133). This may be due to the fact that FFQ measures the usual intake of some foods that may only be occasionally consumed. (132). This will overestimate specific nutrient intakes; particularly when these nutrients may be found in a narrow selection of food types. For example, vitamin D is found within a narrow selection of foods (fortified dairy products, fatty fish) which that may result in over/under estimations of vitamin D intake (131). On the other hand, 24-hour recalls and dietary records (3 day food intake) estimate the actual intake of daily food; particularly when used at more than one time point to estimate food intake (132). Moreover, multiple recalls or records are better to detect seasonal variability of micronutrient intake, and daily variation (129,131). They are, however, limited by higher respondent participation and investigator burden. When used in combination with a FFQ, the multi-pass diet recall/food record are good tools at detecting nutrient intake in individuals (131,132). In clinical studies, the use of multi-pass 24 hour- recalls is better than the 3 day prospective food intake records in terms of decreasing the patient burden and the cost of the research (132).

1.6 Conclusion

Infants and children with end-stage liver failure often have severe malnutrition due to anorexia, malabsorption of fat and fat-soluble vitamins and altered nutrient utilization. This results in significant risk for poor bone health

and growth failure in the pre-transplant period. LTX is a life saving procedure for infants and children with ESLD. However, this procedure is not without its complications. The major complications include the risk for graft failure, adverse drug effects (e.g. growth failure, suboptimal bone health, renal insufficiency) and an increased risk for the development of lymphoproliferative disease. One of the major variables thought to contribute to poor bone health and suboptimal growth in the post-LTX period is the use of corticosteroid therapy. While the use of corticosteroids as part of an overall immunosuppressive therapy to maintain graft survival in children who have received an organ transplant is very common, there are some medical centers (including the adult and pediatric University of Alberta Liver Transplant Program) that have chosen to develop immunosuppressive drug protocols that do not include the use of corticosteroids. Little is currently known to what extent these changes in immunosuppressive protocols have impacted the bone health in children post-LTX. Some data suggests that early withdrawal of corticosteroid therapy results in improved growth and bone health; while others have shown minimal impact on growth and bone health outcomes (134). Few studies have also examined the impact of lifestyle variables (diet, weight bearing activity, sunlight exposure) on micronutrient (vitamin D, K and calcium) status known to influence overall bone health in this population. This information is critically needed to ensure that evidenced based nutrition focused protocols for prevention of growth failure and poor bone health in infants and children undergoing LTX are developed. The focus of this thesis is to retrospectively examine the impact of a corticosteroid free protocol on growth and bone health

outcomes in infants and children who have undergone LTX within the LTX Program at the Stollery Children's Hospital and to describe some of the potential lifestyle factors (diet) that may influence overall bone health in children with cholestatic liver disease who have had LTX.

Chapter 2: Research Plan

2.1 Study Rationale

Liver transplant (LTX) is an important life saving procedure for infants and children with end stage liver Disease (ESLD). There are many liver diseases in infancy and childhood that result in ESLD and where LTX is a life saving procedure. Biliary Atresia (BA) is the main reason for the need of LTX in children, particularly in the first year of life (43). Children with BA and other cholestatic liver diseases experience several co-morbidities pre-LTX. These include poor bone health (hepatic osteodystrophy) and severe growth delays. All of these are typically due to malnutrition related to anorexia, malabsorption of key nutrients essential for growth and health (such as fat and fat-soluble vitamins), and altered nutritional requirements (5,30). While post -LTX, these issues improve substantially, some children continue to experience growth failure and suboptimal bone health (49,134-137).

There are many factors that may contribute to poor bone health (Steroid induced osteoporosis) and poor growth in children post-LTX. These include age at LTX (134), period since LTX (134), the number of rejection episodes (49,136), type of immunosuppressant medication (corticosteroid) therapy used (5,30,51,136) and nutritional status pre-and post- LTX (5,30). Children who experience LTX at earlier ages tend to have better bone mineral density (BMD) due to shorter periods of suboptimal nutrition prior to LTX (134). However, the major factor thought to contribute to poor bone health post-LTX, is the type of immunosuppressive therapy used. In particular the use of corticosteroids post-

LTX is thought to be related to a higher risk for reduced BMD and growth failure (136). Corticosteroids are associated with increased calcium excretion and bone turnover; all factors associated with an increased risk for reduced BMD. Other factors that are thought to be contributing factors include suboptimal vitamin D status due to reduced dietary intake and endogenous cutaneous synthesis of vitamin D due to poor sunlight exposure post-LTX (5,30,138-140). While most pediatric LTX centres include corticosteroids within their immunosuppressive regimes, the LTX Program at the Stollery Children's Hospital became a corticosteroid-free program in 2003.

The purpose of this thesis was to retrospectively review the impact of a corticosteroid -free immunosuppressive protocol on bone health parameters and growth post-LTX in infants and children undergoing LTX. To examine the other potential dietary variables contributing to bone health in this population, we also prospectively studied intakes of vitamin D/K and calcium in a pilot study in children post LTX and in healthy children.

2.2 Hypotheses & Objectives:

Hypothesis 1: Infants and children receiving corticosteroids as part of their immunosuppressant protocol will have significantly lower BMD (lumbar and whole-body) and growth than infants and children who were treated without corticosteroids in the post-LTX period.

Study 1: Corticosteroid–free Immunosuppressive Protocols: Effects on Bone Health and Growth in Infants and Children Post Liver Transplantation (Chapter 3)

Objective 1: Determine if there were differences in BMD/bone mineral content (BMC) between children post-LTX who received corticosteroid therapy verses those that did not receive corticosteroids.

Objective 2: Determine the extent to which confounding variables (bone age, age at LTX, number of rejection episodes) may contribute to BMD/BMC in infants and children post-LTX.

Study 2: Vitamin D, Vitamin K and Calcium Intake in Children Post Liver Transplant (Chapter 4)

Objective 1: Compare dietary intakes of calcium, vitamin D/K in children who have undergone LTX with age matched healthy and/or disease control children.

Objective 2: Compare dietary intake of vitamin D, calcium and vitamin K and whether these meet the Dietary Reference Intake (DRI) in children who have undergone LTX and in age matched healthy and/or disease control children.

Chapter 3: corticosteroid-free Immunosuppressive Protocol: Effects on Bone Health and Growth in Infants and Children Post Liver Transplantation

3.1 Abstract

The immunosuppressive protocol for children post-liver transplant (LTX) was changed in 2003 from a "corticosteroid protocol" to "corticosteroid -free protocol" to decrease long-term complications of corticosteroid use in children post-LTX. The study objective was to compare bone mineral density (BMD) (as measured by Dual X-ray absorptiometry) in children undergoing a corticosteroid-free or corticosteroid containing drug regimen post-LTX. We retrospectively reviewed 115 charts of all children post-LTX at the Stollery Children's Hospital (1999-2009). Variables studied included anthropometric, demographic, laboratory parameters and use of corticosteroid therapy (dose, duration). A total of 39 patient charts (20 Female, 19 Male) met study inclusion. Twenty-eight children received corticosteroids (age:100±49 months) and 11 children were corticosteroid-free (age: 110±48 months). The mean weight-for-age and height-for-age z-score on-and-off corticosteroids were -0.31±0.14 (corticosteroid) and 0.22±0.23 (corticosteroid-free) ($p=0.09$); -0.71 ±0.13 (corticosteroid) and 0.23±0.22 (corticosteroid-free) ($p=0.002$). BMD z-score < -2 was in 15% in lumbar and 8% in whole-body. Logarithmic transformation of lumbar Bone Mineral Content (BMC) was inversely related to corticosteroid doses (>0.2 mg/kg/day) and positively related to bone age ($p<0.001$) ($r^2=0.890$; $p<0.001$). Low dose corticosteroid therapy in children post-LTX is associated with reduced bone mineral content and delayed linear growth.

3.2 Introduction

Children with liver failure typically experience significant growth delay and failure to thrive due to malnutrition and malabsorption of fat and fat-soluble vitamins (30). Although, many children post-LTX experience significant improvements in overall growth and development due to resolution of these issues, some children still continue to experience suboptimal growth and bone status post-LTX (30). This is likely due to the use of immunosuppressive therapy, particularly the use of corticosteroid therapy, as well as other factors such as sustained pre-and-post LTX under-nutrition and fat-soluble vitamin (particularly vitamin D/K) insufficiency (39,89,141). As part of the overall clinical management of infants and children receiving LTX, infants and children receive annual Dual X-ray Absorptiometry (DXA) scans for assessment of overall BMD and bone health.

Chronic corticosteroid use is associated with an up-regulation of protein catabolism and calcium turnover (by decreasing absorption and increasing excretion); all of which are known contribute to decreased BMD (73,126). Bolus or pulse corticosteroid use is used in most transplant centers for the treatment of acute cellular rejection, which is known to occur in up to 80% of children in the first six months post-LTX (1). The extent to which the use of corticosteroids influences childhood growth and BMD in children post-LTX is controversial (134,136). Some studies show that the use of corticosteroids (frequency and dose) has a large impact on growth post-LTX (30,49,136); while other studies show little or no effects of corticosteroids on bone health and growth

(134-136). A systematic review examining the influence of chronic corticosteroids use has shown that chronic doses above or equal to 0.2mg/kg/day for >1-2 years is associated with greater adverse effects on growth in children (49). The pediatric LTX program at the Stollery Children's Hospital established a Corticosteroid -free immunosuppressant program in 2003 following a successful conversion of the LTX adult program to Corticosteroid -free protocol; whereby the majority of LTX recipients receive little or no corticosteroids as part of their immediate post-LTX care (142). Corticosteroid use in this new protocol was predominantly relegated for the use of acute cellular rejection; with pulse steroid administration (intravenous followed by oral) over a short duration; following a rapid taper down (**Table 3-1**) at lower doses. The long-term goal of this new protocol was to minimize or discontinue the use of corticosteroid therapy as part of the overall immunosuppressive management of children post-LTX within the program.

Table 3-1: Corticosteroid Dosing Pre and Post 2003

Pre 2003		**Post 2003***
Maintenance Therapy	**Rejection Therapy (Acute)**	**Rejection Therapy**
10mg/kg/day in the first three days post-LTX	10 mg/kg/day in the first three days	10 mg/kg/day in the first three days
1 mg/kg/day (PO/IV) for 3 months	1 mg/kg/day (PO/IV) for 3 months	1 mg/kg/ day (Po/IV) for 4 weeks
Weaning off Protocol: 0.5 mg/kg/day for 3 months	Weaning off Protocol: 0.5 mg/kg for 3months	Weaning off Protocol: Reduce dose by 5 mg/week until D/C
0.1-0.2 mg/kg/day till 1 year (for approximately 6 months)	Not Applicable	Not Applicable

* Corticosteroids NOT included in maintenance rejection therapy
5 mg prednisone (PO, oral) = 4 mg Methylprednisolone (IV, intravenous)

The purpose of this retrospective review was to investigate whether the implementation of a corticosteroid-free protocol resulted in significant differences in bone health and growth in infants and children undergoing LTX at the Stollery Children's Hospital. Primary outcome variables of study included: BMD/BMC as measured by DXA, bone age, fracture incidence and growth post-LTX (linear and weight). We hypothesized that the use of a corticosteroid-free immunosuppressant protocol in infants and children undergoing LTX would result in significantly greater BMD and improved growth rate than in children who had corticosteroids included in their immunosuppressant protocol post-LTX.

3.3 Methods

3.3.1 Patient Population and Study Design

This is a retrospective chart review, which included a review of 115 patient charts of infants and children who underwent LTX at the Stollery Children's Hospital, Edmonton, Alberta, Canada between January 1999 and December 2009. The inclusion criterion was children and infants (1-17 years post-LTX) who have undergone LTX and had one DXA study performed at least one-year post-LTX. The exclusion criteria included: LTX prior to January 1999 and post December 2009, multi-visceral transplant and no available DXA post-LTX. Patients were also excluded from the analysis if their primary medical care post-LTX was outside of Alberta and lack of available DXA data (**Figure 3-1**).

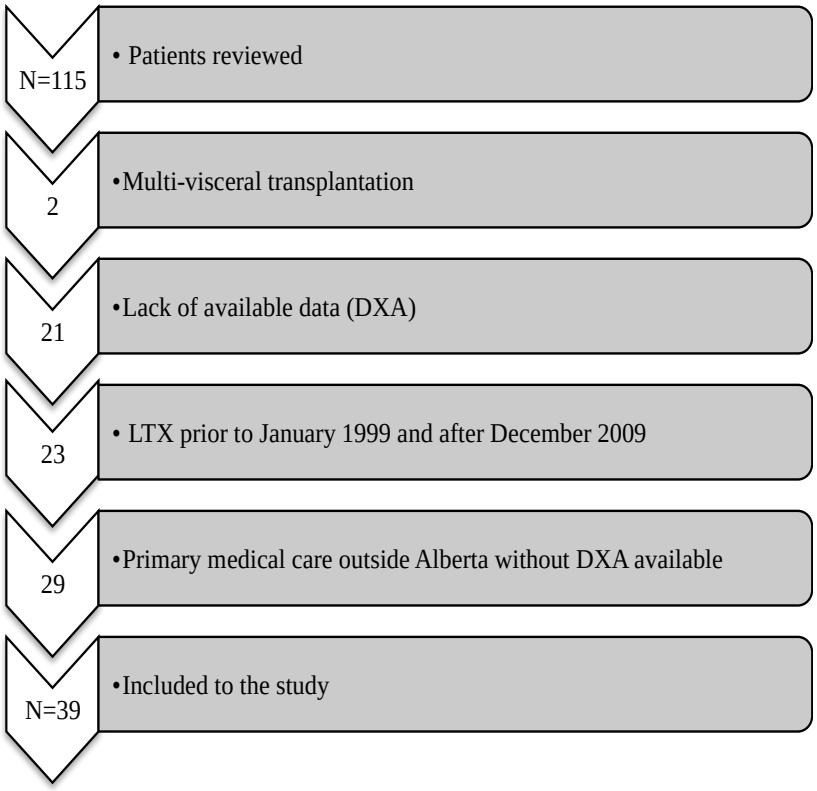


Figure 3-1: The Inclusion and Exclusion Criteria

Ethics Approval was obtained from the Human Research Ethics Board at University of Alberta and Operational and Administrative Approval from the Stollery Children's Hospital, Alberta Health Services (AHS) and the Northern Clinical Trials Centre at AHS/University of Alberta/Covenant Health prior to initiation of this study review.

3.3.2 Immunosuppressive Protocol within the Pediatric LTX Program

In 2003 the Stollery Children's Hospital LTX program adopted a corticosteroid -free immunosuppressant protocol that resulted in significant reductions in the use of corticosteroids for immunosuppressive therapy in the program. The main immunosuppressive therapy used after this period was tacrolimus. The initiation of a corticosteroid -free immunosuppressant protocol

(post 2003) did not preclude the use of corticosteroids where clinically warranted for treatment of acute rejection, but did minimize the use of this medication in the overall management of patients post-LTX and in the treatment of chronic rejection. In this paper, the corticosteroid immunosuppressant protocol will be defined as the protocol pre-2003 (corticosteroid immunosuppressant protocol) versus post-2003 (corticosteroid -free immunosuppressant protocol) (**Table 3-1**).

3.3.3 DXA Protocol within the Pediatric LTX Program

A DXA scan is one method for measurement of BMD and BMC in different parts of the body (lumbar spine and/or whole-body) which can be used starting at age of 2-18 months (143). A DXA scan in lumbar spine is done annually for post-LTX children followed at Stollery Children's Hospital, prior to 2009 (Hologic QDR 4500A and Apex System 2.4.2; Hologic Inc., Walham, MA, USA) at routinely scheduled clinic visits. All BMD z-scores (BMD-z) was adjusted based on chronological age (61). The BMD-z estimates the risk of osteoporosis by comparing the measurement to a healthy child of the same age, gender and ethnicity. According to the World Health Organization definition of bone health in children, BMD-z <-1 indicates an increased risk for poor bone health and BMD-z <-2 is indicative of a diagnosis of osteoporosis (143).

3.3.4 Primary and Secondary Outcome Variables

Demographic data reviewed included age at DXA, age at transplant, number of transplant(s), indication for LTX, number of episodes of rejection post LTX, use of corticosteroids (therapeutic indication, dose, duration and frequency), history of bone fracture (post-LTX). Corticosteroids dose (cumulative lifetime

dose (mg, mg/day, mg/kg and mg/kg/day) and one-year prior to DXA dose) and duration of corticosteroids (days) use were then calculated utilizing this data for each patient.

Anthropometric data (weight and height) was collected from the DXA report. These were all performed according to standard methodologies at the same centre (61). Height-for-age z-score (ht-z), weight-for-age z-score (wt-z), body mass index (BMI) and BMI z-score (BMI-z) were calculated using Epi Info 3.5.1 software (Atlanta, GA, USA) using Centre for Disease Control growth standards (144). Laboratory data were collected within one month of the time the DXA scans were performed. Laboratory variables reviewed included: 25-hydroxycholecalciferol (25(OH)D), Parathyroid Hormone (PTH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), calcium, total bilirubin, creatinine and urea. For purposes of analysis, the optimal serum 25(OH)D for bone health was defined as a value >75nmol/L; serum 25(OH)D level <50nmol/L was considered as deficient; serum 25(OH)D 50 –75nmol/L was considered as suboptimal (86,105). All laboratory data were collected from the medical charts of patients/electronic health record (OTTR) and were performed within the Core Laboratory, University of Alberta Hospital, Alberta Health Services.

3.3.5 Statistical Methods

Values are expressed as mean ± standard error (SE). Data were analyzed using SPSS Statistics (version 19, SPSS Data Collection, Chicago, USA, 2010). Data were analyzed by treating individual patient data as single measures, rather than repeated measures. This was done because the sample size at each time point

was very small; precluding sufficient power to detected differences within an individual. A post-hoc power analysis was run in each time point for the primary outcome variables. The data is described in two different ways: on corticosteroid/ corticosteroid-free and pre/post 2003. Corticosteroid data (dosing and duration of therapy) was calculated in two different ways: as a categorical variable by dosing (on corticosteroid/ corticosteroid-free, above and below 0.2 mg/kg/day, and above and below 365 days) and as a continuous variable (duration of therapy in days and dose (mg, mg/kg, mg/kg/day and days). Wt-z and ht-z were compared between on-and-off corticosteroids over 1-6 years post-LTX using univariate analysis. Groups (on corticosteroids and corticosteroid-free) were compared using univariate analysis as a continuous variable for anthropometric, laboratory and bone health parameters. BMC and BMD were transformed by the natural logarithmic transformation (logBMC and logBMD). A multivariate regression was run using bone health parameters (BMD/BMD-z/BMC: lumbar-spine and whole-body) and anthropometric data. Fisher-exact was used to assess differences (between groups) in frequency. A p value of < 0.05 was considered statistically significant (two-tailed tests).

3.4 Results

3.4.1 Patient Characteristics

A total of 39 patient charts (20 male; 19 female) were extensively reviewed for this study. These 39 charts included a total review of 126 DXA reports that covered a period of time 5.5± 0.3 years post-LTX (1-11 years). Of these 39 charts n=8 had 1 DXA measurement (1-8 years post-LTX); n=5 had 2

DXA measurements (2-9 years post-LTX), n=11 had 3 DXA measurements (3-9 years post-LTX); n=8 had 4 DXA measurements (4-11 years post-LTX); n=4 had 5 DXA measurements (5-10 years post-LTX); n=1 had 7 DXA measurements (10-11 years post-LTX); n=2 had 8 DXA measurements (11 years post-LTX)) (**Figure 3-1**).

3.4.2 Demographic Data

Table 3-2 summarizes the Demographic Data of the 39 charts reviewed. The primary indication for LTX in this cohort was BA. However, there was also a significant increase in the number of children with other liver disorders (6 vs 12) as the indication for LTX post-2003. As a result, many of these children received their first LTX at older ages in the post-2003 time frame compared to the pre-2003 time frame.

Table 3-2: Demographic Data

	Pre 2003 (n=16)[1]	Post 2003 (n=23)[1]
Indication of LTX (n)		
Biliary Atresia	10	11
Alagille Syndrome	2	1
Citrullinemia	1	2
Autoimmune Cirrhosis	-	1
Fulminant Hepatic Failure	2	1
Alpha 1-antitrypsin deficiency	1	1
Autoimmune Hepatitis	-	3
Hepatic Carcinoma	-	1
Wilson Disease	-	1
Crigler-Najjar Syndrome	-	1
PELD Score	14 ± 5	17 ± 4
MELD Score	-	19 ± 4[2]
Age at Transplant (months)[3]	21 ± 6 (5-86)	75 ± 16 (4-201)[4]
<2 years (n)	13	9
>2 years (n)	3	14[5]
No of Transplant		
1	15	17
2	0	5
3	0	1
4	1	0
Fracture (n)	1;3 fractures: femur and other	1; 1 femur fracture

[1] Children pre 2003 were on corticosteroid; only 12 children post 2003 were on corticosteroid
[2] n=6 for MELD score
[3] Mean ± SE
[4] There are six patients who have LTX after age of 12 years (170 ±9) months. When those six patients are removed, the mean age of LTX is 39 ± 11 (4-108) months
[5] 8 patients had LTX between the age of 2-12 years and 6 patients had LTX above 12 years of age

3.4.3 Rejection Data

The reason, frequency and the average graft survival are presented in **Table 3-3.**

Table 3-3: Rejection Data

	Pre 2003 (n=16)[1]	**Post 2003 (n=23)[1]**
Graft Survival for Multiple Transplants (n)		
2-3 days	-	3
125-91 days	-	2
385 days	1	1
Reason (n)		
Hepatic Artery Thrombosis	1	2
Hepatic Artery Stenosis	-	1
Chronic Rejection	-	1
Non-adherence	-	1
Primary non-function and Portal vein thrombosis	-	1 (3 LTX)
Graft Survival for one Transplant (n) [2]		
< 3 years	0	7
> 3 years	15	10
Acute Cellular Rejection (n)		
One rejection episode	5	8
Two rejection episodes	3	3
Four rejection episodes	1	-
Banff Score (mean)		
First Rejection	4	4
Second Rejection	3	4

[1] Children pre 2003 were on corticosteroid; only 12 children post 2003 were on corticosteroid
[2] Calculated from the date of transplant to the date of last DXA

3.4.4 Corticosteroids

Out of a total of 23children who were transplanted post 2003, 11children were totally corticosteroid-free with the remaining 12 children receiving some corticosteroid therapy for the treatment of acute rejection. A total of 28 children (16 pre-2003 and 12 post-2003) (**Figure 3-2**) received corticosteroids. Data regarding the mean duration of corticosteroid therapy are presented in **Figure 3-3**.

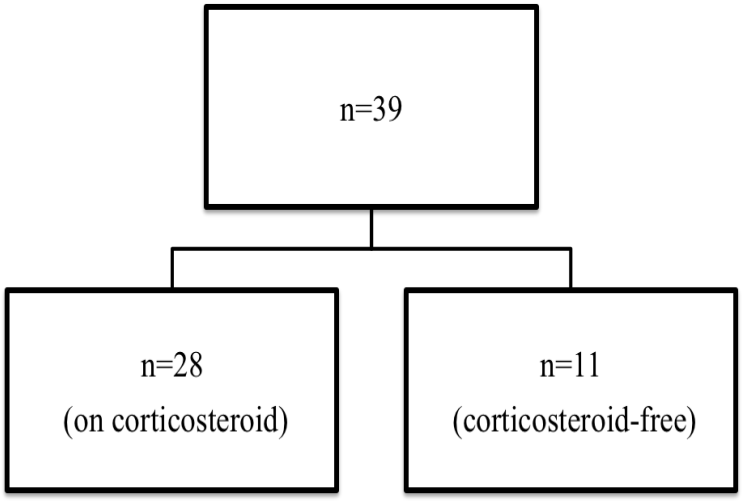
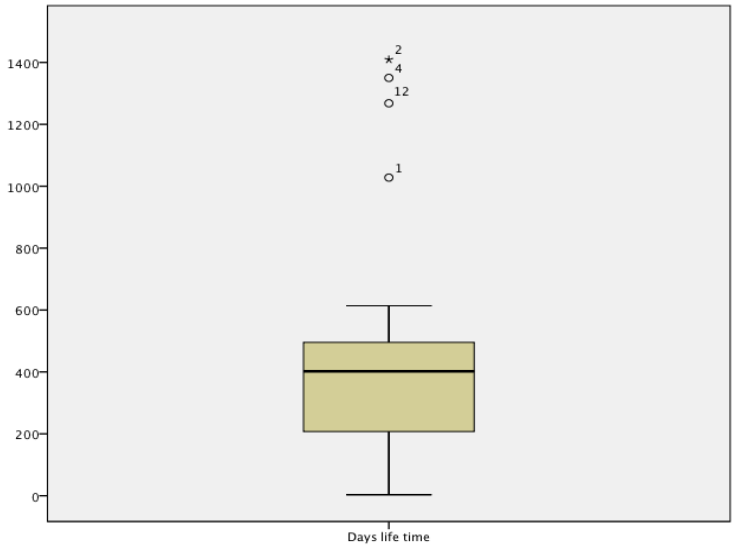


Figure 3-2: Sample size of cohort studied



Twenty-eight patients on corticosteroid; Outliers: Patient No 1 had 4 LTXs (pre 2003); Patient No 2 had chronic rejection (post 2003); Patient No 4 and 12 had Autoimmune Hepatitis (post 2003)

Figure 3-3: The mean duration of corticosteroid therapy in children receiving corticosteroid therapy (n=28) pre-and-post 2003

3.4.5 Laboratory Variables

Laboratory variables are presented in **Table 3-4**. Data regarding serum levels of 25(OH)D and PTH were only available post-2007. A total of 85 measures were available for review for 25(OH)D (63 measures for patients on corticosteroids; 23 measures for children corticosteroid-free) and 12 measures for PTH (n=11 measures on corticosteroid and n=2 measure corticosteroid-free). Of these 20 /63 measurements for children on corticosteroid and 6/23 measurements for children corticosteroid-free had vitamin D <75nmol/L (p=0.792). All patients but three had PTH levels within normal ranges (PTH< 6.8pmol/L is 9/11 (on corticosteroid) and 1/2 (corticosteroid-free; p= 0.769). Renal function parameters (urea and creatinine) were reviewed to assess the potential influence of kidney dysfunction (due to immunosuppressive therapy on overall bone health).

Table 3-4: Laboratory Variables

Models	On Corticosteroid (n=28)	Corticosteroid-free (n=11)	*P*-value
25(OH)D (nmol/L)[1]	94.3 ± 5.9 (27 - 200)	94.8 ± 9.1 (45 - 203)	0.404
PTH (pmol/L)[2]	4.9 ± 0.7 (2.6 -9.7)	9.3 ± 3.1 (3.5 - 14.3)	0.051
Calcium (mmol/L)	2.34 ± 0.01 (2.14 - 2.55)	2.42 ± 0.02 (2.17 - 2.58)	0.004
T. Bilirubin (umol/L)	10.2 ± 1.0 (1 - 49)	10.7 ± 1.5 (4 - 36)	0.518
ALT (U/L)	38 ± 4 (10 - 326)	46 ± 7 (10 - 394)	0.418
AST (U/L)	40.5 ± 3 (4 - 174)	56.8 ± 19 (7 - 478)	0.177
Creatinine (μmol/L)	41.7 ± 1.5 (5 - 85)	47.2 ± 2.1 (14 - 68)	0.041
Urea (mmol/L)	5.1 ± 0.5 (2.1 - 32)	4.9 ± 0.8 (2.4 - 44)	0.984

Values are represented as mean ± standard error
25(OH)D: 25 hydroxycholecalciferol; T. Bilirubin: Total Bilirubin; PTH: Parathyroid Hormone; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase
[1] 33 patients had vitamin D value: 23 and 10 patients on corticosteroid and corticosteroid-free. Vitamin D <75nmol/L was in 9/23 (on corticosteroid) and 5/10 (corticosteroid-free)
[2] 8 patients had PTH value: 6 (corticosteroid) and 2 patients (corticosteroid-free). PTH <6.8pmol/L was in 5/6 (on corticosteroid) and 1/2 (corticosteroid-free).

3.4.6 Anthropometrics Data

The age of children who were on corticosteroid was 100±49 and who were corticosteroid-free was 110±48 months (p>0.05). Anthropometric measurements are summarized in **Table 3-5** and **Figure 3-3**. A total of 86 (corticosteroid) and 30 (corticosteroid-free) measures for weight and height were available for analysis. Out of these, approximately 8 out of 86 measures in children had a wt-z < -2 (corticosteroid) and 2 out of 30 (corticosteroid-free) had wt-z <-2 (p=0.495). Ht-z were <-2 in 15 out of 86 (on corticosteroid therapy) and in 3 out of 30 children (corticosteroid-free) (p=0.397). Children on corticosteroid-free immunosuppressive regimens had higher height-z scores at one and four years post LTX (Figure 3-4A) and higher weight-z scores after 1year LTX (Figure 3-4B) when compared to those children on corticosteroids (P<0.05). A post-hoc power analysis, revealed sufficient power (β >0.8) for the height-z and weight z-scores at these time points only; indicating that insufficient sample size was available at the other times to detect any other differences.

Table 3-5: Anthropometric Measurements

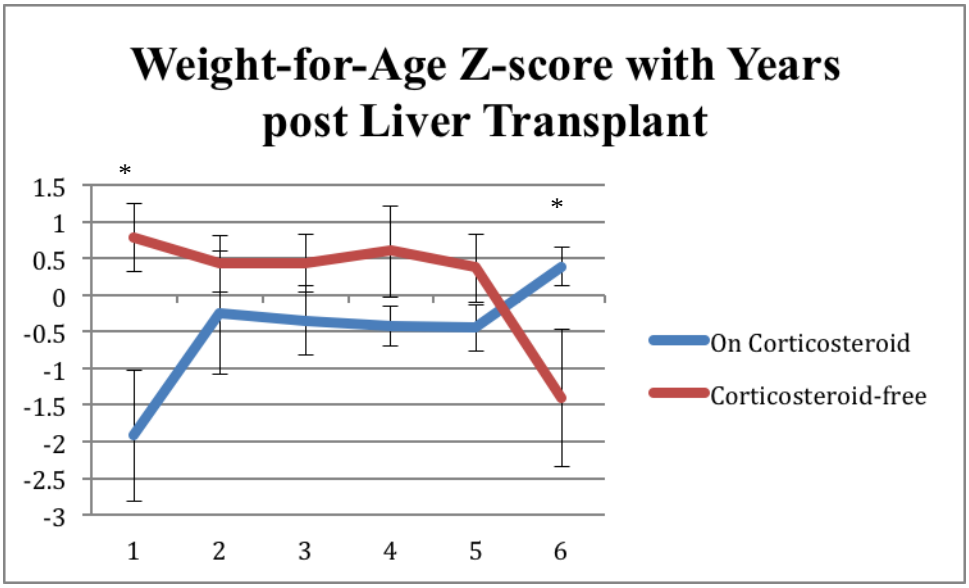
Models	On Corticosteroid (n= 28)	Corticosteroid-free (n=11)	*P*-value
Weight (kg)	26.1 ± 1.5	32.9 ± 2.4	0.020
Weight growth rate (kg/day)	0.015 ± 0.003	0.011 ± 0.005	0.560
Weight for age z-score[1]	-0.31 ± 0.14	0.22 ± 0.23	0.049
Height (cm)	120.0 ± 2.3	132.3 ± 3.6	0.005
Height growth rate (m/day)	0.027 ± 0.004	0.030 ± 0.006	0.629
Height for age z-score[2]	-0.71 ± 0.13	0.23 ± 0.21	0.001
BMI (kg/m^2)	17.1 ± 0.4	17.9 ± 0.6	0.258
BMI for age z-score	0.23 ± 0.11	0.41 ± 0.18	0.391

Values are represented as mean ± standard error

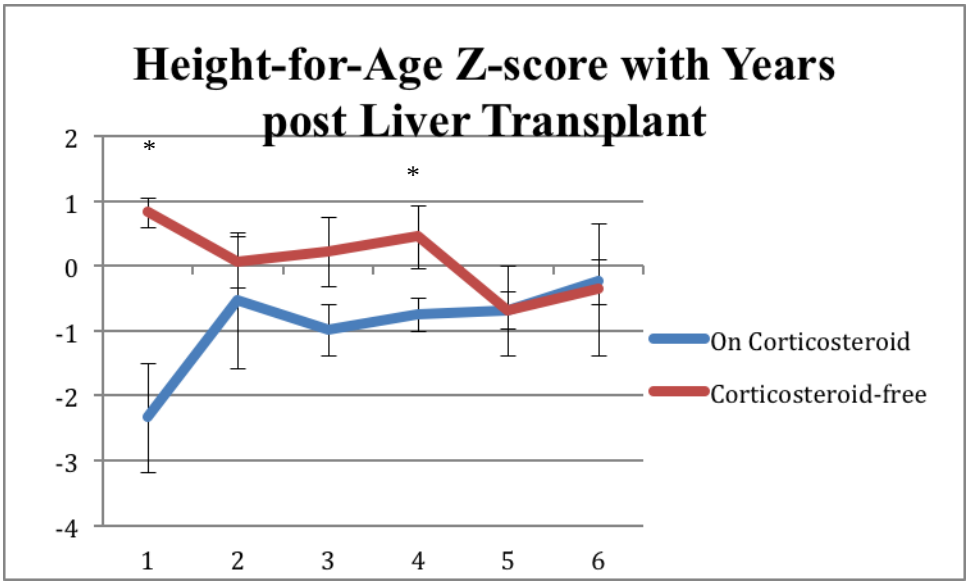
[1] Weight for age z-score <-2 was in 4/28 patients (on corticosteroid) and 2/11 patients (corticosteroid-free)

[2] Height for age z-score <-2 was 5/28 patients (on corticosteroid) and 3/11 patients (corticosteroid-free)

3-4A



3-4B



The number of children 1-6 years post-LTX is 3,3,10,13,16,11(on corticosteroid) and 5,6,8,5,3,2 (corticosteroid-free), respectively

Figure 3-4: **The impact of corticosteroid therapy on weight (3-4A) and height (3-4B) growth post liver transplantation (1-6 years). Values are mean ± standard error (SE). Values with asterisk are significantly different $p<0.05$**

3.4.7 Bone Mineral Density

BMD is summarized in **Table 3-6**. A total of 86 measures were available for lumbar BMD (absolute and z-scores) for children on corticosteroid therapy and approximately 34 measures for children who were on corticosteroid-free. There were no significant differences in the proportion of children with lumbar BMD-z less than -1 when on corticosteroid therapy (n=32/88 measurements) versus corticosteroid-free (n=14/31 measurements) (p=0.400). Lumbar BMD-z less than -2 was (n=5/88 measurements) on corticosteroid therapy versus on a corticoesteroid-free protocol (n=3/31 measurements) (p=0.428). Whole-body BMD-z was available for a total of 30 measurements (on corticosteroid); 17 measurements (corticosteroid-free). No significant differences in whole-body BMD-z were noted between the proportion of measures with z-scores less than -1 (n=9/30 (on corticosteroid) versus 4/11 (corticosteroid-free) or less than -2 (n=5/30 (on corticosteroid) versus 2/17 (corticosteroid-free) (p>0.05). The mean bone age (months) between children on corticosteroid and corticosteroid-free is 101 ± 5 (on corticosteroid) and 112 ± 10 (corticosteroid-free) (p= 0.289).

Multivariate models were run on absolute and log transformed lumbar and whole-body BMD/BMD-z scores in two different ways: corticosteroid doses above and below 0.2mg/kg/day with bone age; corticosteroid doses (continuous variable with bone age; and duration of corticosteroid therapy (above and below 365days) with bone age. The following models were significant: Log transformed BMD whole-body (r^2=0.650;p<0.001) was inversely (not significantly) to corticosteroid doses (above and below 0.2 mg/kg/day; p=0.144) and bone age

(p<0.001). Log transformed BMD lumbar (r^2=0.403;p<0.001) was inversely, but not significantly, related to corticosteroid doses (above and below 0.2 mg/kg/day; p=0.882) and bone age (p<0.001). More models were summarized in **Appendix A Table A-4**, **A-5** and **A-6.**

Table 3-6: Bone Parameters

Model	On Corticosteroid (n=28)	Corticosteroid-free (n=11)	*P*-value of the model	*P*-value of corticosteroid	*P*-value of Bone age
Lumbar BMC (g)	22.2 ± 1.4	23.4 ± 2.02	<0.001	0.165	<0.001
Lumbar log(BMC)	2.98 ± 0.06	3.06 ± 0.09	<0.001	0.418	<0.001
Lumbar BMD (g/cm^2)	0.57 ± 0.02	0.80 ± 0.22	0.007	0.152	0.007
Lumbar log(BMD)	-0.59 ± 0.03	0.48 ± 0.11	<0.001	0.311	<0.001
Lumbar BMD z-score[1]	-0.73 ± 0.11	-0.90 ± 0.20	0.734	0.496	0.752
Whole-body BMC (g)	889 ± 57[3]	944 ± 88	<0.001	0.058	<0.001
Whole-body log(BMC)	6.74 ± 0.06	6.75 ± 0.10	<0.001	0.079	<0.001
Whole-body BMD (g/cm^2)	0.77 ± 0.02	0.79 ± 0.03	<0.001	0.489	<0.001
Whole-body log(BMD)	-0.27 ± 0.02	-0.21 ± 0.03	<0.001	0.432	<0.001
Whole-body BMD z-score[2]	-0.29 ± 0.321	0.11 ± 0.50	0.323	0.488	0.186

Values are represented as mean ± standard error

BMD: Bone Mineral Density; BMC: Bone Mineral Content

[1] Lumbar BMD z-score <-1 was in 17/26 patients and 6/11 patients on corticosteroid and corticosteroid-free; lumbar BMD z-score <-2 was in 3/28 patients and 3/11 patients on corticosteroid and corticosteroid-free

[2] Whole-body BMD z-score <-1 was in 6/28 and 3/11 patients on corticosteroid and corticosteroid-free; whole-body BMD z-score <-2 was in 3/28 patients and 0/11 patients on corticosteroid and corticosteroid-free

[3] One patient has been removed in order to skewed the data (n=27)

3.4.8 Bone Mineral Content

BMC data are summarized in **Table 3-6**. To assess the impact of duration of therapy (above and below 365 days) and bone age on the logarithmic transformation of whole-body and lumbar BMC, we performed multivariate analysis on these variables. Absolute lumbar BMC was inversely ($p<0.001$; $r^2=0.890$) related to corticosteroid dose (above and below 0.2 mg/kg/day; $p=0.014$) and positively to bone age ($p<0.001$). Log transformed lumbar BMC was inversely ($p<0.001$; $r^2=0.878$) related to corticosteroid dose but not significant (above and below 0.2 mg/kg/day; $p=0.160$) and positively to bone age ($p<0.001$). More models were summarized in **Appendix A Table A-4**, **A-5** and **A-6.**

3.5 Discussion

LTX is a life saving procedure in children with end-stage liver disease (ESLD). The most common cause of ESLD in infants and children is Biliary Atresia (BA). BA is a destructive, inflammatory condition that leads to severe cholangiopathy (43). Most infants diagnosed with this condition need corrective surgery within the first two months of life (Kasai portoenterostomy) and a LTX within the first year of life (43). Although LTX is a procedure that saves lives, it contains complications that could affect the graft survival and the need for re-transplantation (2).

The causes of graft failure in children post-LTX are: Hepatic artery thrombosis, hepatic artery stenosis, rejection (acute and/ or chronic), non-adherent patients to immunosuppressive therapy and other causes such as portal vein thrombosis (2,145,146). Other complications following LTX are increased risk

for renal insufficiency, infection and lymphoproliferatie disease (8,41,146). Renal dysfunction is related to the use of immunosuppressive therapy (tacrolimus, sirolimus, mycophenolate) (4). High dose and/ or repeated pulses of immunosuppressive therapy to treat rejection can also result in an increased risk for biliary infection and lymphoproliferative disease (4,146). These complications can all influence overall growth and bone health in children who have undergone LTX and are hence important factors to consider as potential confounding factors.

Children with cholestatic liver disease who undergo LTX have a high prevalence of poor bone health prior to LTX (1,11). Post-LTX, BMD appears to improve, albeit it takes some time for this to occur. The major factors that are thought to contribute to the high prevalence of poor bone health in children with cholestatic liver disease awaiting LTX include alterations in bone turnover induced by vitamin deficiency (vitamins D and K) (5,39,89,141). Typically in the pre-transplant period vitamin deficiency is related to suboptimal intake due to anorexia and malabsorption of fat and fat-soluble vitamins related to impaired bile flow (141). Post-LTX, factors such as the use of immunosuppressive therapy (including corticosteroid therapy), age at transplant and the extent and duration of malnutrition prior to transplantation, time from transplant and the number of rejection episodes requiring high dose immunosuppression are factors that are associated with altered bone turnover and diminished BMD (134-137).

A variety of studies conducted in children who have undergone organ transplantation (kidney, liver, bone marrow, cardiac) have shown that suboptimal

BMD (as defined by BMD z-scores <-2) persists several years post organ transplantation and may occur in up to 15% of children (5,135-137). In our study the number of children with suboptimal BMD status ranged between 15% (lumbar-spine) to 8% (whole-body) over the 10 year period of review. This was comparable to other studies that have found reduced BMD up to 15 years post-LTX (134). The major variables that are associated with suboptimal BMD in these cohorts has been the use of corticosteroid therapy to treat rejection (acute and chronic); as well as other immunosuppressants such as cyclosporine, azathioprine and tacrolimus (137). Uniquely approximately 28% of the children in this cohort were completely corticosteroid-free post-LTX; and the remaining 72% on smaller dose corticosteroid therapy which may explain the slightly lower rates of reduced BMD observed within our study population. However, it is unclear whether or not these differences in rates of low BMD were related to corticosteroid therapy; particularly as we found few relationships between the use of corticosteroid therapy and low BMD. The major differences we observed were in children with corticosteroid doses above 0.2 mg/kg/day and whole-body BMD-z, absolute BMD and lumbar BMC; as well as in linear growth. In contrast, no major effects of corticosteroid therapy (or lack there of) was observed on lumbar BMD or BMD-z. This may be due in part to the smaller sample size of the current cohort; limiting the power to detect these effects.

There are many factors that have an impact on bone health that may have contributed to poor bone health in this population. This includes the potential for suboptimal vitamin D and K status pre-and-post LTX. Suboptimal vitamin K/D

status may be induced by malabsorption and suboptimal intake in children with chronic cholestatis leading to chronic deficiency that may extend into the post-LTX period and thereby contributing to poor bone health (39,89,147). In northern climates, diminished cutaneous synthesis of vitamin D due to poor sunlight exposure may potentiate this issue leading to chronic under-nutrition of vitamin D and poor bone health in the post transplant period (105,148). Although, the children within our study are routinely supplemented with fat-soluble vitamins, poor compliance to vitamin supplementation is reported consistently within the literature (138-140). In addition, vitamin D and calcium intakes are often significantly below recommended levels of intake in children (5,89). Preliminary data in our population has shown that vitamin D/K and calcium intakes were approximately 50% of recommended daily allowances (RDA) (unpublished data: **Chapter 4**) which may have contributed to suboptimal bone health in some children post LTX. However, we are unable to determine the extent to which this may have influenced overall bone health in this cohort. Vitamin D supplementation 3-10 times of RDA is recommended for children post-LTX and is clearly warranted given that intakes of vitamin D in children within our clinics is well below requirements (5).

While 25(OH) vitamin D levels ranged between 40-200 nmol/L in the children in the cohort; only 2/3 of the cohort had serum values (>75 nmol/L) that are associated with optimal bone health (39,148). In addition, most of these measures were not collected at the same time as BMD measurement; making it difficult to determine whether or not a change in vitamin D status may have

contributed to overall bone health. Although routine clinical practice within our clinics is to recommend vitamin D and calcium supplementation (after a comprehensive dietary assessment is completed), we were unable to directly determine the extent to which suboptimal intakes of vitamin K/D and calcium influenced study findings as this data was not available within the clinic charts. Our preliminary review of dietary intakes in our study population shows that these are nutrients (particularly vitamin K) is at risk in our population. Exploring the interrelationships between dietary intake of these key nutrients and bone health within our study population warrants further investigation.

Other factors such as change in weight bearing physical activity in the post-transplant period may also influence bone health (118); particularly in the chronically ill infants where developmental milestones (gross motor function) may have been delayed due to debilitating illness in the first few years of life (149,150). However, it is unlikely that this was a major contributing factor in overall bone health post-LTX in this review as the majority of children in this cohort were ambulatory, greater than five years old and had no major limitations reported related to gross motor function.

Limitations to this study include those that are inherent with most retrospective studies: missing data and a smaller sample size make it difficult to determine the extent to which the primary outcome of interest (the effect of a corticosteroid-free immunosuppressive regimen) had on BMD. While a post-hoc power analysis revealed sufficient sample size ($p>0.8$) to detect differences in height-z scores and bone health parameters (BMD for lumbar and whole body in

those children on corticosteroid verses corticosteroid-free regimens when the cohort was examined as a whole, there was limited sample size to determine the changes that might be observed with time. This is important to understand in terms of developing medical and nutritional therapies aimed at optimizing bone accrual in children and adolescents post LTX. Other factors that may also have influenced the ability to determine the impact of corticosteroid therapy on bone health in this population is changes in other medications such as tacrolimus and the number of episodes of acute rejection. Changes in medication therapies for treatment of acute and chronic rejection using medications such as tacrolimus are common in this population. Tacrolimus is a calceneurin inhibitor known to influence bone health and hence is an important factor to examine in any TX population. While we did not collect any information with regard to tacrolimus dosing, we did collect information on its use, and the number of episodes of acute and chronic rejection and did not find any significant differences ($p>0.05$) pre-and-post 2003. Finally, limited information regarding potential confounding factors (fat-soluble vitamins status, dietary intake, weight bearing activity and sunlight exposure (at time of DXA) was unavailable; making it difficult to determine the extent to which these factors influenced overall bone health post-LTX in this cohort.

3.6 Conclusions and Clinical Implications

This study shows that the use of low dose corticosteroid therapy and/or a corticosteroid-free medication regimen in children who have undergone LTX is associated with low rates of poor bone health and improved linear growth. It is

unclear to what extent lifestyle factors (dietary intakes of calcium, vitamin D/K, weight bearing activity) may influence overall bone health and growth may be contributing factors within our cohort of study. Our preliminary findings suggest that suboptimal vitamin K intake may potentially be a factor contributing to poor bone health; but more research is required. Although vitamin D and calcium intake appears to be relatively low in this population, routine supplementation of vitamin D/calcium is prescribed and therefore is unlikely to have been a major factor in the children who experience poor bone health. Further studies examining the interrelationships between lifestyle factors, immunosuppressive therapy and bone health/bone growth in this population is warranted.

Chapter 4: Vitamin D, Vitamin K and Calcium Intake in Children Post Liver Transplant

4.1 Abstract

The risk for poor bone health in children who have undergone liver transplantation (LTX) is high. Potential factors contributing to poor bone health in this population include decreased intake of fat-soluble vitamins (e.g. vitamin D/K), altered weight bearing activity and/or the use of immunosuppressive medications known to adversely influence bone health (e.g. corticosteroids). The objectives of this study were to compare vitamin D, K and calcium intakes in children post-LTX with age matched healthy children and to compare intakes with the Dietary Reference Intakes (DRI). Dietary intake was assessed using two validated methodologies: food frequency questionnaires (vitamin D/calcium assessment) and using two multi-pass 24-hour dietary recall (one weekday/one weekend day). Eleven children post-LTX and 11 children for the control group participated in the study. No significant differences in vitamin D and calcium intake between the groups were observed ($p > 0.05$). A significant difference in vitamin K intake ($p=0.007$) was observed between children post-LTX (10.1 ± 8.0 μg/day) and the control group (28.8 ± 8.6 μg/day). None of the children in either group met the RDA for vitamin D or the adequate intake (AI) for vitamin K, by diet alone. The mean daily vitamin D intake (diet and micronutrient supplementation) was 564 ± 456 IU in children post-LTX (4 children met the RDA) versus 277 ± 244 IU in the control group (1 child met the RDA). The mean daily calcium intake (diet and micronutrient supplementation) was 1060 ± 364 mg

in children post-LTX (8 children met the RDA) versus 840 ± 296 mg in the control group (8 children met the RDA). In conclusion, children post-LTX were able to meet the RDA of vitamin D and calcium by adding supplementation. Vitamin K needs remained a nutrient at risk with total intake by diet and micronutrient supplementation well below recommended levels of intake.

4.2 Introduction

Children who have undergone liver transplant (LTX) are at high risk for suboptimal growth due to poor dietary intake and malabsorption of fat and fat-soluble vitamins pre-and-post LTX (5,30). At particular risk in the diet are fat and fat-soluble vitamins (A, D, E and K) due to pre-existing deficiency at time of LTX placing children at high risk for poor bone health (5). At the Stollery Children's Hospital, around 38% of all children who have undergone LTX have Bone Mineral Density z-scores (BMD-z) that are less than -1 (**Chapter 3**); suggesting that poor bone health is a persistent issue in the post transplant period. Although, the use of corticosteroids is known to be a major factor in overall bone health, this is unlikely to be the only variable, as the current medical therapy used in transplant care within the Pediatric LTX Program is largely a corticosteroid-free immunosuppressive protocol. The use of corticosteroid therapy has been shown to be associated with suboptimal bone health in a variety of populations (136,151,152), but other factors such as calcium, vitamin D and K intake/status (105,153,154) and weight bearing exercise are also important factors. Weight-bearing exercise is important to enhance peak bone mass in children (117) and contributes to improved BMD (by up to 17%) (118,155,156).

Vitamin D in particular is a nutrient at risk due to the reduced levels of sunlight exposure in Northern Alberta (91) resulting in diminished endogenous synthesis of vitamin D, particularly in the winter months (94). Recent surveys suggest that the prevalence of vitamin D deficiency, as assessed by serum levels of 25 hydroxycholecalciferol (25(OH)D) less than 75 nmol/L (a level associated with suboptimal bone health) occurs in approximately 35 % of Albertan children (91). The major factors that are thought to contribute to low vitamin D levels are poor sunlight exposure and insufficient vitamin D intake. Although vitamin D is found in food products (primarily fatty fish and vitamin D fortified dairy products), vitamin D intake in the Canadian population has been shown to be very low (92). Hence, the potential for suboptimal vitamin D status to contribute to poor bone health in children is likely to be very high. Other nutrients such as vitamin K and calcium are also likely contributing factors as these are known to influence overall BMD and growth in childhood (153). Vitamin K is a co-factor that is responsible for the gamma carboxylation of an important protein within the bone matrix: osteocalcin (105,153). Supplementation of vitamin K has been associated with significant improvements in BMD and appears to have a positive synergistic effect with calcium on overall bone remodeling (157). While pre-LTX intakes and overall status of vitamin D/K are known to be highly prevalent in children with cholestatic liver disease (39,89), little is known about the extent to which poor intakes of vitamin D, calcium and vitamin K may contribute to suboptimal bone health in infants and children who have undergone LTX (5,153) particularly in northern climates like Alberta.

The objective of the study was to determine if current intakes of vitamin D/calcium and vitamin K in children post-LTX meet recommended levels of intake (DRI) and to compare intakes with age matched healthy children. This information is important to understand to determine what potential lifestyle factors (if any) that may contribute to bone health (BMD) in children in the post-LTX.

4.3 Methods

4.3.1 Patient Population and Study Design

A prospective, case control study design was utilized to compare dietary intakes (calcium, vitamin D/K) in children who have undergone LTX (at the Stollery Children's Hospital, Edmonton Alberta) and in age matched healthy children (2-18 years of age). Children in the post-LTX group were recruited on the annual visit of LTX clinic at Stollery Children's Hospital. Age-matched controls were healthy children recruited from either the community (healthy children) and/or general GI clinics. **Table 4-1** summarizes the inclusion/ and exclusion criteria.

Table 4-1: The Inclusion and Exclusion Criteria

Group	Inclusion/Exclusion Criteria
The Cases	• Children who have undergone LTX between January 1996 and December 2011 (at least 6 months post-LTX) • Children who were medically stable and had not experienced any recent episodes of acute allograft rejection within the last 3-6 months necessitating high dose corticosteroid therapy • Children who were not currently treated with antibiotics known to interfere with calcium absorption (penicillin) and/or medication affecting vitamin D metabolism: Aluminum, Anticonvulsant medications, Bile acid sequestrants (Cholestyramine, Colestipol), Calcipotriene (Dovonex), Cimetidine (Tagamet) and hormone replacement therapy • Children who not were on parenteral support
The Controls	• Children recruited from the General GI clinics typically had referral history of functional constipation, mild abdominal pain and/or gastroesophageal reflux and who had undergone screening to rule out a diagnosis of celiac disease, liver disease, cystic fibrosis, short bowel syndrome • All children in the control group were ambulatory, growing at age-appropriate rates (158) • All children in the control group did not have a history of food allergies (e.g. peanut/milk allergy) or any other condition known to influence dietary intake

A sample size of 40 participants of each group was determined to be sufficient to detect whether or not our study population had vitamin D/K intakes of approximately 65-70% of the DRI with an alpha of 0.05 and Beta of 0.8. Only preliminary results for cases (n=11) and controls (n=11) are presented within this current thesis chapter.

Ethics Approval was obtained from the Human Research Ethics Board at University of Alberta and Operational and Administrative Approval from the Stollery Children's Hospital, Alberta Health Services (AHS) and the Northern Clinical Trials Centre at AHS/University of Alberta/Covenant Health. Informed consent/assent was obtained for all children and/or their responsible caregivers prior to subject enrollment into the study.

4.3.2 Anthropometric and Demographic Data

Anthropometric data was measured at time of routine clinic visits in both cases/controls using validated methodologies. Weight was measured by an upright scale (Sunbeam Products, Inc., Pelstar LLC, Alsip, IL, USA) to the nearest 0.1 kg. Height was measured, without shoes, by using a wall-mounted stadiometer (Holtain Ltd, Crymych, Dyfed, UK) to the nearest 0.1cm.

Height-for-age z-score (ht-z), weight-for-age z-score (wt-z), body mass index (BMI) and BMI z-score (BMI-z) were calculated using Epi Info 3.5.1 software (Atlanta, GA, USA) using Centre for Disease Control growth standards (144). Additional data (age at LTX, current age at time of assessment, dates of transplants) and medication history were reviewed in the cases only.

4.3.3 Dietary Intake

Vitamin D and calcium intake were examined in both cases/controls using a validated food frequency questionnaire in children (159). In addition, dietary intake (Vitamin K/D and calcium) was assessed by 24-hour recalls (using the multi-pass technique) for two different days (weekend and weekday). These methods have been used previously in children to determine patterns of dietary intake (160). Evaluating intake included an assessment of use of vitamin supplementation. Actual food intake (24-hour recall) was analyzed by Food Processor ® (SQL 10.6 ESHA Research, Salem, OR, USA) (105); vitamin K was assessed using the United States Department of Agriculture (USDA) database. Dietary intake was also analyzed by food group based on the food serving size for children by comparing to the Alberta Nutrition Guidelines for Children and Youth

for children in the same gender and same age (161). Calcium and vitamin D intake in the cases and the control were compared to the RDA and the Estimated Average Requirements (EAR); vitamin K for Adequate Intake (AI) for age and gender (103).

4.3.4 Laboratory Variables

Vitamin D and calcium status were assessed by measurement of serum levels of 25 hydroxycholecalciferol (25(OH)D) and calcium. In addition, parathyroid hormone (PTH), levels were reviewed. Other routine clinical blood work collected included serum levels of: phosphorus and liver function tests (AST, ALT, total bilirubin and albumin). These were also collected at the time of routine clinic visits and did not require any additional draws by the researchers. For the purposes of analysis, the optimal serum 25(OH)D was defined as a value >75nmol/L; serum 25(OH)D level <50nmol/L was considered as deficient; serum 25(OH)D 50 –75nmol/L was considered as suboptimal (86,105). Other routine clinical blood work collected included serum levels of: phosphorus and liver function tests (AST, ALT, total bilirubin and albumin). These were also collected at time of routine clinic visits and for descriptive purposes.

4.3.5 Statistical Method

A *p* value of less than 0.05 was considered statistically significant by using SPSS Statistics (version 19, SPSS Data Collection, Chicago, USA, 2010). The data were assessed for normality by Shapiro-Wilk Test. Independent T-test analysis was used to compare between the groups and paired T-test was used to compare between 24-hr recall and FFQ. Non-parametric test (Mann Whitney)

was run in food serving. Bland Altman test was performed to test agreement between FFQ and 24 HR recall data for vitamin D and calcium (162) (**Figure 4-2A** and **4-2B**).

4.4 Results

4.4.1 Anthropometric and Demographic Data

Data are present for the first 22 children enrolled into the study (11 children post-LTX; 11 control children). Anthropometric and demographic data are presented in **Table 4-2**. Among the 11 children who had LTX, nine children had a diagnosis of Biliary Atresia (82%) as a reason of LTX and the other two indications for LTX were Acute Fulminant Hepatic Failure (n=1) and Familial Intrahepatic Cholestatsis Type 2 (n=1). Eight children had one transplant (73%) and three children had two LTX. Among 11 children in the control group, 6 out of 11 control children were healthy children recruited from the community and five were children recruited from the General GI Clinics. No significant differences in anthropometric data were found between children recruited from the community and the children recruited from GI clinic ($p>0.05$).

Table 4-2: Anthropometric & Demographic Data

	Case (n=11) (range) 6F/5M	**Control (n=11) (range) 10F/1M**	***P*-value**
Age (years)	7.6 ± 3.4 (2.3-12.5)	7.4 ± 3.5 (2.0 -12.1)	0.880
Weight (kg)	25.2 ± 10.1 (11.2 – 42.3)	29.6 ±15.0 (13.4 – 61.6)	0.154
Height (cm)	122.9 ± 22.8 (83.1 – 155.7)	125.9 ± 22.9 (87.2 – 153.6)	0.788
BMI (kg/m^2)	16.1 ± 1.4 (13.6 – 17.6)	17.5 ± 3.7 (14.3 – 26.7)	0.068
Weight-for-age z-score	-0.29 ± 1.29 (-2.58 – 1.77)	0.49 ± 0.89 (-1.41 – 2.02)	0.286
Height-for-age z-score	-0.24 ± 1.35 (-2.58 – 1.61)	0.65 ± 1.11 (-1.18 – 2.65)	0.319
BMI-for-age z-score	-0.15 ± 0.99 (-2.25 – 1.33)	0.24 ± 0.91 (-1.01 – 19.96)	0.792

4.4.2 Laboratory Data

Laboratory data are summarized in **Table 4-3** for children post-LTX only. All the children post-LTX had normal vitamin D level (n=3) but only 1 out of 3 children post-LTX had an elevated PTH level. It was only 1 out of 10 children had an elevated AST and ALT and 3 out of 10 children had abnormal creatinine. Four out of 10 children had decreased magnesium level outside of the reference range.

Table 4-3: Laboratory Data in Children post Liver Transplantation

	Mean ± SD (range)	Reference Range
AST (U/L)[2]	41 ±16 (28- 82)	5-35
ALT (U/L)[2]	40 ± 26 (15- 104)	<50
Alb (g/L)[2]	43 ± 2 (40- 46)	35 - 50
Cr (umol/L)[2]	57 ± 35 (22-107)	62-106
Urea (mmol/L)[2]	5.4 ± 1.9 (2.5- 9.7)	2.2 – 7.0
PTH (pmol/L) [1]	5.9 ±1.8 (4.8- 8.0)	1.4 – 6.8
25(OH)D (nmol/L)[1]	86.2 ±15.4 (76.6 -104.0)	>75
Ca (mmol/L) [2]	2.4 ± 0.1 (2.2 - 2.6)	2.2 - 2.7
Mg (mmol/L) [2]	0.72 ± 0.09 (0.58 - 0.88)	0.70 -1.00

25(OH)D :25 hydroxycholecalciferol
[1] n=3
[2] n=10

4.4.3 Dietary Data

4.4.3.1 Twenty-four Hour Recall: Vitamin D, K and Calcium Intake

Dietary data are presented in **Table 4-4**. The variability between the weekend day and weekdays were 5.6%. Eight children met the EAR for calcium in the cases and 6 in the control but none of the cases and the controls met the EAR for vitamin D by diet alone. For vitamin K, only three children met the AI for vitamin K in the control group. The remaining children (n=11 cases/8 controls) did not meet the AI for vitamin K by diet alone. Although many of the children received a pediatric multivitamin supplement (n=5), this did not result in any of the children meeting the AI for vitamin K.

Table 4-4: Twenty-four Hour Recall Data

	Case (n=11)	Control (n=11)	*P*-value	DRI
Energy (kcal)[1]	1660 ± 667	1168 ± 456	0.084	900-2600
Protein (g)[2]	56.9 ± 3.3	47.6 ± 7.8	0.068	13-52
Carbohydrate (g)[2]	234.0 ± 75.5	183.5 ±71.4	0.211	130
Fat (g)[2]	56.9 ± 35.9	40.5 ±12.2	0.124	-
%Fat (%)[3]	29.7 ± 7.3	29.8 ± 7.9	0.729	25-40
% Carbohydrate (%)[3]	58.0 ± 8.8	56.7 ± 8.0	0.742	45-56
% Protein (%)[3]	13.3 ± 2.0	18.8 ± 12.1	0.151	5-30
Vitamin D (IU)[2]	215 ± 95	166 ± 71	0.235	600
Vitamin K (μg)[4]	10.1 ± 8.0	28.8 ± 38.6	0.007	30-75
Calcium (mg) [2]	1003 ± 358	813 ± 298	0.653	700 - 1300
Magnesium (mg) [2]	163 ± 76	1723 ± 56	0.162	80 - 360
Phosphorus (mg) [2]	863 ± 384	828 ± 207	0.792	460 - 1250
Sodium (mg) [4]	2798 ± 2583	1748 ± 828	0.185	1000 -1500
Zinc (mg) [2]	5.45 ± 2.34	4.94 ± 0.95	0.519	3 - 11
Caffeine (mg)[5]	3.98 ± 4.69	0.87 ± 2.15	0.065	-

DRI: Dietary Reference Intakes
[1]EER Estimated Energy Requirement (103)
[2]RDA: Reference Daily Intake (103)
[3]AMDR: Acceptable Macronutrient Distribution Range (103)
[4]AI: Adequate Intake (103)
[5]No more than 45–85 mg/day for 7–12 years; 2.5 mg/kg body weight for 13–18 years (Health Canada: http://www.hc-sc.gc.ca.login.ezproxy.library.ualberta.ca/hl-vs/iyh-vsv/food-aliment/ caffeine-eng.php#he)

4.4.3.2 Micronutrient Supplementation in the Children post Liver Transplant

The mean dose of calcium supplementation was 62 ± 99 mg in children post-LTX versus 27 ± 65 mg in the control group (p=0.358). Supplementation with calcium resulted in a total calcium intake of 1060 ± 364 mg (8 children met RDA and 5 children met EAR) in the cases and a total intake of 840 ± 296 mg (8 children met RDA and 9 children met EAR) in the controls. Seven children post-LTX versus 3 healthy children were on vitamin D supplementation. The mean dose of vitamin D supplementation was 384 ± 478 IU in children post-LTX versus 111 ± 203 IU in the control group (p=0.069). Supplementation of vitamin D

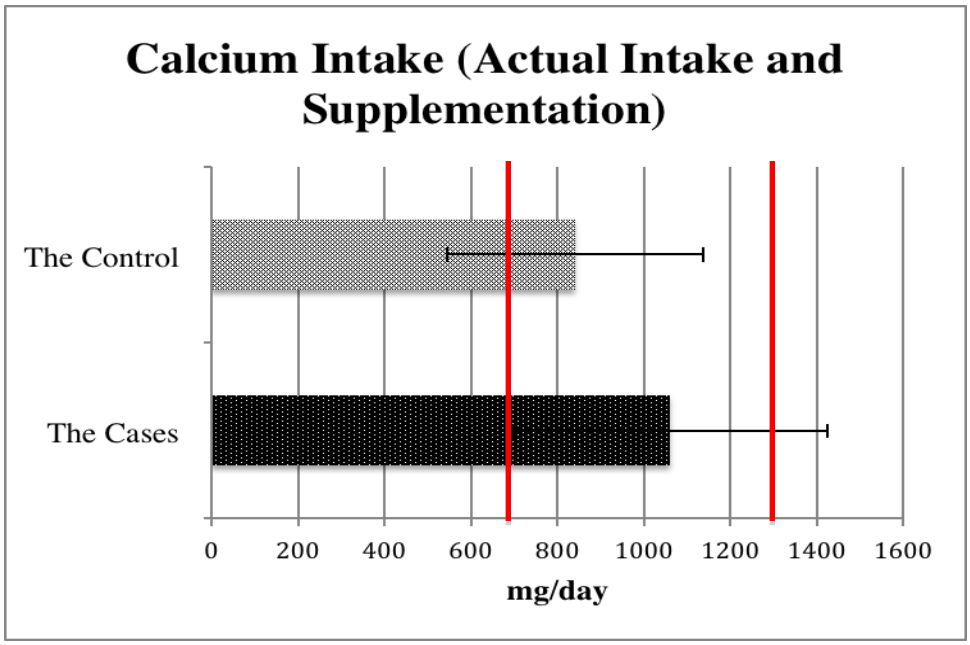
resulted in a total vitamin D intake of 564 ± 456 IU in children post-LTX (4 children met RDA and 5 children met EAR) and 277 ± 244 IU in the control group (1 child met RDA and 3 children met EAR) (**Figure 4-1**).

Table 4-5: Multivitamins Supplementation in Children

	Multivitamin A	**Multivitamin B**	**Multivitamin C**
Vitamin D (IU)	400	400	400
Vitamin K (µg)	10	0	0
Calcium (mg)	108	160	0

After assessing dietary intake and reviewing pertinent laboratory variables (such as serum levels of 25(OH) vitamin D levels) to assess overall micronutrient status, children within the LTX program at the Stollery Children's Hospital may be prescribed supplemental vitamin D by the interdisciplinary health care team. This typically is in the form of a single vitamin preparation (400 IU/drop or 1000 IU tablets) and/or pediatric multivitamin supplement. **Table 4-5** summarizes the most common multivitamins that are prescribed (not an exclusive list). Of note, is that most of these preparations do not contain vitamin K in these preparations.

4-1A



4-1B

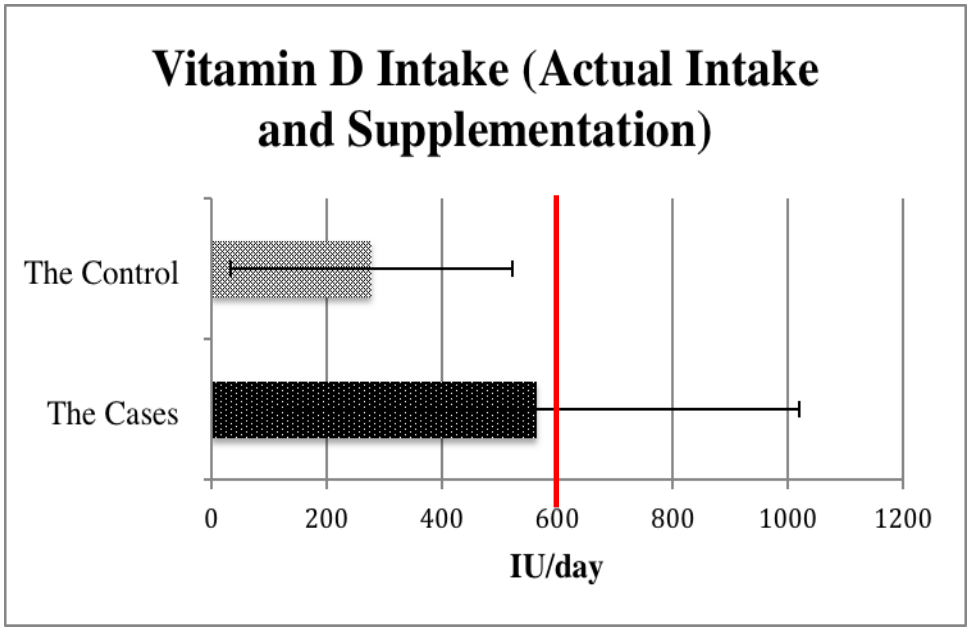
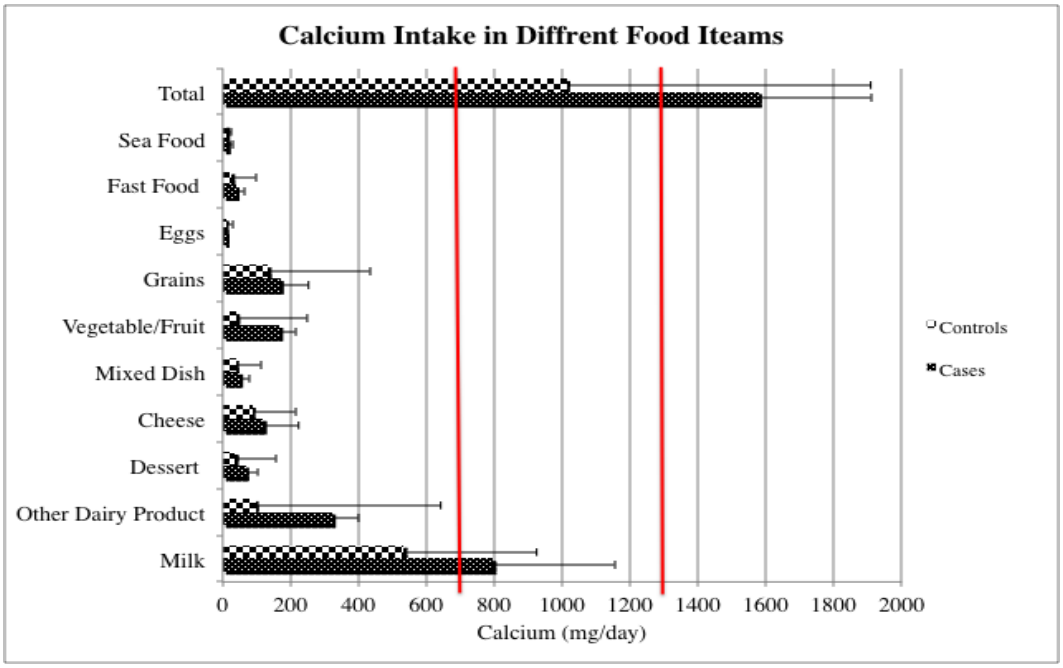


Figure 4-1 Calcium (4-1A) and Vitamin D (4-1B) Intake (Actual Intake and Supplementation) in Children post-LTX and The Control Group. The green line represents the RDA for calcium and Vitamin D for age and gender

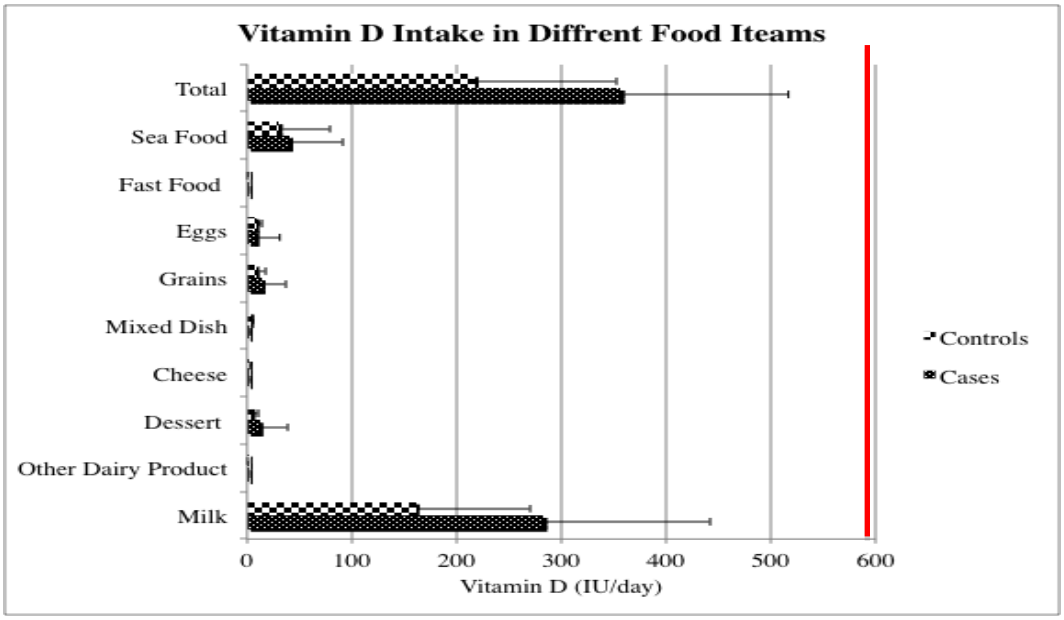
4.4.3.3 Food Frequency: Vitamin D and Calcium Intake

Data regarding the primary food sources for calcium and vitamin D are presented in **Figure 4-2A and 4-2B** and **Table 4-6.** The major food sources of calcium were liquid milk, dairy products (e.g. vitamin D fortified yogurt) and grains; accounting for 51, 16 and 12%, respectively. No significant differences in the sources of calcium from different food types were observed between the cases/controls ($p>0.05$). In contrast, the major food source of vitamin D was liquid milk (fortified) and sea-food; accounting for 77 and 12% of total vitamin D intake, respectively. This did not differ between cases versus controls. One child post-LTX did not meet the RDA of calcium versus 5 children in the control group. In contrast, 1 child post-LTX met the vitamin D requirement but none of the children in the control group met the RDA for vitamin D.

4-2A



4-2B



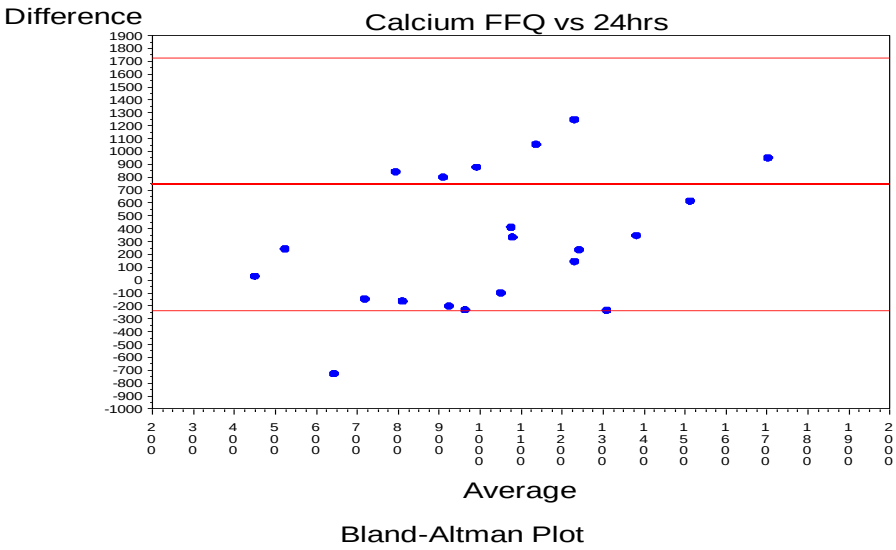
Dairy Product: Butter Milk, Yogurt
Dessert: Ice-cream, Frozen yogurt, Pudding or custard, Cake, chocolate
Cheese: Mozzarella, Cheddar, Cream cheese, Cottage cheese
Mixed Dishes: Macaroni cheese, Lasagna, Spaghetti with tomato sauce, Cream or cheddar soup
Grains: Bread, Waffles, Pancakes, Muffins
Fast Food: Hamburger, Pizza
Sea-food: Oysters, Shrimp, Crab, Salmon, Sardines
Vegetable / Fruit: Broccoli, Greens, Calcium fortified Juice

Figure 4-2: Food Sources of Calcium (mg) (A-2A) and Vitamin D (IU) (A-2B) from the diet. The green line represents the RDA for calcium and Vitamin D for age and gender

4.4.3.4 Comparison between Food Frequency Questionnaire and 24-hour Recall of Vitamin D and Calcium

There were no significant differences in the amounts of vitamin D and calcium consumed between children post-LTX and the control group (p=0.235 and 0.653) by using the 24-hour recall tool (**Table 4-4).** There were no significant differences in vitamin D and calcium intake between 24-hour recall and FFQ in the cohort (**Figure 4-3**) by Bland Altman. However, there were some differences noted between the determination of calcium and vitamin D between the two different dietary assessment tools in the children with LTX only (**Table 4-6**).

4-3A



4-**3B**

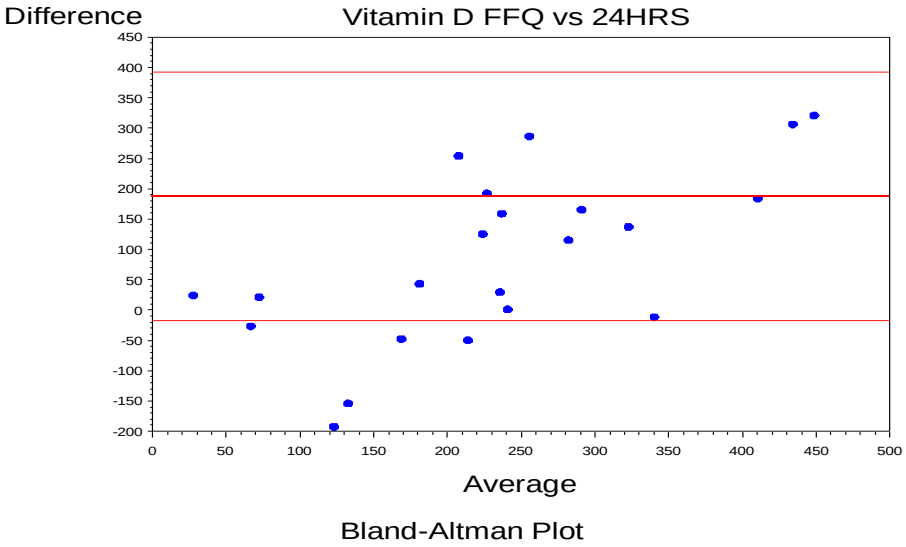


Figure 4-3: The Differences Between 24-hour Recall and Food Frequency Questionnaire of Calcium (4-3A) and Vitamin D (4-3B) in the Cohort by Bland Altman

Table 4-6: Comparison between Food Frequency Questionnaire and 24-hour Recall (Case versus Control)

	Case	*P*-value	Control	*P*-value
Calcium (mg)				
FFQ	1591 ± 858	0.267	999 ± 346	0.130
24-hour recall	1003 ± 358		813 ± 293	
Vitamin D (IU)				
FFQ	336 ± 183	0.080	217 ± 136	0.214
24-hour recall	215 ± 96		166 ± 70	

4.4.3.5 Food Groups from 24-hour Recall Data

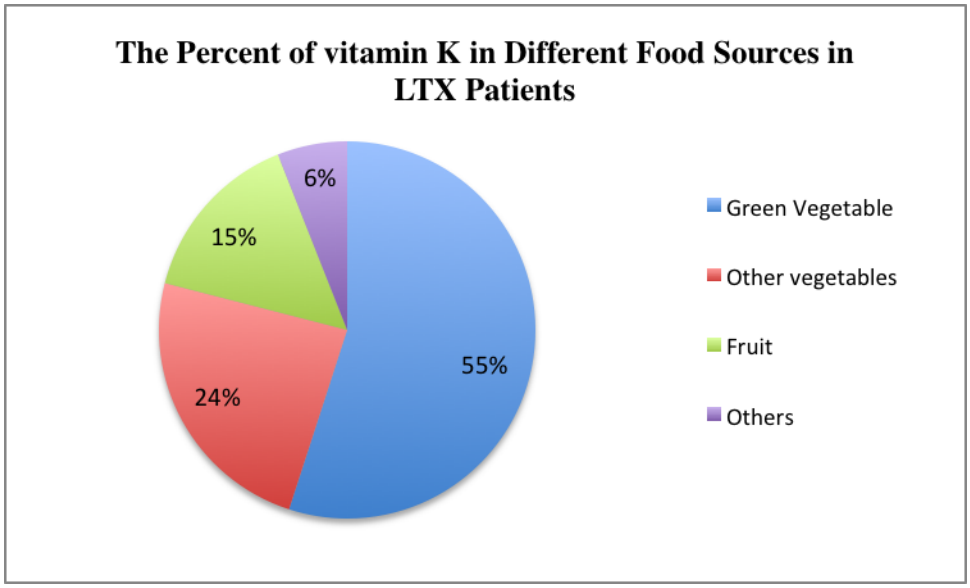
Table 4-7 is a comparison of intakes in cases and controls by food group. **Figure 4-4A** and **4-4B** demonstrate food sources of vitamin K by 24-hour recall in children post-LTX and the control group.

Table 4-7: Food Group Intakes in Cases/Controls

	Case (n=11)	Control (n=11)	*P*-value	Alberta Nutrition Guidelines
Vegetable/ Fruit	2.3 ±1.3	2.3 ±1.6	0.576	4-8
Meat	0.75 ± 0.4	1.1 ± 0.6	0.167	1-3
Grains	4.7 ± 1.0	4.4 1.2	0.562	3-7
Dairy products	2.4 ± 1.2	2.2 ± 0.9	0.847	2-4
Milk only	1.6 ±1.1	1.5 ± 0.7	0.748	-

Serving size based upon Alberta Nutrition Guidelines: The Alberta Nutrition Guidelines for Children and Youth (161)

4-4A



4-4B

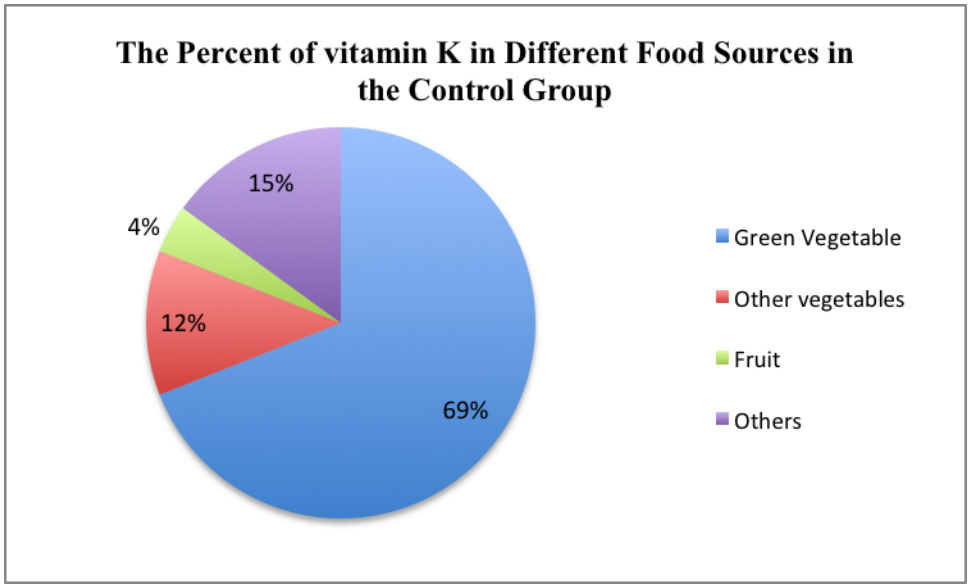


Figure 4-4 shows the percent of Vitamin K in Different Food Sources from 24-hour Recall Data in children post-LTX (4-4A) and the control group (4-4B); others indicate to another sources such as (such as food from other food groups and mixed foods)

4.5 Discussion

This pilot study examined some of the dietary factors (vitamin D, K and calcium) that are known to affect bone health in children who have undergone LTX and in healthy children. Estimates of poor health in children post-LTX indicate that up to 24% of children post-LTX may have bone mineral densities that are indicative of osteoporosis; six months after LTX (6,50). This can be even higher pre-LTX (37) indicating the need for careful evaluation of all contributing factors to overall bone health in this vulnerable population. Osteoporosis can lead to an increased bone fracture risk throughout the life cycle, as childhood and adolescence is the period of peak bone accrual (163). For the child who has faced a life saving surgery, it is imperative that all modifiable risk factors (such as diet and physical activity) are evaluated and treatment protocols initiated to prevent such serious co-morbidities. At the Stollery Children's Hospital, this includes a protocol for yearly evaluation of bone mineral density and fat-soluble vitamins status (A, E, D and K) by the interdisciplinary team.

The dietary factors that are known to influence bone health include dietary intake of vitamin D/K, calcium (118,153). Other factors that are known to influence bone health include - age, pubertal status, smoking, alcohol intake, using medication and the presence of chronic disease (5,30). In children who undergo LTX, a variety of other factors such as the use of corticosteroids for treatment of organ rejection and other immunosuppressive therapy (such as tacrolimus), malnutrition prior to LTX and age at transplantation are other factors that are associated with suboptimal bone health (136). A major factor of overall bone health in children and adults is overall vitamin D/K and calcium status. In

northern climates such as Alberta, this can pose serious issues with diminished cutaneous synthesis of vitamin D due to poor sunlight exposure (94,164). Hence, evaluation of these factors is important when evaluating the lifestyle factors that influence bone health in children. The purpose of the current study was to determine vitamin D, vitamin K and calcium intakes in children who have undergone LTX are and to compare this with intakes in healthy children.

There are limited studies regarding dietary intake (vitamin D, vitamin K and calcium) in different transplant populations. Our preliminary findings indicate that the majority of children in our study did not meet the RDA of either vitamin D or calcium through oral intake alone. The major sources of vitamin D and calcium in the diet were obtained from milk, dairy products and seafood (153). However, children in both study groups consumed insufficient serving sizes from either milk products to meet the EAR of vitamin D; indicating that vitamin D is a nutrient at risk for both healthy children and children who have undergone LTX. These results are consistent with some studies in the literature that show that healthy school aged children in Canada have suboptimal intakes of vitamin D from food alone (95). In contrast, when factoring in the effect of vitamin D supplementation, 5 children who had undergone LTX met the EAR and 4 met the RDA for vitamin D. This result was consistent for the healthy controls who were taking daily vitamin D supplementation, indicating the need for routine vitamin D supplementation to meet micronutrient needs in this population. Of interest, this most of the children in our cohort had sufficient intakes of calcium; likely reflecting the consumption of other non-dairy sources of calcium in the diet

such as soy and calcium fortified fruit juices; rather than consumption of vitamin D fortified dairy products (153). These results are consistent with another study in children that have undergone LTX. This study demonstrated that more than 60% of children had intakes of vitamin D significantly lower than 200 IU; but when supplemented with 200-400 IU/D of vitamin D daily were able to meet the RDA for vitamin D (134).

From a vitamin K perspective, our data show that the majority of healthy children and children who undergo LTX did not meet requirements for vitamin K in their diet. Although, the main source of vitamin K is green leafy vegetables (153), most of the children in our cohort did not meet the AI of vitamin K, even when taking a pediatric multivitamin supplement. Very little data are available regarding dietary intakes of vitamin K in children; particularly in children who have undergone LTX. The data available is more focused on the pre-LTX period or in children with other GI disorders. It has been shown that 45% of cholestatic children were vitamin K deficient (by PIVKA-II) values, even when supplemented with either 0.2 mg/day or 5 mg q 2-7 days a week (39). Malabsorption of vitamin K combined with anorexia was likely the major factor contributing to this high prevalence of vitamin K insufficiency. In children with celiac disease and cystic fibrosis, suboptimal intakes of vitamin K have been reported and have been related to poor bone health (105,165,166). While vitamin K supplementation appears to correct nutritional deficiency in these populations, it is unclear the extent to which supplementation is needed to promote optimal bone health. Some data suggest vitamin K intakes in the order of 1 mg/day is

needed to promote bone accrual in children (167,168) a magnitude of over 100 times the current level of intake noted in our population.

Weight- bearing exercise has been shown to increase bone mineralization (119). A study examined the physical activity effect on children at the mean age of 10-11 years showed that BMD, BMC and bone size were higher in professional Tennis players (playing 11-25 hours/ week for 5-7 days/week) than healthy children (120). Another study investigated the impact of 3 hours per week of weight bearing physical activity in children with diabetes; the study resulted in improving total body BMD (169). A study examined the physical activity in children post-LTX, and 88% of children post-LTX participated in physical activities on average 3 days/week for 45 minutes (170). Hence, it is possible that children may have reduced BMD due to suboptimal levels of weight bearing activity; suggesting that this needs to be examined in children post-LTX. The average amount of time spent in physical activity within our cohort was less than 60 minutes per day in children post-LTX (data not shown).

A major limitation in this study was that we did not measure vitamin K/D status and /or the interrelationships between vitamin K/D status and vitamin K/D intake and overall bone health. While we have preliminary data regarding adequacy of vitamin D status in laboratory data (25 (OH)D), we did not include any assessment of vitamin K status within the protocol. Protein induced in vitamin K absence (PIVKA-II) is a sensitive marker of vitamin K status and could be used to assess vitamin K status (39). Our study results suggest that suboptimal vitamin K status due to low intakes could be a potential factor influencing lower bone

health. However, it is unclear the extent to which this occurs in the post transplant period. Suboptimal vitamin K status is highly prevalent pre-transplant; occurring in up to 45% of children (39,89).

Another potential limitation of the study is the measurement of dietary intake at time of clinic visits because this may not represent the 'usual intake' of participants; particularly in the LTX group who travel over considerable geographical distances to attend routine clinic visits. While we conducted two 24-hour recalls (one weekend/one weekday) to overcome this limitation, it is possible that the foods cited as part of 'usual' intake might be more reflective of the foods available within the hospital environment and not usual intake and hence an under/over-estimation of typical food intake may have occurred. In addition, these methodologies require a high level of responder knowledge regarding food portion size and interviewer ability to convey standard food portion size to the respondent (171). A conferred strength was that only one interviewer, reviewed questionnaires and the dietary 24-hour recalls with the participants. The 24-hour recalls were also conducted for two different days: one weekend and one weekday, which would account to some extent for daily variations in intake; albeit to a lesser extent than if both interviews had been conducted outside of the routine clinical visit. Moreover, FFQ for vitamin K is missing in this study that could have estimation of usual vitamin K intake (172). Finally, a selection bias with the control population could have influenced study outcomes. Healthy controls were recruited in two ways: from the General GI clinics and from the community. It is possible that food intake might differ between the two groups. Although the

children in the GI clinics were screened for liver and other gastrointestinal disorders such as celiac disease, it is possible that their intake may have not represent intakes of 'healthy' children in the community. To examine this possibility, we compared vitamin D/K and calcium intakes and did not find any overall differences in intake of these particular nutrients.

Both healthy children and children who have undergone LTX have suboptimal intakes of vitamin D, calcium and vitamin K. Suboptimal intakes of vitamin D were only corrected with routine supplementation of vitamin D in both groups. Current intake of vitamin D fortified foods (liquid milk) was insufficient to meet vitamin D needs without supplementation in both groups. Calcium, an important nutrient for overall bone health, appeared to be the nutrient that was more likely to be consumed at adequate levels in both groups; primarily due to high intakes of dairy products such as yogurt. Although vitamin K intakes met recommended levels in around 13% of the cohort, many children still had insufficient levels of intake. This was particularly evident in the children who have had LTX. While some children reported the use of a pediatric multivitamin preparation, most contain relatively low levels of vitamin K in comparison to the levels that are thought to be needed for optimal bone health (173).

In conclusion, suboptimal vitamin D and K in both healthy children and children who have undergone LTX may potentially contribute to suboptimal bone health in children. Routine annual monitoring of vitamin D and K status and bone health is warranted in children who have undergone LTX, particularly in the first 6 months post-LTX when the highest rates of poor bone health are observed

(127). Vitamin D is a nutrient of particular concern in both healthy children and children who have undergone LTX, particularly in northern climates where rates of cutaneous synthesis are substantially reduced. Childhood and adolescence is the peak period for bone accrual, and optimizing nutritional intake in this part of the life cycle is critical to prevent future co-morbidities in vulnerable populations at high risk for suboptimal bone health.

Chapter 5: Conclusions and General Discussion

5.1 Summary and Principle Findings

This thesis retrospectively examined the impact of corticosteroid therapy on bone health parameters (bone mineral density (BMD), bone mineral content (BMC)) and growth outcomes (weight, height) in infants and children who have undergone LTX at the Stollery Children's Hospital, Edmonton, Alberta. The rationale for this study was to examine if the change in clinical practice within the liver transplant (LTX) program to discontinue the use of corticosteroids as part of the immunosuppressive medical protocol at the Stollery Children's Hospital in 2003 resulted in improved growth and bone health parameters (BMC/BMD) in infants and children in the post transplant period. The use of corticosteroid therapy has been associated with an increased risk for osteoporosis and growth failure in this population. Poor bone health has been recognized as a significant risk factor in children who have undergone LTX (134-136), particularly as infants and children with cholestatic liver disease have experienced significant periods of protein energy malnutrition due to malabsorption, anorexia and altered nutrient utilization prior to LTX (5,30). All of these factors are known to increase the risk for poor bone health and growth failure in children and are important to understand to ensure effective treatment strategies in children undergoing LTX are developed.

The overall study hypothesis was that the transition to a corticosteroid free immunosuppressive protocol in children who had undergone LTX would result in improved growth (height, weight) and bone health outcomes (BMD/BMC)

compared to infants and children who received corticosteroids in the post transplant period. A secondary objective of this thesis was to describe some of the lifestyle factors (vitamin D/K, calcium intake) that may be contributing factors to suboptimal bone health in children undergoing LTX.

5.1.1 Growth Outcomes

Overall our study findings demonstrate that the use of corticosteroids in children who have undergone LTX had some effects on linear growth in infants; particularly in the first year post-LTX in weight and first and sixth years post-LTX in height. Most children treated with corticosteroids had lower rates of height growth when treated with corticosteroid therapy in the first and fourth years post-LTX. This occurred particularly in children who received corticosteroid doses in excess of 0.2 mg/kg/day for a mean duration of one year. However, limited effects of corticosteroid therapy on rates of weight gain were observed between those that were treated with corticosteroids and those who were not treated with corticosteroid therapy post LTX. This is likely due to the some of the major side effects of therapy which include increased appetite/food intake and modest fluid retention; all factors known to contribute to increased weight gain (5). These findings are similar to what has been observed in other pediatric LTX programs (49). Whether or not rates of weight gain were due to these factors remains undetermined within this study, as no data were available within the charts regarding food intake.

5.1.2 Bone Health Outcomes

The prevalence of suboptimal bone health in our population (on and off corticosteroid therapy) approximated 15%; similar to the findings of other pediatric LTX centers in North America (134,136). Another finding in this study was that the children who were treated with corticosteroids as part of their immunosuppressive protocol had lower BMC (lumbar and whole-body); but varying effects on BMD. This appeared to be somewhat dose dependent (those children given doses greater than 0.2 mg/kg/day had lower BMC's than children treated with lower doses). One of the major reasons for these differences in BMC could be that corticosteroids influence calcium metabolism; contributing to increased calcium excretion in the urine and increasing bone re-modeling therapy contributing to decreased rates of bone accrual (BMD). The other potential factors that could have contributed to detected differences of corticosteroid therapy on bone health parameters could have been due to differences in duration of therapy and route of administration (oral versus intravenous). Most children in this cohort were treated with corticosteroids for treatment of acute rejection, rather than chronic rejection and hence the dose, duration and route of administration varied substantially (as clinically warranted) and hence the ability to detect effects of therapy on bone health outcomes is difficult. In addition, most of the children that were treated with corticosteroids were being treated for 'pulse' corticosteroids therapy over short periods of time, with variable frequency of therapy; all of which may have resulted in different effects on bone health. While cumulative dosing with corticosteroids may have potentially decreased BMC and

linear growth, these changes may have potentially occurred without directly affecting BMD. Recent evidence suggests that pulse corticosteroid therapy (once per week) for treatment of various disorders over 3-6 months has a smaller effect on BMD in adults when compared with daily dosing (174).

Other factors that may have contributed to overall bone accrual/bone health in our population may have been overall vitamin D/K and calcium status; all factors known to contribute to bone health. For this reason, we prospectively studied intakes of vitamin D/K and calcium in children who have undergone LTX, and in healthy age matched children (**Chapter 4**). Our preliminary evidence indicates that intakes of all three of these nutrients are substantially lower than recommended levels of intakes in both healthy children and in children who have undergone LTX. While most children within the LTX clinics at the Stollery Children's Hospital are routinely prescribed vitamin D and calcium supplements in a multivitamin preparation; few of these supplements have sufficient vitamin K to meet needs for optimal bone health (39,105). Whether or not suboptimal vitamin D/K and calcium intakes were major contributors to the reductions of BMD we found in the retrospective cohort is unknown, as no dietary data was available to us within the medical charts reviewed. In addition, we do not have any data regarding overall vitamin K status in children who have undergone LTX. What limited data we have available regarding vitamin D and calcium status, suggests that patients were receiving sufficient levels intake (diet and supplementation). However, how this related to overall bone health is not clear; as vitamin D/calcium levels were not routinely measured on the same day as the

DXA measures were done and therefore it is challenging to draw specific conclusions about this point within our cohort.

5.2 Study Limitations

The retrospective study contains several limitations even though it has repeated measurements. The main limitation in this study is the small sample size and missing data that affect the over all ability to detect differences in primary outcome variables. This was particularly evident with regard to the sites of DXA scan. Although most children had whole body scans performed; not all had spinal or hip scans done in a consistent fashion. Moreover, Dual-energy X-ray absorptiometry (DXA) software has been changed several times since 1999 (61) which could affect the result of BMD z-score. This made it difficult to determine the effect of corticosteroid therapy on different bone types (cortical versus trabecular) bone; particularly since software changes to determine BMD-z scores in these regions varied (61). In addition, using different scales to measure weight causes slight differences in weight accuracy especially for children who have a decline in growth. Moreover, the study does not measure the difference in bone health parameters between the routes of corticosteroid (intravenous and oral).

Another study limitation is that dietary assessment data and other data regarding lifestyle factors (weight bearing activity) were not routinely available within the medical charts. A comprehensive questionnaire on physical activity will help to examine weight-bearing exercise on bone health in children post-LTX. A validated vitamin K food frequency questionnaire would help to understand the usual intake of vitamin K in this population. Finally information

regarding corticosteroid therapy (medical chart, electronic data base) sometimes contained contradictory or missing information, making it challenging to be able to calculate total corticosteroid exposure for the individual patients. This was particularly evident during the period of transition in medication protocols (2002-2004) to a corticosteroid-free immunosuppressive protocol. To minimize the potential for these influencing overall study conclusions, a comprehensive review of all patient charts was performed.

5.3 Clinical Implications

Results from these studies indicate that children with end-stage liver disease who undergo LTX are at risk for poor bone health and growth failure; particularly in the first few years post-LTX. This may be due in part to suboptimal intakes of vitamin D/K and calcium; all important nutrients for bone health. While current recommended levels of vitamin D/calcium supplementation by the interdisciplinary health care team appear to be sufficient to meet recommended levels of dietary intake, vitamin K appears to be a nutrient at risk within the diets of children post-LTX. However, further work needs to be done to confirm this finding. It is currently unknown the extent to which suboptimal vitamin K intake may be influencing suboptimal bone health in children who have undergone LTX. There is however, substantial evidence within the literature that vitamin K is an important nutrient in skeletal health in children (39).

5.4 Further Research

It is difficult to manage the factors that affect bone health in children post-LTX, for example, age at transplant (above/ below 2 and 10 years), duration after

transplant, corticosteroid (dose, route and duration). Thus, there are some studies that could be done with children post-LTX to understand the factors influencing bone health and growth in children. A multi-centre retrospective review that compares growth rates and bone health parameters (BMD, BMC) in children following LTX could be done to increase the power to determine if the use of corticosteroids influences growth rates/bone health. This could be conducted within the context of the North American SPLIT (Studies of Pediatric Liver Transplantation) group with existing clinical data. The SPLIT group is a consortium of different pediatric liver transplant centres across North American (n=30) who share data regarding liver transplant outcomes and hence represents a unique opportunity to examine data from a multi-centre perspective.

A prospective study examining the impact of vitamin D/calcium and vitamin K supplementation to ensure intakes approximately at the DRI, on bone health parameters (BMD, BMC) over 1-3 years post LTX could be done to examine the impact of these nutrients on overall bone health. This should include a protocol to include DXA scans of two types of bones: trabecular (e.g. spine) and cortical (e.g. forearm). A multi-site perspective (those using corticosteroids vs those not using corticosteroids) would help us to understand the effect of corticosteroid on both BMC and BMD. Measuring body composition in the DXA scans would also help to address the influence of corticosteroid therapy on growth patterning as well. When done in conjunction with measurement of biomarkers of vitamin D (25 OH vitamin D), vitamin K (PIVKA-II), bone turnover (osteocalcin, bone alkaline phosphatase, N-telopeptide collagen type 1),

and other measures of liver function, this would provide important information regarding the interrelationships between dietary factors and bone health in this population. Including an evaluation of weight bearing activity and sunlight exposure in this population would be important as this is known to influence overall bone health as well. This may be done using a variety of validated questionnaires such as the Habitual Activity estimation Scale and Physical Activity Questionnaire for Children. Moreover, a better questionnaire of sunlight exposure would help to address the duration of sunlight and the estimation of vitamin D production endogenously. Monitoring other nutrition factors affecting bone health (such as zinc, magnesium and sodium) is another factor to complete the picture of modifiable factors in bone health.

5.5 Conclusion

In conclusion, initiation of a "corticosteroid-free protocol" for immunosuppressive therapy in children undergoing LTX shows short and long-term benefits (1 to 6 years post-LTX) in growth (weight and height). The protocol shows that children who were on corticosteroids had lower BMC and slower height velocity than who corticosteroid-free. This demonstrates that the "corticosteroid-free protocol" enhanced growth and bone health in children post-LTX. Inclusion of an interdisciplinary approach to clinical care in the infant and child undergoing LTX is critical to ensure all the nutritional and medical needs of the child is met. Routine evaluation of all factors (diet, physical activity, medical therapies) influencing bone health is an important component in the overall care of the child with cholestatic liver disease; pre-and-post LTX. This thesis provides

important information about the influence of initiation of a corticosteroid-free protocol on overall bone health and growth in infants and children undergoing LTX.

Appendix A: Tables

Table A-1: Rejection Activity Index (RAI)

Criteria	Description	Score
Portal inflammation	Most lymphocytic inflammation involving but not noticeably expanding, a minority of the triads	1
	Expansion of most or all of the triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils and eosinophils	2
	Marked expansion of most or all of the triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the periportal parenchyma	3
Bile duct inflammation damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear:cytoplasmic ratio of the epithelial cells	1
	Most or all of the ducts infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity and cytoplasmic vacuolization of the epithelium	2
	As above for 2, with most or all of the ducts showing degenerative changes or focal lumenal disruption	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltration involving some, but not a majority of the portal and/or hepatic venules 1	1
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules 2	2
	As above for 2, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis	3

Reference: Banff schema for grading liver allograft rejection: an international consensus document. Hepatology 1997 Mar;25(3):658-663.

Table A-2: Macronutrient and Micronutrient Requirements for Children with End Stage Liver Diseases

Nutrient	Daily requirement
Energy	Infants < 12 months: up to 150% EER (NRV for age), or 120–150 kcal/kg; older children: 120–170% EER (NRV for age)[1] 130% of requirement based on ideal weight [2]
Total fat	30–60% total energy[1]
MCT	30–70% of total fat[1] 30–50% of total fat[2]
PUFA	> 10% total energy[1]
EFA	ESPGHAN guidelines suggest infant formula should contain 4.5–10.8% energy as linoleic acid and linoleic: α-linolenic ratio should be 1:5–15[1]
Protein	3–4 g/kg, 9% energy from protein for catch up growth[1] 2-4g/kg/day unless encephalopathy[2]
BCAA	10% total amino acid[1]
CHO	40–60% total energy[1]
Vitamin A	< 10 kg 5000 IU/day, > 10 kg 10 000 IU/day[1] 5000–25,000 U/d [2]
Vitamin D	Cholecalciferol > 400 IU/day[1] 400 IU daily[2]
Vitamin E	25 IU/kg/day[1] 15-25 IU/kg/day[2]
Vitamin K	2 mg/kg weekly[1] 2.5-5 mg/day[2]
Calcium	25–100 mg/kg/day[1]
Zinc	1 mg/kg/day[1,2]

BCAA: Branched-chain amino acid; CHO: Carbohydrate; EER: Estimated energy requirement; EFA: Essential fatty acid; ESPGHAN: European Society for Pediatric Gastroenterology, Hepatology, and Nutrition; MCT: Medium chain triglyceride; NRV: Nutrient reference values; PUFA: Polyunsaturated fatty acid.

Adapted from:

[1] Zhao VM, Ziegler TR. Nutrition support in end-stage liver disease. Crit Care Nurs Clin North Am 2010 Sep;22(3):369-380.

[2] Sultan MI, Leon CD, Biank VF. Role of nutrition in pediatric chronic liver disease. Nutr Clin Pract 2011 Aug;26(4):401-408.

Table A-3: Diet Therapy for Nutrient Deficiency and/or Malnutrition in Children with End Stage Liver Diseases

Nutrient Deficiency	Diet Therapy
Energy	130-180% of RDA based on weight for height at 50th percentile MCT oil: 1-2 mL/kg/day in 2-4 doses, add glucose polymers and supplemental nighttime nasogastric drip feedings
EFA	Oral vegetable oil or intravenous fat emulsions
Vitamin A Serum retinol <20 mcg/dL	5000-25,000 units/day orally of water-miscible preparation
Vitamin D Serum vitamin D (25(OH)D) <30 ng/mL	Ergocalciferol: 3-10 times RDA Cholecalciferol based on weight and vitamin D levels: Weight >40 kg <10 ng/mL: 5000IU/day 11-19 ng/mL: 4000 IU/day 20-29 ng/mL: 3000 IU/day Weight <40 kg <10 ng/mL: 100 IU/kg/day, 11-19 ng/mL: 75 IU/kg/day, 0-29 ng/mL: 50 IU/kg/day
Vitamin E Vitamin E–total lipid ratio: <0.6 mg/g (age <1 y) <0.8 mg/g (age >1 y)	α-Tocopherol (acetate): 25-200 IU/kg/day Tocopherol polyethylene glycol (TPGS): 15-25 IU/kg/day
Vitamin K	Vitamin K: 2.5-5 mg, 2-7 times/week, Intravenous vitamin K might be required
Zinc Plasma zinc <60 mcg/dL	Elemental zinc: 1 mg/kg/day

EFA: Essential fatty acid; MCT: Medium chain triglyceride; RDA: Recommended dietary allowance.

Adapted from: Sultan MI, Leon CD, Biank VF. Role of nutrition in pediatric chronic liver dissease. Nutr Clin Pract 2011 Aug;26(4):401-408.

Table A-4: Multivariate Analysis of Bone Health Parameters with Corticosteroid Dose (mg/kg) and (0.2mg/kg/day) and Bone Age

Dependent Variable	Independent Variables	R^2	*P*-value of The Model	*P*-value of Corticosteroid	*P*-value of Bone Age
Log whole-body BMD	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.650	<0.001	0.144	<0.001
Log BMD lumbar	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.413	<0.001	0.814	<0.001
Lumbar BMC	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.890	<0.001	0.014	<0.001
Log whole-body BMC	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.787	<0.001	0.415	<0.001
Whole-body BMD	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.675	<0.001	0.212	<0.001
Whole-body BMD	1) Corticosteroid (0.2mg/kg/day) 2) Bone age	0.675	<0.001	0.212	<0.001
Whole-body BMC	1) Corticosteroid (mg/kg) 2) Bone age	0.923	<0.001	0.025	<0.001
Whole-body BMD-z	1) Corticosteroid (mg/kg) 2) Bone age	0.048	0.355	0.589	0.193
Log Whole-body BMC	1) Corticosteroid (mg/kg) 2) Bone age	0.809	<0.001	0.007	<0.001
Lumbar BMC	1) Corticosteroid (mg/kg) 2) Bone age	0.891	<0.001	0.007	<0.001

Table A-5: Multivariate Analysis of Bone Health Parameters with Duration of Corticosteroid Use (365 day) and Bone Age

Dependent Variable	Independent Variables	R^2	*P*-value of The Model	*P*-value of Duration of Corticosteroid Use	*P*-value of Bone Age
Whole-body BMC	1) Duration used corticosteroid 2) Bone age	0.930	<0.001	0.001	<0.001
Whole-body BMC	1) Duration used corticosteroid (365 day) 2) Bone age	0.866	<0.001	0.001	<0.001
Whole-body BMD	1) Duration used corticosteroid (365 day) 2) Bone age	0.686	<0.001	0.055	<0.001
Lumbar BMC	1) Duration used corticosteroid (365 d) 2) Bone age	0.884	<0.001	0.123	<0.001
Whole-body BMD-z	1) Duration used corticosteroid (365 day) 2) Bone age	0.055	0.308	0.448	0.143

Table A-6: Multivariate Analysis of Bone Health Parameters with Number of Rejection and Bone Age

Dependent Variable	Independent Variables	R^2	*P*-value of The Model	*P*-value of No of Rejection	*P*-value of Bone Age
Whole-body BMC	1) No of Rejection 2) Bone age	0.840	<0.001	0.885	<0.001
Lumbar BMC	1) No of Rejection 2) Bone age	0.881	<0.001	0.884	<0.001
Whole-body BMD	1) No of Rejection 2) Bone age	0.733	<0.001	0.002	<0.001
Whole-body BMD-z	1) No of Rejection 2) Bone age	0.082	0.164	0.177	0.206
Lumbar BMD	1) No of Rejection 2) Bone age	0.887	<0.001	0.377	<0.001

Table A-7: Food Analysis of 24-hour Recall in Children post Liver Transplant

	Case (n=11)	Control (n=11)	*P*-value	*RDA/ AI*[3]
Fiber (g)[1]	14.7 ± 9.0	9.3 ± 3.1	0.082	19-38
Saturated Fat (g)	23.3 ± 19.0	13.8 ± 5.4	0.140	-
Monounsaturated Fat (g)	11.2 ± 5.3	12.6 ± 7.5	0.565	-
Polyunsaturated Fat (g)	5.6 ± 2.3	6.1 ± 3.5	0.250	-
Cholesterol (mg)	168.6 ± 217.5	149.1 ± 117.2	0.410	-
Vitamin A-RAE (RAE)[1]	335.8 ± 188.1	290.9 ±125.8	0.395	300-900
Vitamin B1 (mg)[1]	1.05 ± 0.54	1.03 ± 0.29	0.899	0.5-1.2
Vitamin B2 (mg)[1]	1.66 ± 0.82	1.90 ± 0.32	0.331	0.5-1.3
Vitamin B3 (mg)	10.33 ± 6.52	10.00 ± 4.23	0.295	-
Vitamin B3-Niacine Equiv (mg)[1]	18.28 ± 7.19	17.98 ± 5.77	0.655	6-14
Vitamin B6 (mg)[1]	0.87 ± 0.44	0.87 ± 0.35	0.826	0.5-1.3
VitaminB12 (µg)[1]	2.62 ±1.48	2.38 ± 1.14	0.397	0.9-2.4
Biotin (µg)[2]	12.53 ± 17.5	0.93 ± 0.72	0.124	8-25
Vitamin C (mg)[1]	107.9 ± 88.6	122.0 ± 149.5	0.534	15-90
Vitamin E- Alpha- Toco (mg)[1]	1.99 ± 1.24	1.93 ± 0.77	0.102	6-15
Folate (µg)1	198.51 ± 123.29	179.23 ± 58.73	0.647	150-400
Pantothenic Acid (mg)2	3.18 ± 1.19	3.12 ± 0.99	0.547	2-5
Chromium (mcg)2	2.03 ± 2.91	0.27 ± 0.38	0.256	11-35
Copper (mg)1	0.64 ± 0.36	0.72 ± 0.21	0.198	340-890
Fluoride (mg)2	0.017 ± 0.017	0.061 ± 0.056	0.473	0.7-3
Iodine (mcg)1	22.66 ± 44.29	9.92	-	90-150
Iron (mg)1	11.98 ± 8.41	9.02 ± 3.42	0.298	10-15
Manganese (mg)2	1.6 ± 1.3	1.7 ± 0.7	0.289	1.2-2.2
Molybdenum (mcg)1	9.8 ± 12.1	5.2 ± 0.4	0.168	17-43
Potassium (mg)2	1825.8 ± 215.7	1847.1 ± 558.3	0.332	3-4.7
Selenium (mcg)1	52.0 ± 23.4	50.9 ± 22.1	0.632	20-55
Omega 3 (g)	0.23 ± 0.37	0.08 ± 0.11	0.292	-
Omega 6 (g)	1.78 ± 1.78	2.05 ± 1.80	0.983	-
Choline (mg)2	41.39 ± 46.34	8.66 ± 12.59	0.073	200-550

[1] RDA: Reference Daily Intake

[2] AI: Adequate Intake

[3] **Reference:** Meyers LD, Hellwig JP, Otten JJ. DRI, dietary reference intakes: the essential guide to nutrient requirements / Jennifer J. Otten, Jennifer Pitzi Hellwig, Linda D. Meyers, editors. : Washington, D.C.: National Academies Press, c2006; 2006.)

Appendix B: Questionnaires and Forms

June 4 2012
V1 Readability 9.2

UNIVERSITY OF ALBERTA

Information Form (Healthy Children)

Title of Project: Dietary intake in Children who have undergone liver transplantation.

Principal Investigator: Diana Mager PhD RD Telephone: 492-7687
Co-Investigator Jason Yap, MD FRACP Telephone: 248-5420
Susan Gilmour MD FRCPC Telephone: 248-5409

This letter is intended for the study subject. If you are signing on behalf of your child, the words 'you' and 'your' should be read as 'your child'.

Purpose of the Study

Liver transplantation is an important life saving surgery for children with some liver diseases. Children post liver transplantation can have slow growth and poor bone health. Some of this may be due to low intakes of certain vitamins that are important for bone health. We would like you to participate in a research study that will help us understand if you are getting enough in your diet to grow healthy bones. This information will help us understand whether or not children with liver disease who have had a liver transplant are meeting their nutritional needs.

Procedure(s) of the study

1. **Food Intake**

We will ask you to tell us about what you are typically eating in clinic so we can understand what types of nutrients you get in from the foods you eat. The way we will do this is that we will ask you to tell us about what types of foods you eat every day. We will also ask you questions about how often you eat different types of foods and how much you eat. All of this information will help us to understand how much nutrition you receive in your diet. The total amount of time this will take is about 30 minutes.

2. **Sun Exposure Questionnaire**

We will also ask you to fill out a Sun Exposure Questionnaire. You can fill this out in clinic or you can take this home and mail it back to us. We will give you a stamped envelope to mail it back to us if you want to do this at home. This questionnaire consists of some questions that tell us about your daily outdoor activities. This will only take about 5 minutes of your time. Our research assistant will answer any questions you might have about this questionnaire.

3. **Child Feeding Questionnaire**

If you are less than 12 years old, we will also ask your parents to fill out a questionnaire about the types of food that they believe are healthy. This will help us understand what types of foods are offered at home. This questionnaire will only take about 5 minutes to fill out. If you or your parents have any questions about this questionnaire, our research assistant will be happy to answer any questions you might have.

4. Weight & Height
We would like to record down your weight and height and your age from your clinic visit. This information is important for us to collect so we can compare your daily intake of nutrients to the recommended levels of intake for your age.

5. Medical Record
We would also like to look at your medical records to find out about medications, relevant lab work (for example your blood work results related to your vitamin D levels or your liver). This will help us understand everything about you having liver transplant and how your diet is helping you to meet your needs for vitamin D and calcium.

Phone calls: We may need to call you at home to get more information from our questionnaires to answer any further questions you may have.

Benefits of this Study: The benefit to you in this study is that we will be able to tell you if you are meeting all of your nutritional needs in the foods you eat. Your participation in this study will help us learn more about vitamin D status in children and adolescents who have had liver transplants and how what they eat might affect this. It is important to know this so you have enough vitamin D and calcium in your body.

Risks: The additional tests in this study are the questionnaires that ask you about your usual food intake, how much you eat vitamin D and calcium rich food, your feeding pattern, how much you exposed to the sun and your physical activity. All of the *additional* tests used in this study are harmless.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate.
By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 5 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You don't have to take part in this study at all or you can quit at any time. No one will be upset with you if you decide that you don't want to do this or if you decide to stop part way through. You should tell your doctor or the other study investigators if you or your child does not want to participate in this study. This will not affect the care that you and your child will receive by anyone within the clinic or at the Stollery Children's Hospital. You can still continue to see the dietitian, nurse and doctor without participating in this study.

Do you have more questions?

You can ask your dietitian about anything you don't understand. You can also talk to Dr Diana Mager or Dr Yap or Dr Gilmour. Dr Diana Mager's phone number is 492-7687. Dr Jason Yap's phone number is 248-5420 and Dr Gilmour's number is 248-5409. If you have any problems or concerns about any part of this study please call the Research Ethics Office at 780-492-2615. This office has no connection with the study researchers.

Principal Investigator: Dr Diana Mager,PhD RD Telephone: 780-492-7687

Co- Investigator: Dr. Jason Yap, MD FRACP Telephone:780-248-5420
Dr. Susan Gilmour MD FRCPC Telephone: 780-248-5409

Version 1 Parent Consent Form
June 4 2012

UNIVERSITY OF ALBERTA

PARENT CONSENT FORM (Healthy Controls)

Title of Project: **Dietary Intake in Children who have undergone Liver Transplantation**

Principal Investigator(s): Dr Diana Mager PhD RD , Phone Number: 780-492-7687
Co-Investigator (s): Dr. Jason Yap MD FRACP Phone Number: 780-248-5420
Dr. Susan Gilmour MD FRCPC Phone Number: 780-248-5409

	Yes	No
Do you understand that your child has been asked to participate in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved for your child in taking part in this research study?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to withdraw your child from the study at any time, without having to give a reason and without affecting your child's future medical care?	☐	☐
Do you understand who will have access to your child's records, including personally identifiable health information?	☐	☐
Do you want the investigator(s) to inform your child's family doctor or pediatrician that your child is participating in this research study?	☐	☐

Doctor's name _____

Who explained this study to you?

_____

Child's Name _____
I agree for my child to take part in this study: YES ☐ NO ☐

Signature of Parent or Guardian _____ Date & Time _____

(Printed Name) _____

Signature of Parent or Guardian _____ Date & Time _____

(Printed Name) _____

Signature of Witness _____ Date & Time _____

Signature of Investigator or Designee _____ Date & Time _____

Version 1 June 4 2012
Readability 6.3

UNIVERSITY OF ALBERTA

Assent Form (Healthy Controls)

Title of Project: Dietary intake in children who have undergone liver transplantation.

Principal Investigators: Diana Mager, PhD RD Telephone: 492-7687
Dr. Jason Yap MD FRACP Telephone: 248-5420
Dr. Susan Gilmour MD FRCP Telephone: 248-5409

We would like you to participate in a research study that will help us to know if you are getting enough nutrients in the foods that you eat,

What will you have to do?
If you and your parents agree that its okay to take part in this study we will ask you to:

1. About what you eat every day. In total answering these questions might take 30-40 minutes of your time.
2. Sunlight Exposure Survey.
3. Your parents to fill out a survey that asks them questions about what they think are healthy foods.
4. To record down your weight, height and age from your clinic visit. We want to do this so we can compare what nutrients you get from your food to your requirements for these nutrients.
5. To review your medical chart for your blood work to see how your diet is related to your vitamin D status.
6. To mail us a copy about what you eat every day.

Will it help?
We do know that some children who have had liver transplants don't get enough vitamins and minerals for their age. Vitamin D and calcium are particularly important as it can be hard to get enough in the diet. We want to find out how much vitamin D and calcium you eat so we can find out if we need to recommend more.

Will it hurt?
None of the questions will hurt. You do not have to answer all the questions if you don't want to.

Can you quit?
You don't have to take part in the study at all, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or nurse that you want to quit.

Who will know?
No one except your parents and the doctor will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors, research study employees and nurses during the study.

Your signature
We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions?
You can ask your parent or guardian about anything you don't understand. You can also talk to Dr Yap or Dr. Mager. Dr. Mager's phone number is 492-7687 and Dr. Yap phone number is 248-5420 and Dr Gilmour's phone number is248-5609. If you have any problems or concerns about any part of this study please call the Human Research Ethics Board at 492-2615, This office has no connection with the study researchers.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of investigator:_____ Date:_____

June 12 2012
V3 Readability 9.2

UNIVERSITY OF ALBERTA

Information Form

Title of Project: Dietary intake in Children who have undergone liver transplantation.

Principal Investigator: Diana Mager PhD RD Telephone: 492-7687

Co-Investigator Jason Yap, MD FRACP Telephone: 248-5420
Susan Gilmour MD FRCPC Telephone: 248-5409

This letter is intended for the study subject. If you are signing on behalf of your child, the words 'you' and 'your' should be read as 'your child'.

Purpose of the Study

You have had a liver transplant. We would like to study the diets of children and adolescents who have had liver transplantation so we can understand if children are getting enough nutrition from their diets to grow. We would like you to participate in a research study that will help us understand if you are getting enough vitamin D and calcium you are getting in your diet as these are important nutrients to have strong bones and to grow well.

Procedure(s) of the study

1. **Food Intake**

We will ask you to tell us about what you are typically eating in clinic so we can understand what types of nutrients you get in your diet especially about how much vitamin D and calcium you eat. We will also ask you if you have any food allergies. The way we will do this, is that we will ask you to tell us about what types of foods you eat every day. This will help us understand what types of nutrients you receive from your diet. We will also review a questionnaire with you that will ask you questions about how often you eat different types of foods and how much you eat of these types of foods. We would also like to give you a copy of the 24-hour recall questionnaire to fill it out in any appropriate weekday and then email it to us.

2. **Sun Exposure Questionnaire**

We will also ask you to fill out a Sun Exposure Questionnaire. You can fill this out in clinic or you can take this home and mail it back to us. We will give you a stamped envelope to mail it back to us if you want to do this at home. This questionnaire consists of some questions that tell us about your daily outdoor activities. This will only take about 5 minutes of your time. Our research assistant will answer any questions you might have about this questionnaire.

3. **Child Feeding Questionnaire**

If you are less than 12 years old, we will also ask your parents to fill out a questionnaire about the types of food that they believe are healthy. This will help us understand what types of foods are offered at home. This questionnaire will only take about 5 minutes to fill out. If you or your parents have any questions about this questionnaire, our research assistant will be happy to answer any questions you might have.

4. Weight & Height
We would like to record down your weight and height and your age from your clinic visit. This information is important for us to collect so we can compare your daily intake of nutrients to the recommended levels of intake for your age.

5. Medical Record
We would also like to look at your medical records to find out about medications, relevant lab work (for example your blood work results related to your vitamin D levels or your liver). This will help us understand everything about you having liver transplant and how your diet is helping you to meet your needs for vitamin D and calcium.

Phone calls: We would need to call you get your more information or any further questions. That will not take more than 5 minutes.

Benefits of this Study: The benefit to you in this study is that we will be able to tell you if you are meeting all of your nutritional needs on your diet. Your participation in this study will help us learn more about vitamin D status in children and adolescents who have had liver transplants and how what you eat might affect this. It is important to know this so you have enough vitamin D and calcium in your body.

Risks: The additional tests in this study are the questionnaires that ask you about your usual food intake, how much you eat vitamin D and calcium rich food, your feeding pattern, how much you exposed to the sun and your physical activity. All of the *additional* tests used in this study are harmless.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate.

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 5 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your

health records and request changes if your personal information is incorrect.

Voluntary Participation: You don't have to take part in this study at all or you can quit at any time. No one will be upset with you if you decide that you don't want to do this or if you decide to stop part way through. You should tell your doctor or the other study investigators if you or your child does not want to participate in this study. This will not affect the care that you and your child will receive by anyone within the clinic or at the Stollery Children's Hospital. You can still continue to see the dietitian, nurse and doctor without participating in this study.

Do you have more questions?
You can ask your dietitian about anything you don't understand. You can also talk to Dr Diana Mager or Dr Yap or Dr Gilmour. Dr Diana Mager's phone number is 492-7687. Dr Jason Yap's phone number is 248-5420 and Dr Gilmour's number is 248-5409. If you have any problems or concerns about any part of this study please call the Research Ethics Office at 780-492-2615. This office has no connection with the study researchers.

Principal Investigator: Dr Diana Mager, PhD RD Telephone: 780-492-7687

Co- Investigator: Dr. Jason Yap, MD FRAC Telephone:780-248-5420
Dr. Susan Gilmour MD FRCPC Telephone:780-248-5409

Version 1 Parent Consent Form
Oct 14 2011

UNIVERSITY OF ALBERTA

PARENT CONSENT FORM

Title of Project: **Dietary Intake in Children who have undergone Liver Transplantation**

Principal Investigator(s): Dr Diana Mager PhD RD Phone Number: 780-492-7687
Co-Investigator (s): Dr. Jason Yap MD FRACP Phone Number: 780-248-5420
Dr. Susan Gilmour MD FRCPC Phone Number: 780-248-5409

	Yes	No
Do you understand that your child has been asked to participate in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved for your child in taking part in this research study?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to withdraw your child from the study at any time, without having to give a reason and without affecting your child's future medical care?	☐	☐
Do you understand who will have access to your child's records, including personally identifiable health information?	☐	☐
Do you want the investigator(s) to inform your child's family doctor or pediatrician that your child is participating in this research study?	☐	☐

Doctor's name _____

Who explained this study to you?

_____

_____

Child's Name _____

I agree for my child to take part in this study: YES ☐ NO ☐

Signature of Parent or Guardian _____ Date & Time _____

(Printed Name) _____

Signature of Parent or Guardian _____ Date & Time _____

(Printed Name) _____

Signature of Witness _____ Date & Time _____

Signature of Investigator or Designee _____Date & Time____

Jan 12nd 2012 v3
Readability:5.4

UNIVERSITY OF ALBERTA

Assent Form

Title of Project: Dietary intake in Children who have undergone liver transplantation

Principal Investigator: Diana Mager PhD RD Telephone: 492-7687

Co-Investigator Jason Yap, MD FRACP Telephone: 248-5420
Susan Gilmour MD FRCPC Telephone: 248-5409

You have had a liver transplant. We would like you to participate in a research study that will help us understand whether or not you are getting enough nutrients in the foods that you eat. We are particularly interested in finding out how much vitamin D and calcium you get in your diet as these are important for your bones to grow well.

What will you have to do?

If you and your parents agree that it's okay to take part in this study we will ask you:

1. About what you eat every day. In total answering these questions might take 30-40 minutes of your time.
2. Your parents to fill out a survey that asks them questions about what they think are healthy foods.
3. To record down your weight, height and age from your clinic visit. We want to do this so we can compare what nutrients you get from your food to your requirements for these nutrients.
4. To review your medical chart for your blood work to see how your diet is related to your vitamin D status.
5. To mail us a copy about what you eat every day.

Will it help?

We do know that some children with who have had liver transplants don't get enough vitamins and minerals for their age. Vitamin D and calcium are particularly important as it can be hard to get enough in the diet. We want to find out how much vitamin D and calcium you eat so we can find out if we need to recommend more.

Will it hurt?

None of the questions will hurt. You do not have to answer all the questions if you don't want to.

Can you quit?
You don't have to take part in the study at all, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or nurse that you want to quit.

Who will know?
No one except your parents and the doctor will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors, research study employees and nurses during the study.

Your signature
We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions?
You can ask your parent or guardian about anything you don't understand. You can also talk to Dr Yap or Dr. Mager. Dr. Mager's phone number is 492-7687 and Dr. Yap phone number is 248-5420 and Dr Gilmour's phone number is248-5609. If you have any problems or concerns about any part of this study please call the Human Research Ethics Board at 492-2615, This office has no connection with the study researchers.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of investigator:_____ Date:_____

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24-Hour Recall

Participant ID: **Interviewer Name:**
Date:

Time, Location, Meal# or Snack#	Foods, Beverages, Condiments, Sauces, Spreads	Brand, Preparation Method	Portion Size

Instructions

The 24-hour recall will be conducted in four stages ("Multiple Pass Method").

Multiple Pass Method

1) Obtain a complete list of all foods and beverages consumed from the previous day, together with the time and place of consumption. Begin by asking about the first food and/or drink consumed in the morning. Avoid asking questions about specific meals (eg. breakfast, lunch, or supper). Rather, use neutral questions such as "*Tell me what you had to eat or drink after you woke up yesterday morning. What was the time? Did you eat that food at home? What did you have next and when was that?*" Proceed through the day, repeating these questions as necessary, and record each food or drink consumed. Prompting the subject about his or her activities during the previous day may help in recalling food intake.

2) Go over each of the responses, probing for more specific descriptions of all the foods and drinks consumed, including cooking methods and brand names. Information on the place and time of eating should also be recorded. Examples of prompts for specific food items are:

i) Meat: type of meat, description of cut, method of cooking, lean or lean + fat, sauces

ii) Poultry: type of poultry, parts or pieces eaten, method of cooking, white or dark meat, meat + skin or meat only, sauces

iii) Milk products: type of dairy product, brand name, percentage fat

iv) Bread/rolls: type of grain (eg. rye, whole wheat, etc.), homemade/store bought, size, toasted, condiments (eg. butter, jam, etc.)

v) Vegetables: fresh/frozen/canned, peeled/unpeeled, method of cooking, topping

vi) Beverages: volumetric or fluid ounces, size of can or bottle, sweetened/unsweetened, water

3) Obtain estimates of the amounts of all foods and beverages consumed. Record as volumes (eg. cups, tablespoons, millilitres) or as weights (eg. grams, pounds, ounces).

i) If the interview is in person, refer to the food models, measuring cups and spoons, plates, glasses, bowls, and serving sizes handout.

ii) If the interview is via phone ask the participant to take out a set of measuring cups and spoons, as well as the cup(s), bowl(s), glass (es) that they used the previous day. Send them a copy of the serving sizes handout in advance and ask them to refer to it during the conversation.

4) Review the recall with the subject to ensure that all items have been recorded correctly. A statement such as "*I will read back to you what I have recorded to make sure that I have not made any mistakes*" can be used. Finally, the subject should be asked about the use of any vitamin and mineral supplements, protein or diet drinks, and any alcohol consumed. Inquire about foods/beverages consumed in the middle of the night. Check for missing condiments, food groups (eg. meat, milk products), and fluids.

Additional Notes:

* The same interviewer should do all four interviews of the same participant.
* Standardized household dishes and utensils and food models should be used as much as possible.
* If the recall is done by phone, ask the individual to set out some of their own dishes/utensils of known quantities.

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Vitamin D and Calcium Intake Questionnaire

Name: Date:

If you eat any of the following foods, write how much you would have in a serving, then check off how often.

Food	Medium Serving	Patient's serving	Never or < once per month	1-2 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	> 1 per day
Cold cereal	*1 cup*										
Milk (whole, Low Fat, skim, choc, soy)	*8 oz (1 cup)*										
Milk over cereal	*4 oz (1/2 cup)*										
Milk, cream in coffee	*1 oz (2 tbsp)*										
Buttermilk, whole	*8 oz (1 cup)*										
Yogurt (fruited or flavored)	*1 cup 2containers*										
Yogurt (plain)	*1 cup 2containers*										
Ice cream or ice milk	*1/2cup*										
Frozen yogurt	*8 oz*										

	(1 cup)										
Ice cream bar, fudge bar	*1 each*										
American or mozzarella	*1 oz*										
Cream Cheese	*1 oz*										
Macaroni and cheese, lasagna	*1 cup*										
Cheese pizza	*1/8 large*										
Cottage cheese	*1 cup*										
Cheese food or spread	*1 oz*										
Pudding or custard made with milk	*1/2 cup*										
Cream soup, chowders, cream sauces	*1 cup*										
Broccoli	*1/2 cup*										
Greens: mustard, turnip, collard, beet, spin.	*1/2 cup*										
Calcium Fortified Juice (orange, others)	*8 oz*										
Bread (white/wheat/pita)	*1 slice*										
Muffins	*1 medium*										
Biscuit, cornbread	*2" cube*										
Pancakes/waffles frozen	*4"*										
Pancakes/waffles-HM	*4"*										
Spaghetti w/tomato sauce	*1 cup*										
Eggs	*1 small each*										
Fast Food Hamburger	*1 each*										

Fast Food Cheeseburger	*1 each*										
Oysters, shrimp, crab, crawfish, herring	*3 oz*										
Canned Salmon w/Bones	*3.75oz can*										
Sardines	*3.75oz can*										
Cake	*3×3×2"*										
Almonds	*1/4 cup*										
Milk Chocolate	*1.6oz bar*										

	Brand Name	Serving	Serving/ day	Serving/ week
Multivitamins		1 Tablet		
Calcium Supplement		1 Tablet		
Vitamin D supplement		1 Tablet		

Validated Questionnaire adapted from Taylor et al (2008): Validation of a Food Frequency Questionnaire for Determining Calcium and Vitamin D Intake by Adolescent Girls with Anorexia Nervosa. J Am Diet Association. 109(3): 479-85.

Measurement Conversions

1 cup = 250 mL
1 ounce = 2 tbsp = 30 mL; 1 tbsp = 0.5 ounce = 15 mL
1 can of pop = 355mL = 1.5 cups
1 single container yogurt=100 g = 0.5 cup

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