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EARLY NUTRITION MATTERS

Clinical studies on the effects of nutritional intake in the early postnatal period of Very Low Birth Weight Infants

Viola Christmann
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In Gedanken bei meinen Eltern
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Chapter 4 Effect of early nutritional intake on long-term growth and bone mineralization of former very low birth weight infants Bone 2017;108:89-97 95
Chapter 5 The early postnatal nutritional intake of preterm infants affected neurodevelopmental outcomes differently in boys and girls at 24 months Acta Paediatr 2017; 106:242-249
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PART 1

General introduction
Nutritional care for the preterm infant
Definition of preterm birth
Prematurity is defined as being born alive before 37 weeks of pregnancy are completed. The subcategories of preterm birth are based either on weeks of gestational age or weight at birth. Moderate to late preterm infants are born 32 up to 37 weeks of gestation, while very preterm infants have a gestation at birth ranging from 28 to below 32 weeks and extremely preterm infants are born below 28 weeks of gestation. For categorization according to birth weight, the following subcategories are used: low birth weight (LBW < 2500 grams), very low birth weight < 1500 grams, and extremely low birth weight (ELBW < 1000 grams). A subdivision is important since lower gestational age and birth weight are associated with greater risk of developing both, short and long-term health complications, or even death. The major cause of neonatal mortality, which is defined as death within the first 28 days after birth, is preterm birth.

Prevalence and outcome of preterm birth
Figure 1 presents the global rates of preterm birth with distribution of subcategories of prematurity. Despite a reduction in live births over the past decades, the number of preterm births is still increasing. Approximately five million children are born every year in the EU, of whom about half a million are preterm infants requiring care in the

**Figure 1** Estimated numbers and rates of preterm births by region and by gestational age grouping for the year 2010. (1, 5)
neonatal period. In 2008 the incidence of prematurity varied from 5.5% to 11.1% of all births among European Countries. Between 2004 and 2010 neonatal mortality declined in Europe by 29% (95% CI 23% to 39%) with a range of 9 to 67% by country. This was mainly based on increase of survival of preterm infants with similar changes for all subcategories of prematurity as described above. Despite this progress, prematurity has been recognized as a severe public health problem with an increasing impact on the annual societal economic costs caused by long hospital stays, high first year medical costs, educational and lost productivity costs. According to Petrou et al ‘neonatal costs tended to be higher for preterm infants who survive compared to those who die’ and are related to the need for surgical intervention and the level of assisted ventilation needed by the infant. Table 1 presents a summary of long-term impact of preterm birth.

The most comprehensive information with regard to long-term outcomes of survivors in Europe has been collected in a number of cohort studies such as the Dutch nationwide prospective study (POPS), certain regions of France (EPIPAGE), the United Kingdom (EPICURE) and Sweden (EXPRESS). The EPIPAGE and EPICURE study were repeated and thus included infants during two time periods. All of these studies followed high risk preterm infants up to the age of five years and evaluated survival, morbidity and neurodevelopmental outcomes. Summarizing the results of the cohort studies of different European countries showed that, despite the high level of health care available in these countries, preterm birth still bears a high risk of unfavorable outcome. On the other hand these studies also showed, that improvement of outcome over the years was possible, that a substantial number of infants achieved a normal development and outcome differed among countries. The evaluation of different treatment strategies among different countries could be helpful to improve treatment for preterm infants.

The care for very preterm infants implies a complex treatment concept taking into account the immaturity of all organ systems but also the rapid growth and maturation at that stage of the development, demanding adequate nutritional supply. With regard to the long-term consequences of preterm birth, it should be taken into consideration that the immature gastrointestinal system may prohibit the supply of adequate nutritional intake, leading to undernutrition with consecutively growth retardation affecting not only growth of the body but also the brain. Over the past 30 years, neonatal care has become highly technical and sophisticated leading to fully equipped intensive care units. The use of antenatal steroids, respiratory support with mechanical ventilation, surfactant replacement therapy as well as hemodynamic support, treatment with antibiotics among other things caused a significant decrease in neonatal mortality and improved outcomes for ill born and very preterm infants. Nevertheless with regard to optimal treatment concepts, the recent EPICE study, that evaluated clinical practices in European NICU centers, found that treatment

<table>
<thead>
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<th>Table 1 Long-term impact of preterm birth on survivors</th>
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<tr>
<td><strong>Specific physiological effects</strong></td>
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<tr>
<td>Chronic lung disease</td>
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<td>Long-term neurological and non-communicable disease</td>
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<td>Visual impairment</td>
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<td>Neurodevelopment/Behavior</td>
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<td>Impact on health services</td>
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<td>Adapted from Wieruszewski et al 2013</td>
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33 Nevertheless with regard to optimal treatment concepts, the recent EPICE study, that evaluated clinical practices in European NICU centers, found that treatment
protocols varied greatly and consensus on best practices appeared to be lacking, as well as standardized protocols or guidelines on national or regional levels. This study showed that outcome was improved when evidence based treatments were applied. Thus, to optimize the treatment of preterm infants, and thereby improve long-term outcome, uniform treatment guidelines based on adequate research should have a priority.

This thesis will focus on nutritional aspects for the very low birth weight preterm infant, including very and extreme preterm infants, with the aim to provide more knowledge with regard to the efficacy of current recommendations for nutritional supply.

**Historical perspective of preterm infant nutrition**

The history of neonatology shows that in the first place the survival of preterm infants was related to the awareness that thermoregulation on the one hand was an important factor for survival, while on the other hand feeding was necessary to keep preterm born infants alive. At the end of the 19th century Pierre Budin, a French obstetrician, combined the incubator care with the focus on mother-child bonding to improve breastfeeding, very likely to currently reestablished practices. According to his ideas, warmth and the ability to provide own mother’s milk, were the key-principles for preterm infant survival. He became an international authority on the care of preterm infants. His colleague introduced gavage feedings for preterm infants who were too weak to receive breast milk from a spoon or the breast.

The energy content of human milk was first determined in 1887. The first energy requirements for preterm infants were based on a number of studies at the beginning of the 20th century and recommended a range between 95 and 160 kcal/kg/day, while most infants received intakes between 150 and 200 kcal/kg/d, because it was believed that rapid weight gain would be beneficial. Twenty years later the energy requirements were reevaluated based on 11 preterm infants with a birth weight ranging from 1130 to 2220 grams between 10 and 44 days after birth. The daily energy requirement was determined to be 120 kcal/kg/day with an average daily weight gain of 16 g/kg/day.

Recommendations used nowadays, are mainly based on these early calculations, while it is not known whether this is valid for the extremely preterm infants treated in modern neonatal intensive care units.

During the last century techniques to provide nutrition to preterm infants, as well as the knowledge concerning nutritional needs have improved. However, there are still controversies in recommendations and clinical practice. Knowledge on specific requirements are often derived from clinical observations, animal experiments, short-term observational or interventional studies. The recommendations based on these results may not always have contributed to the improvement of long-term outcome of preterm infants. As an example, fluid restriction and delayed feedings that were proposed in the 40ies of the last century, revealed to be associated to dehydration, electrolyte disturbances and finally serious growth retardation with impaired neurodevelopment. Despite the fact that these practices were revisited twenty years later, nowadays, the fear of fluid overload and feeding intolerance still exists. Fluid restriction often implies a reduction in the amount of nutritional intake. Consequently many preterm infants born nowadays develop postnatal growth retardation, remain growth retarded and perform less in later life compared to their term born pears. This impaired development has been associated with early postnatal growth retardation. Most early studies looked at more mature
Nutrients – The ingredients

Nutrition in general is an agglomeration (collective term) of all constituents or nutrients the body needs for optimal functioning, growth, metabolism and repair. Nutrients are subdivided into macronutrients and micronutrients. Carbohydrates, protein and fat are the primary macronutrients, while micronutrients comprise minerals, trace elements and vitamins.

The requirements of macro- and micronutrients as well as the amount of fluid not only depend on the weight of a child, but gestational age, immature gastrointestinal and renal function have to be taken into consideration to guarantee adequate nutritional supply. The common recommendation for postnatal nutrition of very preterm infants is that the nutritional intake should meet the nutritional requirements of the growing human fetus and lead to a comparable growth as term born infants. Fetal requirements have been estimated by the factorial method based on analyses of carcasses of deceased fetuses and from animal studies. These recommendations are insufficient since postnatal nutritional requirements exceed the fetal needs by energy needed for thermoregulation, respiratory work load, illness and other adverse conditions. Furthermore the way of nutrient administration differs, as the fetal nutrient supply occurs as a constant flow through the umbilical cord influenced by maternal nutrition, endocrinology, placental metabolism and perfusion. Fetal nutrition is a complex interaction between genetic growth potential, the ability of the maternal-placental system to transfer nutrients to the fetus and the endocrine environment determining whether the fetus will follow this growth potential through intra-uterine life. In contrast, during the postnatal situation the nutritional supply to the preterm infant is dependent on intake based on standard nutritional guidelines, impaired by incomplete nutrient absorption through the gastrointestinal tract and suboptimal composition of either enteral or parenteral nutrition.

Macronutrients

Macronutrients provide energy to all organs to sustain various functions of the body including thermoregulation, growth, pulmonary function, circulation, and physical activity. Energy provided by carbohydrates, protein and fat is converted to ATP by oxidation in mitochondria or lost as heat production, while ATP is hydrolyzed to ADP to maintain vital functions, growth and storage of energy. Energy loss can occur through losses in stool, mostly as fat and in small proportions through carbohydrates and protein, and through urine as urea. Energy stores comprise primarily fat as adipose tissue, protein as structural component of all organs, and in small amounts as glycogen derived from glucose and other carbohydrates.

Glucose is the major carbohydrate and the most important energy substrate for the fetus and newborn infant. Glucose is particularly necessary as primary energy source for the brain but also for the heart, liver and kidney. Furthermore, glucose serves as a source of carbon for the de novo synthesis of fatty acids and a number of non-essential amino acids. Fetal energy requirements are high and the driving force for glucose transfer is a constant fetal glucose concentration to ensure a constant supply. Glucose utilization rate is twice as high in very preterm compared to term born infants, mainly as a result of the decreasing contribution of brain and heart to the metabolic rate in older infants. Alternative substrates, such as ketones, are low in concentrations in the fetus and preterm infant and glycogen synthesis and storage does not develop before the third trimester and is low in the preterm infant. Immediately after birth hormonal changes occur that stimulate gluconeogenesis, namely to achieve a postnatal growth rate similar to fetal growth combined with satisfactory functional development, is still valid.

CHAPTER 1 INTRODUCTION

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CHAPTER 1 INTRODUCTION
of impaired protein synthesis. The non-essential amino-acids can be synthesized by the body from intermediates of the tricarboxylic acid cycle and other metabolic pathways.

The formation of protein is a process of synthesis and degradation of amino acids with no storage pool but continuous turnover of proteins. Shortage of a certain amino acid will impair protein synthesis, while a surplus of amino acids can either be used for glycogenesis and energy production or will lead to renal losses. Protein synthesis exceeds five times the rate of protein accretion, meaning that one gram of protein synthesis requires the accretion of 5 gram of protein. This also means that protein synthesis is a highly energy consuming activity. Depending on the calculation used, protein metabolism requires approximately 40% of the resting energy expenditure. Amino acid requirements have been estimated based on animal studies, veno-arterial differences in amino acids in the umbilical cord, the factorial method, stable isotope tracer method or human milk analysis. Amino acid requirements have been estimated based on animal studies, veno-arterial differences in amino acids in the umbilical cord, the factorial method, stable isotope tracer method or human milk analysis.

Quantitative balance studies showed that amino acid uptake exceeds the amount necessary for protein accretion, indicating that the human fetus oxidizes amino acids to generate energy. Fetal amino acid requirements are high to provide sufficient substrate to enable a high protein synthesis rate with adequate growth during early gestation. The preterm infant has amino acid requirements comparable to the substrate to enable a high protein synthesis rate with adequate growth during early gestation. (102) The preterm infant lacks adipose tissue and thereby has no stores to produce energy. This may result in lipolysis of available long-chain polyunsaturated fatty acids and fatty acid oxidation to produce the necessary energy and potentially lead to abnormal structural brain development with consequence for long-term outcome. (107)

Micronutrients

All electrolytes such as sodium, potassium, chloride, magnesium as well as the trace elements and vitamins belong to the group of micronutrients. As part of this thesis focuses on nutritional interventions to improve bone mineralization, this description is limited to the electrolytes and hormonal regulation that play a crucial role for the development of bone tissue namely the metabolic homeostasis of calcium and phosphorus.

The primary constituents of skeletal tissue are calcium, phosphorus and magnesium. They compose 0.07 to 2.7% of the body weight of an appropriate for gestational age term newborn infant. These minerals are also important components of extra-cellular fluid, intracellular structures, cell membranes, and soft tissue. The circulating fraction of calcium and phosphorus as measured in blood is less than 1% of the total amount of the mineral. (Table 2)

Fetal bone mineral homeostasis

The fetal bone formation starts around the second part of the gestation and mainly follows two stages: 1. Proliferation and growth of mesenchymal structures and cartilage. 2. Progressive ossification and mineralization of these structures. Growth is directly related to protein and energy supply, but also to the hormonal environment including insulin and IGF 1 and 2, while bone formation and mineralization are related to mineral supply of calcium and phosphorus as well as hormonal factors, like parathyroid hormone (PTH) and vitamin D. The process of bone mineralization increases rapidly only in the later stages of pregnancy, limiting to significant increase...
supplement in parenteral nutrition. The circulating concentrations of calcium and phosphorus in blood are regulated within narrow limits through changes in intestinal absorption, renal excretion or reabsorption, mainly under influence of PTH and calcitriol. (138) PTH is activated as a result of low extracellular concentration of ionized calcium (Ca\(^{2+}\)). PTH stimulates the resorption of calcium in bone tissue and increases the renal tubular reabsorption of calcium, while on the other hand tubular reabsorption of phosphorus is reduced leading to increase of phosphaturia. Furthermore PTH stimulates the enzyme 25(hydroxy) cholecalciferol-1-alpha hydroxylase in the kidney that regulates the hydroxylation of 25-hydroxy cholecalciferol (calcidiol) into the active 1,25-dihydroxy cholecalciferol (calcitriol). Calcitriol stimulates the intestinal absorption of calcium and phosphorus, the resorption of calcium and phosphate from bone tissue and indirectly the renal reabsorption of phosphorus. The hormonal regulation of the bone mineral homeostasis of the preterm infant is functionally comparable to the adult regulation. (139)

## Intestinal absorption of calcium and phosphorus

The intestinal absorption of calcium occurs through passive and active mechanisms. The passive absorption is not hormonally regulated while the active absorption of calcium is regulated by calcitriol mainly in the duodenum. More than half of the nutritional intake is passively absorbed by the jejunum. This means that directly after birth, even for preterm infants, intestinal calcium absorption is effective. Bile salts and lactose stimulate the intestinal absorption of calcium, while some medications such as xanthine derivates reduce absorption. About 80 - 90% of the administered amount of phosphorus is absorbed through the passive pathways. In case of low serum concentration this amount can be increased by activation of intestinal absorption through calcitriol. (137)

## Renal excretion and reabsorption of minerals

In general, 98% of the calcium excreted by the glomeruli is reabsorbed by the renal tubuli. Increasing nutritional intake will lead to an increase of calcium excretion. A number of drugs enhance calcium excretion such as furosemide and xanthine derivates, while thiazide diuretics limit excretion. Renal calcium excretion depends on the acid base status. Metabolic acidosis will increase calcium excretion while it is reduced with metabolic alkalosis. Renal excretion of phosphorus is primarily regulated by the amount of phosphorus in the nutritional intake. A reduction in nutritional intake can lead to renal tubular reabsorption of more than 90% of phosphorus excreted by the glomeruli. Phosphorus depletion leads to increase in renal calcium excretion in favor of phosphorus reabsorption while an increase in nutritional supply of phosphorus will decrease the calcium excretion. (140, 141)

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Distribution</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>Bone (99%)</td>
<td>Skeletal structural integrity</td>
</tr>
<tr>
<td></td>
<td>Extracellular fluid</td>
<td>Receptor activation for metabolic events</td>
</tr>
<tr>
<td></td>
<td>Intracellular structures</td>
<td>Neurromuscular excitability</td>
</tr>
<tr>
<td></td>
<td>Cell membranes</td>
<td>Blood coagulation</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>Bone (80 - 85%)</td>
<td>Skeletal structural integrity</td>
</tr>
<tr>
<td></td>
<td>Skeletal muscle (ca 9%)</td>
<td>Component lipoproteins cell membrane</td>
</tr>
<tr>
<td></td>
<td>Viscera, extracellular fluid (ca 11%)</td>
<td>Bone mineralization – matrix formation</td>
</tr>
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Adapted from (124-127)
Postnatal supply of minerals and vitamin D

After birth, human milk is the major source for bone minerals and vitamin D. For the preterm infant human milk does not provide the quantity of minerals as during fetal development, and necessary for adequate bone mineralization. Insufficient supply of nutrients will lead to bone resorption to maintain stable blood concentrations of calcium and phosphorus. In preterm infants the insufficient supply of nutrients leads to bone mineralization called ‘osteopenia of the premature’ with an increased risk of bone fractures. Vitamin D needs to be supplemented postnataally since the exposure to sunlight is negligible and vitamin D content of human milk is low. The postnatal vitamin D stores are dependent on the maternal vitamin D concentrations. Recent studies showed that an increasing number of pregnant women, especially if veiled and dark skinned, are vitamin D deficient. Placental insufficiency and preeclampsia are also associated with impaired placental-fetal transfer of vitamin D. In conclusion, after preterm birth, the infant is at risk of impaired bone mineralization and supplementation of calcium, phosphorus and vitamin D is necessary directly after birth.

Enteral and parenteral nutrition

The natural way to provide and ingest food for any infant is the gastrointestinal tract, or enteral pathway. The alternative route is the parenteral route via venous access through application of liquid nutrients. Both ways of administration bare benefits and risks that should be taken into account prescribing nutritional intake for the individual patient. The preterm infant lacks stores of all nutrients. An extremely preterm infant is composed of more than 85% of water, with no adipose tissue, low stores of liver glycogen, very few amounts of minerals, while proteins available in muscles and organs may function as the only source of energy. The potential energy stores merely comprise enough energy to meet the basal metabolic energy requirements for more than 24 hours while in contrast the energy requirements exceeds those of the term born infant based on energy needed for heat loss, respiratory distress and loss of stools. Therefore separating the preterm infant from the placental circulation means that full supply of all nutrients should be started immediately after birth to prevent catabolism.

Enteral nutrition

In preterm infants the enteral pathway is partly compromised in the early postnatal period, based on immature gastrointestinal motility and immature intestinal epithelium with impaired digestion and absorption of food. Initially, most preterm infants tolerate only small volumes, insufficient to provide all nutrients necessary to continue fetal growth in the postnatal period. The disorganized gastrointestinal motility may cause a delayed passage.

Even the extremely preterm intestine is capable of digesting and absorbing milk feeds but not as well as term infants. Protein digestion is not limited, while lipid digestion and absorption are relatively inefficient caused by low concentrations of lipolytic enzymes as lingual and pancreatic lipases as well as bile acids. While human milk improves lipid digestion and absorption, because of the presence of two human milk lipases, this may be less effective with tube feeding. Short and medium chain fatty acids are absorbed and transported more readily than long-chain fatty acids. Lipid absorption has been estimated to vary between 58 to 89 % of the intake in preterm infants compared to 72 to 95% for term infants. High intake of calcium impairs digestion of fat, but high intake of fat impairs the absorption of calcium. Carbohydrates such as lactose and sucrose need to be hydrolyzed by lactase and sucrase. Lactase activity has been found to be very low in preterm infants while other disaccharidases seemed to be higher. This led to the assumption that preterm infants are lactose intolerant. Later studies showed that, even in preterm infants, lactase activity increased soon after the introduction of human milk and was sufficient to digest human milk carbohydrates sufficiently. The activity to absorb monosaccharides is available in early gestation.

The initiation and increment of volumes of enteral feeding for preterm infants has been a matter of debate for many years. There have been concerns about feeding intolerance, gastrointestinal reflux, and necrotizing enterocolitis (NEC). A number of studies suggested a link between NEC and rapid advancement of enteral feeding rates. The undigested content was thought to function as a chemoattractant for neutrophils and thus might initiate an inflammation cascade, which was proposed to increase the risk of necrotizing enterocolitis. Consecutively, this led to delay in introduction of enteral feeds for the first postnatal days or even until the end of the first week in combination with very cautious increments. Numerous studies suggested disadvantages in the delay of enteral nutrition. Withholding enteral feeds has been shown to impair gastrointestinal motility, hormonal activity, diminish structural integrity and growth of the intestinal mucosa and thus can delay the time to achieve full enteral feeding, while on the contrary the introduction of enteral feeds has been shown to promote the development of the intestinal mucosa. Several studies have shown that specifically human milk increases the epithelial proliferation of the immature intestine and the intestinal function.

Up to date there is a great variation in clinical practice in the initiation of the first enteral feeding. Meta-analyses of available studies by Cochrane reviews concluded that delayed introduction of enteral feeding and moderate advancing of enteral feed volumes did not reduce the risk of NEC, while slow advancement delayed the time to full enteral feeding and increased the risk of infection. It is not known...
whether this holds true specifically for the group of extremely preterm infants, since only few studies included infants below 1000 grams. \textsuperscript{(176, 177)} Furthermore, a number of studies have shown that irrespective of the details of recommendations, having a standardized feeding protocol will reduce necrotizing enterocolitis and improve outcome of patients. \textsuperscript{(160, 166, 178-181)}

**Parenteral nutrition**

Nowadays parenteral nutrition (PN) is able to provide all nutrients necessary to achieve adequate growth and functional development. \textsuperscript{(182)} Thereby parenteral nutrition is of vital importance for all preterm infants and sick neonates who are unable to tolerate enteral nutrition. The availability of adequate PN has contributed to a decrease in mortality and improvement of outcome of very preterm infants. \textsuperscript{(183)} However, there is no consensus on the optimal composition and requirements of fluid nutrients, and the way to administer parenteral solutions. \textsuperscript{(184)} Furthermore, the application of PN bares a number of risks, such as increased prevalence of infections, thrombosis and hepatic failure that may have life-long consequences for the individual patient. \textsuperscript{(183)}

In the early years of PN, protein was supplemented as hydrolysate of fibrin or casein and later as synthetic amino acids solutions. \textsuperscript{(185)} A number of studies reported side-effects in combination with these solutions like hyperammonaemia, hyperchloremic metabolic acidosis, hepatic failure, hyperphenylalaninemia and other imbalances of plasma amino acids and mineral concentrations. \textsuperscript{(186-192)} Despite the fact that studies showed that early commencement of more recent amino acid solutions was safe and led to a positive nitrogen balance, the fear of adverse events caused a delayed introduction and supplementation of restricted amounts of amino acids. \textsuperscript{(105,106,108,110)} Current available amino acid formulations have been designed to result in blood concentrations of amino acids similar to either cord blood or breast-milk fed infants. \textsuperscript{(193-197)} Nevertheless, the optimal composition, the ideal preparation of the solution, as well as the daily requirements have not been found for all parenteral amino acids. All 20 amino acids are required equally for a normal protein synthesis. The combination of macro-nutrients may affect the hormonal and metabolic regulation. The optimal amount and combination of macro-nutrients in PN subsequently led to restricted or delayed use of parenteral lipid emulsions. Parenteral lipid emulsions have been associated with increased pro-inflammatory eicosanoid production and lipid peroxidation and consecutively with chronic lung disease, increase in pulmonary vascular resistance and retinopathy of prematurity as well as impaired immune response with increased risk for nosocomial infections. \textsuperscript{(203-208)} Cholestatic liver disease is frequently recognized with long-term PN and has been associated with an accumulation of phytosterols that can be found in vegetable oil. \textsuperscript{(209,212)}

Parenteral lipid emulsions have predominately been based on soybean oil that contains a high amount of n-6 fatty acids (52-55\%). \textsuperscript{(211)} Due to the adverse effects, subsequently different lipid emulsions were developed aiming at a reduction of the amount of n-6 fatty acids and a more physiologic fatty acid profile. \textsuperscript{(202)} The optimal composition has not been developed yet, neither the optimal time to initiate parenteral lipids nor the optimal requirements. Systematic reviews and meta-analyses did not find benefits of early introduction of lipid emulsions with regard to postnatal growth but also no differences in adverse effects such as chronic lung disease, duration of supplemental oxygen, retinopathy of prematurity and sepsis. \textsuperscript{(214-216)} Further the alternative lipid emulsions in comparison with soybean emulsion showed no statistically significant differences in clinically important outcomes including death, growth, chronic lung disease, sepsis, retinopathy of prematurity ≥ grade 3 and PN associated liver disease. \textsuperscript{(215,217)}

The third macronutrient and major source of non-protein calories in PN is D-glucose (dextrose) that contributes most to the osmolality of the PN solution, thereby a risk factor for adverse effects of venous access. \textsuperscript{(211)} While glucose provides about 40 - 60\% of the energy in nutrition and is utilized by all cells, administration of an excess of glucose may be responsible for hyperglycemia, and will lead to mitochondrial damage with lipogenesis, fat deposition with finally development of steatosis and cholestasis. Excessive glucose intake causes increased CO\textsubscript{2} production which may result in respiratory distress. \textsuperscript{(218,219)} Glucose administration affects the hormonal regulation of the glucose metabolism and may lead to impaired sensitivity for insulin. Adding lipid emulsions to PN decreases the need of carbohydrates as non-protein energy, while in combination with a high amount of amino acids the amount of carbohydrates should be adequately high enough to allow optimal protein synthesis. The combination of macro-nutrients may affect the hormonal and metabolic regulation. The optimal amount and combination of macro-nutrients in PN solutions is still a matter of debate. For enteral nutrition it has been demonstrated that the composition of the feeding affects the body composition of the infant. \textsuperscript{(220, 221)} Abnormal deposition of fat mass may have adverse consequences in later life with increased risk of type II diabetes, hypertension and cardiovascular disease. (‘postnatal origin hypothesis’) \textsuperscript{(222-224)}
The supplementation of calcium and phosphorus in PN has been a challenging subject since the introduction of PN solutions, because both minerals combined in solution easily precipitate, in quantities necessary to meet the requirements of preterm infants. Alternate infusion of both minerals, cyclic infusions but also low quantities of both minerals have been used to prevent central venous line occlusions seen as result of calcium phosphate crystal formation. These practices led to metabolic disturbances with hyper- and hypocalcaemia or -phosphataemia. Metabolic bone disease and rickets have been reported in combination with long-term parenteral nutrition. The solubility of calcium and phosphorus depends on the source of mineral that is used for the preparation of the PN solution. Organic calcium and phosphorus compounds, such as calcium gluconate or sodium glycerol-phosphate, are more stable in PN solutions than the PN solution. Organic calcium and phosphorus depends on the source of mineral that is used for the preparation of the PN solution. Organic calcium and phosphorus compounds, such as calcium glucone-phosphate, are more stable in PN solutions than inorganic minerals like calcium chloride, potassium or sodium phosphate.

More recent studies have shown that high concentrations of calcium in combination with sodium-glycerol-phosphate safely can be provided in PN solutions without precipitation. A number of studies demonstrated that adding calcium and phosphorus to the PN solution caused a positive nutrient balance, and increasing the amount increased the retention of both minerals. None of these studies provided amounts that would equal the fetal accretion rate. Current recommendations of daily requirements of mineral intake by international committees are based on either the factorial approach or balance studies and aim to achieve a postnatal mineral accretion equivalent to that of the intrauterine gain of a normal fetus. In contrast to enteral nutrition, parental supply of minerals is independent of intestinal absorption and the amount of minerals is directly available for the metabolic needs. Consecutively parenteral daily requirements are assumed to be lower than enteral needs. Furthermore in comparison, the recommendations for enteral Ca/P intake ratios differ compared to parenteral. The recommended Ca/P intake ratios have not been evaluated systematically. Only few studies evaluated the effect of parenteral mineral supplementation on bone status in preterm infants. Although these studies showed a positive effect on bone mineralization, the intervention period was short, the amount of minerals supplied was below current recommendations, the numbers of infants evaluated was small, and infants were either at term age or at higher gestational age than that of infants currently cared for in NICUs. Up to date it has not been proven that mineral supplementation in PN will lead to normal bone mineralization in preterm infants.

Breast milk or Formula

Human milk

Human milk has a unique composition of nutrients and is recognized as the preferred nutrition for all newborn infants. Human milk contains functional nutrients that help provide the optimal microenvironment for protection and maturation of the gut and passively provide protection from infections. The composition of the bioactive factors of human milk differs not only among women but also during the lactation period from colostrum with the highest concentrations, through transitional to mature milk. The amount of these factors also seems to be dependent on the degree of prematurity. Depending somewhat on the agent investigated, colostrum of preterm infants seems to have higher concentrations of immuno-active substances than colostrum from term mothers, while colostrum of mothers of extremely preterm infants has the lowest concentrations.

Besides its contribution to host defense and trophic effects on the gastrointestinal tract, it is generally accepted that human milk contains all nutrients necessary for optimal growth and development of newborn infants. Human milk has a high amount of lactose and polyunsaturated fatty acids. The benefits of both nutrients for optimal digestion and development of the child have been described above. However, the exact composition of breast milk is usually unknown and is dependent on gestational age and time of lactation. The amount of carbohydrates, protein and fat of breast milk of mothers who deliver preterm is usually higher compared to mothers of term infants during the transitional phase of the lactation period. In both groups the content of protein decreases with lactation duration while carbohydrates and fat seem to increase. Nevertheless, the overall nutritional content of human milk has been shown to be inadequate for very preterm infants. A number of clinical trials showed that feeding pure human milk led to impaired postnatal growth in very preterm infants. On the other hand, Polberger et al. found in a randomized trial that enrichment of human milk with protein in very preterm infants improved growth in weight and length. This study also found large fluctuations in daily nutrient intake due to a varying content of protein and fat content in human milk. This has also been found in a number of studies that evaluated the composition of human milk. Overall, regarding the high nutritional needs of preterm infants, human milk has an insufficient content of protein and minerals namely sodium, calcium and phosphorus. Finally, this may lead to nutritional deficiencies in very preterm infants and consecutively result in growth retardation and impaired bone mineralization.
Preterm formula

Special preterm formulas were developed several decades ago since the nutritional adequacy of human milk for preterm infants was questioned. These special preterm formulas were enriched with protein as well as relatively high amounts of carbohydrates, fat, sodium, calcium, phosphorus and vitamins. A number of clinical trials demonstrated that postnatal weight gain improved with preterm formula compared to human milk. Meta-analyses of Cochrane reviews concluded that higher protein intake with formula accelerated weight gain and formula compared to donor breast milk resulted in higher rates of short-term growth.

The advantage of formula feeding is the ability to provide a guaranteed and standardized nutritional intake of all nutrients. However, the composition of preterm formulas is artificial and has been developed according to the nutritional requirements as defined by Ziegler based on the reference fetus. Over the years the formula composition has been adapted to improve feeding tolerance. Initially high doses of protein led to elevated blood urea nitrogen, blood ammonia, urine osmolarity and metabolic acidosis while weight gain did not further improve above an intake of 4.5 g/kg/d of protein. Longitudinal studies to determine the effects of high protein intake on long-term outcome of growth, body composition, neurodevelopment and possible adverse conditions of obesity, diabetes and cardiovascular disease are still needed. Short-term studies have shown that body composition can be altered by changes in the composition of preterm formula. The protein source of preterm formulas is cow’s milk. Milk proteins can be divided into caseins and whey proteins with a ratio of 80:20 for cow’s milk and 40:60 for human milk. Casein easily coagulates when acidified and therefore is more difficult to digest, leads to slower gastric emptying and amino acid absorption. More recent preterm formulas have a whey-dominant composition that aims at more rapid gastric emptying and production of free amino acids that are comparable to those produced by human milk. The fat source of formula usually is a blend of vegetable oils and medium chain triglycerides (MCT) and is less complex than human milk fat. Human milk contains ‘bile-salt-stimulated lipase and palmic acid in the β position that increase the bioavailability of milk fat and increase digestion and absorption of nutrients. More recent preterm formulas have a whey-dominant composition that aims at more rapid gastric emptying and production of free amino acids that are comparable to those produced by human milk.

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Table 3 Immun agents in human milk

<table>
<thead>
<tr>
<th>Immune functions</th>
<th>Agents</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-microbial</td>
<td>SigA</td>
<td>Interferes attachment of microbial pathogens to epithelium, neutralizes bacterial toxins</td>
</tr>
<tr>
<td></td>
<td>Lactoferrin</td>
<td>Blocks multiplication of siderophiles by chalting Fe+++</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>Digests peptidoglycans from certain bacterial cell walls</td>
</tr>
<tr>
<td></td>
<td>Alpha-lactalbumin</td>
<td>Destroys streptococcus pneumoniae</td>
</tr>
<tr>
<td></td>
<td>Lactadherin</td>
<td>Blocks binding rotavirus to intestinal epithelium</td>
</tr>
<tr>
<td></td>
<td>MUC1</td>
<td>Blocks binding E. coli to intestinal epithelium</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>Precursor of opsonins C3b and C3bi</td>
</tr>
<tr>
<td></td>
<td>Fibronectin</td>
<td>Opsonin</td>
</tr>
<tr>
<td></td>
<td>CCL28</td>
<td>Destroys C albicans and certain bacteria</td>
</tr>
<tr>
<td></td>
<td>MIF</td>
<td>Supports killing M. tuberculosis</td>
</tr>
<tr>
<td></td>
<td>Defensins</td>
<td>Inhibits HIV-1 replication, and destroys E.coli</td>
</tr>
<tr>
<td></td>
<td>Oligosaccharides</td>
<td>Interferes attachment of microbial pathogens to epithelium</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>Prostaglandins E2, F2a</td>
<td>Cytoprotecives</td>
</tr>
<tr>
<td></td>
<td>EGF, Lactoferrin, polyamines</td>
<td>Epithelial growth factors</td>
</tr>
<tr>
<td></td>
<td>Cortisol</td>
<td>Maturational factor</td>
</tr>
<tr>
<td></td>
<td>PAF-acetylhydrolase</td>
<td>Degradation inflammatory mediators</td>
</tr>
<tr>
<td></td>
<td>α-1-antichymotrypsin</td>
<td>Binder of enzymes</td>
</tr>
<tr>
<td></td>
<td>Lysozyme to elastin</td>
<td>Binder of substrates of enzymes</td>
</tr>
<tr>
<td></td>
<td>Lactoferrin to lipid A of LPS</td>
<td>Binders of toxin</td>
</tr>
<tr>
<td>Immun-modulatory</td>
<td>IL-10, TGF-β1</td>
<td>Modulators of inflammatory leukocytes</td>
</tr>
<tr>
<td></td>
<td>Uric acid, β-carotene, ascorbate</td>
<td>Anti-oxidants</td>
</tr>
<tr>
<td></td>
<td>IL-7</td>
<td>Stimulates T-cell production</td>
</tr>
<tr>
<td></td>
<td>Interferon-γ, TNF-α, IL-12, IL-18</td>
<td>Enhancement of cellular immunity</td>
</tr>
<tr>
<td></td>
<td>TGF-β1, IL-4, IL-10</td>
<td>Enhancement of humoral immunity</td>
</tr>
<tr>
<td></td>
<td>IL-β1,IL-6, MIF</td>
<td>Macrophage stimulation</td>
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<tr>
<td></td>
<td>IL-8, RANTES, MIP-1, CCL28</td>
<td>Chemokine activities</td>
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<tr>
<td></td>
<td>IP-10, MIG</td>
<td>Interferon inducible proteins</td>
</tr>
<tr>
<td></td>
<td>TGF-β1, IL-10</td>
<td>Anti-inflammatory actions</td>
</tr>
<tr>
<td></td>
<td>EGF, M-CSF, G-CSF, erythropoietin</td>
<td>Growth stimulation</td>
</tr>
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Adapted from (233, 260)
able to suppress the growth of opportunistic bacteria. However, preterm formulas also contain glucose polymers, like maltodextrin, to reduce the osmolality load. In animal studies maltodextrin in formula was associated with increased risk of necrotizing enterocolitis. (159, 284)

The greatest concern with regard to preterm formula is the association of formula feeding with necrotizing enterocolitis (NEC) and late-onset sepsis. (50) It has been suggested that this may be related to an inflammatory response in relation to cow’s milk protein but also to changes in the intestinal microbiome in relation to formula feeding. (285, 286) A number of studies found that exclusive diets with human milk shortened the duration of parenteral nutrition, decreased the incidence of NEC including the need for surgical intervention. (287, 288) Formula feeding does not provide the immunological protection of human milk. A retrospective study found that the amount of own mother’s milk during the first 10 days of life was associated with decreased morbidity and mortality in very preterm infants. (308, 290)

Fortified Human milk
The alternative way to provide the required higher amount of nutrients to very preterm infants is to enrich own mother’s milk specifically with nutrients insufficient available in human milk such as protein and minerals especially sodium, calcium and phosphorus as well as several trace elements and vitamins. The idea was to combine the advantages of non-nutritional factors of human milk with an extra amount of nutrients. Consecutively a number of powdered and liquid human milk supplements or ‘fortifiers’ (HMF) were developed. A number of small studies evaluated the effect of fortification of human milk on biochemical mineral status, weight gain and bone mineralization in comparison to either pure human milk or preterm formula. (267, 291-304) Most studies found improved mineral balances with fortified human milk compared to pure human milk and improved weight gain and a few found improved bone mineralization. Some of the studies reported that the use of HMF led to growth equivalent to growth seen in infants fed formula feeding. This led to the recommendation that human milk for very preterm infants needed to be enriched with supplements. (305) However, the above mentioned studies used different products, the initiation and duration of the fortification were diver and most infants evaluated were more stable than preterm infants currently treated in the NICU. Studies that could not demonstrate an improvement in bone mineralization with higher mineral intake had a short intervention period. (292, 293, 295) There is no evidence based recommendation available with regard to either the optimal time to start or the optimal volume of human milk to commence fortification. Early enrichment at low volumes of enteral intake may accelerate the enteral nutritional intake and thereby support postnatal growth and decrease the duration of parenteral nutrition. On the other hand most multi-nutrient fortifiers are based on cow’s milk protein and fortification has been shown to increase the osmolality of human milk. (306) Both factors are assumed to increase the risk of feeding intolerance, specifically impaired gastric emptying and increased risk for necrotizing enterocolitis. (287, 307) Though recent systematic reviews did not find an increase in adverse effects among infants who received HMF, the available data are limited to small trials and usually more stable infants. (306) It is not known whether these statements are applicable for extremely preterm infants, the ones that should mostly take advantage of human milk fortification.

Outcome measures in relation to nutritional practices
The key processes of intra-uterine as well as postnatal development of preterm born infants are growth, neurodevelopment and bone development. All three have been shown to be associated with nutritional intake and thereby are often used as outcome parameters for the evaluation of nutritional interventions.

Growth
For growth assessment the optimal expected postnatal growth of a preterm infant needs to be defined. In general, pediatric societies recommend that postnatal growth of preterm infants should approximate the fetal intra-uterine growth. (65, 305) Clinical measures of growth commonly compare weight, length and head circumference. Estimates of fetal growth may be obtained from large population studies. Uniform standardized fetal growth charts are not available currently. For the analyses of postnatal growth evaluation as performed for this thesis, the Dutch references by gestational age, the Swedish growth reference from 24 weeks of gestation to 24 months by gender and the revised Fenton growth chart for preterm infants were used. (309-311)

The period of fastest human growth occurs between 22 and 40 weeks of gestation requiring the comparatively highest nutritional supply during lifetime for achievement of optimal fetal growth. While after preterm birth the energy expenditure exceeds the fetal needs, the infant is physically unable to tolerate the full required amount of enteral nutrition and technically it can be difficult to provide the high requirements as described above. Many clinical studies have demonstrated that most very preterm infants develop a severe growth retardation in the postnatal period. (80, 312-314) Dancis et al. who developed one of the first growth curves for preterm infants, reported nearly 70 years ago that ‘the chief variable in determining the weight curve of …a premature is the feeding policy’. (315) Embleton et al. demonstrated that most very preterm infants did not receive the recommended nutritional intake during the first two postnatal weeks and consecutively developed a cumulative nutritional deficit
that was not regained during the following weeks. The same infants demonstrated an increasing growth retardation during the same period which led to the assumption that the postnatal growth retardation as seen in these infants could be associated with insufficient nutritional intake. The postnatal growth retardation has been shown to persist until early childhood. The ELGAN study, a prospective cohort study evaluating nutritional practices and postnatal growth of extremely preterm infants, found that first week nutritional practices were positively associated with weight gain during the first month. While follow up studies showed that postnatal morbidities such as intraventricular hemorrhage and cerebral palsy, chronic lung disease, infectious diseases and necrotizing enterocolitis was negatively associated with growth, several studies indicated that improved growth specifically was associated with higher protein intake in the early postnatal period. Irrespective of nutritional intake or other co-variables, infants may follow different growth trajectories depending on either being born as appropriate or small for gestational age. The size of a newborn infant is determined by the genetic potential of each parent but is also dependent on the intra-uterine environment or chromosomal abnormalities. The term ‘appropriate for gestational age’ describes a newborn whose size is appropriate for gestational age according to a standardized growth chart, while small for gestational age generally is considered if the weight falls below the tenth percentile. The optimal postnatal nutritional treatment of intra-uterine growth restricted preterm infants is a matter of debate. Most follow up studies have shown that small for gestational age born infants do not catch up and remain smaller than infants born with appropriate weight at the same gestational age. This impaired postnatal growth could be a result of a persisting postnatal undernutrition as seen in appropriate for gestational age preterm infants, indicating increased nutritional requirements compared to infants with appropriate fetal weight. On the other hand, intra-uterine growth restriction may have programmed fetal growth towards adaptation to poor nutritional environment. Accelerated postnatal growth may have adverse consequences for the growth restricted preterm infant, setting the stage for long-term obesity and metabolic problems. Currently it is not known whether small for gestational age preterm infants have a window of opportunity to catch-up to normal growth during the early postnatal period or that moderate postnatal growth should be accepted to prevent obesity, diabetes and cardiovascular disease.

Neurodevelopment
Satisfactory functional development is the second important goal of nutritional management. This implies optimal growth and development of the brain. The human brain is the most complex system of the body and at the time of preterm birth in a rapid developmental process of synaptogenesis and axonal growth and beginning myelination. Recent functional MRI studies have shown that preterm birth will have impact on this complex process of brain development with potentially effects on the brain architecture. Studies found that preterm birth, at term equivalent age, was associated with reduced brain volumes, diminished cortical gyification and delayed maturation in gray matter structures. These deficits were found in the absence of focal brain injury and seemed to persist throughout childhood. These recent morphological findings seem to confirm the results of many international follow up studies that evaluated the neurobehavioral outcome of very preterm infants up to childhood and school age. Very preterm infants showed more behavioral problems, attention problems, less academic achievements and impaired executive functions compared to term born infants. Until now it has been difficult to prove a causative relationship between nutritional support and neurodevelopment. A number of studies demonstrated that more aggressive nutritional support led to improved extra-uterine weight gain for very preterm infants and this seemed to be associated with improved neurodevelopment in childhood. Ehrenkranz et al and Belfort et al found that postnatal weight gain was positively associated with mental and psychomotor development at 12 and 18 month corrected age. Furthermore, head growth seemed to be positively associated with neurodevelopment. This improvement has specifically been related to higher amino acids intake. However, there is always more than one single determinant of neurodevelopmental outcome. Risk factors for adverse development include antenatal conditions like maternal illness, prenatal drug exposure, reduced fetal oxygen and nutrient supply as a result of placental insufficiency, while furthermore socioeconomic status and maternal education have been associated with neurodevelopmental outcome. Perinatal events such as asphyxia, intra-ventricular hemorrhage, infectious diseases and other prematurity related morbidities have been associated to impaired neurodevelopmental outcome. All of these determinants may contribute to the overall outcome of a child and therefore may hamper the determination of the effect of nutritional support.

Besides perinatal conditions gender has been reported to be a factor for outcome after preterm birth. In general, long-term follow-up evaluations of preterm infants have shown that boys are more at risk for unfavorable outcome than girls. Hintz et al evaluated risk factors for adverse neurodevelopmental outcomes of preterm born children and found male gender to be an independent risk factor for severe impairment of psychomotor development at two years of age. On the other hand, nutritional intervention studies have indicated that boys probably may take more advantage of improvements of nutritional intake than girls. Placental insufficiency, the most common cause of small for gestational age, implies a period of fetal undernutrition and thereby also an increased risk of altered brain development that consecutively may cause altered neurodevelopment.
Most studies found lower cognitive scores, or neurological abnormalities in small for gestational age children compared to children with appropriate birth weight.\(^{351-355}\) It is not known whether postnatal nutritional interventions in preterm infants are able to ameliorate the unfavorable intra-uterine development. Overall, studies looked only at term born small for gestational age infants. A systematic review reported of two randomized trials that found no effect of nutrient enriched formulas on neurocognition, whereas early growth increased fat mass, lean mass and blood pressure.\(^{356}\) It is not known whether preterm infants are born within a window of opportunity for favorable catch up in growth and neurodevelopment. A systematic review evaluating prognostic factors for poor neurodevelopment in children born very preterm found that low birthweight was a significant prognostic factor in early infancy with lack of an association in later years.\(^{353}\) This could indicate a ‘catch-up’ in neurodevelopment but currently it is not known whether this is related to nutritional interventions.

**Bone development**

**Bone mineralization**
The formation of bone occurs in several steps. Bone matrix is synthesized and deposited by osteoblasts. Minerals are incorporated into the pre-existent bone matrix, the cortex or trabeculae may be thickened or new trabeculae may be synthesized. Bone mineralization can only occur when bone matrix has previously been deposited.\(^{357}\) Decreased bone mineralization may reflect two situations: either not enough organic matrix has been deposited or not enough mineral has been incorporated. Both conditions correspond to the most common pathological situations seen in the development of the preterm skeleton – either osteomalacia or osteopenia.\(^{357-359}\) (Figure 2)

**Osteomalacia**
Osteomalacia describes the situation of accumulation of un-mineralized bone matrix with osteoblasts continuing to secrete osteoid. Therefore the average mineral content is low and the bone is soft. A clinical example for this situation may be seen in the occipital flattening of preterm infants. If this process involves the growth plate it is called rickets. Bone mineral density and bone mineral content are decreased in osteomalacia and trabecula have a washed out appearance on radiographs.\(^{357,360,361}\)

**Osteopenia**
This term describes a decreased amount of bone tissue with a decreased thickness or number of trabeculae and/or decreased thickness of the bone cortex.\(^{357}\) Osteopenia is caused by either insufficient deposition or increased resorption of organic bone matrix. In contrast to osteomalacia, the incorporation of calcium and phosphate into organic bone matrix is not affected and the mineralization of the growth plate cartilage proceeds normally, thus there is no sign of rickets. Bone mineral density and bone mineral content are physically and radiologically decreased.

**Osteoporosis**
Osteoporosis has only been defined for adults and reflects a situation where the areal bone mineral density is below -2.5 standard deviations from the mean of young healthy adults.\(^{362,363}\) This definition has been shown to predict a future fracture risk. In large epidemiological studies a reduction of 1 standard deviation score predicted a two-to threefold increase in the risk of fractures.\(^{364}\) The areal bone mineral density depends on bone size and therefore this definition cannot be used in neonatology, since all infants would be diagnosed as having osteoporosis.\(^{357}\) Currently there is no definition of osteoporosis available in pediatrics. It has been suggested to use the term osteoporosis if bone stability is not adapted to mechanical requirements because bone mass is inadequately low and fractures occur after minor trauma.\(^{357}\)

<table>
<thead>
<tr>
<th>Bone mineral content</th>
<th>Normal</th>
<th>Low</th>
<th>Low</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteomalacia</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

Figure 2 Conditions of bone development in preterm infants. Black: mineralized bone matrix; grey speckled: unmineralized bone matrix adapted from\(^{357}\)

**Osteopenia of prematurity**
Preterm infants are at significant risk to develop reduced bone mineral content (BMC) with subsequent development of osteopenia of prematurity or metabolic bone disease (MBD).\(^{365,366}\) Osteopenia results from diminished synthesis and/or increased
resorption of organic bone matrix. (357) There are numerous reasons for impaired bone development in preterm infants but adequate supply of substrate is obviously a prerequisite for synthesis of bone tissue. Up to 80% of the body calcium of a term infant is accrued during the last trimester of pregnancy. (347) Infants born preterm miss this active foetal mineralization in the third trimester and instead are reliant on parenteral and enteral sources of minerals. (368) In clinical practice it is difficult to meet the high foetal needs either because of limited solubility of parenteral fluids, low content of calcium and phosphate in human milk and impaired intestinal absorption of calcium or phosphorus through formula feeding. (142, 241) Besides insufficient supply of nutrients, impaired organic bone matrix formation can be caused as a result of severe systemic disease, drug side effects (corticosteroids, sedativa), increased losses of minerals based on immature renal function, suboptimal calcium/phosphate intake ratio, diuretics and vitamin D deficiency based on renal or hepatic disease. (357, 365) Intra-uterine growth restriction has been associated with increased risk of osteopenia. (369) Furthermore, it has been suggested that skeletal development is driven by functional requirements, meaning that bone strength increases when and where it is required to maintain bone stability, resulting mainly from muscle contraction and not from passive gravity. (357, 370) Nutritional and mechanical factors seem to have different roles in bone development and cannot substitute for each other, meaning that lack of mechanical challenge posed by muscle contractions will decrease the need for bone formation and therefore high amounts of minerals may not be used. On the other hand inadequate supply of minerals will prevent mineralization of bone matrix. (357)

Metabolic bone disease has declined since the 1980’s, probably due to improved nutritional management. (365) However, the incidence of MBD still is increasing with decreasing gestational age and birth weight. The infants most at risk are those under 28 weeks and less than 1500 gram. For infants less than 1000 gram an incidence of 55% has been reported. (128) Depending on the severity, MBD may remain clinically silent or result in fractures. (371) Postnatal development of MBD has been related to myopia of prematurity, and impaired respiratory function as a result of bone softening and poor growth in childhood. (372-374) For the long-term development preterm infants have been shown to be smaller in length with significantly lower lumbar spine bone mass compared to population reference data. (375-377) Inadequate bone mineralization is seen as a risk factor for the development of osteoporosis in later life, which is an important cause of morbidity and mortality in elderly people and a considerable factor of healthcare expenditure. (378-380) The peak bone mass is attained before skeletal maturity. (381) Any factor that influences the acquisition of peak bone mass may represent a mechanism to affect later osteoporosis risk. Although medical treatment of preterm infants has improved over the years, the optimal treatment to prevent bone mineral disease is still a matter of debate. Only a few studies have evaluated long-term bone development of VLBW children until childhood and adolescence. (376, 382-389) Their findings were diverse, but most of the studies showed that these former preterm infants remained smaller later in life, some with lower bone mineralization, others with low mineralization but normal in proportion to their small body size. Several studies found impaired bone mineral content in boys compared to girls as well as for small for gestational age born children compared to children born with appropriate weight. (390, 391) Despite the low content of minerals, human milk seemed to have a positive effect on bone development. (377, 392) All of the participants of these studies received diets that provided nutrients markedly below the current recommendations and therefore the results may not be representative for the population of preterm infants treated nowadays.

Screening methods
Metabolic bone disease is often defined as decreased bone mineral content relative to the expected level of mineralization for a fetus or of an infant of a comparable size in combination with either biochemical or radiographic changes. (393) As MBD is asymptomatic in most infants, screening seems necessary for a timely diagnosis. Despite the recognition of the importance of early intervention to prevent MBD there is a lack of consistency and practices concerning screening and monitoring. (366, 394) Currently there is no consensus on diagnosis, treatment or timing of initiation of treatment. This is partly based on the lack of evidence based parameters to evaluate bone status. (395, 396) Standard screening methods currently used, comprise determination of biochemical parameters of the calcium-phosphorus homeostasis including serum and urine analysis as well as imaging methods.

Biochemical parameters
Evaluation of electrolyte disturbances is standard of care in many neonatal units assuming that biochemical parameters of Ca-P homeostasis within a normal range will lead to optimal bone mineralization. (140,141,397-410) However, there is currently neither a consensus on the appropriateness of either parameter nor the frequency of measurements. (395, 396) Reference values in relation to adequate nutritional intake have not been developed. Urinary excretion of minerals in spot urine samples has been shown to be an easy tool for routine evaluation. Pohlandt proposed to aim for a small ‘surplus of minerals’ in urine samples, while Aladangady et al developed reference values for urinary Ca-/P-creatinine ratios for preterm infants. (400, 406) Staub et al compared both methods with regard to an agreement between their results and found neither method to be superior. (411) None of the studies evaluated the direct effect of nutritional intake on biochemical parameter of Ca-P homeostasis.
Imaging
Osteopenia can be discovered on a plain radiograph, but this usually only provides visible changes of ‘thin bones’ if bone mineral content has decreased by at least 20 - 40%. These radiographic signs occur late and furthermore are not quantifiable. Since it also uses radiation this method is not suitable for routine practice. Dual-Energy-x-ray absorptiometry (DEXA) currently is recognized as the gold standard for bone measurements in adults and is currently also used in infants. This method is sensitive to detect small changes in bone mineral content. Nevertheless, the availability is limited, it is not suitable for daily screening and it also uses a small amount of radiation. Although the use is validated for preterm and term infants, the reference values depend on the method used. Therefore it may be difficult to compare the results of different studies. Quantitative ultrasound is a promising new method based on the idea that changes in ultrasound are related to changes in bone mineral density and bone structure. This method is a non-invasive, cheap bedside test, and suitable for repeated measurements. Depending on the method used, changes in ultrasound are measured at the tibia, calcaneus, radius of metacarpal phalanges. It is not known whether this method can replace DEXA.
Outline of the thesis
The overall purpose of the work presented in this thesis is to determine the effect of nutritional intake during the first two weeks of life in very low birth weight infants with regard to three key processes of postnatal development, namely growth, neuro-development and bone mineralization.

In 2003 the department of Neonatology of the Radboudumc introduced a standard nutritional protocol for all infants admitted to the NICU. This protocol mainly provided recommendations concerning the initiation and increment of enteral nutrition. The use of human milk was strongly advised and otherwise preterm formula was used. In 2004 the regular use of human milk fortifier was introduced. A standard protocol for registration of feeding tolerance, growth and mineral status including blood and urine samples was introduced. During the same time the composition of the standard parenteral solution was adapted according to the recommendations of international guidelines. The new parenteral nutrition provided a higher amount of amino acids, carbohydrates and minerals, specifically calcium and phosphorus and was introduced and used from January 1st in 2005 onwards. Furthermore, the parenteral nutrition protocol was adapted to achieve the full amount of parenteral intake earlier than compared to the previous guideline, while at the same time more emphasis was put on fast increment of enteral nutrition. The aim of the new nutritional protocol was to provide the full nutritional requirements for all infants as soon as possible after birth.

The studies presented in this thesis are based on two cohort studies. The first study is observational and included the data collection of two consecutive year cohorts (2004, 2005) of very preterm infants admitted to the neonatal intensive care unit of the Radboudumc. The two cohorts differed with regard to the composition of parenteral nutrition provided after birth. After the second week of life infants of both cohorts usually received the same nutritional intake, mainly fortified human milk or preterm formula. The surviving infants of both cohorts who fulfilled the criteria for the national follow-up were included for a long-term follow up of growth and neuro-development. The same infants were finally invited at the age of nine to 10 years for an evaluation of growth and bone mineralization.

The second study was performed in combination with the multi-center double-blind randomized controlled trial the ‘Early Nutrition Study’ (ENS) that evaluated the effect of human milk on sepsis, necrotizing enterocolitis and death. This study randomized preterm infants with a birth weight below 1500 gram to receive either donor milk in addition to mother’s own milk or preterm formula if mother’s milk was not available. According to the study protocol human milk was not fortified until day 10 of life. For participants of the Radboudumc the study protocol was extended (Early Supplementation Study; ESS). A third group was included in a randomized fashion. This third group received enteral nutrition according to the nutritional protocol of the NICU of the Radboudumc. Further, this group received early fortification of human
milk and preterm formula if mother’s own milk was not available and thereby had a higher enteral nutritional intake of protein, calcium and phosphorus during the first 10 days compared to infants who received unfortified human milk during the same period. The aim of this study was to evaluate the effect of higher enteral mineral intake during the first 10 days, on growth and bone mineralization in preterm born infants at term corrected age.

The following hypotheses were tested:
• Increase in protein and energy intake during the first postnatal week improves postnatal weight gain and leads to improved long-term growth at nine to 10 years of age
• Early enteral feeding improves weight gain
• The nutritional intake of calcium and phosphorus during the first two weeks of life of very low birth weight infants affects biochemical parameters of the calcium – phosphorus homeostasis
• Higher amount of enteral supplementation of calcium and phosphorus during the first 10 days of life leads to improved growth and bone mineralization at term corrected age of very low birth weight infants
• Nutritional intake during the first week affects head growth and neurodevelopmental outcome of very low birth weight infants at 24 months
• Increase in calcium and phosphorus intake during the first postnatal week improves long-term growth and bone mineralization at nine to 10 years of age
• Boys take more advantage of nutritional improvements than girls
• Phalangeal quantitative ultrasound can replace dual-x-ray absorptiometry for determination of bone mineralization in children born very preterm

Part 2 | Different amino acid and mineral intakes
In chapter 3 the effect of different nutritional intake of the two observational cohorts (2004, 2005) are evaluated with regard to weight gain and length for the short-term outcome until term corrected age. In chapter 4 the effect of early nutritional intake on long-term growth and bone mineralization of former preterm infants is evaluated. In chapter 5 the effect of early nutritional intake on neurodevelopmental outcome at 24 months corrected age is evaluated.

Part 3 | Calcium and Phosphorus metabolism and bone mineralization in preterm infants
Chapter 6 presents an observational study describing the longitudinal changes of biochemical parameters of the calcium-phosphorus metabolism during the first five postnatal weeks based on data collected from the 2005 cohort. In chapter 7 data recorded during the ESS, were used to quantify changes in biochemical parameters of the calcium-phosphorus homeostasis in relation to nutritional intake and other clinical parameters. In chapter 8 we analyzed growth and bone mineralization at term corrected age of participants of the randomized ESS in relation to enteral nutritional intake during the first 10 days of life. Chapter 9 presents a study that investigated whether the method of phalangeal quantitative ultrasound is able to replace the method of dual-energy-x-ray absorptiometry for determination of bone mineralization in children born as very-low birth weight infants.

Part 4 | Discussion and Summary
Chapter 10 will summarize the results of all studies, put them into perspective and provide recommendations for further studies or improvements. Chapter 11 summarizes all parts of the thesis.
References


38. Butler P. The nursing; the feeding and hygiene of premature and full-term infants. Caxton; 1907.


CHAPTER 2 OUTLINE THESIS

1. Introduction to Premature Infants
   - Early Nutrition and Development

2. Nutrition during Prematurity
   - Nutrient Requirements
   - Growth and Development

3. Early Feeding and Enteral Nutrition
   - Techniques and Recommendations

4. Protein Metabolism in Premature Infants
   - Amino Acid Metabolism

5. Immune Function and Nutritional Interventions
   - Nutrition and Infection

6. Long-term Outcomes
   - Neurodevelopmental and Growth Milestones

7. Conclusions and Future Directions
   - Integration of Clinical and Research Perspectives

References


113. Clark RH, Chao DH, Spitzer AR. Effects of two different doses of amino acid supplementation on growth and blood amino acid levels in premature neonates admitted to the neonatal intensive care unit: a randomized, controlled trial. Pediatrics. 2007;120:1286-96.


CHAPTER 2

OUTLINE THESIS


Broadhurst M, Beddix K, Black J, et al. Effect of gestation length on the levels of five innate


PART 2

Different amino acid and mineral intakes
The enigma to achieve normal postnatal growth in preterm infants – using parenteral or enteral nutrition –

V Christmann, R Visser, M Engelkes, AM de Grauw, JB van Goudoever, AFJ van Heijst

Published in:
Abstract

Aim
To evaluate whether increasing the amount of amino acids and energy in parenteral nutrition combined with rapid increment of enteral feeding improves postnatal growth in preterm infants.

Methods
Observational study; two consecutive year-cohorts of preterm infants; Cohort 2 received higher supplementation of parenteral amino acids and energy with more rapid enhancement of enteral feeding than Cohort 1. Nutritional intake, weight and head circumference (HC) were compared.

Results
Cohort 2 (N:79, gestational age (GA): 29.8±2.2 weeks, birth weight (BW):1 248±371g) achieved full enteral feeds earlier (p < 0.001) and had a higher protein/energy intake during the first week (p < 0.001) than Cohort 1 (N: 68, GA: 29.5±2.3 weeks, BW: 1261±339g). Both cohorts developed cumulative protein/energy deficits, but less in Cohort 2 (p < 0.01). Appropriate for gestational age infants (AGA) of Cohort 2 improved weight gain until week 5 (p < 0.01) compared to AGA of Cohort 1, nevertheless all infants demonstrated a decline in mean standard deviation score (>1) for weight at term. Small for gestational age infants failed to improve HC.

Conclusions
Improved parenteral intake may lead to improved short-term postnatal weight gain. Faster increase of enteral nutrition was well tolerated but failed to prevent nutritional deficits. Practising early enteral feeding with higher supplementation of nutrients may be needed and requires further study.

Introduction
The main goal of nutrient supply to preterm infants is to achieve growth similar to intrauterine fetal growth combined with satisfactory functional development. (1) Many preterm infants face long-term detrimental effects. (2,3) Even first week parenteral nutritional management seems pivotal in determining long-term outcomes. (4)

Protein intake and enteral feeding have been considered important growth-promoting factors in very low birth weight (VLBW) infants. Several trials have demonstrated that increasing protein intake can lead to a positive nitrogen balance, albumin synthesis, whole body protein synthesis and improved postnatal weight gain. (5)

Recently it was demonstrated that early aggressive nutrition directly after birth led to reduced postnatal weight loss and reduced postnatal growth restriction. (6, 7) Nutrients can be provided by either parenteral or enteral route. Central venous catheter and parenteral nutrition (PN) allow administration of adequate nutrients soon after birth but both have been shown to be significant risk factors for late-onset sepsis. (8,9) Early establishment of enteral feeding, especially of human milk, may be protective against infection. (10) Several studies demonstrated that rapid advancement of enteral feeding in preterm infants led to an earlier regain of birth weight and a shorter hospital stay. (11,12)

To improve our nutritional management of preterm infants, we increased the amount of amino acids and energy provided by parenteral nutrition in combination with the intention to rapidly increase enteral feeding. We compared the new feeding regimen to the previous standard protocol, hypothesizing that the increase in protein and energy intake as well as early full enteral feeds would lead to improved postnatal weight gain.

Method

Study design
This prospective cohort study was conducted during two consecutive years, 2004 (Cohort 1) and 2005 (Cohort 2). The study was approved by the local ethics committee. Informed consent of parents was not necessary according to our institutional review board because the parenteral nutrition solution was a standard medical prescription that was changed to improve quality. The nutritional protocol and record-keeping were part of the standard care applied in our department.
Study population
Preterm infants born below 34 weeks gestational age (GA), admitted to our tertiary neonatal intensive care unit (Radboud University Nijmegen Medical Center, The Netherlands) were recruited on the first day of life if it was assumed that PN was needed for at least 5 days. Infants with major congenital malformations or asphyxia were excluded from evaluation.

Nutritional protocol
During both time periods, all infants received nutrition according to standard institutional nutritional protocols. No other major changes in clinical practice occurred during either period.

Parenteral nutrition
Parenteral nutrition was started directly after birth in both cohorts. Nutritional intake was increased by daily increment of the amount of fluid. Our PN consisted of standard prepared components. The cohorts differed for the intake schedule. Cohort 2 achieved full parenteral nutrition earlier than Cohort 1 (4 vs. 6 days). At full PN Cohort 2 received more amino acids (21 vs. 17.5 g/kg/week) than Cohort 1. Carbohydrate intake of Cohort 2 exceeded Cohort 1 if the total fluid intake increased above the recommended 150 mL/kg/day (C1: 95.9 vs. C2: 96.6 – 117.6 gram/kg/day). The total amount of parenteral lipid intake was not changed. (Table 1). Details are presented in Table 1.

Enteral nutrition
In both cohorts enteral feeding was started on the first day of life. The amount was increased daily, while the PN was gradually reduced to maintain a daily fluid intake within the protocol range. The maximum enteral fluid intake was the same as for the PN (Table 1). The stepwise increase of enteral volume was left at the discretion of the neonatologist in charge. For the second cohort we intended to increase the enteral amount of nutrition as soon as possible. Gastric residuals up to 3 ml per feeding were generally accepted. Human milk was enriched using a commercially available fortifier (Nutrilon Nenatal BMF, Nutricia, Zoetermeer, NL) above an intake of 50 mL/d. The fortifier (HMF) added 0.8 g/dL of protein. If human milk was not available, infants received preterm formula (Nutrilon Nenatal Start, Nutricia, Zoetermeer, NL) with a protein content of 2.5 g/dL.

Data collection and Analysis
The intake of all nutrients via both, PN and enteral feeding as well as growth characteristics was recorded daily during the first 2 weeks, weekly until week 5 and at term corrected age (TCA), calculated and analysed according to the information in the patient charts.

Table 1 Parenteral nutritional intake

<table>
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<tr>
<td>Fluid ml/kg/d</td>
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<td>100</td>
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<tr>
<td>CH g/kg/d</td>
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<td>5.9</td>
<td>6.8</td>
<td>7.8</td>
<td>8.83</td>
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<td>2</td>
<td>2.25</td>
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<td>2.5</td>
</tr>
<tr>
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<td>1.5</td>
<td>2</td>
<td>2.5</td>
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</tr>
<tr>
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<td>47</td>
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<td>67</td>
<td>86</td>
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<td>74</td>
<td>94</td>
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<td>94</td>
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</table>

Standard protocol for parenteral nutrition of Cohort 1 and Cohort 2 with different standardized parenteral solutions. CH: carbohydrates; AA: amino acid; EQ: energy quotient. The parenteral nutrition consisted of two standard prepared components, a mixture of amino acids/glucose/minerals and lipid emulsion plus vitamins. Amino acid solutions and lipid emulsion: Cohort 1: Aminovenos N paed 10% (Fresenius Kabi), Intralipid 20% (Fresenius Kabi) Cohort 2: Primene (Clintec, Brussels), Intralipid 30% (Fresenius Kabi).

Nutritional intake of human milk was calculated assuming a protein content of 1.4 g/dL, a fat content of 3.2 g/dL, and a carbohydrate content of 7 g/dL as used for comparable studies (6, 7) and published previously (13). To evaluate nutritional intake in relation to recent recommendations, a mean daily intake of 3.8 g protein and 120 kcal/kg/d was assumed to be adequate. (1) Actual intake was subtracted from the recommended intake to calculate the daily deficit, which was then summed to calculate the cumulative deficit. Because intake during weeks 3, 4, and 5 was only recorded once a week, the mean daily intake was considered as the weekly intake divided by 7.

A BW below the 10 th percentile of the Dutch reference curves for BW by gestational age was defined as small for gestational age (SGA). (14) We performed analyses between subgroups of GA and between appropriate for gestational age (AGA) and SGA infants.

The mean daily weight gain was calculated weekly from birth for the first 5 weeks and until TCA. Postnatal weight gain was calculated using the exponential formula described by Patel et al. (15). To quantify postnatal growth, we calculated the standard deviation score (SDS) for weight changes using the Dutch reference curves as a reference for fetal growth and, thus, the estimated optimal postnatal growth. (14) The head circumference was recorded during the first week, at TCA, 6 months, 1 and 2 years of corrected age. The SDS for HC were calculated using the Swedish growth reference for preterm infants and the Dutch reference of the nationwide growth study. (16,17)
Statistics
The primary outcome of our study was postnatal growth. The power calculation to determine the size of the study group, was based on the study of Pauls et al., who investigated postnatal weight gain in VLBW infants on a comparable feeding regimen. A difference in weight change of 0.5 SD was assumed to be clinically relevant. We calculated that each group should contain at least 66 patients to find a statistical difference with an α of 0.05 and a power of 0.80 between the 2 cohorts. Data are presented as the mean ± SD, except otherwise mentioned. Paired Student t-tests were performed to compare the mean variables between subgroups of each cohort. Fisher’s exact test was used to evaluate categorical data. The level of significance was set at p < 0.05

Results

Patient selection and characteristics
The patient recruitment and cohort inclusion are presented in Figure 1. Finally, 68 infants in Cohort 1 and 79 infants in Cohort 2 were evaluated. The mean GA (29.5 ± 2.3 weeks vs. 29.8 ± 2.2 weeks) and mean BW (1261 ± 339 g vs. 1248 ± 371 g) were not different between the 2 cohorts. In Cohorts 1 and 2, respectively, 7.5 and 11% of infants were classified as SGA (Table 2). Mortality before day 8 was unrelated to nutritional intake. Morbidity and mortality before discharge are described in Table 2. The incidence of common diseases related to prematurity and mortality were not significant different between both cohorts.

Nutritional intake
Both cohorts started PN directly after birth, but the mean duration was significantly shorter and full enteral feeding was achieved significantly earlier in Cohort 2 (p < 0.001) (Table 3). Ninety-six and 91% of infants in Cohorts 1 and 2, respectively, received human milk, which was generally supplemented with human milk fortifier by day 5.

Daily changes in protein and energy intake are presented in Figure 2. The mean daily protein intake (g/kg/d) was higher in Cohort 2 up to day 5 (p < 0.005), whereas energy intake (kcal/kg/d) was higher up to day 7 (p=0.01) (Figure 2). This difference was mainly caused by the increased parenteral intake (Figure 3). Cohort 2 had a significant increased protein and energy intake during the first week (protein: 14.5 ± 4 vs. 18.1 ± 4 g/kg/week; energy: 512 ±93 vs. 609 ± 127 kcal/kg/week; p < 0.001) This difference was reduced during the second week of life (Table 3). The mean maximum protein intake was higher in cohort 2 (4.0 ± 0.5 vs. 4.3 ± 0.9 g/kg/d; p < 0.01) whereas the day to achieve the maximum intake demonstrated a wide range in both cohorts (Table 3). Although the achievement of the assumed recommended intake of 3.8g protein/kg/d was reached earlier in Cohort 2 than Cohort 1 (12.3 ± 7.4 vs.14.7 ± 8.0 days; non-significant), a considerable number of infants in both cohorts never achieved this intake during the first five postnatal weeks (14/68 (20.5%) vs. 13/79 (16.5%)). Energy intake increased continuously. At the end of the five week follow up Cohort 1 received a mean energy intake of 117 ± 24 kcal/kg/d whereas  cohort 2 received 123 ± 24 kcal/kg/d (Figure 2).

Infants of both cohorts developed cumulative deficits of protein and energy intake compared to the recommended intake (Figure 2). Nevertheless, these deficits were significantly lower in Cohort 2 during the observational period (p < 0.01). Protein deficits were not compensated, whereas the cumulative energy deficit tended to catch-up from the third week on. (Figure 2).

Postnatal growth
Maximum weight loss was 9.5% in both cohorts. The day that infants reached the nadir for weight was achieved earlier in Cohort 2 than Cohort 1 at mean 3.3 ± 0.6 days vs. 4.1 ± 1.2 days (p < 0.001). The mean number of days to regain birth weight was not different between both cohorts (8.9 ± 0.7 days vs. 8.3 ± 1.3 days).
At birth the mean SDS for weight was positive and similar between AGA infants of both cohorts (Table 2). SGA infants had a significant lower mean SDS for weight than AGA infants of their respective cohort. (p < 0.0001) (Table 2).

For AGA infants daily weight gain (g/kg/d) increased from the second week on but was significantly higher in Cohort 2 (p < 0.01). However, at TCA, the daily weight gain was no longer different between both cohorts (Figure 2). SGA infants demonstrated a higher growth velocity from birth until week 5 and until term age compared to AGA infants of the same cohort (birth - week 5: SGA C1/C2: 12.2 ± 3.1 / 12.7 ± 3.1 g/kg/d vs. AGA C1/C2: 8.9 ± 2.7 / 10.7 ± 3.0; p = 0.03; birth – term corrected age: SGA C1/C2: 15.5 ± 4.2 / 14.5 ± 1.5 vs. AGA C1/C2: 11.1± 2.3 / 11.8 ± 2.3 g/kg/d; p < 0.001) (Figure 2). Weight gain was not different between SGA infants of Cohort 1 and Cohort 2.

Despite the positive weight gain from the second week onward, all infants demonstrated a steady decrease in SDS for weight during the 5-week follow up, which continued until TCA (Figure 2). For AGA infants this decrease resulted in a mean

### Table 2 Cohort characteristics

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<th>Cohort 1</th>
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<td>57</td>
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</tr>
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<td>SGA</td>
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<tr>
<td>AGA</td>
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<tr>
<td>AGA</td>
<td>906 (154)</td>
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<td>837 (165)</td>
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Cohort characteristics concerning patient selection, morbidity and mortality. AGA = appropriate for gestational age; BW = birth weight; GA = gestational age; SGA = small for gestational age. SGA was defined as birth weight below the 10th percentile in the Dutch reference (14). HC = head circumference during the first postnatal week (16). Chronic lung disease (CLD) was defined as need for supplemental oxygen for more than 28 days. Sepsis was defined as clinical signs in combination with positive results from blood cultures ≥ 48 hours after birth, and necrotizing enterocolitis (NEC) was defined according to the criteria given by Bell et al. (32). IRDS = Infant respiratory distress syndrome; PDA = Patent ductus arteriosus; IVH III-IV = Intra-ventricular hemorrhage grade III-IV. None of the items were significantly different between the cohorts. None of the parameters were significantly different.
loss of 2.0 ± 1 SDS from birth until term corrected age in Cohort 1 and 1.9 ± 1.2 SDS in Cohort 2 (not significant). In contrast, SGA infants demonstrated a lower decline in mean SDS for weight than AGA infants (C1/C2: 1.16 (± 1.2)/1.64 (± 0.7) (not significant). At birth the mean SDS for HC was significant lower in AGA infants of C2 compared to AGA C1 (Table 2). At term corrected age this difference did no longer exist (AGA TCA C1/C2: -1.05 ± 1.25/ -0.35 ± 1.96; p < 0.11) and by 6 months both groups demonstrated a complete catch up in mean SDS for head circumference compared to the normal Dutch population at 6 months (AGA 6m C1/C2: 0.09 ± 1.01/ 0.12 ± 1.07; p< 0.88). AGA infants of C2 had a significant greater increase of mean SDS for head circumference than AGA infants of C1 by 6 months (C1/C2: 1.12 ± 0.74/ 1.57 ± 0.78; p < 0.01). The mean SDS for HC of SGA infants was not different at birth between both cohorts but significant lower than their respective AGA group (Table 2). Both cohorts of SGA infants showed an improvement of mean SDS for head circumference by 6 months, but the difference remained significant compared to AGA infants (p < 0.02) (SGA term corrected age C1/C2: -2.1 ± 1.91/ -2.69 ± 1.78; p < 0.69; SGA 6m C1/C2: -2.07 ± 1.69/ -0.73 ± 0.77; p <0.11). While AGA infants achieved a catch up to normal head circumference within 6 months the mean SDS of SGA infants remained below 1 SD for the normal Dutch population at the age of two years. (SGA 1y C1/C2: -1.36 ± 1.49/ -1.07 ± 1.19; p< 0.49; 2y C1/C2:-1.32 ± 1.68/ -0.98 ±0.98).

Subgroup analyses within a cohort revealed no differences between subgroups of GAs of AGA infants (data not shown). The number of SGA infants was too small to perform sub analyses.

---

**Table 3 Nutritional Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Cohort 1</th>
<th>Cohort 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN, mean (SD) days, (range)</td>
<td>12.8 (3.8) (7-25)</td>
<td>10.6 (3.6) (6-25)*</td>
</tr>
<tr>
<td>Full enteral feeding, mean (SD), days</td>
<td>13.6 (3.4)</td>
<td>11.6 (3.6)*</td>
</tr>
<tr>
<td>Protein week 1, mean (SD), g/kg/week</td>
<td>14.5 (3.7)</td>
<td>18.1 (4.0)*</td>
</tr>
<tr>
<td>Protein week 2, mean (SD), g/kg/week</td>
<td>21.3 (4.8)</td>
<td>22.4 (5.5)</td>
</tr>
<tr>
<td>Energy week 1, mean (SD), kcal/kg/week</td>
<td>512 (93)</td>
<td>609 (127)*</td>
</tr>
<tr>
<td>Energy week 2, mean (SD), kcal/kg/week</td>
<td>718 (165)</td>
<td>768 (178)</td>
</tr>
<tr>
<td>Age at protein intake ≥ 3.8 g/kg/d, days, mean (SD), (med, min, max)</td>
<td>14.7 (8.0)</td>
<td>12.3 (7.4)</td>
</tr>
<tr>
<td>Intake never above ≥ 3.8g/kg/d, No. (%)</td>
<td>14 (20.5)</td>
<td>13 (16.5)</td>
</tr>
<tr>
<td>Max protein intake, g/kg/d, mean (SD)</td>
<td>4.0 (0.5)</td>
<td>4.3 (0.9)**</td>
</tr>
<tr>
<td>Age at max protein intake, days, mean (SD), (med, min, max)</td>
<td>18.2 (9.0)</td>
<td>16.7 (9.7)</td>
</tr>
<tr>
<td>(14;4.35)</td>
<td>(13;4.35)</td>
<td></td>
</tr>
</tbody>
</table>

PN = parenteral nutrition; * = p < 0.001; ** = p < 0.01
Appropriate for GA and SGA infants in this study demonstrated a different growth pattern (Figure 2). AGA infants, in general, have demonstrated a normal growth potential until birth, and therefore ideally should be able to continue this growth thereafter. For AGA infants in this study, the nutritional deficits led to growth retardation of body weight with brain sparing with even catch up in the cohort with improved intake. Tan and Cooke evaluated nutritional intake and head growth in a randomized trial and demonstrated a positive correlation between head growth and nutritional intake but failed to demonstrate a significant improvement. This study did not separate between AGA and SGA infants. Our SGA infants of both cohorts mainly used energy for weight gain. The same phenomenon has been described in other studies. There is increasing evidence that SGA infants follow a different growth trajectory with negative long-term consequences. Therefore, the postnatal weight changes in growth parameter we observed in our study can be related to nutritional changes. We speculate that further improvement of nutritional intake might have improved head growth in SGA infants and weight gain in AGA infants. We are aware that our cohort of SGA infants is too small for further conclusions, but we suggest that with regard to probably different utilization of nutritional supply, future studies should evaluate differences in nutritional requirements between AGA and SGA infants.

The protein requirement of preterm infants includes the amount required for growth plus replacement of inevitable losses and ‘catch-up’ growth caused by morbidity or a failure to provide sufficient intake. Analysis of data collected in the NICHD glutamine supplementation study revealed that significantly improved growth occurred in infants who received at least 3 g/kg/d of parenteral protein by the fifth day of life. In our study, Cohort 1 had a mean intake of 3 g/kg/d by day 8, whereas in Cohort 2, this was achieved by day 5. According to recent international recommendations, enteral protein intake for preterm infants should range between 3.5 and 4.5 g/kg/d (1). Nevertheless, our assumed optimal intake of 3.8 g/kg/d was never achieved by a considerable amount of infants in both cohorts. The significant improved intake in Cohort 2 occurred mainly as a result of higher parenteral protein intake. Early introduction of human milk fortifier did not prevent cumulative protein and energy deficits, resulting in growth retardation at term age in both cohorts.

Postnatal growth is influenced by morbidity. The incidence of major morbidities in our cohorts was comparable to that of other epidemiological studies. Senterre and Rigo demonstrated improved postnatal growth with minimal loss of SDS by providing the recommended intake, especially amino acids, by continuation of long-term parenteral nutrition. In their study infants received parenteral nutrition for more than 21 days whereas our Cohort 2 achieved full enteral feeding at a mean of 11.6 days (Table 3). These differences in clinical practice reveal the essential scientific
question concerning optimal feeding for preterm infants. It is obvious that provision of sufficient nutrients is necessary to achieve optimal growth and development in preterm infants. It is not known what the optimal way of feeding is, either by parenteral or enteral route. Both ways have their (dis-)advantages. In contrast, we assumed that human milk is the optimal source for feeding preterm infants. Nearly all studies demonstrate that human milk stimulates gastrointestinal maturation, supports infant host defense, improves neurodevelopmental outcome and may be protective against the development of necrotizing enterocolitis. (27,28) We therefore provided an aggressive enteral feeding regimen with fast increment of enteral nutrition and early fortification of human milk. This was well-tolerated and did not lead to increased morbidity but unfortunately did not prevent growth retardation. The optimal intake for enteral nutrition as recently recommended cannot be achieved even with an aggressive enteral feeding regimen. (1)

For the use of early enteral feeding, especially with human milk, we suggest that the currently available HMFs do not contain enough protein. Assuming an average protein content of less than 2 g/dL in human milk over a longer period of lactation in combination with a fluid intake of 150 ml/kg/d, a minimum of at least 0.5 g/dL of protein is needed as an extra supplement for fortified human milk to achieve the currently recommended intake.

Our study has several limitations. Firstly, for this study, human milk was not analysed. Therefore, our calculations concerning human milk are mainly based on assumptions and therefore remain hypothetical. Different studies have reported varying milk compositions depending on infant prematurity and the time at which samples were collected. Milk composition differs between the mothers of infants born preterm or at term and the composition changes during the lactation period. (13,29-31) We decided to use assumptions that have been used by Senterre and Rigo for evaluation of comparable studies with different results. (6) Using different assumptions may influence the results. Bauer and Gerss found a higher content of macronutrients than usually found in most other studies. (31) To rule out an underestimation of nutritional intake we also calculated the intake using the data provided by Bauer et al. with a varying protein intake between 2.5 and 2.0 g/dL. (31) (data not shown) Although with this calculation the nutritional intake was higher and the optimal intake was achieved earlier, both cohorts still had cumulative deficits that were not resolved by the end of the observational period. Furthermore, since infants of both cohorts had similar GA, it is not plausible that differences in growth were caused by general differences in human milk content between cohorts.

A second limitation of our study is that patients were not randomly assigned to one treatment, but rather were recruited over two consecutive time periods. We reasoned that attempting two different feeding protocols in one department might pose an increased risk for patient safety. In both cohorts, all patients received nutrition according to a standardized protocol, data were recorded in the same standardized manner and patient characteristics were comparable. In accordance to our results, the randomized trial performed by Tan and Cooke correlated impaired head growth to persisting cumulative protein and energy deficits caused by insufficient supply with current available formulas and human milk fortifiers. (22)

In conclusion, we confirm that VLBW infants develop protein and energy deficits during the first two weeks of life. Aggressive enteral nutrition is well-tolerated but not sufficient for adequate postnatal growth. Practising early enteral feeding with higher supplementation of nutrients may be needed and requires further study.
References

10. Lavoie PM. Earlier initiation of enteral nutrition is associated with lower risk of late-onset bacteremia only in most mature very low birth weight infants. J Perinatol 2009; 29:448-54
Reply to commentary of Maas et al.:

Yes, we can – achieve adequate early postnatal growth in preterm infants

V Christmann
R Visser
M Engelkes
AM de Grauw
JB van Goudoever
AFJ van Heijst

Published in:
Acta Paediatrica 2013; 102 (12):e530
In reply:

Sir,

We appreciate the commentary made by C. Maas and coworkers and fully agree that a comparison of recent cohort studies demonstrates that the first week nutritional intake is a major determinant for further postnatal development. (1-3)

Nevertheless we remain at our conclusion that enteral formulas and supplements may not provide sufficient nutrients for preterm infants. We disagree that the 'near adequate' growth as described by Maas et al. was predominately achieved by accelerated enteral nutrition. (1)

Both our studies aimed for early full enteral feeding, provided predominantly by human milk, but resulted in different outcome. In contrast to our study, fortification of human milk in the Maas cohorts added more protein and energy (protein: 1.1 – 1.6 vs. 0.8 g/dL; energy: 15 – 20 vs. 15 kcal/dL). (1-3) This may have improved early growth. On the other hand, Maas et al started fortification only at 140/100 ml/kg/day, thus nearly full enteral feeding. This occurred around the end of the first week. Therefore parenteral amino acids were the major contributor to the total protein intake during the first week. In contrast to our study, they also provided by far earlier and higher amounts of parenteral amino acids. Theoretically, this may have led to less cumulative deficit with minimal loss of SDS in the early postnatal period.

We would like to point at the significant increased postnatal weight gain in our SGA infants compared to AGA infants. This seems to indicate different growth trajectories with different nutritional requirements for certain groups. With regard to long-term developmental outcome the optimal postnatal growth has still not been defined.

We agree that optimal growth and development is mainly dependent on sufficient supply of nutrients which can be provided as parenteral nutrition. While there are many advantages to provide feeding through the enteral route, no study has demonstrated that the recommended intake for preterm infants during the first week can be provided by enteral nutrition. Nevertheless, our studies show that rapid advancement of enteral nutrition is feasible, well tolerated and that small differences in nutritional protocols may have a remarkable effect on postnatal growth. Further improvement may be achieved by advanced or individualized fortification of human milk and optimization of the composition of fortifiers.

In conclusion, we agree that it is possible to improve postnatal growth in preterm infants with enteral nutrition, but further studies are required to improve nutritional protocols, preferably as randomized controlled trials.
References

Effect of early nutritional intake on long-term growth and bone mineralization of former very low birth weight infants

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Abstract

Background
Preterm infants are at risk for impaired bone mineralization and growth in length later in life due to inadequate nutritional intake in the early postnatal period.

Objective
To investigate whether increased nutritional supplementation of calcium, phosphate and protein in Very Low Birth Weight (VLBW) infants during the first 14 days after birth was associated with improvement in length and bone development until 9-10 years of age.

Design
Observational follow-up study of VLBW infants (birth weight < 1500 grams or gestational age < 32 weeks) born in two consecutive years (eligible infants: 2004 n: 63 and 2005: n: 66). Cohort 2005 received higher intake of calcium, phosphate and protein with parenteral nutrition compared to Cohort 2004. Anthropometric data were collected during standard follow-up visits until five years, and additionally at 9-10 years of age including measurements of bone mineral content, bone mineral density of the whole body and lumbar spine determined by dual-energy X-ray absorptiometry. Long-term growth trajectories of both cohorts were evaluated separately for participants born appropriate (AGA) and small for gestational age (SGA), stratified by gender. Multivariate linear regression was used to examine the effect of nutritional intake and clinical covariates on length and bone mineralization.

Results
Both cohorts achieved a catch-up in length to SDS within the normal range by 6 months (length SDS estimated mean (95% confidence interval (CI)): 6 months: Cohort 2004: -0.7 (-1.1, -0.3) Cohort 2005: -0.5 (-0.8, -0.2)). Bone mineral content and density were within the normal range and not different between the cohorts. SGA children achieved a catch-up in length at 5 years with bone mineralization comparable to AGA children. Only for girls birth weight was significantly associated with length SDS (per gram: β 0.001; 95% CI (0.000, 0.003); p = 0.03) There was no evidence of an association between early nutritional intake and bone mineralization.

Conclusion
Children born as appropriate or small for gestational age preterm infants are able to catch up in length after the postnatal period, and achieve a normal length and bone mineralization at age nine - ten years. An improvement of calcium and phosphate intake during the first 14 days after birth was not associated with improvement in length and bone development.

Introduction
Achieving growth and development comparable to healthy term born infants has been a challenge for the treatment of preterm born infants for many decades. (1) As the survival of Very Low Birth Weight (VLBW) infants has increased significantly during the last years, it is important to evaluate their long-term outcomes, especially since recommendations and policies with regard to nutritional intake have been changed to improve postnatal growth. (2-4) While early cohort studies demonstrated that VLBW infants experienced a significant growth retardation during the early postnatal period without catch-up to the initial birth percentile, more recent studies showed that improvement of early nutritional intake diminishes the cumulative nutritional deficit and thereby may prevent growth retardation. (5-7) Growth and skeletal development seem to be closely related. (8-10) Adequate bone mineralization is necessary for optimal development of the bones. (11-13) Given the difficulties associated with meeting the nutritional needs of VLBW infants and to provide sufficient nutritional supply of minerals, VLBW infants are especially at risk of impaired bone mineral content. (14-17) While early studies showed that exclusive feeding of human milk in preterm infants leads to deficiencies of calcium and phosphate, it is nowadays generally recommended to fortify human milk with additional minerals, protein and vitamins. (18-20) Furthermore, parenteral supplementation of calcium and phosphate has been improved through the inclusion of organic phosphate in parenteral nutrition (PN). (21)

Only a few studies have evaluated long-term bone development of VLBW children until childhood and adolescence. (22-30) Their findings were diverse but most of the studies showed that these former preterm infants remained smaller later in life, some with lower bone mineralization, others with low mineralization but normal in proportion to their small body size. Several studies found impaired bone mineral content in boys compared to girls. (22,31) Despite the low content of minerals, human milk seemed to have a positive effect on bone development. (32,33) As the survival of preterm infants has increased significantly, the population of preterm infants treated nowadays is more diverse than ever before. A more recent randomized trial in VLBW infants found a positive effect of post-discharge feeding on BMC in comparison to human milk and term formula at the corrected age of 6 months, irrespective of gain in weight and length. (34)

Previously we reported the short-term outcome results for two consecutive year-cohorts of VLBW infants that differed with regard to the nutritional intake during the first two weeks of life. (35) The second cohort received a higher intake of protein, energy as well as calcium and phosphate and this was associated with improved weight gain during the early postnatal period and at the corrected age of two years.
there was a tendency of improved growth in length. A secondary analysis revealed that this was mainly based on improvement in boys. Small for gestational age (SGA) infants had a higher postnatal weight gain than appropriate for gestational age (AGA) born infants. For the current study we describe the long-term growth in length for the surviving infants of the original cohorts and analyze the effect of the postnatal nutritional intake on length and bone mineralization at the age of 9 to 10 years. We hypothesized that increased nutritional intake would lead to improved length and bone mineralization.

Methods

Study population and design
This observational follow-up study evaluated the long-term outcomes of growth and bone mineralization of a previously described prospective cohort study that was conducted in 2004 and 2005, in order to evaluate changes in the composition of parenteral nutrition (PN). Surviving participants of both cohorts who were eligible for the standard follow-up schedule provided to VLBW infants born prior to 32 weeks of gestation, or those with a birth weight below 1500 grams were included in the study. (Eligible children: Cohort 2004: n = 63; Cohort 2005: n = 66). These children were invited for an additional outpatient clinic visit that included an evaluation of bone mineralization by dual energy X-ray absorptiometry (DEXA). The parents of all participants provided written informed consent for the additional investigation. The study was approved by the local ethics committee (2013/594) and registered within the Dutch Trial Registry (NTR=TC4842).

In accordance with the standard follow-up program, growth and general health status were recorded at the corrected ages of six months, and 1, 2 and 5 years. For the current study, we extended the follow-up by inviting children who were previously seen during the national follow-up program to return for further testing around the ages of nine and 10 years.

Nutritional protocol
The 2004 and 2005 cohorts included preterm infants admitted to the level III neonatal intensive care unit (Radboud university medical center, The Netherlands) on the first day of life after it was estimated that parenteral nutrition would be needed for at least 5 days. Infants with major congenital malformations or asphyxia were excluded. The nutritional protocols for the two cohorts primarily differed in terms of the parenteral nutritional intake with higher amounts of protein, calcium and phosphate provided to the 2005 Cohort. (Table 1) Following the recommendation of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition the 2005 PN provided 3 mmol per kg per day of calcium and 1.92 mmol per kg per day of glycerophosphate. According to the nutritional protocol of 2005, full PN was achieved four days following birth, while the maximum amount of PN in Cohort 2004: Aminovenos N paed 10% (Fresenius Kabi), Intralipid 20% (Fresenius Kabi), Ca gluconate 10% (Braun Melsungen), Sodium-glycerophosphate (Glycophos; Fresenius Kabi).
Anthropometric measurements and questionnaire
At the last visit length and weight were determined, and the health condition evaluated using a questionnaire. We specifically focused on morbidities and the use of medication that could have affected growth and bone mineralization, for example corticosteroids, asthma and fractures. For the preceding follow-up visits anthropometric measurements were collected either from the patient charts or requested from the local pediatric out-patient clinics specifically for measurements at term corrected age (TCA). Body weight was measured to the nearest 0.1 kg using an electronic digital scale (SECA MOD701) and body height to the nearest 0.1 cm by a wall-tapered height meter (SECA MOD240).

Dual energy X-ray absorptiometry
Bone mineralization of the whole body and lumbar spine (L1-L4) was evaluated using dual energy X-ray absorptiometry (QDR Discovery 85606, Hologic, Inc., USA) (DEXA). The measurements of this DEXA-scan were analyzed using the APEX system software version 3.3. The DEXA estimated the bone mass at the measurement site as Bone Mineral Content (BMC) in grams. Bone Mineral Density (BMD) is an aerial measurement and was recorded as grams per square centimeter. BMC and BMD were measured for the Whole Body (WB) and the Lumbar Spine (LS). For both sites, the BMD standard deviation scores (SDS) were calculated. The SDS of the WB were based on the reference data of the National Health and Nutrition Examination Survey (NHANES 2008)(38), while the SDS of the LS were based on reference data from the Bone Mineral Density in Childhood Study (BMDCS)(39).

Data handling
Patient characteristics including growth, clinical course and intake of all nutrients via both parenteral nutrition and enteral feeding were recorded daily for the first two years, and then weekly until week five, as described previously. The amounts of daily intake of all nutrients were calculated for each patient. From term age onwards the SDS were calculated for length and BMI using the Dutch reference of the nationwide growth study. A birth weight below the 10th percentile of the Dutch reference curves for birth weight by gestational age was defined as small for gestational age (SGA). A birth weight above the 10th percentile was defined as appropriate for gestational age (AGA). Differences in patient characteristics between responders and non-responders at corrected age of 5 years and at nine to 10 years are presented in the supplementary materials. (Table 5a, Table 5b, Table 6a, Table 6b).

Statistical analysis
The required sample size was not estimated for the current study as the study involved the follow-up of surviving children who were included in the original two cohorts and fulfilled the criteria for standard follow-up. The average length at each time point was estimated separately for the 2004 and 2005 cohorts, as well as for the subgroups of SGA/AGA at birth and gender within the cohorts. Age related differences in growth and bone mineralization were accounted for by using age and gender specific standard deviation scores. Associations between nutritional intake and the outcomes of length SDS, whole body and lumbar spine BMD and BMD SDS at 9-10 years of age were examined using multivariable linear regression for all eligible children and separately for boys and girls. As potential confounders cohort, sex, gestational age, birth weight, SGA/AGA status, and use of corticosteroids at 5 years of age were included as co-variables in the analyses.

Not all children who were eligible for follow-up had data collected at each time point (Figure 1). Missing data were handled using multiple imputation. We generated 100 imputed datasets using the method of chained equations. The imputation model included length at each time point, BMD and BMD SDS measurements for whole body and lumbar spine, and the full set of nutrition variables and potential confounders included in the regression models. All estimates were obtained by averaging results across the 100 imputed datasets with inferences under multiple imputation obtained using Rubin’s rules. Statistical analyses were conducted using Stata Corp (2015). Stata Statistical Software: Release 14. StataCorp LP: College Station, TX and IBM SPSS statistics, version 22 for Windows (IBM SPSS Inc., IL, USA).

Results
Cohort description
Recruitment
Figure 1 presents the number of children for whom length could be measured. Two infants in the 2004 cohort and four infants in the 2005 cohort had died before discharge, while three and nine infants of the respective cohorts did not fulfill the criteria for the follow-up program, thus 63 and 66 children of 2004 and 2005 respectively were eligible for the current analysis. Since the national follow up program aimed at the evaluation of health care and had a more scientific character, participation was voluntary, thereby not all parents followed the invitation and a varying number of children were seen over the time period. At term corrected age, measurements of length were available for about half of the participant sample. At the age of five years, 52 (83%) and 54 (82%) children were seen in the 2004 and 2005 cohorts respectively. Finally, at 9-10 years of age, growth was evaluated for 32 and 30
CHAPTER 4 FOLLOW UP GROWTH AND BONE MINERALIZATION

Figure 1: consort diagram

N: number of patients; TCA: Term corrected age; 6m: six months, 12 m: 12 months, 24 m; 24 months, incomplete data: missing data with regard to weight or length.

Cohort 2004

Admissions <34 weeks N = 226

Inclusion N = 115

Follow-up criteria yes N = 73

Follow-up at TCA N = 30

Follow-up at 6m N = 59

Follow-up at 12 m N = 55

Follow-up at 24 m N = 54

Follow-up at 5 years N = 52

Follow-up at 9-10 years N = 31

DEXA scan N = 29

Inclusion criteria yes N = 66

Transfer < d 10: N = 26

Death < d 8: N = 7

Incomplete data: N = 9

Follow-up before discharge N = 68

Follow-up at 6m N = 55

Follow-up at 12 m N = 54

Follow-up at 24 m N = 54

Follow-up at 5 years N = 52

Follow-up at 9-10 years N = 31

DEXA scan N = 29

Inclusion criteria yes N = 33

No show, incomplete data N = 11

No show, incomplete data N = 11

No show, incomplete data N = 3

No show, incomplete data N = 12

Follow-up before discharge N = 69

Follow-up at 6m N = 55

Follow-up at 12 m N = 55

Follow-up at 24 m N = 54

Follow-up at 5 years N = 52

Follow-up at 9-10 years N = 31

DEXA scan N = 29

Inclusion criteria yes N = 33

Death before discharge N = 1

Complete data: N = 114

Follow-up before discharge N = 68

Follow-up at 6m N = 55

Follow-up at 12 m N = 54

Follow-up at 24 m N = 54

Follow-up at 5 years N = 52

Follow-up at 9-10 years N = 31

DEXA scan N = 29

Inclusion criteria yes N = 63

Transfer < d 10: N = 20

Death < d 8: N = 9

Incomplete data: N = 9

Follow-up before discharge N = 72

Follow-up at 6m N = 59

Follow-up at 12 m N = 55

Follow-up at 24 m N = 54

Follow-up at 5 years N = 52

Follow-up at 9-10 years N = 31

DEXA scan N = 29

Inclusion criteria yes N = 66

Death before discharge N = 4

Complete data: N = 111

Follow-up before discharge N = 69
children, and bone mineralization in 31 and 29 children (49 and 43%) of the 2004 and 2005 cohorts respectively.

**Characteristics and morbidity**

Table 2 presents the characteristics of the children of both cohorts seen at 9-10 years of age. The postnatal characteristics of the children were similar between the cohorts. However, SGA born children of both cohorts had a higher gestational age at birth and a significant lower birth weight compared to their respective AGA group. Cohort 2005 also had a greater weight gain during the first five weeks of life compared to children of Cohort 2004, which was significant for the AGA boys, while all SGA infants demonstrated the highest weight gain. Since children of both cohorts differed by one year of age at the last visit, the mean length and BMI of cohort 2004 were higher. For both groups the respective SDS were within the normal range for the Dutch reference population. More than one third of the children in both cohorts reported bronchial hyper reactivity including the use of medication, while 22% in Cohort 2004 and 14% Cohort 2005 reported to have had at least one incident with bone fractures. These incidences and the use of other medication were similar between both cohorts. None of the children received growth hormone therapy.

**Length and bone mineralization**

**Longitudinal development of length and BMI**

Figure 2 presents the estimation of change in length SDS from TCA until 9-10 years after multiple imputation. Both cohorts were severely growth retarded by the time they had reached term corrected age (TCA) (SDS length: estimated mean (95% CI) TCA: Cohort 2004 versus Cohort 2005: -3.0 (-3.8, -2.2) vs. -3.0 (-3.7, -2.6)). Both cohorts showed a catch-up in length within the first 6 months with an improvement in mean SDS up to the normal range for the reference population (SDS length: estimated mean (95% CI) 6 months: Cohort 2004: -0.7 (-1.1, -0.3) Cohort 2005: -0.5 (-0.8, -0.2)). Both cohorts showed a catch-up in length within the first 6 months with an improvement in mean SDS up to the normal range for the reference population (SDS length: estimated mean (95% CI) 6 months: Cohort 2004: -0.7 (-1.1, -0.3) Cohort 2005: -0.5 (-0.8, -0.2)). Cohorts of Cohort 2005 seemed to have slightly improved length SDS compared to Cohort 2004 until the age of two years. This was more pronounced for the group of children born as AGA and specifically for the AGA girls at five years. The group of AGA boys of Cohort 2005 showed a decrease in length SDS from two years onwards and remained below the SDS of AGA boys of Cohort 2004. The SGA groups did not achieve a normal length at corrected age of six months but in contrast to AGA children continuously improved their SDS for length until the end of the observational period reaching the normal range for the reference population.

BMI developed in the same manner as the length with a major catch-up during the first six months. There were no differences seen between both cohorts or among subgroups of AGA and SGA children. For the group of SGA children SDS BMI developed within the same range as SDS length with mean lower values.

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**Table 2 Patient characteristics at 9 – 10 years**

<table>
<thead>
<tr>
<th></th>
<th>Cohort 2004</th>
<th>Cohort 2005</th>
</tr>
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<tbody>
<tr>
<td>Numbers</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>Sex (boy/girl), n</td>
<td>18/14</td>
<td>15/15</td>
</tr>
<tr>
<td>Exact age, years</td>
<td>10.3 (0.3)</td>
<td>9.5 (0.3)*</td>
</tr>
<tr>
<td>Postnatal characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age, weeks, n</td>
<td>29.4 (1.9)</td>
<td>29.4 (1.4)</td>
</tr>
<tr>
<td>Appropriate / small for gestational age, n</td>
<td>29/3</td>
<td>27/3</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>1182 (254)</td>
<td>1160 (328)</td>
</tr>
<tr>
<td>Birth weight SDS</td>
<td>0.2 (1.0)</td>
<td>0.04 (1.1)</td>
</tr>
<tr>
<td>GV week 1-S, gram/kg/day</td>
<td>8.6 (2.6)</td>
<td>11.1 (2.8)*</td>
</tr>
<tr>
<td>Nutrition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN days, median (IQR)</td>
<td>26.0 (14.0 – 38.8)</td>
<td>10 (8.0 – 16.0)*</td>
</tr>
<tr>
<td>Phosphate, mmol/kg/14 days</td>
<td>16.0 (8.8)</td>
<td>35.7 (9.3)*</td>
</tr>
<tr>
<td>Calcium, mmol/kg/14 days</td>
<td>22.6 (10.1)</td>
<td>40.9 (7.4)*</td>
</tr>
<tr>
<td>Protein, grams/kg/14 days</td>
<td>35.3 (7.7)</td>
<td>41.6 (6.2)*</td>
</tr>
<tr>
<td>Energy, kcal/kg/14 days</td>
<td>1185 (238)</td>
<td>1403 (205)*</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>32.2 (5.6)</td>
<td>30.2 (5.5)</td>
</tr>
<tr>
<td>Length, cm</td>
<td>141 (6)</td>
<td>137 (5)*</td>
</tr>
<tr>
<td>BMI</td>
<td>16.1 (2.2)</td>
<td>16.0 (2.5)</td>
</tr>
<tr>
<td>SDS length</td>
<td>-0.6 (0.9)</td>
<td>-0.5 (0.7)</td>
</tr>
<tr>
<td>SDS BMI</td>
<td>-0.4 (1.2)</td>
<td>-0.2 (1.2)</td>
</tr>
</tbody>
</table>

Data presented as mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1 until week 5 according to Patel’s formula [58]; PN days: number of days of parenteral nutrition; BMI: body mass index; *: p-value < 0.01

for further follow-up at 9-10 years were similar to those for the full cohort. Comparisons of the characteristics of responders and non-responders at five years of age, and at 9-10 years are provided in tables 5a and 5b and tables 6a and 6b in the supplementary materials.

In agreement with the original full sample postnatal cohorts, children of Cohort 2005 seen at the follow-up, had received a significant higher amount of calcium, phosphate, protein and energy during the first 14 days of life, with a shorter duration of parenteral nutrition, compared to Cohort 2004. (Table 2) AGA born children of Cohort 2005 also had a greater weight gain during the first five weeks of life compared to children of Cohort 2004, which was significant for the AGA boys, while all SGA infants demonstrated the highest weight gain. Since children of both cohorts differed by one year of age at the last visit, the mean length and BMI of cohort 2004 were higher. For both groups the respective SDS were within the normal range for the Dutch reference population. More than one third of the children in both cohorts reported bronchial hyper reactivity including the use of medication, while 22% in Cohort 2004 and 14% Cohort 2005 reported to have had at least one incident with bone fractures. These incidences and the use of other medication were similar between both cohorts. None of the children received growth hormone therapy.
Figure 2 Follow up in length SDS using imputed data
AGA: appropriate for gestational age at birth; SGA: small for gestational age at birth; SDS: standard deviation scores; bars represent estimates of the mean and 95% confidence intervals; The analysis represents the full sample of children who were eligible for standard follow up.
CHAPTER 4 FOLLOW UP GROWTH AND BONE MINERALIZATION

Bone development

The results of the Whole Body (WB) and Lumbar spine (LS) scan for the sub-groups of both cohorts are presented in Table 3. Comparing the total cohorts, the mean bone mineral content (BMC) of both, WB and LS, tended to be higher for Cohort 2004 (n = 31), which is likely to be related to the average younger age and lower weight of Cohort 2005 (n = 29). The mean whole body BMC of Cohort 2004 was on average 90.0 gram higher compared to Cohort 2005 (95% confidence interval (CI): (8.1, 161.8); p-value = 0.015). The mean whole body BMD only differed by 0.032 g/cm² (95% CI: (0.00, 0.06); p-value = 0.049). The mean lumbar spine BMC and BMD were only slightly higher for Cohort 2004 compared to 2005 respectively, with 2.1 gram (95% CI: (0.05, 4.3); p-value = 0.045) and 0.026 g/cm² (95% CI: (-0.02, 0.07); p-value = 0.230). While the differences for the lumbar spine were relatively small, the more relevant age dependent mean BMD SDS for both measurement sites were similar between both cohorts and within the normal range for the reference population (2004 versus 2005: mean WB BMD SDS 0.43 vs. -0.35 (mean diff: 0.08; 95% CI: (-0.43, 0.58); p-value = 0.75); mean LS BMD SDS -0.036 vs. -0.15 (mean diff: 0.11; 95% CI: (-0.48, 0.71); p-value = 0.70).

The evaluation of subgroups of children (Table 3) showed that the lower bone mineral content (WB and LS) of Cohort 2005 was specifically associated with a lower mean BMC of AGA boys of 2005, while in contrast the mean BMC of AGA girls of 2005 nearly attained the same amount as the respective subgroup of 2004. Thus, of Cohort 2005, girls had a non-significant higher BMC than boys despite a comparable body weight. This was also seen for the BMD. The subgroup of SGA girls was found to have the highest BMC and BMD compared to all other subgroups.

The effect of early nutrition on length and bone mineralization

Table 4 presents the results of the adjusted associations between nutritional intake during the first 14 days of life, clinical characteristics and length SDS and BMD and BMD SDS for whole body and lumbar spine, separately for boys and girls. For the total eligible group, there was some evidence that birth weight was associated with length, with a 1 gram increase associated with 0.001 SDS increase in length (95% CI: 0.0002, 0.002; p-value 0.046), (table 4a, supplementary material). This was mainly based on the association between birth weight and length for girls with 0.001 SDS increase in length (95% CI: 0.000, 0.003; p-value 0.03). For boys as well as for girls, there was no evidence of an association between length at nine to 10 years of age and nutritional intake of the first 14 days or any of the potential confounders.

For the total group as well as both sexes, there was no evidence that mean BMD of the whole body and lumbar spine and BMD SDS was associated with nutritional intake or any of the other clinical characteristics that were measured.

**Table 3 Bone mineralization at 9 -10 years**

<table>
<thead>
<tr>
<th></th>
<th>Boys</th>
<th>Girls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exact age, years</td>
<td>10.3 (0.3)</td>
<td>9.5 (0.26)</td>
</tr>
<tr>
<td>Whole Body: BMC g</td>
<td>1208.8 (156.1)</td>
<td>1042.7 (117.6)</td>
</tr>
<tr>
<td>BMD g/cm²</td>
<td>0.882 (0.06)</td>
<td>0.831 (0.05)</td>
</tr>
<tr>
<td>Lumbar spine: BMC g</td>
<td>25.6 (4.9)</td>
<td>22.3 (3.5)</td>
</tr>
<tr>
<td>BMD g/cm²</td>
<td>0.606 (0.08)</td>
<td>0.548 (0.09)</td>
</tr>
<tr>
<td>BMD SDS</td>
<td>0.73 (1.09)</td>
<td>0.19 (0.84)</td>
</tr>
<tr>
<td></td>
<td>-0.64 (1.31)</td>
<td>-0.37 (0.74)</td>
</tr>
</tbody>
</table>

Data presented as mean (SD); SDS: standard deviation score; BMC: bone mineral content; BMD: bone mineral density

4
Table 4: Effect of nutritional intake and clinical characteristics on length and bone mineralization

<table>
<thead>
<tr>
<th></th>
<th>Boys (n = 63)</th>
<th>Girls (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SDS Length at 9-10 years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2005 (vs. 2004)</td>
<td>-0.195 (-1.091, 0.701) 0.662</td>
<td>-0.421 (-1.310, 0.468) 0.341</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.007 (-0.040, 0.054) 0.768</td>
<td>0.029 (-0.016, 0.075) 0.201</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.015 (-0.071, 0.041) 0.597</td>
<td>-0.003 (-0.060, 0.053) 0.900</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>0.007 (-0.056, 0.070) 0.832</td>
<td>-0.008 (-0.066, 0.051) 0.796</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>0.089 (-0.137, 0.316) 0.430</td>
<td>-0.009 (-0.217, 0.199) 0.932</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.001 (-0.001, 0.002) 0.378</td>
<td>0.001 (0.000, 0.003) 0.030</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-0.053 (-1.237, 1.130) 0.928</td>
<td>0.490 (-0.626, 1.606) 0.379</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.387 (-1.133, 0.359) 0.297</td>
<td>0.020 (-1.156, 1.196) 0.972</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Whole Body BMD</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 2005 (vs. 2004)</td>
<td>-0.028 (-0.107, 0.050) 0.459</td>
<td>-0.059 (-0.146, 0.027) 0.169</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.001 (-0.004, 0.003) 0.732</td>
<td>0.003 (-0.002, 0.008) 0.256</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.002 (-0.007, 0.003) 0.438</td>
<td>-0.002 (-0.008, 0.005) 0.558</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>0.001 (-0.005, 0.008) 0.611</td>
<td>0.000 (-0.006, 0.006) 0.953</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>0.008 (-0.012, 0.028) 0.399</td>
<td>0.008 (-0.012, 0.029) 0.402</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.000 (0.000, 0.000) 0.352</td>
<td>0.000 (0.000, 0.000) 0.208</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-0.012 (-0.115, 0.090) 0.808</td>
<td>0.006 (-0.099, 0.110) 0.911</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.007 (-0.070, 0.056) 0.808</td>
<td>0.025 (-0.055, 0.106) 0.529</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Whole Body SDS</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 2005 (vs. 2004)</td>
<td>-0.253 (-1.474, 0.969) 0.672</td>
<td>-0.444 (-1.767, 0.878) 0.493</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.007 (-0.056, 0.070) 0.823</td>
<td>0.033 (-0.009, 0.105) 0.350</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.019 (-0.100, 0.062) 0.627</td>
<td>-0.015 (-0.110, 0.079) 0.734</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>0.016 (-0.076, 0.108) 0.717</td>
<td>-0.004 (-0.094, 0.087) 0.935</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>0.163 (-0.147, 0.472) 0.291</td>
<td>0.123 (-0.182, 0.427) 0.414</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.001 (-0.001, 0.003) 0.373</td>
<td>0.001 (-0.001, 0.003) 0.156</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-0.272 (-1.840, 1.296) 0.723</td>
<td>0.177 (-1.385, 1.740) 0.817</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.139 (-1.119, 0.842) 0.722</td>
<td>0.412 (-0.873, 1.697) 0.520</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Lumbar Spine BMD</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 2005 (vs. 2004)</td>
<td>-0.044 (-0.156, 0.067) 0.418</td>
<td>-0.078 (-0.187, 0.032) 0.155</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.000 (-0.006, 0.006) 0.992</td>
<td>0.004 (-0.003, 0.001) 0.236</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.002 (-0.010, 0.007) 0.690</td>
<td>-0.001 (-0.011, 0.008) 0.763</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>0.003 (-0.006, 0.012) 0.472</td>
<td>-0.002 (-0.010, 0.007) 0.669</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>0.009 (-0.019, 0.036) 0.522</td>
<td>0.015 (-0.013, 0.043) 0.276</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.000 (0.000, 0.000) 0.896</td>
<td>0.000 (0.000, 0.000) 0.948</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>-0.021 (-0.115, 0.157) 0.751</td>
<td>0.019 (-0.121, 0.160) 0.777</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.029 (-0.115, 0.157) 0.751</td>
<td>0.022 (-0.106, 0.151) 0.728</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Lumbar Spine SDS</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohort 2005 (vs. 2004)</td>
<td>-0.451 (-2.067, 1.164) 0.567</td>
<td>-0.624 (-2.195, 0.946) 0.419</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>-0.003 (-0.089, 0.083) 0.941</td>
<td>0.045 (-0.050, 0.140) 0.336</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.016 (-0.136, 0.013) 0.772</td>
<td>-0.014 (-0.149, 0.121) 0.818</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>0.042 (-0.088, 0.172) 0.499</td>
<td>-0.021 (-0.146, 0.103) 0.718</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>0.139 (-0.263, 0.541) 0.487</td>
<td>0.179 (-0.208, 0.565) 0.347</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.000 (-0.002, 0.002) 0.820</td>
<td>0.000 (-0.002, 0.002) 0.923</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>0.267 (-1.690, 2.224) 0.782</td>
<td>0.333 (-1.634, 2.300) 0.728</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.485 (-1.717, 0.746) 0.424</td>
<td>0.239 (-1.456, 1.935) 0.776</td>
</tr>
</tbody>
</table>

Multivariable linear regression with adjustment for the potential confounders: cohort, gestational age, birth weight, AGA/SGA status, and use of corticosteroids at 5 years of age; SDS: standard deviation score; BMD: bone mineral density (g/cm²)
Discussion

Our evaluation of two cohorts of VLBW infants suggested that growth retardation achieved in the postnatal period returned to a normal range length within the first 6 months. At nine to 10 years the mean BMD SDS measured for Whole Body and Lumbar Spine was within the normal range and seemed not related to nutritional intake. Only birth weight appeared to be associated with length at 9-10 years of age for girls. While a number of previous studies found a decreased length and bone mineralization in former preterm infants, both cohorts in our study had SDS for length, and BMD SDS for whole body and lumbar spine within the normal range for age. (47,48) Several studies pointed at the positive effect of human milk on bone mineralization despite the low mineral content. (27,28) More than 90% of all infants in both cohorts received own mother’s milk. For Cohort 2004 the use of human milk may have compensated for the low parenteral mineral intake. (35)

Although both cohorts had achieved mean SDS for length and BMI within the normal range for the reference population, this was still below the initial SDS for weight at birth. Any long-term effect of early nutrition in our study may be questioned, as in both cohorts a substantial growth retardation was observed by the time infants were discharged. Besides postnatal illness, this phenomenon can be explained by the fact that fortification of human milk at levels that were used more than 10 years ago did not prevent nutritional deficits. A considerable number of infants of both cohorts never achieved the recommended enteral intake. (4,35) It is possible that further improvement of early postnatal nutritional intake may have prevented postnatal growth retardation and consequently may have led to childhood growth SDS equivalent to growth at birth.

In contrast to our hypothesis, the significant different nutritional intake did not lead to a higher bone mass. The results from the linear regression showed no effect of mineral intake on bone mineralization, neither for boys nor for girls. The nutritional increment of calcium, phosphate in combination with more protein may not have been adequate for bone development. Recently, studies demonstrated that high protein intake may be related to hypophosphatemia indicating an increased need for phosphate supplementation. (47,48) Despite the significant increased amount of phosphate supplementation, Cohort 2005 had hypophosphatemia during the first week of life. (36) This may have been the result of an imbalanced nutritional intake of amino acids and phosphate. (49) On the other hand the mean BMD SDS was within the normal range of the reference population for both cohorts. As mentioned above this may have been the positive effect of human milk, but it may also indicate that preterm infants have the capability for catch-up for bone mineralization. Follow up studies of a randomized trial in preterm infants evaluating post-discharge feeding showed that higher nutritional intake positively affected BMC at six months corrected age, while this was not maintained until the age of eight years suggesting a catch up in children who received a lower mineral intake in the post-discharge period. (34,50)

A varying number of children could not be evaluated at the standard follow up visits, leading to lower numbers of patients than originally eligible and thereby questioning the representativeness of the current data. Patient characteristics of the responders to follow up at five and nine to 10 years were comparable to the respective original cohorts and characteristics of non-responders differed only slightly compared to responders. We used multiple imputation to reduce the potential for bias from due to missing data and thus calculated estimates for the total eligible cohorts (2004: n = 63; 2005: n = 66). This narrowed the differences between the groups but the results based on analyses using observed data and those from multiple imputation were generally consistent. The original postnatal evaluation of growth revealed a significant higher weight gain until week 5 for AGA infants of Cohort 2005, mainly based on improved growth in boys. (35,45) The same group of boys of 2005 seemed to deteriorate with regard to length from two years of age onwards in contrast to the respective AGA girls and AGA boys of 2004. This was in contrast to the early reported Dutch follow up study of VLBW infants where boys showed a significant improved catch-up between 5 to 10 years compared to girls. (51) The repeated use of inhaled corticosteroids has been shown to negatively affect linear growth and bone mineralization. (32-54) Further, an increasing obstructive airway disease from childhood into adolescence has been demonstrated in follow up studies of VLBW infants. (55) While a considerable number of children of both cohorts reported bronchial hyper-reactivity, for the current study corticosteroid use seemed not to affect length and bone mineralization. Nevertheless, we cannot exclude effects of childhood morbidities that were not accounted for in the current analysis.

The Dutch nationwide prospective study found stunning of SGA children at 10 years of age, while a 20 year follow up of SGA preterm born young adults found lower SDS for height and lumbar spine bone mineralization. (51,56) In contrast, our SGA children of both cohorts demonstrated the highest growth velocity during the first 5 postnatal weeks, had a lower decline in SDS for weight compared to AGA infants at TCA, and continuously improved growth with a full catch-up in height at the last visit at age nine to 10 years. (35) Bone mineral density of the whole body and lumbar spine were higher than the respective AGA groups. According to the multivariate regression analysis being SGA did not significantly affect SDS for length and BMD. It has been suggested that SGA infants are not able to compensate the intra-uterine incomplete bone mineral accretion in the postnatal period, we speculate that the first postnatal weeks may represent the critical window for SGA infants to start the catch-up growth and thereby indicating the need for sufficiency of nutritional intake directly after birth. (57)
This study has several limitations. First, the patients were not randomly assigned to a treatment, but recruited over two consecutive time periods. However, nutritional protocols and data collection were standardized, patient characteristics were comparable even for the follow-up. For the last visit we were unable to invite all children at the same age which led to age dependent differences in growth and bone mineralization. We accounted for these differences by using age dependent standard deviation scores for comparisons. The major limitation of this study was the fact that the power calculation was not performed for differences in bone development or morbidities in childhood. We tried to overcome a considerable loss of follow up by evaluating characteristics of responders and non-responders to evaluate the representativeness of the children seen at follow up and used the method of multiple imputation. The latter may even be seen as a strength of this study since the few recent studies that evaluated long-term bone mineralization in former preterm infants mostly evaluated lower numbers of patients. However, unknown morbidities during the childhood period and individual lifestyles that were not accounted for may have biased the results.

Conclusions

Children born preterm as appropriate or small for gestational age are able to catch up in length after the postnatal period, and achieve a normal length and bone mineralization. An improvement of calcium and phosphate intake during the first 14 days of life was not associated with improvement in length and bone development.

Acknowledgments

All authors gratefully thank I. Vermeer and C. Lageweg, medical students at the Radboud university medical center, for their dedicated support in the data acquisition.

**Supplementary material**

**Table 4a** Effect of clinical and nutritional characteristics on length and bone mineralization

<table>
<thead>
<tr>
<th>Measure</th>
<th>β</th>
<th>95% Confidence Interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SDS Length at 9 - 10 years</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2005 (versus 2004)</td>
<td>-0.331</td>
<td>(-0.974, 0.312)</td>
<td>0.307</td>
</tr>
<tr>
<td>Sex (being girl)</td>
<td>-0.070</td>
<td>(-0.510, 0.369)</td>
<td>0.751</td>
</tr>
<tr>
<td>Gestational age at birth, weeks</td>
<td>0.034</td>
<td>(-0.119, 0.187)</td>
<td>0.658</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.001</td>
<td>(0.0002, 0.002)</td>
<td>0.046</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>0.211</td>
<td>(-0.607, 1.030)</td>
<td>0.607</td>
</tr>
<tr>
<td>Any corticosteroids (5 years)</td>
<td>-0.229</td>
<td>(-0.873, 0.415)</td>
<td>0.479</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.019</td>
<td>(-0.015, 0.055)</td>
<td>0.274</td>
</tr>
<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.006</td>
<td>(-0.050, 0.038)</td>
<td>0.783</td>
</tr>
<tr>
<td>Protein grams/kg/14 days</td>
<td>-0.001</td>
<td>(-0.047, 0.046)</td>
<td>0.974</td>
</tr>
<tr>
<td><strong>Whole Body BMD SDS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2005 (versus 2004)</td>
<td>-0.400</td>
<td>(-1.331, 0.532)</td>
<td>0.391</td>
</tr>
<tr>
<td>Sex (being girl)</td>
<td>-0.052</td>
<td>(-0.702, 0.599)</td>
<td>0.873</td>
</tr>
<tr>
<td>Gestational age at birth, weeks</td>
<td>0.147</td>
<td>(-0.086, 0.380)</td>
<td>0.210</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>0.001</td>
<td>(-0.0004, 0.002)</td>
<td>0.147</td>
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<tr>
<td>Small for gestational age</td>
<td>-0.068</td>
<td>(-1.28, 1.148)</td>
<td>0.910</td>
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<tr>
<td>Any corticosteroids (5 years)</td>
<td>0.038</td>
<td>(-0.759, 0.836)</td>
<td>0.923</td>
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<tr>
<td>Phosphate mmol/kg/14 days</td>
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<td>(-0.032, 0.070)</td>
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<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.011</td>
<td>(-0.085, 0.064)</td>
<td>0.766</td>
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<tr>
<td>Protein grams/kg/14 days</td>
<td>0.005</td>
<td>(-0.070, 0.080)</td>
<td>0.900</td>
</tr>
<tr>
<td><strong>Lumbar Spine BMD SDS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2005 (versus 2004)</td>
<td>-0.627</td>
<td>(-1.800, 0.544)</td>
<td>0.285</td>
</tr>
<tr>
<td>Sex (being girl)</td>
<td>0.140</td>
<td>(-0.621, 0.901)</td>
<td>0.714</td>
</tr>
<tr>
<td>Gestational age at birth, weeks</td>
<td>0.172</td>
<td>(-0.134, 0.477)</td>
<td>0.263</td>
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<tr>
<td>Birth weight, grams</td>
<td>0.0002</td>
<td>(-0.002, 0.001)</td>
<td>0.786</td>
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<tr>
<td>Small for gestational age</td>
<td>0.179</td>
<td>(-1.289, 1.648)</td>
<td>0.806</td>
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<td>Any corticosteroids (5 years)</td>
<td>-0.253</td>
<td>(-1.254, 0.747)</td>
<td>0.613</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.018</td>
<td>(-0.055, 0.092)</td>
<td>0.612</td>
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<tr>
<td>Calcium mmol/kg/14 days</td>
<td>-0.004</td>
<td>(-0.114, 0.107)</td>
<td>0.946</td>
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<tr>
<td>Protein grams/kg/14 days</td>
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<td>(-0.100, 0.112)</td>
<td>0.908</td>
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CHAPTER 4  FOLLOW UP GROWTH AND BONE MINERALIZATION

Table 4a Continued

<table>
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<th>β</th>
<th>95% Confidence Interval</th>
<th>p-value</th>
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<tr>
<td>Whole Body BMD</td>
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<td></td>
<td></td>
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<tr>
<td>Cohort 2005 (versus 2004)</td>
<td>-0.046</td>
<td>(-0.106, 0.014)</td>
<td>0.128</td>
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<td>Sex (being girl)</td>
<td>-0.009</td>
<td>(-0.052, 0.033)</td>
<td>0.661</td>
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<tr>
<td>Gestational age at birth, weeks</td>
<td>0.009</td>
<td>(-0.006, 0.024)</td>
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<td>Birth weight, grams</td>
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<td>(-0.00003, 0.0001)</td>
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<td>Small for gestational age</td>
<td>-0.004</td>
<td>(-0.084, 0.075)</td>
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<td>0.004</td>
<td>(-0.046, 0.055)</td>
<td>0.865</td>
</tr>
<tr>
<td>Phosphate mmol/kg/14 days</td>
<td>0.002</td>
<td>(-0.002, 0.005)</td>
<td>0.361</td>
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<td>Calcium mmol/kg/14 days</td>
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<td>(-0.006, 0.004)</td>
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<tr>
<td>Protein grams/kg/14 days</td>
<td>0.001</td>
<td>(-0.004, 0.006)</td>
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<td>Lumbar Spine BMD</td>
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<tr>
<td>Cohort 2005 (versus 2004)</td>
<td>-0.067</td>
<td>(0.148, 0.014)</td>
<td>0.103</td>
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<td>Sex (being girl)</td>
<td>0.033</td>
<td>(-0.021, 0.088)</td>
<td>0.228</td>
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<tr>
<td>Gestational age at birth, weeks</td>
<td>0.013</td>
<td>(-0.008, 0.035)</td>
<td>0.220</td>
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<tr>
<td>Birth weight, grams</td>
<td>-0.00002</td>
<td>(-0.0001, 0.00001)</td>
<td>0.791</td>
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<td>Small for gestational age</td>
<td>0.012</td>
<td>(-0.091, 0.116)</td>
<td>0.811</td>
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<td>Any corticosteroids (5 years)</td>
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<td>0.740</td>
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<td>Phosphate mmol/kg/14 days</td>
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<td>(-0.004, 0.007)</td>
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<tr>
<td>Calcium mmol/kg/14 days</td>
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<td>(-0.008, 0.007)</td>
<td>0.898</td>
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<tr>
<td>Protein grams/kg/14 days</td>
<td>0.0003</td>
<td>(-0.007, 0.008)</td>
<td>0.930</td>
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</table>

SDS: standard deviation score; BMD: bone mineral density

Table 5a Follow up at 5 years; Responders and Non-responders to Follow – up

<table>
<thead>
<tr>
<th>Appropriate for gestational age</th>
<th>2004</th>
<th>2005</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>44</td>
<td>8</td>
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<tr>
<td>Gender (F/M)</td>
<td>22/22</td>
<td>4/4</td>
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<tr>
<td>Gestational age; weeks, mean (SD)</td>
<td>28.6 (1.7)</td>
<td>30.0 (1.7)</td>
</tr>
<tr>
<td>Birth weight; grams, mean (SD)</td>
<td>1115 (251)</td>
<td>1352 (371)</td>
</tr>
<tr>
<td>Birth weight SDS</td>
<td>0.3 (0.7)</td>
<td>0.4 (0.7)</td>
</tr>
<tr>
<td>Growth until week 5; g/kg/day, mean (SD)</td>
<td>8.9 (2.7)</td>
<td>9.4 (2.2)</td>
</tr>
<tr>
<td>PN; days, mean (SD)</td>
<td>28.4 (13.4)</td>
<td>18.7 (9.2)</td>
</tr>
<tr>
<td>P 14 days; mmol/kg/day, mean (SD)</td>
<td>15.4 (8.1)</td>
<td>23.2 (11.6)</td>
</tr>
<tr>
<td>Ca, 14 days; mmol/kg/day, mean (SD)</td>
<td>22.8 (9.3)</td>
<td>32.1 (14.1)</td>
</tr>
<tr>
<td>Protein, 14 days; grams/kg/day, mean (SD)</td>
<td>346 (6.8)</td>
<td>405 (6.5)</td>
</tr>
<tr>
<td>Energy, 14 days; kcal/kg/day, mean (SD)</td>
<td>1202 (218)</td>
<td>1333 (248)</td>
</tr>
<tr>
<td>IRDS; n (%)</td>
<td>36 (82)</td>
<td>5 (65)</td>
</tr>
<tr>
<td>CLD; n (%)</td>
<td>8 (18)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>PDA treatment; n (%)</td>
<td>23 (52)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>Late onset sepsis; n (%)</td>
<td>25 (57)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Cortico_5y; n (%)</td>
<td>6 (13)</td>
<td>-</td>
</tr>
<tr>
<td>Bronchial hyper reactivity at 5y; n (%)</td>
<td>11 (25)</td>
<td>-</td>
</tr>
<tr>
<td>Data presented as; mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1 until week 5 according to Patel’s formula [58]; PN: parenteral nutrition; P_14 days: total phosphate intake during the first 14 days; Ca_14 days: total calcium intake during the first 14 days; IRDS: infant respiratory distress syndrome; CLD: chronic lung disease defined as oxygen dependency at 28 days; PDA treatment: medical and or surgical treatment for patent ductus arteriosus; Cortico_5y: any treatment with steroids post discharge until 5 years of age.</td>
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### Table 5b Follow up at 5 years; Responders and Non-responders to Follow – up

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<tr>
<th>Small for gestational age</th>
<th>2004</th>
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<td>Responders</td>
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<td>No</td>
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<tr>
<td>Number</td>
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<td>3</td>
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<tr>
<td>Gender (F/M)</td>
<td>7/1</td>
<td>2/1</td>
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<td>Gestational age; weeks, mean (SD)</td>
<td>31.9 (1.9)</td>
<td>30.0 (3.6)</td>
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<tr>
<td>Birth weight; grams, mean (SD)</td>
<td>915 (162)</td>
<td>766 (80)</td>
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<td>Birth weight SDS</td>
<td>-1.7 (0.6)</td>
<td>-1.7 (1.1)</td>
</tr>
<tr>
<td>Growth until week 5; g/kg/day, mean (SD)</td>
<td>12.8 (3.4)</td>
<td>12.2 (1.5)</td>
</tr>
<tr>
<td>PN; days, mean (SD)</td>
<td>15.9 (7.8)</td>
<td>18.6 (14.5)</td>
</tr>
<tr>
<td>P_14 days; mmol/kg/day, mean (SD)</td>
<td>18.4 (11.6)</td>
<td>9.9 (9.3)</td>
</tr>
<tr>
<td>Ca_14 days; mmol/kg/day; mean (SD)</td>
<td>26.2 (11.4)</td>
<td>13.3 (2.1)</td>
</tr>
<tr>
<td>Protein_14 days; grams/kg/day; mean (SD)</td>
<td>37.6 (7.6)</td>
<td>27.3 (1.0)</td>
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<td>Energy_14 days; kcal/kg/day; mean (SD)</td>
<td>1227 (286)</td>
<td>1099 (75)</td>
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<td>IRDS; n (%)</td>
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<td>2</td>
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<tr>
<td>CLD; n (%)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PDA treatment; n (%)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Late onset sepsis; n (%)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cortico_5y; n (%)</td>
<td>0 - 3</td>
<td>- 3</td>
</tr>
<tr>
<td>Bronchial hyper reactivity at 5y; n (%)</td>
<td>0 - 3</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1 until week 5 according to Patel’s formula [58]; PN: parenteral nutrition; P_14 days: total phosphate intake during the first 14 days; Ca_14 days: total calcium intake during the first 14 days; IRDS: infant respiratory distress syndrome; CLD: chronic lung disease defined as oxygen dependency at 28 days; PDA treatment: medical and or surgical treatment for patent ductus arteriosus; Cortico_5y: any treatment with steroids post discharge until 5 years of age.

### Table 6a Follow up at 9 - 10 years; Responders and Non-responders to Follow – up

<table>
<thead>
<tr>
<th>Appropriate for gestational age</th>
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<th>2005</th>
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</thead>
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<td>Responders</td>
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<td>No</td>
</tr>
<tr>
<td>Number</td>
<td>29</td>
<td>23</td>
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<td>Gender (F/M)</td>
<td>11/18</td>
<td>15/8</td>
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<td>Gestational age; weeks, mean (SD)</td>
<td>29.0 (1.6)</td>
<td>28.5 (1.9)</td>
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<td>Birth weight; grams, mean (SD)</td>
<td>1199 (258)</td>
<td>1092 (305)</td>
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<tr>
<td>Birth weight SDS</td>
<td>0.4 (0.8)</td>
<td>0.3 (0.6)</td>
</tr>
<tr>
<td>Growth until week 5; g/kg/day, mean (SD)</td>
<td>8.5 (2.7)</td>
<td>9.6 (2.6)</td>
</tr>
<tr>
<td>PN; days, mean (SD)</td>
<td>29.4 (15.5)</td>
<td>23.8 (8.8)</td>
</tr>
<tr>
<td>P_14 days; mmol/kg/day, mean (SD)</td>
<td>15.9 (8.8)</td>
<td>17.5 (9.4)</td>
</tr>
<tr>
<td>Ca_14 days; mmol/kg/day; mean (SD)</td>
<td>22.2 (10.3)</td>
<td>26.7 (10.7)</td>
</tr>
<tr>
<td>Protein_14 days; grams/kg/day; mean (SD)</td>
<td>34.5 (7.7)</td>
<td>36.8 (6.1)</td>
</tr>
<tr>
<td>Energy_14 days; kcal/kg/day; mean (SD)</td>
<td>1174 (240)</td>
<td>1283 (191)</td>
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<td>IRDS; n (%)</td>
<td>24 (83)</td>
<td>17 (74)</td>
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<tr>
<td>CLD; n (%)</td>
<td>6 (21)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>PDA treatment; n (%)</td>
<td>16 (55)</td>
<td>8 (34)</td>
</tr>
<tr>
<td>Late onset sepsis; n (%)</td>
<td>12 (42)</td>
<td>15 (65)</td>
</tr>
<tr>
<td>Cortico_5y; n (%)</td>
<td>6 (21)</td>
<td>- 5 (19)</td>
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<tr>
<td>Bronchial hyper reactivity at 5y; n (%)</td>
<td>10 (35)</td>
<td>- 6 (22)</td>
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<tr>
<td>Cortico_10y; n (%)</td>
<td>12 (42)</td>
<td>- 8 (31)</td>
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<tr>
<td>Bronchial hyper reactivity at 10y; n (%)</td>
<td>18 (64)</td>
<td>- 17 (65)</td>
</tr>
<tr>
<td>Fractures at 10y; n (%)</td>
<td>22 (79)</td>
<td>- 23 (89)</td>
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</table>

Data presented as mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1 until week 5 according to Patel’s formula [58]; PN: parenteral nutrition; P_14 days: total phosphate intake during the first 14 days; Ca_14 days: total calcium intake during the first 14 days; IRDS: infant respiratory distress syndrome; CLD: chronic lung disease defined as oxygen dependency at 28 days; PDA treatment: medical and or surgical treatment for patent ductus arteriosus; Cortico_5y: any treatment with steroids post discharge until 5 years of age.
CHAPTER 4 FOLLOW UP GROWTH AND BONE MINERALIZATION

Post discharge until 5 years of age.

- Medical and or surgical treatment for patent ductus arteriosus; Cortico_5y: any treatment with steroids
- Distress syndrome; CLD: chronic lung disease defined as oxygen dependency at 28 days; PDA treatment:
- Until week 5 according to Patel’s formula; (58) PN: parenteral nutrition; P_14 days: total phosphate intake
- Data presented as mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1

Table 6b Follow up at 9 - 10 years; Responders and Non-responders to Follow – up

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2004</th>
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<th>2004</th>
<th>2005</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>8</td>
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<tr>
<td>Gender (F/M)</td>
<td>0/6</td>
<td>0/6</td>
<td>1/2</td>
<td>1/2</td>
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<td>Gestational age; weeks, mean (SD)</td>
<td>53.6(2)</td>
<td>53.6(2)</td>
<td>39.9(1.0)</td>
<td>39.9(1.0)</td>
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<td>Birth weight; grams, mean (SD)</td>
<td>2.07(1.7)</td>
<td>2.07(1.7)</td>
<td>2.22(1.2)</td>
<td>2.22(1.2)</td>
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<td>Birth weight SDS</td>
<td>-1.5(0.4)</td>
<td>-1.5(0.4)</td>
<td>-1.6(0.2)</td>
<td>-1.6(0.2)</td>
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<tr>
<td>Growth until week 5; g/kg/day, mean (SD)</td>
<td>9.8(2.4)</td>
<td>9.8(2.4)</td>
<td>13.2(1.2)</td>
<td>13.2(1.2)</td>
</tr>
<tr>
<td>PN days, mean (SD)</td>
<td>16.3(10.7)</td>
<td>16.3(10.7)</td>
<td>9.3(2.3)</td>
<td>9.3(2.3)</td>
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<tr>
<td>P_14 days; mmol/kg/day; mean (SD)</td>
<td>16.9(11.0)</td>
<td>16.9(11.0)</td>
<td>44.8(16.1)</td>
<td>44.8(16.1)</td>
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<td>Ca_14 days; mmol/kg/day; mean (SD)</td>
<td>26.1(9.6)</td>
<td>26.1(9.6)</td>
<td>37.6(8.0)</td>
<td>37.6(8.0)</td>
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<td>Protein_14 days; grams/kg/day; mean (SD)</td>
<td>42.1(3.8)</td>
<td>42.1(3.8)</td>
<td>32.1(7.5)</td>
<td>32.1(7.5)</td>
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<tr>
<td>Energy_14 days; kcal/kg/day; mean (SD)</td>
<td>1297(226)</td>
<td>1297(226)</td>
<td>1360(142)</td>
<td>1360(142)</td>
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<tr>
<td>IRDS; n (%)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CLD; n (%)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PDA treatment; n (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Late onset sepsis; n (%)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cortico_5y; n (%)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bronchial hyper reactivity; n (%)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BHR_10y; n (%)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fractures_10y; n (%)</td>
<td></td>
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</tbody>
</table>

Data presented as mean (SD); SDS: standard deviation score; GV: growth velocity from postnatal week 1 until week 5 according to Patell’s formula. PN: parenteral nutrition; P_14 days: total phosphate intake during the first 14 days; Ca_14 days: total calcium intake during the first 14 days; IRDS: infant respiratory distress syndrome; CLD: chronic lung disease defined as oxygen dependency at 28 days; PDA treatment: medical or surgical treatment for patent ductus arteriosus; Cortico_5y: any treatment with steroids post discharge until 5 years of age.

References

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less than 1500 g at birth. Early Hum Dev 2002;67:101-112.
The early postnatal nutritional intake of preterm infants affected neurodevelopmental outcomes differently in boys and girls at 24 months

Viola Christmann
Nel Roeleveld
Reina Visser
Anjo J.W.M. Janssen
Jolanda J.C.M. Reuser
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Arno F.J. van Heijst

Published in:
Acta Paediatrica 2017;106:242-249
CHAPTER 5 EARLY NUTRITION AND NEURODEVELOPMENTAL OUTCOME

Abstract

Aim
This study assessed whether increased amino acid and energy intake in preterm infants during the first week of life was associated with improved neurodevelopment at the corrected age (CA) of 24 months.

Methods
We evaluated preterm infants from two consecutive cohorts in 2004 (Cohort 1) and 2005 (Cohort 2) with different nutritional intakes in The Netherlands. Nutritional intake and growth was recorded until week five and after discharge. Neurodevelopment was determined using the Bayley Scales of Infant Development – Second Edition at a CA of 24 months.

Results
Compared to Cohort 1 (n = 56), Cohort 2 (n = 56) received higher nutritional intake during week one (p < 0.001). The weight gain in Cohort 2 was higher until week 5, especially among boys (p < 0.002). The mean Mental Developmental Index (MDI) scores did not differ, but Cohort 2 was associated with an increased chance of having an MDI ≥ 85, with an odds ratio of 6.4 and 95% confidence interval (CI) of 1.5-27.4, among all girls with a higher protein intake (5.3, 1.2-23.3). The Psychomotor Developmental Index increased with increasing nutritional intake, especially among boys (β-coefficient 3.1, 95% CI 0.2-6.0).

Conclusion
Higher nutritional intake was associated with different improvements in growth and neurodevelopment in boys and girls.

Introduction
In addition to promoting growth similar to intra-uterine foetal growth, adequate functional development is the main goal of nutritional supplementation for preterm infants. Postnatal growth has been associated with neurodevelopmental outcomes in very low birth weight (VLBW) infants. In addition to postnatal weight gain, head growth may serve as a predictor of brain growth and therefore of neurodevelopmental outcomes. Follow-up studies of children born preterm have indicated that subnormal head growth predicted poorer intelligence quotient scores at three and eight years of age. Poindexter et al associated low early amino acid intake with increased risks of having a small-for-age head circumference (HC) at 18 months of age, specifically in boys.

In a retrospective study, Stephens et al determined that higher nutritional intake in the first week of life was associated with higher Mental Developmental Index (MDI) scores and related this increase to higher protein and energy intakes. Van den Akker et al suggested that the effects of nutritional interventions in the first few days following birth may be gender specific. VLBW boys, but not girls, were affected by low amino acid and energy intakes when assessed at a corrected age of 24 months. Previously, we demonstrated that increased amino acid and energy intakes during the first week of life were associated with a statistically significant short-term improvement in weight gain in VLBW infants. A secondary analysis of these data revealed that this was mainly based on improvement in male infants.

In the current study, we investigated the neurodevelopment of the surviving infants of the original cohorts in relation to early nutritional intake. We hypothesised that nutritional supplementation during the first week would not only improve weight gain in the early postnatal period, but would also lead to improved head growth and neurodevelopmental outcomes at a corrected age of 24 months, specifically in boys.

Methods
This study evaluated the long-term outcome data of a previously described prospective cohort study that was conducted during two consecutive years, 2004 (Cohort 1) and 2005 (Cohort 2), to evaluate a change in the composition of standard parenteral nutrition (PN). The study was approved by the local ethics committee. The cohort characteristics, the nutritional protocol and its modification and the short-term results were described in detail in the previous paper. The original study included preterm infants born before 34 weeks of gestation who were admitted to our tertiary neonatal intensive care unit at the Radboud university medical center in The Netherlands on the first day of life on the assumption that PN would be needed.
for at least five days. Infants with major congenital malformations or asphyxia were excluded from the study. During both time periods, no major changes in clinical practice occurred and all infants received nutrition according to standard institutional protocols. The two cohorts primarily differed in PN intake which consisted of standard components, with higher amounts of amino acids and carbohydrates for Cohort 2. According to the new protocol, Cohort 2 achieved full PN two days earlier than Cohort 1, at postnatal day four versus six, respectively. (10)

The intake of all nutrients via both PN and enteral feeding, as well as growth characteristics, were recorded daily during the first two weeks, weekly until week five and at term corrected age. Based on the information in the patient charts, the mean daily weight gain was calculated weekly for the first five weeks, according to Patel’s formula. (11)

This study included the surviving participants of the original cohorts who received standard follow-up care provided for VLBW infants born prior to 32 weeks of gestation or with a birth weight of less than 1,500 grams. In accordance with the national follow-up programme, anthropometric data were recorded at the corrected ages of six, 12 and 24 months. The head circumference standard deviation score (HC SDS) was calculated using the Swedish growth reference for preterm infants and the Dutch reference of the nationwide growth study from term corrected age onwards. (12,13) Standard deviation scores for weight and length were calculated using the Dutch reference. (12) In addition, a neurodevelopmental assessment using the Bayley Scales of Infant Development - Second Edition (BSID-II) was performed at 24 months of corrected age, resulting in Mental and Psychomotor Developmental Indices (MDI and PDI). Scores of 85 or above were categorised as normal outcomes, whereas scores between 70 and 85 reflected moderate impairment and scores below indicated 70 severe impairment. (14) We assumed the diagnosis of normal neurodevelopmental outcome as clinically relevant and therefore dichotomised the MDI and PDI scores with a cut-off score of ≥ 85 reflecting normal outcome and as score of below 85 reflecting neurodevelopmental impairment.

The original cohort study was powered to assess differences in postnatal growth. Since this study only evaluated the surviving infants of these cohorts, no power calculation was carried out. The statistical analyses were performed using IBM SPSS statistics, version 22.0 for Windows (IBM SPSS Inc., Chicago, Illinois, USA). As the values calculation was carried out. The statistical analyses were performed using IBM SPSS for the dichotomous outcomes MDI ≥ 85 and PDI ≥ 85. All analyses were performed for the total study population and separately for the subgroups of boys and girls.

**Results**

The original Cohort 1 comprised 68 infants (38 girls), while Cohort 2 comprised 79 infants (37 girls). The detailed cohort characteristics have previously been reported. (10) For the current study, we evaluated all 112 surviving children who visited the routine follow up at the corrected age of 24 months. A further six infants had died, two from Cohort 1 and four from Cohort 2, while three and nine infants from the two respective cohorts were not invited, according to the criteria of the follow-up programme, and seven and 10 infants did not participate in the 24-month follow up. (Figure 1)

**Cohort characteristics**

The cohort characteristics for the participating children, and the potential determinants of the outcomes, are presented in Table 1. Mean gestational age (GA) and birth weight did not differ between the two cohorts, but the duration of mechanical ventilation was 1.4 days shorter for infants in Cohort 2. Concerning nutritional intake, Cohort 2 received 3.3 (95% CI 2.0-4.6) grams/kg/week more protein than Cohort 1 and had a 92 (54-131) kcal/kg/week higher energy intake during the first week. These differences were slightly smaller during the second week.

Only small differences were seen between the two cohorts with regard to gender, the numbers of infants with prematurity-related morbidities and maternal education. The only exception was patent ductus arteriosus (PDA), and its treatment, which occurred more frequently among infants in Cohort 1. Independent of cohort status, male infants seemed to have had an increased risk of infant respiratory distress syndrome (IRDS) and chronic lung disease (CLD) compared to female infants (OR 2.5, 95% CI 1.1-5.6 and 3.1, 1.0-9.6, respectively).

**Growth and neurodevelopmental outcomes**

The outcome parameters postnatal growth velocity, head circumference standard deviation scores (HC SDS) and BSID-II scores are presented in Table 2. The infants in Cohort 2 exhibited a higher mean daily weight gain from birth through to week five compared to Cohort 1, with a mean difference of 1.8 (95% CI 0.7-3.0) grams/kg/day.
The HC SDS in week one was lower in Cohort 2 than Cohort 1, but at the corrected age of six months, the HC SDS was normal compared to the Dutch population for both cohorts. This means that infants in Cohort 2 achieved greater catch-up growth for HC (0.6 SDS; 0.2-0.9) compared to Cohort 1. At the corrected age of 24 months, the weight, length and head circumference were not different between the cohorts and within the normal range compared to the reference population.

In Cohort 1, 10 infants could not be tested for MDI adequately, because they were unable to complete the tests or were easily distracted. As their parents reported concerns regarding their behavior, and all had PDI scores of less than 70, these infants were categorised into the group with an MDI of less than 85. One infant refused to cooperate with MDI testing and was excluded from the analysis. The mean MDI and PDI in Cohort 1 were 97.3 (9.2) and 98.2 (9.4), respectively. In Cohort 2, the mean MDI and PDI were 96.8 (9.3) and 97.8 (9.3), respectively.

### Table 1: Cohort characteristics and potential determinants of the outcomes

<table>
<thead>
<tr>
<th></th>
<th>Cohort 1 (n = 56)</th>
<th>Cohort 2 (n = 56)</th>
<th>Mean difference (C2 - C1)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age in weeks*</td>
<td>29.3 (2.2)</td>
<td>29.6 (1.8)</td>
<td>0.3</td>
<td>-0.4; 1.1</td>
</tr>
<tr>
<td>Birth weight in grams*</td>
<td>1124 (290)</td>
<td>1153 (330)</td>
<td>29</td>
<td>-87; 146</td>
</tr>
<tr>
<td>Head circumference of week 1 in cm</td>
<td>26.5 (2.2)</td>
<td>26.7 (1.8)</td>
<td>-0.3</td>
<td>-1.0; 0.6</td>
</tr>
<tr>
<td>Days of mechanical ventilation*</td>
<td>3.8 (4.3)</td>
<td>2.4 (3.5)</td>
<td>-1.4</td>
<td>-2.9; 0.1</td>
</tr>
<tr>
<td><strong>Nutritional intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein week 1 (grams/kg/week)</td>
<td>14.7 (3.4)</td>
<td>18.0 (3.6)</td>
<td>3.3</td>
<td>2.0; 4.6</td>
</tr>
<tr>
<td>Protein week 2 (grams/kg/week)</td>
<td>21.1 (5.0)</td>
<td>22.8 (4.8)</td>
<td>1.7</td>
<td>-0.1; 3.6</td>
</tr>
<tr>
<td>Energy week 1 (kcals/kg/week)</td>
<td>513 (94)</td>
<td>605 (111)</td>
<td>92</td>
<td>54; 131</td>
</tr>
<tr>
<td>Energy week 2 (kcals/kg/week)</td>
<td>710 (173)</td>
<td>777 (165)</td>
<td>68</td>
<td>5; 131</td>
</tr>
<tr>
<td><strong>Potential determinants of the outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender, female*</td>
<td>32 (57)</td>
<td>27 (48)</td>
<td>1.4</td>
<td>0.7; 3.0</td>
</tr>
<tr>
<td>IUGR</td>
<td>9 (16)</td>
<td>11 (20)</td>
<td>0.8</td>
<td>0.3; 2.1</td>
</tr>
<tr>
<td>IRDS*</td>
<td>38 (68)</td>
<td>33 (59)</td>
<td>1.5</td>
<td>0.7; 3.2</td>
</tr>
<tr>
<td>CLD</td>
<td>10 (18)</td>
<td>6 (11)</td>
<td>1.8</td>
<td>0.6; 5.4</td>
</tr>
<tr>
<td>PDA</td>
<td>26 (50)</td>
<td>17 (30)</td>
<td>2.3</td>
<td>1.1; 5.0</td>
</tr>
<tr>
<td>PDA treatment*</td>
<td>25 (45)</td>
<td>12 (21)</td>
<td>3.0</td>
<td>1.3; 6.8</td>
</tr>
<tr>
<td>LDS*</td>
<td>26 (50)</td>
<td>26 (46)</td>
<td>1.0</td>
<td>0.5; 2.1</td>
</tr>
<tr>
<td>NEC &gt;1b</td>
<td>3 (5)</td>
<td>5 (9)</td>
<td>0.6</td>
<td>0.1; 2.5</td>
</tr>
<tr>
<td>IVH grade 3/4</td>
<td>3 (5)</td>
<td>1 (2)</td>
<td>3.1</td>
<td>0.3; 30.9</td>
</tr>
<tr>
<td>Maternal education* low/high (missing)</td>
<td>10 / 37 (9)</td>
<td>8 / 37 (11)</td>
<td>1.3</td>
<td>0.4; 3.5</td>
</tr>
</tbody>
</table>

Table 1: C1 = Cohort 1; C2 = Cohort 2; OR = odds ratio; 95% CI = 95% confidence interval. * = included in potential confounder set in multivariable analyses (IUGR, CLD and PDA not included because of large overlap with other variables and NEC and IVH included because of small numbers). IUGR = intrauterine growth retardation defined as a birth weight < 10th percentile; IRDS = infant respiratory distress syndrome; CLD = chronic lung disease defined as supplemental oxygen for >28 days; PDA = patent ductus arteriosus diagnosed by echocardiography; PDA treatment = including up to three courses of Indomethacin and/or surgical ligation; LDS = late onset sepsis defined by positive blood culture and clinical signs of infection occurring more than 72 hours after birth; NEC = necrotising enterocolitis defined by Bell’s stage > 1b; IVH = intraventricular haemorrhage; Low maternal education = ≤ 10 years of education.
PDI scores were not different between the two cohorts, either in the total group or in the subgroups of boys and girls. However, the girls in both cohorts achieved higher scores than the boys, especially in Cohort 1 with a mean difference MDI of 12.7 (95% CI 5.2-20.2), PDI of 9.4 (0.8-18.0). In Cohort 2, the differences between boys and girls were smaller, with a mean difference for MDI of 6.3 (95% CI 1.2-13.8) and 5.5 (1.4-14.9) for PDI.

The children in Cohort 1 also seemed to be at increased risk of having an MDI below 85 (OR 2.1, 95% CI 0.9-5.4) compared to Cohort 2. Subgroup analyses by MDI revealed that children with an MDI below 85 had a lower mean protein intake during the first week of life compared to children with an MDI of ≥85: 14.9 versus 16.8 grams/kg/week with a mean difference of 1.8 (95% CI 0.1-3.6). Furthermore, an MDI below 85 seemed to be associated with having a PDA (OR 1.9, 95% CI 0.8-4.7), IRDS (2.2, 1.0-4.8), CLD (2.6, 0.8-8.5) and with ≤ 10 years of maternal education (4.0, 1.3-13.8). For children with a PDI below 85, the same associations were seen but with slightly lower effect estimates: PDI < 85 PDA (OR 1.6, 95% CI 0.8-3.8), IRDS (2.2, 1.0-4.8), CLD (2.6, 0.8-8.8) and maternal education (2.5, 0.8-7.6). Compared to girls, boys seemed to have increased risks of having an MDI below 85 (OR 5.1, 95% CI 1.8-14.0) and a PDI below 85 (1.9, 0.9-4.0).

Effect of early nutrition on neurodevelopmental scores and outcomes

Table 3 presents the results of the linear regression analysis of the effects of early nutritional intake on the continuous neurodevelopmental outcome scores adjusted for confounders. Table 4 presents the results of the logistic regression analysis of the chance to achieve a normal neurodevelopmental outcome in relation to early nutritional intake adjusted for confounders. The different nutritional protocols in Cohorts 1 and Cohort 2 did not influence the mean MDI scores, either in the total study population or in the subgroups of boys and girls. However, a one gram/kg higher intake of protein adjusted for energy in week one was associated with an increase in the PDI score of 2.4 (95% CI 0.6-4.3), especially among the boys (3.1, 0.2-6.0). This led to an approximately 1.5 higher chance of having a PDI ≥ 85 per gram/kg higher protein intake (OR 1.4, 95% CI 1.1-1.8) and this was also more pronounced among the boys (Table 4). Cohort 2 was associated with a markedly higher chance of having an MDI ≥ 85 in the total study population (6.4, 1.5-27.4), as well as for boys and girls separately. Among girls, a positive influence of protein intake on the chance of having an MDI ≥ 85, with or without an adjustment for energy intake, was also observed (5.3, 1.2-23.3 and 2.8, 1.1-6.8, respectively).

### Table 2 Postnatal growth and neurodevelopmental outcomes

<table>
<thead>
<tr>
<th>Measure</th>
<th>Cohort 1 (n = 56)</th>
<th>Cohort 2 (n = 56)</th>
<th>Mean difference (C2 - C1)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth velocity from birth through week 5 (grams/kg/day; mean (SD))</td>
<td>9.4 (3.0)</td>
<td>11.3 (2.9)</td>
<td>1.9</td>
<td>0.7; 3.2</td>
</tr>
<tr>
<td>Boys</td>
<td>8.8 (3.4)</td>
<td>11.8 (2.9)</td>
<td>3.0</td>
<td>1.3; 4.8</td>
</tr>
<tr>
<td>Head Circumference (standard deviation score; mean (SD))</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.3)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Week 6 - week 1</td>
<td>1.1 (0.3)</td>
<td>1.2 (0.3)</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Month 6 - week 1</td>
<td>1.1 (0.3)</td>
<td>1.2 (0.3)</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Head circumference</td>
<td>98.9 (1.3)</td>
<td>99.1 (1.4)</td>
<td>0.1</td>
<td>-1.0; 1.2</td>
</tr>
<tr>
<td>Weight</td>
<td>9.3 (1.0)</td>
<td>9.7 (1.0)</td>
<td>0.4</td>
<td>-1.2; 1.1</td>
</tr>
<tr>
<td>BMI (continuous score; mean (SD))</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.3)</td>
<td>0.0</td>
<td>-0.4; 0.4</td>
</tr>
<tr>
<td>BMI &gt; 85 (n)</td>
<td>16 (29)</td>
<td>23 (41)</td>
<td>1.6</td>
<td>0.6; 2.6</td>
</tr>
<tr>
<td>MDI Total cohort</td>
<td>98 (1.3)</td>
<td>99 (1.4)</td>
<td>1.1</td>
<td>-1.0; 3.2</td>
</tr>
<tr>
<td>Girls</td>
<td>103 (1.2)</td>
<td>103 (1.2)</td>
<td>0.0</td>
<td>-4.4; 5.5</td>
</tr>
<tr>
<td>Boys</td>
<td>95 (1.6)</td>
<td>95 (1.6)</td>
<td>0.0</td>
<td>-6.4; 5.5</td>
</tr>
<tr>
<td>PDI Total cohort</td>
<td>81 (1.0)</td>
<td>87 (1.1)</td>
<td>6.0</td>
<td>-3.5; 15.4</td>
</tr>
<tr>
<td>MDI &gt; 85 (n)</td>
<td>39 (71)</td>
<td>47 (84)</td>
<td>1.2</td>
<td>0.6; 2.6</td>
</tr>
<tr>
<td>Girls</td>
<td>33 (59)</td>
<td>47 (84)</td>
<td>1.2</td>
<td>0.5; 2.5</td>
</tr>
<tr>
<td>Boys</td>
<td>23 (41)</td>
<td>26 (46)</td>
<td>0.3</td>
<td>0.0; 0.6</td>
</tr>
</tbody>
</table>

C1 = Cohort 1, C2 = Cohort 2; OR = odds ratio, 95% CI = 95% confidence interval. Month six – week one = change in SDS from birth until six months of corrected age. Head circumference standard deviation score (HC SDS) calculated using the Swedish growth reference for preterm infants and the Dutch reference of the nationwide growth study at six months corrected age (week one n = 45/42; month six = 52/51; month six – week one n = 46/40). BSID II = Bayley Scores of Infant Development- Second Edition at 24 months corrected age. MDI = mental developmental index (n = 45/56). PDI = psychomotor developmental index (n = 54/54). MDI and PDI < 85 = moderately to severely impaired mental or psychomotor development, including not being able to perform the BSID- II.
Table 3 Effects of early nutritional intake on neurodevelopmental outcome scores

<table>
<thead>
<tr>
<th></th>
<th>MDI score</th>
<th>PDI score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude β</td>
<td>Adjusted β</td>
</tr>
<tr>
<td><strong>Total population (n = 112)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>1.12</td>
<td>2.56</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>-0.17</td>
<td>0.37</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>Protein week 1</td>
<td>0.12</td>
<td>0.18</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Boys (n = 53)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>5.98</td>
<td>6.79</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>0.21</td>
<td>0.52</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein week 1</td>
<td>0.88</td>
<td>0.64</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Girls (n=59)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>-0.41</td>
<td>-0.57</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>-0.41</td>
<td>0.06</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>-0.00</td>
<td>-0.05</td>
</tr>
<tr>
<td>Protein week 1</td>
<td>-0.44</td>
<td>-0.38</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
</tbody>
</table>

MDI = Mental Developmental Index. PDI = Psychomotor Developmental Index. 95% CI = 95% confidence interval. Bold numbers = indicative of an effect. Linear regression effect sizes were estimated for four separate situations: 1) being a member of Cohort 2, 2) the combination of protein and energy intake in week one, 3) protein intake in week one alone and 4) energy intake in week one alone. * = all analyses were adjusted for the potential confounder set: gender, gestational age, birth weight, maternal education, infant respiratory distress syndrome, days on ventilator, indomethacin treatment and late onset sepsis, unless otherwise indicated.

*1 = adjusted for gender, maternal education, and days on ventilator only.
*2 = adjusted for maternal education only.

Table 4 Effect of early nutrition on neurodevelopment

<table>
<thead>
<tr>
<th></th>
<th>MDI score ≥85*</th>
<th>PDI score ≥85*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude OR</td>
<td>Adjusted OR</td>
</tr>
<tr>
<td><strong>Total population (n=112)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>2.14</td>
<td>6.41</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>1.12</td>
<td>1.16</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Protein week 1</td>
<td>1.14</td>
<td>1.17</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Boys (n=53)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>2.41</td>
<td>6.32*2</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>1.06</td>
<td>1.10</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>Protein week 1</td>
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<td>0.92</td>
</tr>
<tr>
<td>Energy week 1</td>
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<td>1.00</td>
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<tr>
<td><strong>Girls (n=59)</strong></td>
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<td></td>
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<td>Cohort 2</td>
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<td>6.10*4</td>
</tr>
<tr>
<td>Protein week 1 &amp;</td>
<td>1.27</td>
<td>5.33*4</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>Protein week 1</td>
<td>1.23</td>
<td>2.75*4</td>
</tr>
<tr>
<td>Energy week 1</td>
<td>1.01</td>
<td>1.02</td>
</tr>
</tbody>
</table>

MDI = Mental Developmental Index. PDI = Psychomotor Developmental Index. Scores ≥ 85 reflect normal neurodevelopment. Bold numbers = indicative of an effect. Logistic regression odds ratios were analysed for four independent variables 1) being a member of Cohort 2, 2) the combination of protein and energy intake in week one, 3) protein intake in week one alone and 4) energy intake in week one alone. * = all analyses were adjusted for the potential confounder set: gender, gestational age, birthweight, maternal education, infant respiratory distress syndrome, days on ventilator, indomethacin treatment and late onset sepsis, unless otherwise indicated.

*1 = adjusted for maternal education only.
*2 = adjusted for gestational age, birth weight, maternal education, indomethacin treatment and late onset sepsis.
*3 = adjusted for maternal education, infant respiratory distress syndrome, days on ventilator, indomethacin treatment, and late onset sepsis.
*4 = adjusted for maternal education and days on ventilator only.
Discussion

Our evaluation of two cohorts at the corrected age of 24 months indicated that infants who received more protein and energy during the first week of life demonstrated a short-term improvement in postnatal weight gain, especially among male infants, while catch-up growth in head circumference seemed to be improved as well. The mean BSID-II scores were not different between the two cohorts and the MDI scores did not seem to be influenced by the nutritional protocol. However, we found differences in the subgroups of children, namely boys and girls. Children in Cohort 2 were more likely to have an MDI ≥ 85, which was clearly associated with a higher protein intake among girls, while higher protein intake had a positive effect on the PDI score among boys.

While head circumference growth has been related to neurodevelopmental outcome, two randomised trials demonstrated that impaired head growth was associated with the persistence of cumulative protein and energy deficits and that postnatal abnormal head growth was ameliorated via the optimisation of nutritional intake, especially parenteral intake. The improved catch-up in head circumference we saw with improved nutrition was in accordance with the results of the above mentioned trials.

A systematic review evaluated the effects of increased nutritional intake in the neonatal period on neurodevelopmental outcomes in VLBW infants and the results were comparable to our cohorts. This review evaluated 15 studies with regard to differences in neurodevelopmental scores and survival without impairment. The relationship between increased nutrition and neurodevelopmental outcomes remained unclear, probably due to the variety of nutritional interventions and neurodevelopmental outcome measures. In contrast, we not only compared the differences between the cohorts, but evaluated the mental developmental and psychomotor indices in relation to the individual nutritional intake of the first week, adjusting for a number of confounders of neurodevelopment and evaluated subgroups of children. In general, preterm infants have been shown to be at risk for adverse neurodevelopmental outcomes associated with socioeconomic status and gender, as well as with a number of clinical factors that may be related to each other. Therefore, it is difficult to evaluate the effect of a single determinant, such as nutritional intake. In our study, for instance, the diagnosis and treatment of PDA seemed to be important risk factors for adverse outcome, but children with a PDA also had a lower protein and energy intake during the first two weeks compared to children without PDA. However, by including treatment for PDA in our confounder set, we were able to estimate the effects of protein and energy intake independent of PDA and other determinants of neurodevelopmental outcomes in preterm born children. The same holds true for the other relevant confounders included in our analyses. However, some residual confounding by maternal education may still have been present in our results due to the relatively large number of missing values for this variable.

There is no consensus on whether improvements in postnatal weight gain and neurodevelopment are a result of a higher intake of amino acids. A Cochrane review found no evidence that high doses of amino acids had a positive effect on neurological outcomes. In contrast, van den Akker et al presented the long-term outcome results of a randomised trial that evaluated the early administration of amino acids and indicated that the intervention group, especially the male infants who received amino acids immediately after birth, experienced fewer disabilities at the corrected age of 24 months. Vlaardingerbroek et al demonstrated that high levels of amino acids and non-protein energy sources were needed to achieve an anabolic state in VLBW infants. Our findings are in accordance with the latter study because Cohort 2 received not only more amino acids, but also an increase in energy.

Gender seemed to be an important factor that determined the consequences of nutritional interventions, both in our study on short-term growth effects, as in other studies. Even short-term interventions may have significant effects for male and female infants are vulnerable to nutritional deficits, but may have had different needs. Even short-term interventions may have significant effects for male and female infants, either inducing harm if the nutritional intake is inadequate or preventing poor neurological outcomes if the nutritional needs are met.

In addition to the strengths mentioned above, this study also had some limitations. First, the patients were not randomly assigned to a treatment, but were recruited over two time periods. However, all patients received nutrition according to a standardised protocol, the data were recorded in the same standardised manner and the results were corrected for disparities in patient characteristics and morbidities. Second, although we improved the nutritional intake in Cohort 2, these infants did not receive the intake recommended by guidelines issued after the two study periods. This factor may have attenuated our results, for both boys and girls. Furthermore, this study was not designed and powered to evaluate gender
differences. Nevertheless, our data are consistent with recent studies and thus indicate that it may be necessary to develop different nutritional strategies for specific subgroups of preterm infants.

Conclusion

This study showed that an increase in the intake of amino acids and energy during the first week of life was associated with improved short-term weight gain, possibly improved catch-up of head growth and several specific long-term neurodevelopmental outcomes among preterm born boys and girls at the corrected age of two years. Our findings indicate a need for adequately powered and randomised studies to evaluate gender-specific nutritional needs in preterm infants.

References

Neurodevelopmental outcome in relation to treatment of Patent Ductus Arteriosus

Viola Christmann
Nel Roeleveld
Arno F. J. van Heijst

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To the editor,

With interest we read the article by Weisz et al evaluating the association between ductal ligation and neurodevelopmental outcome in preterm infants.\(^1\) We fully agree that postnatal events may affect outcome. Although the authors included an extensive list of morbidities and therapies as possible confounders, we suggest nutritional intake should be considered as a covariate for neurodevelopmental outcome.

We previously evaluated neurodevelopmental outcomes of 112 preterm infants at two years of corrected age, but related this to protein and energy intake of the first two weeks of life.\(^2\) Male sex and patent ductus arteriosus were found to be the greatest negative determinants of neurodevelopment, with an increased risk for adverse outcome (Bayley Scales of Infant Development II: Mental and Psychomotor Developmental Indices (MDI and PDI) below 85) for patients with PDA. (MDI Odds Ratio 1.9; 95% CI: 0.8 - 4.7, PDI (1.9; 0.9 - 4.0). Infants with patent ductus arteriosus (n = 45) received statistically significant less protein and energy during the first two weeks compared to infants without patent ductus arteriosus (n = 67) (protein (g/kg/week) week 1: (mean diff: -3.0; 95% CI -4.4, -1.6) week 2: (-2.3; -4.1, -0.4); energy (kcal/kg/week) week 1: (-84; -124, -44); week 2: (-84; -148, -20).

While a number of studies, as mentioned by Weisz et al, associated ductal ligation with impaired outcome, none of these studies accounted for nutritional intake.\(^1\) Wickremasinghe et al discontinued enteral feedings in the ‘early ductal ligation’ group, while with the more ‘selective approach’ enteral feedings were continued.\(^3\) Fluid restriction and cautious increment of enteral feeding is a generally accepted measure of conservative treatment of patent ductus arteriosus. In many cases this is at the expense of the daily required nutritional intake. Ductal ligation usually serves as the last treatment option and thereby prolonging the period of low nutritional intake.

We speculate that the impaired outcome after ductal ligation, as mentioned in many studies, is not only a result of surgery itself, but also the consequence of undernutrition in a period of rapid brain growth. Weisz et al\(^1\) did not report nutritional intake; therefore, any effect of nutrition on their positive outcome for ductal ligation compared to other studies remains speculative.

In light of the previously mentioned data, we suggest that future studies should evaluate outcome of prematurity related clinical conditions, such as treatment of patent ductus, in relation to nutritional intake.
References


PART 3

Calcium and phosphorus metabolism and bone mineralization in preterm infants
Early postnatal calcium and phosphorus metabolism in preterm infants

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Arno F.J. van Heijst

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Abstract

Objectives
Bone mineralisation in preterm infants is related to supply of calcium (Ca) and phosphorus (P). We increased the amount of minerals in parenteral nutrition (PN) for preterm infants and evaluated postnatal Ca and P metabolism in relation to mineral and vitamin D (vit D) intake.

Methods
Preterm infants, included on their first day of life, received standard parenteral nutrition providing a maximum Ca/P intake of 3/1.92 mmol/kg/d on day 3. Ca/P content of formula was 2.5/1.6 mmol/dl, and fortified human milk supplied 2.4/1.95mmol/dl. PN supplied 80 IU/kg/d vit D. Formula and fortified human milk contained 200 IU vitamin D/dl. Over a 5-week period serum concentrations and urinary excretion of Ca/P were registered and related to intake of minerals and vit D.

Results
During twelve months 79 infants (mean gestational age: 29.8 ± 2.2 weeks, mean birth weight: 1248 ± 371 grams) were included. The recommended intake for minerals was achieved by day 5 and for vitamin D by four weeks. Infants developed hypercalcaemia, hypercalciuria, and hypophosphataemia during the first postnatal week, leading to additional phosphorus supplementation in 49 infants. The renal tubular reabsorption of phosphorus was > 95% until day 9 but decreased below 70% after the second week. Alkaline phosphatase was normal at birth, increased to a maximum of 450 IU/l by day 14 and remained above the normal range for the remaining period.

Conclusions
Parenteral intake of phosphorus appeared to be too low leading to mineral imbalances in the early postnatal period, while also vitamin D intake was below recommendations.

Introduction
Preterm infants are at risk for the postnatal development of impaired bone mineralisation. This is primarily caused by an insufficient supply of calcium (Ca) and phosphorus (P) at the developmental stage during which these infants exhibit the fastest mineral accretion and thus have the highest requirements during their lifetime. Therefore minerals should be supplemented in preterm infants directly after birth, either through parenteral nutrition (PN), formula feeding or as supplement to human milk. In addition to minerals, vitamin D (vitD) needs to be supplemented because human milk contains only low amounts of vitD, and infants usually do not obtain enough through contact with sunlight during the first months of life. Vitamin D is essential for the adequate regulation of mineral homeostasis and bone mineralisation. During the last decennia international guidelines presented recommendations of optimal supplementation of calcium and phosphorus and vitamin D (6-12)(Table 1). Daily requirements of minerals have been determined either using the factorial approach or balance studies in stable growing preterm infants. International recommendations concerning vitD requirements are subject of debate but there is consensus that the daily intake should be increased in comparison to former recommendations. Studies evaluating these recommendations in the early postnatal period of very low birth weight infants have not been performed recently. In 2005, we implemented a parenteral solution (PN) that contained more Ca and P to improve the mineral supply for preterm infants. At that time, the Dutch recommendations for vitD supplementation were 80 IU/kg/d with PN and 400 IU/d with enteral feeding. As a quality control in relation to the implementation of the revised PN solution, we performed an observational study evaluating mineral homeostasis during the first five postnatal weeks. In this study, we evaluated mineral homeostasis in relation to postnatal mineral and vitD intake. We hypothesised that the use of this PN solution would not lead to disturbances in calcium and phosphorus metabolism.

Methods
Study population
This prospective cohort study was conducted throughout 2005. Preterm infants born at less than 34 weeks of gestational age (GA) who were admitted to our level III neonatal intensive care unit (Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands) were recruited on the first day of life. Infants with major congenital malformations or asphyxia were excluded from evaluation.
mmol/dl P. The HMF provided minerals as calcium glycerophosphate and calcium lactate. If human milk was not available, the infants received a preterm formula (Nutrilon Neonatal Start, Nutricia, Zoetermeer, NL). The formula contained 2.5 mmol/dl Ca, 1.6 mmol/dl P and 200 IE/dl vitD. Because fortified human milk and preterm formula were both supplemented with vitD, the infants received no further supplements.

Additional Ca and P were administered using 10% calcium gluconate or sodium glycerophosphate for PN or a potassium phosphate (KPO₄) and calcium chloride (CaCl₂) suspension for enteral supplementation. The decision to start additional supplementation of minerals was left to the discretion of the attending neonatologist and based on serum mineral concentrations, urinary excretion of Ca and P in spot urine samples and serum alkaline phosphatase (18). We aimed for sP ≥2.0 mmol/l, sAF < 300 IU/l and urinary excretion of uP > 0.4 mmol/l and uCa >1.2 mmol/l and a tubular reabsorption of P (trP) > 80%. If one of the parameter was below or beyond the target this could lead to changes in additional supplementation of Ca and/or P. Usually 0.5 –1.0 mmol/kg/d were added.

### Data registration
Nutritional intake was registered on a daily basis during the first two weeks and weekly until week five. Serum values of calcium (sCa, mmol/l), phosphorus (sP, mmol/l), alkaline phosphatase (AF, IU/l) and creatinine (sCreat, µmol/l) were measured according to a standard protocol. Spot urine samples were used to evaluate Ca (uCa, mmol/l), P (uP, mmol/l) and Creat (uCreat, mmol/l) excretion.

### Data analysis
Nutritional intake was calculated according to notations in the patient charts, including parenteral or enteral intake and additional supplementation of Ca, P and vitD. Ca and P metabolism was evaluated by comparing the concentrations in blood (sCa and sP) and urinary excretions (uCa and uP). Our laboratory reference served as the normal range for sCa (2.2 – 2.6 mmol/l), sP (2.03 - 2.9 mmol/l) and AF (80 – 280 IU/l).

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### Table 1 Recommended intake of calcium and phosphorus for preterm infants

<table>
<thead>
<tr>
<th></th>
<th>AAP ‘85</th>
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<tr>
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<tr>
<td>Ca : P (molar)</td>
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<td>1.4:1 – 2.0:1</td>
<td>1.7:1 – 2.0:1</td>
<td>1.4:1 – 1.6</td>
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**Table 2 Parenteral intake of fluid, calcium and phosphorus**

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<th>D</th>
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<th>Phosphorus (mmol/kg/d)</th>
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<tr>
<td>D4</td>
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</table>

Parenteral intake of fluid, calcium and phosphorus. D0 was defined as day of the admission, and day 1 was defined as the next morning, when the nutritional intake regimen was routinely started.

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**Nutritional protocol**
Our PN consisted of standard prepared components. The PN solution contained 2.5 mmol/dl calcium gluconate (calcium gluconate 10%; B. Braun, Melsungen, Germany) and 1.6 mmol/dl sodium-glycerophosphate (Glycophos; Fresenius Kabi BV, Zeist, NL). All of the infants received nutrition according to the standard institutional nutritional protocol. PN was started directly after birth. Mineral intake was increased daily by incremental increases in the amount of PN (Table 2). The maximum parenteral intake according to the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommendations in 2005 was achieved at day 3 with 3 mmol/kg/d Ca and 1.92 mmol/kg/d P. (10) The recommendations provided by Tsang et al were used for enteral intake (9). Vitamin D was supplemented with Vitintra infant (40 IE/ml vitamin D, Fresenius Kabi’s Hertogenbosch, NLL). The infants received the generally advised doses of 2 ml/kg/day, resulting in a parenteral intake of 80 IE/kg/d by day 4. (10)

Enteral feeding was started on the first day of life with daily increments, while PN was gradually reduced to maintain a daily fluid intake within the protocol range. The maximum enteral fluid intake for enteral nutrition and PN was the same (Table 2). The stepwise increase in the enteral volume was left to the discretion of the attending neonatologist. Human milk was enriched with a commercially available human milk fortifier (HMF) (Nutrilon Neonatal BMF, Nutricia, Zoetermeer, NL) from an intake of 50 ml/d onwards. HMF added 1.6 mmol/dl Ca, 1.5 mmol/dl P and 200 IE/dl vitD, therefore fortified human milk was assumed to contain 2.4 mmol/dl Ca and 1.95 mmol/dl P. The HMF provided minerals as calcium glycerophosphate and calcium lactate. If human milk was not available, the infants received a preterm formula (Nutrilon Neonatal Start, Nutricia, Zoetermeer, NL). The formula contained 2.5 mmol/dl Ca, 1.6 mmol/dl P and 200 IE/dl vitD. Because fortified human milk and preterm formula were both supplemented with vitD, the infants received no further supplements.

Additional Ca and P were administered using 10% calcium gluconate or sodium glycerophosphate for PN or a potassium phosphate (KPO₄) and calcium chloride (CaCl₂) suspension for enteral supplementation. The decision to start additional supplementation of minerals was left to the discretion of the attending neonatologist and based on serum mineral concentrations, urinary excretion of Ca and P in spot urine samples and serum alkaline phosphatase (18). We aimed for sP ≥2.0 mmol/l, sAF < 300 IU/l and urinary excretion of uP > 0.4 mmol/l and uCa >1.2 mmol/l and a tubular reabsorption of P (trP) > 80%. If one of the parameter was below or beyond the target this could lead to changes in additional supplementation of Ca and/or P. Usually 0.5 –1.0 mmol/kg/d were added.
The tubular reabsorption of P (trP) was used as an indicator of P availability (normal range: 85–95%). The ratios of Ca and P to creatinine (uCa/Creat and uP/Creat) were calculated retrospectively and compared with the percentiles based on the data presented by Aladangady et al (19). AF was used as a marker for changes in bone metabolism. All of the data were categorised into subgroups of infants according to GA.

Statistics
The data were collected within a cohort study evaluating weight gain in relation to different nutritional intakes. For the primary objective of weight gain, we determined that a study group size of 66 patients was required to identify a significant difference with an α = 0.05 and a power of 0.80. The data are presented as the mean ± SD, unless otherwise indicated.

Ethical considerations
The study was approved by the local ethics committee. The informed consent of parents was not necessary according to our institutional review board because the PN solution was a standard medical prescription that was changed to improve quality. The nutritional protocol, record keeping, and blood and urine sampling were part of the standard care used in our department.

Results
Patient recruitment and cohort inclusion are presented in Figure 1. A total of 79 infants were evaluated. The mean GA was 29.8 ± 2.2 weeks, and the mean birth weight was 1248 ± 371 g. The nutritional characteristics are presented in Table 2. Only the total group data are presented.

The nutritional intake of Ca, P, vitD and protein is shown in Figure 2. The daily intake increased with time according to the nutritional protocol. The mean duration of PN was 10.6 ± 3.6 (range 6 – 25) days (Table 3). The highest mean daily intake of calcium was 3.3 ± 1.5 mmol/kg, which was achieved at week 3. The mean total calcium intake was within the recommended range. Only seven infants received additional calcium supplementation over short periods to treat hypocalcaemia (Figure 2A). The mean total P intake was within the lower range of the ESPGHAN PN recommendations at day 2 but increased to just above the maximum intake for PN (2.3 mmol/kg/d) after day 6 when enteral and parenteral nutrition provided a similar amount of intake. The maximum daily intake including parenteral , enteral and the additional supplement (3.3 ± 1.2 mmol/kg) occurred in week 3 (Figure 2B). The total Ca and P intake was slightly above the more recent ESPGHAN recommendations for enteral nutrition. Infants received a total protein intake of mean (SD) 2.6 ± 0.6 and 3.2 ± 0.7 gram/kg per day during the first and second week (Figure 2C).

Vitamin D intake was calculated according to the recorded intake. Human milk was provided to 91% of our infants and reached a mean enteral intake of >50 ml per day at day 4. Subsequently, human milk was fortified and enriched with vitD. After day 5, the daily vitD intake increased above 160 IU. The recommended daily intake of 400 IU was only reached four weeks after birth (Figure 2D).

The calcium, phosphorus and alkaline phosphatase (sAF) concentrations in blood and urine are presented in Figure 3. Directly after birth, the mean sCa was 2.2 ± 0.24 mol/l, which continuously increased to a maximum of 2.7 ± 0.24 mmol/l on day 5. Between days 4 and 8, the mean sCa remained above the upper reference limit of 2.6 mmol/l. Forty-five percent of all infants had a sCa >2.6 mmol/l on day 5. At the same time 34% infants had a sP level below 1.8 mmol/l. For the remaining observational period, the mean sCa remained below the upper normal range. The mean sP concentration was below the lower reference range of 2 mmol/l from birth until day 9 and increased to within the normal range for the remaining observational period. At birth, the mean sAF was within the normal range (mean 194 ± 62 IU/l), but thereafter, it steadily increased to a maximum of 480 IU/l by day 7 and remained above 300 IU/l for the remaining observation period, thus exceeding the upper reference limit.

The mean uP, evaluated with spot urine samples was low until day 9, but generally above the recommended surplus of 0.4 mmol/l. The urinary excretion of P increased...
steadily until week 5. The mean urinary excretion of Ca was below the recommended surplus of 1.2 mmol/l until day 2. The mean uCa remained above the surplus for the rest of the observational period and tended to increase towards the end of the observational period. The highest uCa excretion occurred between day 3 and day 8 (Supplemental Digital Content 1: jpeg; Figure S1 Urinary excretion of calcium and phosphorus determined from spot urine samples).

The mean uCa/Creat ratio increased steadily after day 3, remaining above 3.8 mmol/mmol (the 95th percentile) between days 4 – 8 and above 2 mmol/mmol (the 75th percentile) for the rest of the follow-up (Figure 3)(18). The mean uP/Creat ratio remained below 0.5 mmol/mmol (the 10th percentile) until day 9 and increased above 16 mmol/mmol (75th percentile) after the second week (Figure 3) (19). In accordance with the low mean uP/Creat ratio, the tubular reabsorption of phosphate (trP) remained above 95% during the first week. After the second week, the mean trP decreased to below 70% (range 58 – 68%).

During the second week 49 of 79 infants received additional supplementation of P. This was continued in 25 infants until week 5. Infants who received additional P until week 5 had a significant lower gestational age, and lower birth weight (p < 0.001) and received enteral nutrition earlier. They had lower sP concentrations and lower urinary excretion of P during the first week, and higher sAF concentrations compared to infants without additional P. The trP was low in all infants during the last three weeks independent of any P supplementation (Supplemental Digital Content 2: doc; Word File S1: Sub- analysis additional phosphorus supplementation).

Table 3 Nutritional characteristics

| PN, mean (SD) days, (range) | 10.6 (3.6) (6-25) |
| First enteral feeding, mean (SD) day | 0.91 (0.22) |
| Full enteral feeding, mean (SD) days | 11.6 (3.6) |
| Infants receiving human milk, (n) (%) | 72 (91%) |

Nutritional characteristics. Full enteral feeding was defined as an enteral intake of 150 ml/kg/day; PN: parenteral nutrition
Discussion

Our results demonstrate that the mineral intake of our infants met the recent recommendations for parenteral and enteral nutrition for preterm infants. 

Nevertheless, we observed hypercalcaemia, hypophosphataemia, hypercalciuria, and high tubular phosphate reabsorption during the first week and hyperphosphaturia with a low trP during the second half of the study period. Furthermore, the sAF increased above the normal range. The recommended vitD intake was only achieved by week 5. These data indicate a great need for phosphorus supplementation in the early postnatal period and a mineral imbalance in the second half of the observation period, probably related to low vitD intake.

Increasing Ca and P intake in PN has been shown to improve bone mineral content in preterm infants. The use of sodium-glycerophosphate in PN offers the opportunity to increase the mineral contents of standard PN solutions.

Despite the increased need for minerals, the supplementation of high amounts of minerals is often regarded as a potential risk factor for the development of hypercalcaemia, hypercalciuria and, thereafter, nephrocalcinosis.

Aladangady et al. demonstrated a significant relationship between a high Ca/Creat ratio and the use of xanthine derivatives, such as caffeine. All of the infants received caffeine directly after birth or shortly before extubation from ventilation. Additionally, hypophosphataemia will lead to increased urinary Ca excretion. Several studies recently suggested that this increased need may be related to a higher amount of amino acids administered with parenteral nutrition nowadays, in comparison to older studies used for current guidelines.

During the second half of the observation period, we recognised a different pattern with still elevated uCa excretion, an increasing phosphaturia, low trP, and elevated AF, whereas the mineral intake mainly remained the same. Since many infants received additional P at that time this may be interpreted as a result of an
unnecessary supplementation. Furthermore, we used potassium phosphate as oral supplement, while organic salts probably may have had an improved bioavailability. Nevertheless, infants who did not receive additional P during the last week, demonstrated the same increase in urinary excretion of phosphorus and calcium (Supplemental Digital Content 2: doc; Word File S1: Sub-analysis additional phosphorus supplementation). At that time, the mean vitD intake was below the recommended daily intake of 400 IU vitD. Insufficient vitamin D may explain the high Ca excretion while indirectly this may have inhibited the renal tubular reabsorption of phosphorus. Our data are in accordance with previously performed balance studies in preterm infants that demonstrated that vitD is absorbed well and positively influences mineral homeostasis. In these studies, infants received 1200 IU vitD/d.

Present recommendations concerning nutritional intake and mineral homeostasis are developed for either parenteral or enteral nutritional intake and the recommended Ca : P ratio encloses a wide range. There is a discrepancy between the guidelines presented by Tsang et al and the ESPGHAN ESPEN recommendation. According to Tsang et al the lower limit of parenteral P intake is higher and the range of Ca : P ratio lower than recommended by the ESPGHAN. (Table 1). Looking at our data the American recommendations seem to be more appropriate.

Nowadays, most very low birth weight infants receive a combination of parenteral and enteral nutrition with an increased amount of protein and energy directly after birth. Current recommendations are based on studies performed in stable growing infants of several weeks old, whereas recent studies indicate that nutritional deficits developed during the first postnatal weeks may have a long-lasting effect for future development. Our study has several limitations. We evaluated mineral homeostasis but did not evaluate bone mineral content using Dual-energy X-ray absorptiometry. Nevertheless mineral imbalance, especially hypercalciuria, has been related to impaired bone mineralisation. We observed a high urinary excretion of minerals in our patients. Therefore we believe that despite the high mineral intake, our patients were at risk of developing suboptimal bone mineralisation, with the long-term consequence of reduced length. In addition, we did not measure vitD concentrations although the intake could be calculated from charts and a positive relationship between enteral vitD intake and the vitD concentration in blood has been demonstrated in preterm infants. The latest ESPGHAN guidelines recommend a daily intake of 800 – 1000 IU vitD/day in enterally fed preterm infants. This recommendation does not state at what time enteral vitD supplementation should be initiated. Furthermore, the recommendation for parenteral supplementation has not been re-evaluated with regard to the revised target value of 75 mmol/l (30 ng/ml) 25-hydroxy vitamin D.

Early postnatal mineral homeostasis with high mineral intake and high doses of vitamin D in preterm infants has not yet been investigated. Our data suggest that optimal dosing of minerals and vitamin D should be re-evaluated in very low birth weight infants in the early postnatal period in combination with more aggressive parenteral and enteral nutritional intake.

In conclusion, our data demonstrate that the parenteral intake of phosphorus appeared to be too low, leading to mineral imbalances in the early postnatal period, while also vitamin D intake was below recommendations.
Supplemental Digital Content 2
Word File S1: Sub analysis additional phosphorus supplementation

Sub analysis of infants who received additional phosphorus supplementation
We performed a sub analysis to clarify our conclusion that the hyperphosphaturia we saw in our patients after the second week cannot solely be explained by our regimen of phosphorus supplementation.

From the end of the first week onwards an increasing number of infants received additional phosphorus supplementation. During week 3, 4 and 5 the number of infants who received additional supplementation steadily declined. During the 5th week 25 of 79 infants still received additional phosphorus supplementation. For the sub analysis we divided our cohort into a group without phosphorus supplementation (P-; n=54) and with phosphorus supplementation (P+; n=25) during week 5.

Infants who did not receive P suppl had a significant higher GA and higher birth weight than the group of infants who did receive supplementation (P-:  GA 30.5 ± 2 wks; BW: 1293 ± 358gram vs. P+: 28.3 ± 1.5 wks; BW: 1004 ± 302gram; p< 0.001). 9/54 and 4/25 infants were SGA at birth (16% both).

Intake:
Group P+ received enteral nutrition somewhat faster than group P- and therefore also received somewhat less parenteral nutrition (both non-significant). In both groups additional supplementation of phosphorus was started by day 2 and 3 but from day 9 on group P+ received significant more extra P compared group P- for rest of the observational period.

Serum control:
Serum P: The sP concentration was below 2 mmol/l until day 7 in P-, and until day 8 in P+. Between day 4 and day 8, sP in P+ remained between mean 1.5±0.2 and mean 1.7±0.5 mmol/l. During this period serum phosphate concentrations were significant lower than phosphate concentrations of P+(p < 0.005). After day 9 sP in both groups remained within the reference range (2.0-2.9 mmol/l).

Table S1 number of infants who received additional P

|   | d0 | d1 | d2 | d3 | d4 | d5 | d6 | d7 | d8 | d9 | d10 | d11 | d12 | d13 | d14 | w3 | w4 | w5 |
|---|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|----|----|----|
| P-| 0  | 0  | 1  | 7  | 22 | 34 | 45 | 47 | 48 | 49 | 49  | 46  | 47  | 49  | 47  | 46  | 31  | 25 |

Table S2 serum alkaline phosphatase (sAF U/L; mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-</td>
<td>321 ± 114</td>
<td>338 ± 91</td>
<td>305 ± 112</td>
</tr>
<tr>
<td>P+</td>
<td>380 ± 152</td>
<td>352 ± 128</td>
<td>323 ± 120</td>
</tr>
</tbody>
</table>

Table S3 urinary excretion of phosphorus from spot urine (mmol/L; mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-</td>
<td>20.3 ± 15</td>
<td>16.3 ± 18.4</td>
<td>14.4 ± 3.9</td>
</tr>
<tr>
<td>P+</td>
<td>18.4 ± 10</td>
<td>25.5 ± 18.4</td>
<td>22.9 ± 23.5</td>
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</tbody>
</table>

Table 4 urinary excretion of calcium from spot urine (mmol/L; mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-</td>
<td>1.95 ± 1.4</td>
<td>2.24 ± 1.8</td>
<td>3.18 ± 3.16</td>
</tr>
<tr>
<td>P+</td>
<td>2.56 ± 1.6</td>
<td>1.85 ± 1.12</td>
<td>3.3 ± 3.33</td>
</tr>
</tbody>
</table>

Serum Ca: Serum calcium concentrations were not different between both groups, but slightly higher until day 4 in group P- (non-significant). Data are presented in Figure 3.

sAlkaline Phosphatase (sAF): sAF was not different and within the normal range (80 -280 IU/L) until day 3 in both groups. In P+ the AF increased up to 665 ± 38 IU/L by day 7 whereas in P- the sAF was 434±123 at the same time (p=0.01). In P- the AF achieved a maximum level by day 10 of 447 ±95 IU/L. After the peak level the sAF slowly declined in both groups, but somewhat slower in P+ than in P-.

Urinary excretion
uP: Group P+ excreted more P than group P- between d0 and day2 (4.7 vs. 1.1 – 3.1 mmol/l), while at that moment the nutritional intake was not different and below recommended optimal intake. Group P+ had a lower urinary excretion of P (0.1 – 1.2 mmol/l) than in group P- (0.5 -2.5 mmol/l) between day 4 -8. Between W3 and W5 the urinary excretion of P was not different between both groups.

uCa: The urinary excretion of Ca was not different between both groups.
Renal tubular reabsorption:
Tubular reabsorption of P was not different between both groups and remained between 88 and 99% between d0 and day 10. From day 11 on, in both groups the Tr P declined simultaneously.

<table>
<thead>
<tr>
<th>Table S5 renal tubular reabsorption of phosphorus (TrP %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tr P %</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>P-</td>
</tr>
<tr>
<td>P+</td>
</tr>
</tbody>
</table>

Summarizing:
Infants in group P+ were of significant lower GA and BW and received enteral nutrition somewhat earlier. The group P+ had lower sP levels and a faster increase in sAF and also a slightly lower mean uP excretion during the first two postnatal weeks in comparison to group P-. Both groups had initially a high renal tubular reabsorption. This, in combination with elevated sAF, was interpreted as insufficient P supplementation and therefore additional P was subscribed. Additional supplementation was reduced in the following period in relation to laboratory results. Nevertheless phosphaturia remained high and trP low in both groups, even without supplementation.

We therefore believe that unnecessary supplementation is not the main cause of our results.
References


Changes in biochemical parameters of the Calcium-Phosphorus homeostasis in relation to nutritional intake in Very-Low-Birth-Weight infants

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Abstract

Preterm infants are at significant risk to develop reduced bone mineralization based on inadequate supply of calcium and phosphorus (Ca-P). Biochemical parameters can be used to evaluate the nutritional intake. The direct effect of nutritional intake on changes in biochemical parameters has not been studied. Our objective was to evaluate the effect of Ca-P supplementation on biochemical markers as serum (s)/urinary (u) Ca and P; alkaline phosphatase (ALP); tubular reabsorption of P (TrP) and urinary ratios for Ca/creatinin and P/creatinin in Very-Low-Birth-Weight infants on postnatal day 1, 3, 5, 7, 10, and 14. This observational study compared two groups with High (n=30) and Low (n=40) intake of Ca-P. Birth weight: median (IRQ) 948 (772-1225) vs. 939 (776-1163) grams; Gestational age: 28.2 (26.5-29.6) vs. 27.8 (26.1-29.4) weeks. Daily median concentrations of biochemical parameter were not different between the groups but linear regression mixed model analyses showed that Ca intake increased the uCa and TrP (p = 0.04) and decreased ALP (p = 0.00). Phosphorus intake increased sP, uP and uP/creat ratio and ALP (p ≤ 0.02) and caused decrease in TrP (p =0.00). Protein intake decreased sP (p = 0.000), while low gestational age and male gender increased renal excretion of P (p < 0.03). Standardized repeated measurements showed that biochemical parameters were affected by nutritional intake, gestational age and gender.

Introduction

Bone development is one of the key processes of intrauterine and postnatal growth. (1) Preterm infants are at significant risk to develop reduced bone mineral content based on inadequate supply of calcium and phosphorus (Ca-P). (2,3) During normal pregnancies in healthy mothers, there is an active, placental transfer of Ca-P to the fetus leading to a high mineral accretion during the last trimester, while after birth the infant is dependent on nutritional supply of minerals. (4,5) In clinical practice, postnatally it is difficult to meet the high fetal needs, either because of limited solubility of parenteral fluids, low content of Ca-P of human milk and impaired intestinal absorption through formula feeding. (6-9) Balance studies tried to define nutritional requirements but, in clinical practice it is often uncertain whether the nutritional intake of Ca-P provided to preterm infants is sufficient and is actually used for bone mineralization. (10-12)

Assuming that biochemical parameters of Ca-P homeostasis within a normal range will lead to optimal bone mineralization, evaluation of electrolyte disturbances is standard of care in many neonatal units. (13-28) However, there is currently neither a consensus on the appropriateness of either parameter or the frequency of measurements. (29,30) A recent survey among U.S. neonatologists showed a great lack of consensus and variation in practices regarding definition and screening methods for metabolic bone disease. (31) Reference values in relation to adequate nutritional intake have not been developed. Urinary excretion of minerals in spot urine samples has been shown to be an easy tool for routine evaluation. Pohlandt proposed to aim for a small ‘surplus of minerals’ in urine samples, while Aladangady et al developed reference values for urinary Ca-P / creatinine ratios for preterm infants. (17,23) Staub et al compared both methods with regard to an agreement between their results and found neither method to be superior. (32) None of the studies evaluated the direct effect of nutritional intake on biochemical parameter of Ca-P homeostasis. It is not sure whether biochemical parameters are able to indicate sufficiency of nutritional intake.

The aim of this study was to evaluate changes in biochemical parameters of the Ca-P homeostasis in blood and urine in relation to different nutritional intake during the first 14 days of life in Very Low Birth Weight (VLBW) infants. Our hypothesis was that the nutritional intake of calcium and phosphorus would have an effect on biochemical parameters of the calcium – phosphorus homeostasis.
Materials and Methods

Study design and randomization
The current study (Early Supplementation Study (ESS)) was part of the Early Nutrition Study (ENS), a multi-center double-blinded randomized controlled trial. (CMO file number: NL37296.029.11, Dutch Trial Registry: NTR 3225) While the ENS evaluated the effects of human milk on postnatal outcome, the primary objective of the ESS was bone mineralization in relation to early and late enteral supplementation of minerals. (33) The studies were approved by the Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands). Patients were distributed into three groups through two steps of randomization. The first step randomized eligible infants either into the early supplementation group (High) or the late supplementation group (Low) being part of the ENS. The second step of the randomization was only performed if infants were assigned to late supplementation and randomized them to either ‘ENS A’ or ‘ENS B’ as part of the ENS. Both randomization steps were performed before the first enteral nutrition was administered resulting in basically three groups.

Study population
Participants for the ENS/ESS were recruited at the level III neonatal intensive care unit of the Radboud university medical center (Radboudumc), Nijmegen, The Netherlands. The inclusion criteria were a birth weight below 1500 grams and written informed consent of both parents. The exclusion criteria were maternal drugs and/or alcohol use during pregnancy, birth defects, congenital infection within 72 hours after birth, perinatal asphyxia with a pH < 7.0 and any intake of cow’s milk based products prior to randomization. For the current study infants who died or were discharged before the end of the study period of 14 days were excluded from analysis.

Intervention and Nutritional protocol
Parenteral nutrition (PN) was started directly within the first hour after birth. The PN solution consisted of 2.5 mmol/dL calcium-gluconate (calcium-gluconate 10%; B. Braun, Melsungen, Germany) and 1.6 mmol/dL sodium-glycerophosphate (Glycophos; Fresenius Kabi BV, Zeist, The Netherlands) and 2.25 grams/dL amino acids (Primene; Clnitec, Brussels). Additional parenteral supplementation with 10% calcium-gluconate or sodium glycerophosphate was administrated depending on biochemical parameters. Table A1 presents the standard protocol for PN. The decision to start additional enteral supplementation was left to the attending neonatologist based on biochemical parameter and postnatal growth as being a standard procedure of our department. The additional enteral supplementation could comprise of either a supplement of protein (Nutrilon Neonatal BMF; Nutricia, Zoetermeer, The Netherlands) or a potassium phosphate (KPO4) and calcium chloride (CaCl2) suspension for enteral supplementation.

‘Group Low’, comprising of group ENS A and ENS B, received no additional enteral supplementation or fortification of human milk during the first 10 days of life. Group ENS A received donor milk if mother’s own milk (MOM) was not available. Group ENS B received preterm formula (Hero Baby Prematur Start; Hero Kindervoeding, Breda, The Netherlands) if MOM was not available, containing 2.4 mmol/dL calcium, 1.7 mmol/dL phosphorus and 2.6 grams/dL proteins. The additional nutrition in ENS A and ENS B was blinded to all caretakers and parents. After 10 days all infants received nutrition according to the standard protocol of the Radboudumc.

Early supplementation was assumed to provide a high intake (Group High). This group received enteral nutrition from day 1 onwards according to the local protocol. They received additional enteral supplementation and human milk fortifier (Nutrilon Neonatal BMF; Nutricia, Zoetermeer, The Netherlands; BMF) by the time the enteral intake was 50 ml per day. The human milk fortifier added 1.65 mmol/dL calcium, 1.22 mmol/dL phosphorus and 0.8 grams/dL protein to human milk. They received preterm formula if MOM was not available. The decision to start additional enteral supplementation was left to the attending neonatologist based on biochemical parameter and postnatal growth as being a standard procedure of our department. The additional enteral supplementation could comprise of either a supplement of protein (Nutrilon Neonatal Protein Fortifier; Nutricia, Zoetermeer, The Netherlands) or a potassium phosphate (KPO4) and calcium chloride (CaCl2) suspension for enteral supplementation.

Group ENS A received 100% human milk during the first 10 days and reflected a group with low intake of minerals and protein, because human milk has a very low nutrient content. Group High reflected a high intake of nutrients, because human milk was enriched with minerals and protein as soon as possible. Group ENS B could be considered as intermediate depending on the amount of MOM or preterm formula an infant received, since preterm formula contained approximately the same amount of minerals as fortified human milk.

Biochemical parameters of bone mineralization
For this study, blood and urine samples were analyzed according to the local protocol of the department. Samples were taken on days 1, 3, 5, 7, 10 and 14 after birth. Urine was collected through spot samples. (25) The following parameters were analyzed: serum calcium (sCa), serum phosphorus (sP), serum alkaline phosphatase (ALP), urine calcium (uCa), urine phosphorus (uP), urine calcium/creatinin ratio (uCa/Creat), urine phosphorus/creatinin ratio (uP/Creat), and tubular reabsorption of phosphorus (TrP).

Data registration and handling
Patient characteristics, clinical course, growth and intake of all nutrients were recorded daily from the patient records and abstracted for this study. Amounts of enteral,
parenteral and additional supplementation (parenteral and enteral) of all nutrients were calculated separately for each patient. The total intakes were calculated per kg per day per infant. The intake through human milk was calculated based on the reference of Gidrewicz et al. (34) The calcium/phosphorus ratio was calculated per day by dividing the daily intake of calcium in mmol/kg through the daily intake of phosphorus in mmol/kg.

After closure of patient enrollment and de-blinding of the ENS, we performed a reallocation procedure for the intermediate group ENS B. Infants who received more than 90% MOM were considered to reflect a low intake of minerals and were allocated to group Low together with the infants of group ENS A. Infants who received more than 90% of preterm formula were considered to reflect a high intake of minerals and were allocated to High. Infants in between these extremes were not included in the analyses.

Statistical analysis

The primary objective of the ESS was bone mineralization in relation to mineral supplementation, and the original power calculation was based on bone mineral content at term corrected age. For the evaluation of changes in biochemical parameters in relation to nutritional intake the power calculation was based on sP. In a previous evaluation of our nutritional protocol performed at our department, we found a mean of 1.7 mmol/l of sP during the first week. (35) A concentration of 2.0 mmol/l was defined as target for optimal bone mineralization by Hellstern et al. (20) Assuming an expected mean of 1.7 mmol/l, we determined that 24 infants were required in each group to find a difference of 0.3 mmol/l in sP between High and Low with α=0.05 (two-sided) and a power of β=0.80.

The statistical analyses were performed using IBM SPSS statistics 22.0 for Windows (IBM SPSS Inc., Chicago, IL, USA). Differences in patient characteristics, nutritional characteristics and biochemical parameters between the High and Low group were determined using the Mann-Whitney U test or the chi-square test, depending on the variable under examination. Due to non-normality of the continuous variables, the data were presented as median (with interquartile range (IQR)), unless otherwise indicated. A p-value < 0.05 was considered statistically significant.

To account for repeated outcome measurements, we used a mixed model analysis to determine the effects of daily nutritional intake of calcium and phosphorus on each biochemical parameter. We included the total intake of Ca/P and protein, the percentage of enteral amount of Ca/P intake, and a number of clinical parameter that could affect the Ca/P homeostasis such as birth weight, gestational age, gender, caesarian section, multiple births, sepsis, and days of caffeine, furosemide, steroids, and sedation during the first two weeks as co-variables in the initial models. Necrotizing enterocolitis was not included as co-variable because of small numbers.

Using manual backward selection, variables were kept in the model when they contributed statistically significantly with a p value < 0.1.

Results

Patient characteristics

Enrollment of patients occurred between January 2013 and December 2014. The distribution of the infants is presented in the consort diagram (Figure 1). Finally, 109 infants were randomized, either to Late Supplementation (Low; n = 72; distributed into Group ENS A (n = 40) and ENS B (n = 32) or Early Supplementation (High; n = 37). The characteristics of all infants included in the three groups of the ENS/ESS study are presented in Table A2. After de-blinding of group Low, 4 infants of group ENS B were reallocated to High and 13 to Low so that, Low and High consisted of 53 and 41 infants, respectively. Infants who died or were discharged before postnatal day 14 (13 in Low, 11 in High) were excluded. Finally, data of 40 infants of Low and 30 of High were analyzed. The baseline patient characteristics, morbidity, medication and nutritional characteristics for these patients are presented in Table 1. Infant characteristics were well balanced between the groups Low and High and comparable to the original groups.

Nutritional intake

The nutritional characteristics and intake of calcium, phosphorus and protein during week 1 and 2 are presented in Table 1. The median and interquartile range (IQR) for the duration of PN was 12.0 (10.0–14.0) versus 11.0 (9.0–14.0) days for High versus Low, while the median day of reaching an enteral intake of 150 ml/kg was day 13.0 (10.5–20.0) versus day 12.0 (9.8–17.0), respectively. In accordance with the study protocol Low received a higher amount of human milk. The median start day of BMF in group High and Low was 7.9 (5.0–10.0) and 11.0 (11.0–13.0) respectively. As a result, High received a significant higher total intake of calcium and phosphorus during the first two weeks and of protein during week 2 compared to Low. Table A3 presents the nutritional intake divided into 4 routes of administrations: parenteral, enteral and additional supplementation either par- or enteral. This shows that differences in intake were mainly based on differences in enteral intake. Further, both groups received additional parenteral supplementation of phosphorus, based on low sP concentrations.

Figure 2 presents the daily changes in nutritional intake during the first 14 days. Figures 2A, 2B and 2D demonstrate the total calcium, phosphorus and protein intake. High had a steady increase in intake during the study period, whereas Low showed a temporary decrease, and plateau at the end of the observational period, probably due to the decreasing amount of PN and increasing amount of unfortified human...
CHAPTER 7 NUTRITIONAL INTAKE AND CALCIUM PHOSPHORUS HOMEOSTASIS

Figure 1 Consort diagram

Assessed for eligibility (n = 210 )
Excluded:
Not meeting inclusion criteria (n = 36)
Declined to participate (n = 65)
First Randomization ( n = 109)
Late Supplementation ( n = 72)
Early Nutrition Study ( ENS)
Second randomization

Early Supplementation ( n = 37)
Protocol Radboudumc

Group ENS A (n = 40)
Donormilk in addition to MOM
No enteral supplements until day10

Group ENS B (n = 32)
Preterm formula in addition to MOM
No enteral supplements until day10

Group C (n = 37)
Preterm formula in addition to MOM
Fortification of MOM if > 50 ml/day

Reallocation
> 80% MM → Low ( n = 13)
> 80% formula → High ( n = 4)

Low (n=53)
Intermediate (n = 15)
High (n = 41)
Excluded from analysis:
Death < day 14 ( n = 7)
Discharge < day 14 ( n = 6)

Low (n = 40)
Not used for analysis

Excluded from analysis:
Death < day 14 ( n = 3)
Discharge < day 14 ( n = 8)

High (n = 30)
MOM = mother’s own milk

Table 1 Patient characteristics, morbidity, medication, and nutritional characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low (n=40)</th>
<th>High (n=30)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight, grams; median (IQR)</td>
<td>948 (772 – 1225)</td>
<td>939 (776 – 1163)</td>
<td>0.85</td>
</tr>
<tr>
<td>&lt; 1000 gram, n (%)</td>
<td>22 (55.0)</td>
<td>16 (53.3)</td>
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<tr>
<td>Gestational age, median (IQR)</td>
<td>28.2 (26.5 – 29.6)</td>
<td>27.8 (26.1 – 29.4)</td>
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</tr>
<tr>
<td>SGA, n (%)</td>
<td>8 (20.0)</td>
<td>4 (13.3)</td>
<td>0.46</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>19 (47.5)</td>
<td>15 (50.0)</td>
<td>0.84</td>
</tr>
<tr>
<td>Singletons, n (%)</td>
<td>28 (70.0)</td>
<td>16 (53.3)</td>
<td>0.15</td>
</tr>
<tr>
<td>Cesarean section, n (%)</td>
<td>18 (45.0)</td>
<td>20 (66.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>Apgar score (5 min), median (IQR)</td>
<td>7.0 (6.3 – 9.0)</td>
<td>7.5 (7.0 – 8.0)</td>
<td>0.71</td>
</tr>
<tr>
<td>Mortality</td>
<td>10 (25.0%)</td>
<td>6 (20.0%)</td>
<td>0.62</td>
</tr>
<tr>
<td>Sepsis, n (%)</td>
<td>8 (20.0%)</td>
<td>9 (30.0%)</td>
<td>0.33</td>
</tr>
<tr>
<td>NEC ≥ stage 2, n (%)</td>
<td>1 (2.5%)</td>
<td>2 (6.7%)</td>
<td>0.39</td>
</tr>
<tr>
<td>IVH Grade 3 – 4 n (%)</td>
<td>5 (12.5)</td>
<td>3 (10)</td>
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<tr>
<td>Medication</td>
<td>39 (97.5%)</td>
<td>28 (93.3%)</td>
<td>0.39</td>
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<tr>
<td>Caffeine, n (%)</td>
<td>3 (7.5%)</td>
<td>4 (13.3%)</td>
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</tr>
<tr>
<td>Corticosteroids</td>
<td>3 (7.5)</td>
<td>1 (3.3)</td>
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</tr>
<tr>
<td>Sedation, n (%)</td>
<td>8 (20.0%)</td>
<td>9 (30.0%)</td>
<td>0.33</td>
</tr>
<tr>
<td>Nutritional characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN, days, median (IQR)</td>
<td>11.0 (9.0-14.0)</td>
<td>12.0 (10.0-14.0)</td>
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</tr>
<tr>
<td>150 ml/kg enteral, studyday, median (IQR)</td>
<td>12.0 (9.8 – 17.0)</td>
<td>13.0 (10.5 – 20.0)</td>
<td>0.59</td>
</tr>
<tr>
<td>Start day of BMF, median (IQR)</td>
<td>11 (11.0-13.0)</td>
<td>7.9 (5.0-10.0)</td>
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<tr>
<td>Human milk in ml/kg/day*, median (IQR)</td>
<td>50.9 (24.0 – 82.2)</td>
<td>30.0 (8.5 – 54.6)</td>
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<td>Formula in ml/kg/day*, median (IQR)</td>
<td>0.0 (0.0 – 0.2)</td>
<td>1.1 (0.1 – 7.3)</td>
<td>0.00</td>
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<td>Nutritional intake</td>
<td>11.0 (9.0-14.0)</td>
<td>12.0 (10.0-14.0)</td>
<td>0.10</td>
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<tr>
<td>150 ml/kg enteral, studyday, median (IQR)</td>
<td>12.0 (9.8 – 17.0)</td>
<td>13.0 (10.5 – 20.0)</td>
<td>0.59</td>
</tr>
<tr>
<td>Start day of BMF, median (IQR)</td>
<td>11 (11.0-13.0)</td>
<td>7.9 (5.0-10.0)</td>
<td>0.00</td>
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<tr>
<td>Human milk in ml/kg/day*, median (IQR)</td>
<td>50.9 (24.0 – 82.2)</td>
<td>30.0 (8.5 – 54.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>Formula in ml/kg/day*, median (IQR)</td>
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<td>1.1 (0.1 – 7.3)</td>
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<td>Calcium intake</td>
<td>10.7 (9.9-12.0)</td>
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<td>Phosphorus intake</td>
<td>16.4 (12.9-17.7)</td>
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<tr>
<td>Protein intake</td>
<td>10.8 (9.3-12.4)</td>
<td>12.3 (11.1-14.2)</td>
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<tr>
<td>Prot (total)</td>
<td>16.4 (12.9-19.6)</td>
<td>18.2 (16.0-22.1)</td>
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<tr>
<td>Prot (total)</td>
<td>18.6 (15.9-21.1)</td>
<td>20.0 (16.9-23.4)</td>
<td>0.16</td>
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<tr>
<td>Prot (total)</td>
<td>23.2 (21.0-26.6)</td>
<td>27.0 (24.1-30.6)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Low: no enteral supplementation of human milk before day 11; High: standard protocol: enteral supple-
mentation of human milk if intake was ≥ 50 ml/day; IQR: Interquartile range; SGA: small for gestational age: < p10; Sepsis: > 72 hrs postnatally and positive blood culture, prevalence within the first 14 days;
NEC: necrotizing enterocolitis according to Bell stage [36], prevalence within the first 14 days; IVH: In-
traventricular hemorrhage (grade according to Papile) [37]; PN: parenteral nutrition; BMF: breast milk fortifier;
* : during the intervention period; Ca: calcium; P: phosphorus; Prot: protein; W1: week 1; W2: week 2
Both groups showed a decrease in the calcium/phosphorus ratio on day 5 that lasted until day 11 (Figure 2C), most likely caused by the transition from parenteral nutrition to enteral nutrition. For both groups, the ratio was below the recommendations of ESPGHAN on all days. (38,39)

Biochemical parameters

Table A4 summarizes the median daily values of both groups for all biochemical parameters. The median serum concentrations of Ca and P were within the normal range and only showed slight differences between the two groups and an overall increase during the study period. (40) Except for the first day, the median sP concentrations remained below our target of 2 mmol/l until day 5 and 10 for High and Low respectively. The median uCa and uP values were above the recommended surplus (uCa >1.2 mmol/l, uP >0.4 mmol/l) during the entire observational period. (23) The median TRP values were above the lower normal range of 85% until day 5, and decreased thereafter, reflecting a higher loss of phosphorus. The median ALP values were within the normal range (80-330 U/l) until day 5, but increased steadily thereafter. (40)

In both groups, the uCa/Creat ratios were above the reference value (0.5 mmol/mmol) during the complete study period. (32) The uP/Creat ratios were below the reference value (4.0 mmol/mmol) until day 5, but above the reference thereafter. (32)

The results of the mixed model analyses are summarized in Table 2.

- The sCa concentration was not related to intake of Ca/P and was only marginally affected by a number of co-variables except for daily protein intake that caused an increase of 0.107 mmol/L per gram/kg protein.
- The sP concentration increased in relation to phosphorus intake (0.13 mmol/l per mmol/kg phosphorus) and birth weight (0.0004 mmol/l per gram birth weight), whereas protein intake (-0.13 mmol/l per gram/kg/day protein), gestational age (-0.05 mmol/l per week), furosemide (-0.11 mmol/l per day) and caffeine (-0.02 mmol/l per day) decreased in sP concentration.
- The urinary excretion of Ca seemed to increase in relation to Calcium intake (0.35 mmol/l per mmol/kg calcium), and increased in relation to protein (0.36 mmol/l per gram/kg protein) and being born by cesarean section (0.65 mmol/l if born by cesarean section), whereas it was not affected by the phosphorus intake.
- The urinary excretion of P increased in relation to daily phosphorus intake (3.18 mmol/l per mmol/kg phosphorus), gender (1.88 mmol/l if infant was a boy), whereas P excretion lowered in relation to daily intake of protein (-1.18 mmol/l per mmol/kg protein), gestational age (-0.71 mmol/l per week) and caffeine (-0.29 mmol/l per day). Calcium intake did not affect the urinary P excretion.
### Table 2: Mixed Model analysis: Effect of nutritional intake and clinical characteristics on biochemical parameter

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Covariates</th>
<th>Estimate</th>
<th>95% CI</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum Calcium</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>0.004</td>
<td>-0.046 – 0.054</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>-0.036</td>
<td>-0.073 – 0.002</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of P (%)</td>
<td>0.001</td>
<td>-0.000 – 0.001</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>0.107</td>
<td>0.075 – 0.139</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>0.027</td>
<td>0.013 – 0.042</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Singleton (yes)</td>
<td>0.081</td>
<td>0.021 – 0.140</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Sepsis (yes)</td>
<td>-0.092</td>
<td>-0.167 – 0.191</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Sedation (days)</td>
<td>-0.007</td>
<td>-0.016 – 0.001</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Serum Phosphorus</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>0.0345</td>
<td>-0.0473 – 0.1164</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>0.1252</td>
<td>0.0586 – 0.1918</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of Ca (%)</td>
<td>0.0035</td>
<td>0.0023 – 0.0048</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>-0.1274</td>
<td>-0.1825 – 0.0723</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Birth weight (grams)</td>
<td>0.0004</td>
<td>0.0002 – 0.0006</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>-0.0479</td>
<td>-0.0701 – 0.0258</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Singleton (yes)</td>
<td>0.0898</td>
<td>-0.1493 – 0.096</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Caffeine (days)</td>
<td>-0.0215</td>
<td>-0.0554 – 0.0075</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Furosemide (days)</td>
<td>-0.116</td>
<td>-0.2029 – 0.0203</td>
<td>0.02</td>
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<tr>
<td><strong>Urine Calcium</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>0.35</td>
<td>0.01 – 0.70</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>-0.01</td>
<td>-0.29 – 0.27</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of Ca (%)</td>
<td>-0.02</td>
<td>-0.02 – 0.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>0.36</td>
<td>0.12 – 0.61</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Cesarean section (yes)</td>
<td>0.65</td>
<td>0.32 – 0.98</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Urine Phosphorus</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>-0.05</td>
<td>-1.56 – 1.45</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>3.18</td>
<td>2.06 – 4.30</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of P (%)</td>
<td>0.07</td>
<td>0.04 – 0.09</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>-1.18</td>
<td>-2.20 – 0.16</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>-0.71</td>
<td>-1.09 – 0.33</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Singleton (yes)</td>
<td>1.88</td>
<td>0.26 – 3.50</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Caffeine (days)</td>
<td>-0.29</td>
<td>-0.54 – 0.01</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Tubular reabsorption of P</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>3.10</td>
<td>0.160 – 6.04</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>-6.21</td>
<td>-8.78 – 3.65</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of P (%)</td>
<td>-0.09</td>
<td>-0.15 – 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>3.05</td>
<td>1.92 – 4.17</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Gender (boy)</td>
<td>-4.60</td>
<td>-9.22 – 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Cesarean section (yes)</td>
<td>-5.12</td>
<td>-9.95 – 0.29</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Sepsis (yes)</td>
<td>-6.78</td>
<td>-12.72 – 0.85</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Furosemide (days)</td>
<td>4.75</td>
<td>0.53 – 10.03</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Alkaline Phosphatase</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>-44.94</td>
<td>-69.51 – 20.37</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>23.64</td>
<td>4.14 – 43.14</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of Ca (%)</td>
<td>2.07</td>
<td>1.69 – 2.45</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>30.54</td>
<td>14.08 – 47.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>-20.71</td>
<td>-30.37 – 11.05</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Urine Ca/Crea ratio</td>
<td>23.86</td>
<td>-44.29 – 34.3</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Urine Ca/Crea ratio</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>0.138</td>
<td>-0.292 – 0.568</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>0.139</td>
<td>-0.204 – 0.481</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of Ca (%)</td>
<td>0.023</td>
<td>-0.029 – 0.016</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>0.497</td>
<td>0.206 – 0.787</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Sepsis (yes)</td>
<td>0.584</td>
<td>0.003 – 1.166</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Urine P/Crea ratio</strong></td>
<td>Total intake of Ca (mmol/kg/day)</td>
<td>-1.10</td>
<td>-2.51 – 0.31</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Total intake of P (mmol/kg/day)</td>
<td>4.01</td>
<td>2.97 – 5.05</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Enteral intake of P (%)</td>
<td>0.06</td>
<td>0.04 – 0.08</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Intake of protein (grams/kg/day)</td>
<td>-0.81</td>
<td>-1.75 – 0.14</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Gestational age (weeks)</td>
<td>-0.94</td>
<td>-1.32 – 0.55</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Gender (boy)</td>
<td>2.31</td>
<td>0.72 – 3.89</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Sepsis (yes)</td>
<td>1.72</td>
<td>-0.24 – 3.68</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Caffeine (days)</td>
<td>-0.30</td>
<td>-0.56 – 0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

sCa: serum calcium (mmol/l); uCa: urine calcium (mmol/l); sP: serum phosphorus (mmol/l); uP: urine phosphorus (mmol/l); TrP: tubular reabsorption of phosphorus (%); ALP: Alkaline phosphatase (U/L); uCa/Crea ratio: urine calcium/creatinine ratio (mmol/mmol); uP/Crea ratio: urine phosphorus/creatinine ratio (mmol/mmol); 95% CI: 95% confidence interval; Co-variables initially included: daily nutritional intake of calcium, phosphorus, and protein, the enteral amount of calcium and phosphorus intake, caesarean section, multiple births, birth weight, gestational age, gender, necrotizing enterocolitis, sepsis, caffeine, furosemide, steroids and sedation.
• The TrP increased in relation to daily Calcium intake (3.10% per mmol/kg calcium), and gestational age (3.05% per week). The reabsorption of phosphorus lowered in relation to daily phosphorus intake (-6.21% per mmol/kg phosphorus), gender (-4.60% if infant was a boy), being born by cesarean section (-5.12%), and sepsis (-6.78%).
• The ALP increased in relation to protein intake (30.54 U/l per mmol/kg), daily intake of phosphorus (23.64 U/l per mmol/kg phosphorus). A decrease in ALP was related to calcium intake (-44.94 U/l per mmol/kg calcium), gestational age (-20.71 U/l per week) and the number of days of steroid use (-23.86 U/l per day).
• The uCa/creat ratio increased in relation to daily phosphate intake (0.54 l/l per gram/day protein) and sepsis (0.66 l/l), but it was not affected by the total calcium and phosphorus intake.
• The uP/creat ratio increased in relation to daily phosphorus intake (4.01 l/l per mmol/kg phosphorus), gender (2.31 l/l if infant was a boy), while the P/creat ratio seemed lower in relation to daily protein intake (-0.81 l/l per gram/kg protein), and decreased with gestational age (-0.94 l/l per week), and caffeine (-0.30 l/l per day).

Discussion

In this observational study of initially three randomized groups providing different nutritional intake to VLBW infants during the first 10 days of life, we found no differences between group Low and High concerning the biochemical parameters of Ca-P homeostasis. However, the mixed model analysis showed that the intake of calcium was associated with increased urinary calcium excretion and tubular reabsorption of phosphorus and a decrease in the ALP, while the nutritional intake of phosphorus was associated with a decreased sCa and an increase in sP, uP and uP/creat ratio. The nutritional intake of calcium and phosphorus affected the TrP and ALP in opposite directions. Protein intake was greatly associated with a decrease in sP, uP and an increase in ALP, sCa, and uCa, while in addition, gestational age and male gender affected especially the phosphorus metabolism.

VLBW infants belong to one of the most vulnerable patient groups for whom adequate postnatal nutritional intake has life-long consequences. Therefore intervention studies with different nutritional intakes could be seen as unethical in the light of the right of optimal treatment for every patient. On the other hand, in clinical practice a great variation in clinical guidelines has been reported, often based on rather low evidence. While fortification of human milk is generally seen as necessary nowadays, there is also concern about possible risks of introducing cow-milk based products too early. According to our local protocol, fortification is introduced early and additional mineral supplementation is provided based on laboratory results. The intention is to optimize postnatal growth and bone mineralization but the efficacy of our protocol has not been proven. The combination of the Early Nutrition Study and the Early Supplementation Study provided the opportunity to evaluate two different nutritional concepts within the range of nutritional guidelines and therefore within the ethical limits. On the other hand, all infants participating in the ESS, independent of group allocation, received the standard treatment according to the local practice, which frequently led to additional parental supplementation of nutrients in case of electrolyte disturbances or impaired growth. This practice may have ameliorated the differences between the groups and therefore affected the results. By reallocating infants from group ENS B to either group Low or High and excluding infants with intermediate intake from further analysis we tried to maximize the differences in nutritional intake between the two remaining groups. The relocation of infants did not change the baseline patient characteristics. The detailed analysis of nutritional intake showed that additional supplementation was not different between the groups and differences in intake were mainly based on enteral nutrition.

Even though the two groups had a maximum difference in nutritional intake, the comparison of daily concentrations of the biochemical parameters showed no differences between group High and Low, probably by leveling out inter-individual differences on group level. In contrast, the linear mixed model analysis took into account both intra- and inter-individual fluctuations, and thereby enabled us to specify effects of various co-variables.

Despite an increasing intake of phosphorus, sP remained below our target concentration during the first week. Recently, a randomized trial, evaluating nutritional support according to current recommendations in VLBW infants, observed hypophosphatemia in relation to high protein intake. Jamin et al observed electrolyte disturbances, especially hypophosphatemia and hypokalemia, in low-birth weight piglets with a high protein diet. Hypophosphatemia is the hallmark of the refeeding syndrome and a well-known complication in relation to parenteral nutrition of malnourished patients. Bonsante et al proposed the concept of ‘Placental Incompletely Restored Feeding (PI-Refeeding) syndrome for electrolyte disturbances found in VLBW infants. This syndrome is said to be caused by an imbalanced nutritional intake of amino acids and phosphorus. Amino acids and energy are needed to maintain an anabolic state of the cell, while phosphorus is necessary for a number of cellular functions, energy homeostasis as well as for bone mineralization. Phosphorus in blood will preferably be transferred to the cell regardless of bone mineral status. A higher intake of amino acids will enhance the need for phosphorus in growing cells, and in case of low concentrations of phosphorus in blood it will be released from bone. Simultaneously with the release of phosphorus, calcium will also be released from the bone because of an unfavorable Ca/P ratio and will consecutively...
be excreted in urine if the sP concentrations are too low. Our results are in agreement with this concept. According to the mixed model analyses we found that an increasing amount of protein was associated with an increase in the sCa, uCa, ALP and uCa/Creat ratio, whereas it was associated with a decrease in sP, uP and the uP/Creat ratio. Remarkably, in our study an increase in a sP concentration of 0.13 mmol/l occurred per 1 mmol/kg intake of phosphorus and a decrease of -0.13 mmol/l per 1 gram/kg protein intake, meaning that 1 gram/kg of protein intake should be accompanied by 1 mmol/kg of phosphorus in nutrition of VLBW infants to maintain adequate sP concentrations.

The role of ALP in bone mineralization is controversial, but an increase is usually associated with poor bone mineralization. (30,51) According to our results, an increasing intake of protein was associated with an increase in ALP. Again, following the above mentioned mechanisms higher protein intake enhanced the cellular need of phosphorus and thereby decreased the sP concentration and the availability of phosphorus for bone mineralization, leading to activation of ALP. We also found that an increased ALP was associated with increasing phosphorus intake, while one would expect lowering of ALP. An explanation for this phenomenon could be a relatively insufficient intake of calcium in combination with phosphorus intake, since an increasing calcium intake was associated with decrease in ALP concentrations. In this study, for both groups, the calcium/phosphorus ratio was below recommendations, meaning that relatively more phosphorus than calcium was administered.

Gestational age at birth seemed to be an important determinant for the phosphorus metabolism in our study, meaning that infants with a lower gestational age had a higher renal excretion of phosphorus, irrespective of nutritional intake. Immaturity of the kidneys at lower gestational age has been shown to cause impaired tubular reabsorption of phosphorus. (15) Renal losses of minerals may then compromise the effect of nutritional intake on bone mineralization. However, current recommendations for nutritional intake of calcium and phosphorus usually do not take into account differences in renal function based on gestational age.

Further, we found that male gender was related to low serum phosphorus concentrations, low tubular reabsorption and increased renal excretion of phosphorus and uP/creat ratio. We speculate a retardation in maturation of the renal function in male infants compared to females as is known for the development of the pulmonary function. (52)

All parameters evaluated in this study are regularly used to monitor either electrolyte homeostasis or bone mineralization. Practices among units vary greatly, measurements may be performed at later age and greater intervals and not standardized or in combination, leading to inconsistent results and handling. An explanation for the inconsistency in results of other studies could be the underestimation of the effects of inter-relationships between various co-variates. In our opinion these associations can only be discovered with standardized repeated measurements taking into account other clinical factors. To our knowledge this is the first study evaluating changes in biochemical parameters of the calcium-phosphorus homeostasis based on standardized repeated measurements and daily changes in nutritional intake in a mixed model linear regression analysis including also clinical factors.

Our data show that standardized repeated measurements of blood and urine samples can provide useful information with regard to the Ca-P homeostasis. This does not result in a clear advice for nutritional intake. Nevertheless, this study is a first step and its importance lies in the description and quantification of changes in a more ‘physiological way’ that will further enable us to develop new guidelines to improve bone mineral status in preterm infants. Notwithstanding, we confirmed the relationship between the intake of protein and phosphorus, and demonstrated the effect of renal immaturity and gender. Thus, a second step could be, to relate the current results to bone mineralization and provide recommendations for nutritional intake and a third step to develop a concept of target values for biochemical parameter so that these can be used to monitor nutritional intake to achieve optimal bone mineralization in daily practice.

This study had several limitations. The mixed model analysis assumes that the effects of the different variables are linear which has not been proven yet. In addition, the biochemical parameters may have been influenced by factors that were not taken into account in our analysis. Daily sampling of biochemical parameter would have been optimal, but this was judged unethical regarding the amount of blood volume needed. Nevertheless, measurements were performed in a standardized manner and therefore provided a good reflection of changes in blood and urine concentrations for the complete study period. Further, in comparison to other studies, both groups had relatively high daily intakes. This may partly explain the small variations in biochemical parameters. This study only investigated the biochemical parameters during the first 14 days of life. Maturational changes in renal function may alter the results; however repeated measurements will indicate these changes and thereby can be used as guide for optimal supplementation of minerals.

Conclusions

In conclusion, standardized repeated measurements showed that biochemical parameters of Ca-P homeostasis seemed to be affected by nutritional intake of calcium and phosphorus as well as protein, while immaturity of kidneys was related to an increase in urinary excretion of minerals irrespective of nutritional intake. Further studies are needed to define target values to stabilize electrolyte balances and
improve bone mineralization taking into account nutritional intake and gestational age of the patient.

Acknowledgments
All authors gratefully thank W.R.J.C. Jansen, research nurse of the Pediatric Drug Research Center Radboudumc, for the dedicated support in patient recruitment and data collection as well as Dr. T.A.J. Antonius, neonatologist at Radboudumc, for the development of the algorithm to calculate the nutritional intakes.

### Table A1

<table>
<thead>
<tr>
<th>Standard parenteral nutritional intake</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid ml/kg/day</td>
<td>80</td>
<td>100</td>
<td>125</td>
<td>150</td>
</tr>
<tr>
<td>CH grams/kg/d</td>
<td>8</td>
<td>9.6</td>
<td>11.7</td>
<td>13.8</td>
</tr>
<tr>
<td>AA grams/kg/d</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3</td>
</tr>
<tr>
<td>Lipids grams/kg/day</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>EQ Kcal/kg/day</td>
<td>44</td>
<td>62</td>
<td>82</td>
<td>94</td>
</tr>
<tr>
<td>Calcium mmol/kg/day</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3.00</td>
</tr>
<tr>
<td>Phosphorus mmol/kg/day</td>
<td>0.48</td>
<td>0.96</td>
<td>1.44</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Parenteral nutritional intake based on standardized parenteral solutions. For infants below 1000 grams amino acids were additionally added according to current recommendations. Amino acid solution: Primene (Baxter, the Netherlands); Lipid emulsion including vitamins: Clinoleic (20%; Baxter, The Netherlands) or SMOFlipid 20% (Fresenius Kabi; The Netherlands); CH: carbohydrates, AA: amino acids, EQ: energy quotient.

### Table A2

<table>
<thead>
<tr>
<th>Cohort characteristics of all patients included in the Early Supplementation Study</th>
<th>Group ENS A (n = 40)</th>
<th>Group ENS B (n = 32)</th>
<th>Group C (n = 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA, weeks; med (IQR)</td>
<td>28.2 (27.0-30.1)</td>
<td>28.3 (26.5-30.7)</td>
<td>27.9 (26.1-29.7)</td>
</tr>
<tr>
<td>Birth weight, grams; med (IQR)</td>
<td>967 (753-1245)</td>
<td>1012 (847-1199)</td>
<td>1006 (771-1220)</td>
</tr>
<tr>
<td>SGA; n (%)</td>
<td>9 (23)</td>
<td>8 (25)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Male; n (%)</td>
<td>21 (53)</td>
<td>20 (63)</td>
<td>18 (49)</td>
</tr>
<tr>
<td>Singletons; n (%)</td>
<td>25 (63)</td>
<td>24 (74)</td>
<td>25 (68)</td>
</tr>
<tr>
<td>Antenatal Steroids compl.; n (%)</td>
<td>36 (90)</td>
<td>31 (97)</td>
<td>31 (86)</td>
</tr>
<tr>
<td>Cesarean section; n (%)</td>
<td>19 (48)</td>
<td>20 (63)</td>
<td>25 (68)</td>
</tr>
<tr>
<td>Appar score (5 min); med (IQR)</td>
<td>7.5 (6.3-9.0)</td>
<td>8.0 (7.0-9.0)</td>
<td>7.0 (7.0-8.0)</td>
</tr>
<tr>
<td>Appar score (5min) &lt; 7; n (%)</td>
<td>10 (25)</td>
<td>5 (16)</td>
<td>8 (22)</td>
</tr>
<tr>
<td>Mortality; n (%)</td>
<td>6 (15)</td>
<td>3 (9)</td>
<td>7 (19)</td>
</tr>
</tbody>
</table>

#### Morbidity

| IRDS (Days of MV; med (IQR))                                                      | 24 (60)              | 19 (59)              | 23 (62)           |
| Days of N-CPAP; med (IQR)                                                         | 18.0 (6.5-38.8)      | 28.0 (7.0-40.8)      | 16.0 (6.0-36.5)   |
| CLD; n (%)                                                                        | 12 (30)              | 14 (44)              | 10 (27)           |
| PDA; n (%)                                                                        | 20 (50)              | 20 (63)              | 21 (57)           |
| Ductal ligation; n (%)                                                            | 4 (10)               | 2 (6.3)              | 1 (3)             |
| I/VH grade ≤ 2; n (%)                                                             | 15 (38)              | 5 (16)               | 5 (14)            |
| I/VH grade 3; n (%)                                                               | 2 (5)                | 7 (21)               | 4 (11)            |
| Sepsis; n (%)                                                                     | 13 (33)              | 10 (32)              | 14 (38)           |
| NEC; n (%)                                                                        | 4 (10)               | 5 (16)               | 3 (8)             |
| Bell stage 2; n                                                                   | 2                    | 3                    | 1                 |
| Bell stage 3; n                                                                   | 2                    | 2                    | 2                 |
| Laparotomy; n                                                                     | 2                    | 1                    | 2                 |
| ROP; n (%)                                                                        | 4 (10)               | 1 (3)                | 5 (14)            |
| ROP grade ³ 3                                                                    | 1                    | 0                    | 1                 |

#### Medication

| Caffeine; n (%)                                                                   | 38 (95)              | 30 (94)              | 33 (90)           |
| Furosemide; n (%)                                                                 | 11 (28)              | 10 (31)              | 7 (19)            |
| Diuretics (maintenance); n (%)                                                    | 3 (8)                | 0                    | 3 (8)             |
| Corticosteroids; n (%)                                                            | 1 (3)                | 2 (6)                | 4 (11)            |
| Sedation; n (%)                                                                   | 13 (33)              | 11 (34)              | 15 (41)           |

#### Nutritional characteristics

| Days of PN; med (IQR)                                                             | 10.0 (8.0-13.0)      | 10.5 (9.0-14.8)      | 10.5 (8.3-21.0)   |
| 120 ml/kg enteral, day; med (IQR)                                                 | 9.0 (7.0-12.5)       | 9.0 (8.0-13.0)       | 9.0 (7.2-14.8)    |
| 150 ml/kg enteral, day; med (IQR)                                                 | 12.0 (9.0-17.0)      | 11.0 (10.0-17.0)     | 12.0 (10.0-20.0)  |
| Start day of BMF; med (IQR)                                                       | 11.0 (11.0-12.7)     | 12.0 (11.0-14.0)     | 6.0 (4.0-8.0)     |

ENS A: donor milk in addition to mother’s own milk (MOM) and no supplements with enteral feeding until day 10; ENS B: preterm formula in addition to MOM and no supplements with enteral feeding until day 10; Group C: preterm formula in addition to MOM and fortifier if intake ≥ 50 ml/day; med, median; IQR, inter quartile range; GA: gestational age; SGA: small for gestational age according to Fenton et al; IRDS: infant respiratory distress syndrome; MV: mechanical ventilation; N-CPAP: nasal continuous positive airway pressure; CLD: chronic lung disease defined as oxygen dependency at 36 weeks gestational age; PDA: patent ductus arteriosus with need for treatment; I/VH: intra-ventricular hemorrhage; Sepsis: > 72 hrs postnatally and positive blood culture; NEC: necrotizing enterocolitis with staging according to Bell; ROP: retinopathy of prematurity; sedation: morfine and/or midazolam > 24 hrs; PN, parenteral nutrition; BMF: breast milk fortifier.
## NUTRITIONAL INTAKE AND CALCIUM-PHOSPHORUS HOMEOSTASIS

### Table A3: Nutritional intake of calcium and phosphorus by route of administration

<table>
<thead>
<tr>
<th>Nutritional Intake</th>
<th>Low (n = 40)</th>
<th>High (n = 30)</th>
<th>p - Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td>Ca (total) W1</td>
<td>10.7 (9.9 – 12.0)</td>
<td>13.1 (11.1 – 14.6)</td>
<td>0.00</td>
</tr>
<tr>
<td>Ca (total) W2</td>
<td>16.4 (12.9 – 17.7)</td>
<td>21.7 (15.3 – 24.4)</td>
<td>0.00</td>
</tr>
<tr>
<td>Ca (enteral) W1</td>
<td>1.7 (1.1 – 2.2)</td>
<td>3.3 (1.1 – 5.7)</td>
<td>0.00</td>
</tr>
<tr>
<td>Ca (enteral) W2</td>
<td>10.8 (6.2 – 16.0)</td>
<td>17.5 (2.3 – 22.8)</td>
<td>0.07</td>
</tr>
<tr>
<td>Ca (enteral suppl) W1</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 0.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca (enteral suppl) W2</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.22</td>
</tr>
<tr>
<td>Ca (PN) W1</td>
<td>9.4 (8.0 – 10.2)</td>
<td>9.8 (7.9 – 11.5)</td>
<td>0.34</td>
</tr>
<tr>
<td>Ca (PN) W2</td>
<td>3.1 (0.7 – 7.9)</td>
<td>5.1 (1.9 – 10.9)</td>
<td>0.14</td>
</tr>
<tr>
<td>Ca (PN suppl) W1</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 0.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca (PN suppl) W2</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.74</td>
</tr>
<tr>
<td>P (total) W1</td>
<td>10.8 (9.2 – 12.4)</td>
<td>12.3 (11.1 – 14.2)</td>
<td>0.00</td>
</tr>
<tr>
<td>P (total) W2</td>
<td>16.4 (12.9 – 19.6)</td>
<td>18.9 (16.0 – 22.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>P (enteral) W1</td>
<td>1.0 (0.6 – 1.4)</td>
<td>2.1 (0.7 – 3.9)</td>
<td>0.00</td>
</tr>
<tr>
<td>P (enteral) W2</td>
<td>8.1 (5.0 – 12.0)</td>
<td>10.4 (1.7 – 17.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>P (enteral suppl) W1</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 0.9)</td>
<td>0.03</td>
</tr>
<tr>
<td>P (enteral suppl) W2</td>
<td>0.0 (0.0 – 0.0)</td>
<td>0.0 (0.0 – 1.4)</td>
<td>0.33</td>
</tr>
<tr>
<td>P (PN) W1</td>
<td>7.5 (6.4 – 8.2)</td>
<td>7.8 (6.3 – 9.2)</td>
<td>0.34</td>
</tr>
<tr>
<td>P (PN) W2</td>
<td>2.5 (0.6 – 6.3)</td>
<td>4.1 (1.5 – 8.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>P (PN suppl) W1</td>
<td>2.4 (0.5 – 3.6)</td>
<td>1.8 (0.0 – 2.7)</td>
<td>0.16</td>
</tr>
<tr>
<td>P (PN suppl) W2</td>
<td>2.3 (0.0 – 4.2)</td>
<td>0.7 (0.0 – 4.3)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Low: no enteral supplementation of human milk before day 1; High: standard protocol: enteral supplementation of human milk if intake was ≥ 50 ml/day; IQR: inter quartile range; Ca: calcium; P: phosphorus; total: som of all nutritional intake; enteral: enteral intake including standard fortification; enteral suppl: additional enteral supplementation; PN: parenteral intake; PN suppl: additional parenteral supplementation; W1: week 1; W2: week 2
### Table A4: Daily Measurements of Biochemical Parameters of Calcium and Phosphorus Homeostasis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum Ca (mmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>2.2 (2.0 - 2.4)</td>
<td>2.4 (2.2 - 2.5)</td>
<td>2.5 (2.4 - 2.7)</td>
<td>2.6 (2.4 - 2.7)</td>
<td>2.5 (2.4 - 2.7)</td>
<td>2.6 (2.4 - 2.8)</td>
</tr>
<tr>
<td>- High</td>
<td>2.2 (2.0 - 2.4)</td>
<td>2.4 (2.3 - 2.6)</td>
<td>2.5 (2.4 - 2.7)</td>
<td>2.4 (2.3 - 2.6)</td>
<td>2.5 (2.3 - 2.7)</td>
<td>2.6 (2.5 - 2.8)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.94</td>
<td>0.17</td>
<td>0.34</td>
<td>0.15</td>
<td>0.69</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Urine Ca (mmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>1.3 (1.0 - 1.9)</td>
<td>2.1 (1.5 - 3.4)</td>
<td>2.6 (2.0 - 3.9)</td>
<td>2.7 (1.6 - 3.8)</td>
<td>2.0 (1.6 - 3.3)</td>
<td>1.8 (1.4 - 3.5)</td>
</tr>
<tr>
<td>- High</td>
<td>1.5 (1.2 - 1.7)</td>
<td>3.3 (2.0 - 4.7)</td>
<td>3.1 (2.3 - 5.8)</td>
<td>2.5 (1.8 - 3.8)</td>
<td>2.7 (2.0 - 3.4)</td>
<td>2.3 (1.6 - 3.4)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.66</td>
<td>0.03</td>
<td>0.06</td>
<td>0.84</td>
<td>0.21</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Serum P (mmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>1.8 (1.7 - 2.2)</td>
<td>1.8 (1.5 - 2.0)</td>
<td>1.7 (1.5 - 2.0)</td>
<td>1.9 (1.8 - 2.2)</td>
<td>2.0 (1.8 - 2.2)</td>
<td>2.3 (2.1 - 2.4)</td>
</tr>
<tr>
<td>- High</td>
<td>2.1 (1.8 - 2.3)</td>
<td>1.8 (1.5 - 2.0)</td>
<td>1.6 (1.3 - 2.2)</td>
<td>2.1 (1.8 - 2.4)</td>
<td>2.1 (2.0 - 2.4)</td>
<td>2.2 (2.0 - 2.3)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.22</td>
<td>0.88</td>
<td>0.71</td>
<td>0.21</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Urine P (mmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>1.7 (0.3 - 3.8)</td>
<td>2.1 (0.7 - 5.5)</td>
<td>1.3 (0.7 - 3.5)</td>
<td>4.1 (0.7 - 6.8)</td>
<td>5.4 (2.2 - 8.2)</td>
<td>10.4 (6.8 - 18.4)</td>
</tr>
<tr>
<td>- High</td>
<td>2.3 (0.2 - 4.6)</td>
<td>2.6 (0.9 - 4.1)</td>
<td>3.1 (1.6 - 6.9)</td>
<td>5.4 (3.5 - 9.8)</td>
<td>7.4 (4.5 - 13.7)</td>
<td>9.5 (5.5 - 16.4)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.68</td>
<td>0.98</td>
<td>0.04</td>
<td>0.02</td>
<td>0.04</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>Tubular reabsorption of P (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>94.0 (76.8 - 98.2)</td>
<td>86.5 (76.1 - 95.3)</td>
<td>92.2 (83.8 - 96.9)</td>
<td>83.1 (72.4 - 96.3)</td>
<td>80.8 (68.8 - 92.7)</td>
<td>75.9 (64.9 - 85.2)</td>
</tr>
<tr>
<td>- High</td>
<td>85.3 (76.4 - 98.1)</td>
<td>92.7 (73.3 - 95.7)</td>
<td>88.1 (80.8 - 95.1)</td>
<td>79.4 (51.9 - 87.6)</td>
<td>76.6 (59.1 - 83.2)</td>
<td>67.4 (53.9 - 82.4)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.59</td>
<td>0.62</td>
<td>0.21</td>
<td>0.08</td>
<td>0.23</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>Alkaline Phosphatase (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>167.0 (138.0 - 236.0)</td>
<td>213.0 (182.0 - 291.0)</td>
<td>263.5 (202.3 - 368.5)</td>
<td>329 (261.3 - 455.0)</td>
<td>379 (311.0 - 503.0)</td>
<td>423.0 (301.8 - 506.0)</td>
</tr>
<tr>
<td>- High</td>
<td>203 (146.5 - 232.8)</td>
<td>244.0 (174.3 - 270.8)</td>
<td>275.5 (206.3 - 307.0)</td>
<td>304.5 (249.3 - 375.0)</td>
<td>342.0 (213.8 - 414.3)</td>
<td>380.0 (281.8 - 509.8)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.76</td>
<td>0.94</td>
<td>0.76</td>
<td>0.22</td>
<td>0.10</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>uCa/Crea ratio (mmol/mmol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>2.1 (1.3 - 3.5)</td>
<td>2.5 (1.8 - 3.1)</td>
<td>3.6 (2.3 - 5.2)</td>
<td>3.8 (2.3 - 6.2)</td>
<td>2.9 (1.8 - 4.1)</td>
<td>2.3 (1.6 - 3.7)</td>
</tr>
<tr>
<td>- High</td>
<td>1.7 (1.4 - 3.7)</td>
<td>2.7 (2.0 - 6.1)</td>
<td>4.0 (2.9 - 6.3)</td>
<td>3.0 (2.1 - 5.4)</td>
<td>3.8 (2.4 - 5.0)</td>
<td>3.4 (1.9 - 4.8)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.98</td>
<td>0.30</td>
<td>0.35</td>
<td>0.49</td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>uP/Crea ratio (mmol/mmol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Low</td>
<td>1.6 (0.5 - 5.9)</td>
<td>2.8 (1.0 - 6.8)</td>
<td>1.9 (0.8 - 5.4)</td>
<td>5.1 (1.1 - 9.3)</td>
<td>7.2 (2.5 - 12.3)</td>
<td>11.2 (6.2 - 18.6)</td>
</tr>
<tr>
<td>- High</td>
<td>3.8 (0.5 - 6.7)</td>
<td>1.8 (1.1 - 5.8)</td>
<td>3.5 (1.6 - 6.1)</td>
<td>7.7 (5.0 - 10.9)</td>
<td>9.4 (7.0 - 16.5)</td>
<td>14.5 (9.3 - 18.7)</td>
</tr>
<tr>
<td><strong>p-Value</strong></td>
<td>0.64</td>
<td>0.72</td>
<td>0.15</td>
<td>0.14</td>
<td>0.06</td>
<td>0.73</td>
</tr>
</tbody>
</table>

All data are presented as median and interquartile range; sCa: serum calcium (mmol/l); uCa: urine calcium (mmol/l); sP: serum phosphorus (mmol/l); uP: urine phosphorus (mmol/l); TrP: tubular reabsorption of phosphorus (%); ALP: Alkaline phosphatase (U/L); uCa/Crea ratio: urine calcium/creatinine ratio (mmol/mmol); uP/Crea ratio (mmol/mmol).
References


CHAPTER 8

Abstract

Preterm infants often have a reduced bone mineral content (BMC) with increased risk of metabolic bone disease. After birth it is difficult to supply calcium (Ca) and phosphorus (P) comparable to the high fetal accretion rate. It is not known whether high supplementation of minerals in the early postnatal period improves growth and bone mineralization. The aim of this study was to evaluate growth and bone mineralization at term corrected age (TCA) in very and extremely preterm infants who received different enteral Ca and P intakes during the first 10 days of life. Infants (n = 109) with birth weights below 1500 g were randomly assigned to one of three groups that differed in the nutritional protocols delivered until day 10: Group A, mother’s own milk (MOM) and donor milk (unfortified); Group B, MOM (unfortified) and preterm formula; Group C, MOM (start fortification >50 mL/day) and preterm formula. Due to the earlier commencement of fortification, Group C received higher intakes of calcium and phosphorus and protein (p < 0.001) until day 10. At TCA weight, length, BMC and bone mineral density (BMD), measured by dual-X-ray absorptiometry, were not different between the groups. Nutritional intake of P was positively associated with length (β; (95% confidence interval (CI)): 0.20 (0.001; 0.393); p-value = 0.048), whereas Ca intake was negatively associated with BMC (−1.94 (−2.78; −1.09); p-value < 0.001). A small interaction between Ca and P intake was only found for BMD (0.003 (0.00002; 0.00006); p-value = 0.036). The volume of human milk per kg provided during the first 10 days was positively associated with BMC (β; (95% CI): 0.013 (0.002; 0.023); p < 0.017). Higher intakes of Ca and P during the first 10 days, as provided in this study, did not improve bone mineralization at term corrected age.

Introduction

Very and extremely preterm infants are known to have a reduced bone mineral content (BMC) with increased risk of development of metabolic bone disease (MBD). There are numerous reasons for impaired bone development in preterm infants, but an adequate supply of substrates of calcium (Ca) and phosphorus (P) is a prerequisite for normal bone mineral accretion, whereas vitamin D is essential for the adequate regulation of the mineral homeostasis and bone mineralization. (5,7) Up to 80% of the body Ca of a term infant is accrued during the last trimester of pregnancy. (7,8) Infants born preterm miss this active foetal mineralization in the last trimester, and instead are reliant on supplementation of minerals, provided through parenteral and enteral sources. (9,10) In clinical practice, it is difficult to meet the high foetal needs after preterm birth. Parenteral fluids have a limited solubility for high amounts of Ca and P, whereas human milk has low contents of calcium (Ca) and phosphorus (P) and formula feeding has been shown to have an impaired intestinal absorption of minerals. (9,11,12)

Nowadays, it is accepted that early enteral nutrition, and especially human milk (HM), has beneficial health effects. Enrichment of HM with human milk fortifiers (HMF) for preterm infants is the standard of care. (13) However, there is uncertainty with regard to the method of fortification of human milk. The timing and amount of mineral supplementation vary greatly, resulting in varying international practices. (14) Supplementation of Ca and P is often delayed because of fear of nephrocalcinosis, feeding intolerance and necrotizing enterocolitis. (15,16) Early mineral supplementation of human milk at low volumes of enteral intake accelerates the amount of enteral intake, decreases the duration of parenteral nutrition, and may support postnatal growth and bone mineralization in the early postnatal period, whereas delay of fortification may lead to insufficient mineral intake and consecutively impaired bone mineralization. Whether early postnatal high mineral intake will improve bone mineralization has not been evaluated.

The aim of this study was to evaluate bone mineralization and growth at term corrected age (TCA) in very and extreme preterm infants who received either unfortified human milk, preterm formula, or early fortified human milk during the first 10 days of life. We hypothesized that a higher mineral intake would lead to a higher weight and length as well as improved bone mineralization at term corrected age.
Materials and Methods

Study Design and Randomization
This study (Early Supplementation Study (ESS)) was part of a larger multi-center double-blinded randomized controlled trial: the Early Nutrition Study (ENS). The ENS evaluated the effects of human milk on postnatal mortality and morbidity, while the ESS evaluated bone mineralization and growth in relation to the timing of mineral supplementation. The studies were approved by the Ethical Committee of the VU University Medical Center, (Amsterdam, The Netherlands) 23 November 2012 (CMO dossier number: NL37296.029.11, Netherlands Trial Registry: NTR 3225). Participants were assigned into one of three groups through two steps of randomization, based on stratification according to birth weight, below or above 1000 g, and appropriate or small for gestational age status. First, infants were randomized into either late mineral and protein supplementation, as part of the ENS (Group A and B), or early supplementation, as part of the ESS (Group C). The second step was only performed if infants were randomized to the late supplementation group. This step randomized infants to either Group A (mother’s own milk (MOM) and/or donor milk) or Group B (MOM and/or preterm formula). Both randomization steps were performed before the first enteral nutrition was administered.

Study Population
Infants were recruited at the level III neonatal intensive care unit of the Radboud University Medical Center (Radboudumc), Nijmegen, Netherlands. Preterm infants, with a birth weight below 1500 g, were eligible for inclusion, if both parents had given written informed consent before the first enteral feeding. Exclusion criteria were congenital malformations, congenital infection proven within 72 h after birth, perinatal asphyxia with a pH <7.0, maternal drugs and/or alcohol use during pregnancy and any intake of cow’s milk based products prior to randomization.

Intervention and Nutritional Protocol
The nutritional protocol and intervention have previously been described. All infants received parenteral nutrition (PN), according to the standard institutional protocol. PN was started directly within the first hour after birth and consisted of standard components with 2.5 mmol/dL calcium gluconate (calcium gluconate 10%; B. Braun, Melsungen, Germany) and 1.6 mmol/dL sodium-glycerophosphate (Glycophos; Fresenius Kabi BV, Zeist, The Netherlands). Table A1 presents the standard protocol for PN. Additional parenteral mineral supplementation with 10% calcium gluconate or sodium-glycerophosphate was administered, according to the discretion of the attending neonatologist, based on blood and urine chemistry.

Enteral feeding, according to group allocation, was started within several hours after birth, with daily increments, while PN was gradually reduced, to maintain daily fluid intake within the protocol range. Where possible, MOM was used for enteral nutrition. If MOM was not available, Group A received donor milk and Group B and C received formula. Preterm formula (Hero Baby Prematuar Start; Hero Kindervoeding, Breda, The Netherlands) contained 2.40 mmol/dL Ca, 1.70 mmol/dL P and 2.6 g/dL proteins. Groups A and B started fortification of human milk or other enteral enrichment only after day 10. For both groups the additional nutritional intake was blinded to all caretakers and parents. Group C received enteral nutrition from day 1 onwards, according to the local protocol. This group received additional enteral supplementation and human milk fortifier (HMF) by the time the enteral intake was 50 mL per day. (Nutrilon Neonatal BMF; Nutricia, Zoetermeer, The Netherlands) The HMF added 1.65 mmol/dL Ca, 1.22 mmol/dL P and 0.8 g/dL protein. Additional enteral supplementation could comprise of either a supplement of protein (Nutrilon Nanenpatal Fortifier; Nutricia, Zoetermeer, The Netherlands) or a potassium phosphate (KPO4) and calcium chloride (CaCl2) suspension for enteral supplementation. The decision to start additional enteral supplementation was made by the attending neonatologist and according to the department’s protocol, based on biochemical parameters and postnatal growth.

All infants received vitamin D with parenteral nutrition (80 IE/kg/day) directly after birth. Enteral supplementation was 600 IE (15 micrograms) per day for infants with a weight below 1250 g and 400 IE (10 micrograms) per day for all infants with a weight above 1250 g. Human milk fortifier and preterm formula added 200 IE (5 micrograms)/dL vitamin D; thus, infants received, in total, between 600 and 1000 IE vitamin D per day. According to the local protocol (Group C), vitamin D supplementation was started by the day human milk fortification was started. For Groups A and B, enteral vitamin D supplementation was started by day 8 in combination with vitamin K supplementation, according to the national Dutch recommendations.

After 10 days, all infants received nutrition, according to the standard protocol of the Radboudumc, as described above. Around term corrected age (±6 weeks), all surviving participants were invited for an outpatient visit and scheduled for a dual energy X-ray absorptiometry (DXA).

Outcome Measures
All outcome measures were taken up to term corrected age (TCA). Primary outcome measures were bone mineralization and growth. Bone mineralization was measured by dual energy X-ray absorptiometry (DXA), using a whole-body fan beam scanner (Hologic Discovery 85606, software APEX 3.3, Hologic, Vilvoorde, Belgium). Bone mineral content (BMC), bone mineral density (BMD), lean body mass (LBM) and fat mass were determined. Scans showing movement artifacts were classified as
unacceptable. Weight and length were determined from the first week onwards, at least weekly, until discharge. Weight was determined using an electronic scale to the nearest 1 g. Crown–heel length was measured to the nearest 5 mm. For participants who had already been transferred, the anthropometric data at TCA were collected from the local hospitals.

Data Registration and Handling
Patient characteristics, clinical course, growth and intake of all nutrients were recorded from the patient records and extracted for this study—daily during the first 14 days and weekly until discharge from the department. After discharge, anthropometric data at TCA were collected from local hospitals. The amounts of enteral, parenteral and additional supplementation (parenteral and enteral) of all nutrients were calculated separately for each patient. For this study, the nutritional intake from the first 10 days was calculated, because this period comprised the intervention period with the maximum difference in nutritional intake. The total intakes were calculated per kg per day for each infant. The intake of nutrients with human milk was calculated using the reference from Gidrewicz et al. (19) Postnatal growth was evaluated using standard deviation scores (SDS) for weight and length, based on the revised reference chart for preterm infants by Fenton and Kim. (20) Infants with a birth weight below the 10th percentile were classified to be small for gestational age (SGA).

Statistical Analysis
The primary objective of the ESS was to examine whether bone mineralization at TCA differed by type of mineral supplementation at term corrected age (TCA). We performed a power calculation before the enrollment of participants started. We anticipated that a higher intake of minerals would result in a BMC that would, on average, be 5 g higher. Lagemaat et al. found a variability in BMC of 12 g at TCA [21]. Based on two-sided testing with α = 0.05 and β = 0.80, 65 infants per group were required. The statistical analyses were performed using SPSS 22 for Windows (IBM SPSS INC., Chicago, IL, USA). Differences in nutritional characteristics, anthropometric data at TCA and DXA scan measurements were detected using the one-way ANOVA or Kruskal–Wallis test, as appropriate.

The outcomes of interest were two DXA scan measurements, (i.e., BMC and BMD) as well as weight and length. We used Generalized Estimating Equations with an independent correlation structure and robust standard errors to account for the correlation between twins. (22) The stratification factors of birth weight and SGA status as well as gestational age at the time of measurement were included in all analyses. For the primary analysis, separate linear regression models were fitted to examine the association between ESS group and the four outcomes of interest, with Group C being the reference. Continuous predictor variables were centered at their respective means for the analysis. A set of secondary analyses examined the associations between each of the main nutritional variables (i.e., intake of P, Ca, protein per kg per 10 days) and outcomes. An interaction term was included for Ca and P. Finally, the analysis also examined associations between the amount of human milk and the outcomes of interest.

Results

Patient Characteristics
Patients were enrolled between January 2013 and December 2014. The ENS trial was closed when the required number of infants was included nationwide, forcing us to stop the ESS before the anticipated number of patients was included. The distribution of patients and the exact numbers of measurements are presented in the consort diagram (Figure 1). A total of 109 infants were randomized to either early supplementation (Group C, n = 37) or to late supplementation and distributed into Groups A (n = 40) and B (n = 32). All surviving infants (Group A, n = 34, Group B, n = 29, Group C, n = 30) were included in the growth analyses, but only subsets of those were included in the DXA analyses, either because of parent refusal to attend the follow-up visit, or disapproval of the DXA scan as a result of unacceptable movement artifacts. The baseline characteristics of all patients included and the morbidities and relevant medications of patients who survived to TCA are shown in Table 1. The baseline characteristics, frequencies of morbidities and treatments were comparable between the three groups.

Nutritional Intake
The nutritional characteristics for the first 10 days are presented in Table 2. The number of days with parenteral nutrition and the time to achieve full enteral feeding were not different between the three groups. In agreement with the study protocol, Group C started HMF earlier, at a median (IQR) of day 6.0 (4.0–7.0) compared to Group A (day 11.5 (11.0–12.5)) and Group B (day 12.0 (11.0–13.5)) (p-value < 0.001). As a result of the study protocol, Group C received significantly higher mean (SD) intakes of both Ca (Group C: 21.5 (4.8) versus Group A: 15.7 (2.6) and Group B: 17.1 (4.0) mmol/kg per 10 days) and P (Group C: 21.6 (3.7) versus Group A: 16.5 (3.1) and Group B: 16.5 (3.0) mmol/kg per 10 days) during the first 10 days (p-value < 0.001). Furthermore, Group C received significantly higher mean (SD) intakes of protein (Group C: 32.1 (5.5) versus Group A: 26.8 (5.0) and Group B: 27.5 (4.3) g/kg per 10 days; p-value < 0.001) and carbohydrates (Group C: 14.5 versus Group A: 109.1 (10.7) and Group B: 103.6 (11.5) g/kg per 10 days; p-value = 0.02) According to the study protocol, Group A received a significantly higher percentage of human milk during the first 10 days, compared to
Figure 1: Consort diagram

MOM: mother’s own milk; PFM: preterm formula milk; TCA: term corrected age; DXA: dual energy X-ray absorptiometry scan

Assessed for eligibility (n = 210)
Excluded:
- Not meeting criteria (n = 30)
- Declined to participate (n = 65)
- Twin > 1500 gram birth weight (n = 6)
Randomized (n = 109)
- Late Supplementation (n = 72)
  - Early Nutrition Study (ENS)
    - Group ENS A (n = 40)
      - Donor milk in addition to MOM
        - Fortification HM > day 10
    - Group ENS B (n = 32)
      - PFM in addition to MOM
        - Fortification HM > day 10
Outcome at TCA
1: length (n = 31); weight (n = 34)
2: DXA (n = 14; failed n = 7)
Lost to follow up:
- Death (n = 6)
- No show (n = 13)

2nd Randomization
- Group C (n = 37)
  - PFM in addition to MOM
    - Fortification HM > 50 ml/day
Outcome at TCA
1: length (n = 27); weight (n = 29)
Analysis 2: DXA (n = 12; failed n = 7)
Lost to follow up:
- Death (n = 3)
- No show (n = 10)

Table 1: Patient characteristics, morbidity, medication

<table>
<thead>
<tr>
<th>Characteristics (All Infants)</th>
<th>Group A (n = 40)</th>
<th>Group B (n = 32)</th>
<th>Group C (n = 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age, weeks; mean (SD)</td>
<td>28.3 (2.6)</td>
<td>28.4 (2.6)</td>
<td>28.1 (2.4)</td>
</tr>
<tr>
<td>Birth weight; grams; mean (SD)</td>
<td>1002 (275)</td>
<td>1012 (219)</td>
<td>1006 (265)</td>
</tr>
<tr>
<td>Small for gestational age; n (%)</td>
<td>8 (20)</td>
<td>8 (25)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Male; n (%)</td>
<td>21 (53)</td>
<td>20 (63)</td>
<td>18 (49)</td>
</tr>
<tr>
<td>Singleton; n (%)</td>
<td>25 (63)</td>
<td>24 (74)</td>
<td>17 (46)</td>
</tr>
<tr>
<td>Antenatal Steroids; n (%)</td>
<td>36 (92)</td>
<td>31 (97)</td>
<td>32 (87)</td>
</tr>
<tr>
<td>Cesarean section; n (%)</td>
<td>19 (48)</td>
<td>20 (63)</td>
<td>25 (68)</td>
</tr>
<tr>
<td>Apgar score (5 min); med (IQR)</td>
<td>7.5 (6.5, 9.0)</td>
<td>8.0 (7.0, 9.0)</td>
<td>7.0 (7.0, 8.0)</td>
</tr>
<tr>
<td>Apgar score (5 min); &lt;7; n (%)</td>
<td>10 (25)</td>
<td>5 (16)</td>
<td>8 (22)</td>
</tr>
<tr>
<td>Death before discharge; n (%)</td>
<td>6 (15)</td>
<td>3 (9)</td>
<td>7 (19)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morbidities (All Infants Discharged)</th>
<th>Group A (n = 34)</th>
<th>Group B (n = 29)</th>
<th>Group C (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant respiratory distress syndrome; n (%)</td>
<td>19 (56)</td>
<td>17 (59)</td>
<td>17 (57)</td>
</tr>
<tr>
<td>Days of mechanical ventilation; med (IQR)</td>
<td>0 (0, 4.0)</td>
<td>1.0 (0.0, 3.0)</td>
<td>0.0 (0.0, 4.0)</td>
</tr>
<tr>
<td>Days of Nasal-CPAP; med (IQR)</td>
<td>22.5 (11.0, 40.0)</td>
<td>29.0 (8.0, 41.0)</td>
<td>22.0 (9.0, 42.0)</td>
</tr>
<tr>
<td>Chronic lung disease; n (%)</td>
<td>12 (35)</td>
<td>13 (45)</td>
<td>8 (27)</td>
</tr>
<tr>
<td>Patent ductus arteriosus; n (%)</td>
<td>14 (41)</td>
<td>17 (59)</td>
<td>16 (53)</td>
</tr>
<tr>
<td>Intra-ventricular hemorrhage grade ≥ 2; n (%)</td>
<td>5 (14.7)</td>
<td>5 (17)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Sepsis; n (%);</td>
<td>8 (24)</td>
<td>8 (28)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>Necrotizing enterocolitis; Bell stage ≥ 2; n (%)</td>
<td>0</td>
<td>0</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Retinopathy of prematurity; n (%)</td>
<td>4 (12)</td>
<td>1 (3)</td>
<td>5 (17)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medication (All Infants Discharged)</th>
<th>Group A (n = 32)</th>
<th>Group B (n = 27)</th>
<th>Group C (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine; n (%)</td>
<td>32 (94)</td>
<td>27 (93)</td>
<td>28 (93)</td>
</tr>
<tr>
<td>Diuretics; n (%)</td>
<td>9 (27)</td>
<td>10 (35)</td>
<td>6 (20)</td>
</tr>
<tr>
<td>Corticosteroids; n (%)</td>
<td>4 (12)</td>
<td>2 (7)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Sedation; n (%)</td>
<td>7 (21)</td>
<td>8 (28)</td>
<td>8 (27)</td>
</tr>
</tbody>
</table>

Group A: donor milk in addition to mother’s own milk (MOM) and no supplements with enteral feeding until day 10; Group B: preterm formula in addition to MOM and no supplements with enteral feeding until day 10; Group C: preterm formula in addition to MOM and fortifier if intake ≥50 ml/day; SD: standard deviation; med: median; IQR: inter quartile range; small for gestational age: below 10th percentile, according to Fenton et al. [20]; Nasal-CPAP: nasal continuous positive airway pressure; chronic lung disease was defined as oxygen dependency at 36 weeks gestational age; patent ductus arteriosus was classified as need for treatment; sepsis was included if present for >72 h postnatally with positive blood culture; necrotizing enterocolitis was determined with staging, according to Bell [23], diuretics included single doses of furosemide and maintenance diuretics; sedation included morphine and/or midazolam >24 h.
Table 2 Nutritional characteristics during the first 10 days of life

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group A (n = 34)</th>
<th>Group B (n = 29)</th>
<th>Group C (n = 30)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenteral nutrition, days; med (IQR)</td>
<td>10.5 (8.0, 13.0)</td>
<td>10.0 (9.0, 14.0)</td>
<td>10.0 (9.0, 21.0)</td>
<td>0.71</td>
</tr>
<tr>
<td>Enteral (120 mL/kg/day); day; med (IQR)</td>
<td>10.0 (8.0, 13.0)</td>
<td>9.5 (9.0, 12.0)</td>
<td>10.5 (9.0, 16.0)</td>
<td>0.89</td>
</tr>
<tr>
<td>Enteral (150 mL/kg/day); day; med (IQR)</td>
<td>12.0 (9.0, 17.0)</td>
<td>11.0 (10.0, 13.0)</td>
<td>12.0 (10.0, 20.0)</td>
<td>0.89</td>
</tr>
<tr>
<td>Start day of HMF; med (IQR)</td>
<td>11.5 (11.0, 12.5)</td>
<td>12.0 (11.0, 13.5)</td>
<td>6.0 (4.0, 7.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Human milk, mL/kg; mean (SD)</td>
<td>552.8 (248.4)</td>
<td>403.1 (294.5)</td>
<td>432.5 (267.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Formula, mL/kg; med (IQR)</td>
<td>-</td>
<td>33.9 (13.6, 162.9)</td>
<td>12.1 (1.1, 48.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Percentage human milk; med (IQR)</td>
<td>100 (100.0, 100.0)</td>
<td>82.6 (53.2, 97.6)</td>
<td>97.3 (91.8, 99.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium, mmol/kg; mean (SD)</td>
<td>15.7 (2.6)</td>
<td>17.1 (4.0)</td>
<td>21.9 (4.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphorus, mmol/kg; mean (SD)</td>
<td>16.5 (3.1)</td>
<td>16.5 (3.0)</td>
<td>21.6 (3.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein, grams/kg; mean (SD)</td>
<td>26.8 (5.0)</td>
<td>27.5 (4.3)</td>
<td>32.1 (5.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calories, kcal/kg; mean (SD)</td>
<td>836 (108)</td>
<td>838 (86)</td>
<td>832 (168)</td>
<td>0.60</td>
</tr>
<tr>
<td>Carbohydrate, grams/kg; mean (SD)</td>
<td>109.1 (10.7)</td>
<td>103.6 (11.5)</td>
<td>114.5 (18.5)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Enteral: represents the day infants reached an enteral intake of either 120 or 150 mL/kg/day; HMF: human milk fortifier; Ca: calcium; P: phosphorus. All intakes are presented as the sum of the first 10 days; SD: standard deviation; med: median; IQR: inter quartile range. Missing data: Group A (n:2); Group B (n:4); Group C (n:4).

Group B and Group C (p-value < 0.001), although these groups received predominantly human milk as well. The calorie intake differed only slightly between the groups. Table A2 presents the distribution of the route of supplementation of Ca and P. This table demonstrates that differences in mineral intake between the groups were based on enteral intake.

Weight and Length at Term Corrected Age
Table 3 presents the anthropometric data at birth and TCA for all groups, including appropriate (AGA) and small for gestational age (SGA) infants. For both time points, weight and length were similar between the groups. All groups decreased in standard deviation score (SDS) for weight, as well as for length. The decrease in SDS for weight varied between −0.41 and −0.75, and for length between −0.33 and −0.69; for both measurements, there were no significant differences.

DXA-Scan at Term Corrected Age
Table 4 presents the data on body composition, as measured by DXA scan. A total of 35 scans were classified as acceptable. Not all infants were able to visit the outpatient clinic on the scheduled day and for logistic reasons, a number of infants were scanned close before discharge from hospital, therefore the gestational age and weight at the time of the scan varied. Nevertheless, the outcomes were similar across the groups for BMC and BMD with a wide interquartile range.

Determinants of Growth and Bone Mineralization
Table 5 presents the results of the analyses evaluating the effect of being a member of one of the three supplementation groups, and the amount of human milk provided during the first 10 days, on growth and bone mineralization. Groups A and B were analyzed with Group C as the reference. Birth weight, gestational age at measurement and being small for gestational age were significant determinants for all outcomes and thus were used as covariates in all analyses. There was little evidence of an association between the studied groups and the outcomes of weight, length, BMC and BMD. The amount of human milk was associated with a significant effect on BMC, with each mL/kg increase in human milk over 10 days associated with an average increase of 0.013 g (p-value = 0.017) in BMC. Length and weight were not associated with intake of human milk.
Table 6 presents the results of the regression analysis for the effects of Ca, P, and protein intake per kilogram during the first 10 days on growth and bone mineralization at term corrected age, of very preterm born infants. Based on the assumption that these three nutrients have a complex interrelationship in the effect on growth and bone mineralization, we analyzed the association with weight, length, BMC and BMD in three models by introducing step-by-step Ca, P, and protein, thereby aiming to provide insight into—for example, how the effect of Ca might be influenced by the levels of P. In this analysis, the nutritional intakes of Ca, P, and protein for the first 10 days were not associated with weight at term age. Phosphorus intake was positively associated with length, with a significant increase of 0.19 cm ($p$-value = 0.047) length for each mmol/kg received over the first 10 days. This effect remained after inclusion of protein in the model. Calcium intake was associated with a significant negative effect on BMC. Our analysis showed that each mmol/kg of Ca received over the 10 days was associated with a decrease of 1.21 g of BMC ($p$-value: 0.001). The effect persisted and increased slightly with the introduction of phosphorus and protein in the model. The testing on the interaction of Ca and P over the 10 days intake was negative for all outcomes, except for BMD, which showed a small positive effect ($β$ 0.0003; 95% CI (0.00002; 0.0006); $p$-value = 0.036).

### Table 4  Body composition measured by DXA

<table>
<thead>
<tr>
<th>DXA Scan</th>
<th>Group A ($n = 14$)</th>
<th>Group B ($n = 12$)</th>
<th>Group C ($n = 9$)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>40.5 (36.7, 44.0)</td>
<td>42.1 (37.6, 45.7)</td>
<td>43.7 (36.5, 45.7)</td>
<td>0.569</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>3318 (2407, 4290)</td>
<td>3325 (2343, 3856)</td>
<td>3115 (2533, 3920)</td>
<td>0.966</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>48.5 (43.8, 52.8)</td>
<td>48.0 (44.0, 52.1)</td>
<td>49.5 (45.0, 53.0)</td>
<td>0.923</td>
</tr>
<tr>
<td>Bone area (cm$^2$)</td>
<td>314.1 (263.0, 368.3)</td>
<td>326.7 (254.3, 353.5)</td>
<td>286.6 (256.8, 390.6)</td>
<td>0.988</td>
</tr>
<tr>
<td>Bone mineral content (gram)</td>
<td>47.6 (42.0, 66.1)</td>
<td>51.1 (35.4, 65.3)</td>
<td>45.4 (35.3, 63.0)</td>
<td>0.967</td>
</tr>
<tr>
<td>Bone mineral density (g/cm$^2$)</td>
<td>0.164 (0.147, 0.177)</td>
<td>0.157 (0.138, 0.186)</td>
<td>0.157 (0.138, 0.173)</td>
<td>0.819</td>
</tr>
<tr>
<td>Lean body mass (gram)</td>
<td>2862 (2064, 3647)</td>
<td>3164 (2289, 3659)</td>
<td>2576 (2254, 3382)</td>
<td>0.665</td>
</tr>
<tr>
<td>Fat mass (gram)</td>
<td>568 (314, 888)</td>
<td>473 (397, 918)</td>
<td>641 (231, 914)</td>
<td>0.922</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>16.9 (13.5, 20.4)</td>
<td>15.6 (11.0, 21.5)</td>
<td>16.7 (8.7, 23.5)</td>
<td>0.918</td>
</tr>
</tbody>
</table>

All data are presented as median (IQR); GA: Gestational age; DXA: dual energy X-ray absorptiometry.
### Table 6: Associations between nutritional intake and outcomes at term corrected age

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>1.2</td>
<td>0.893</td>
<td>-</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>19.8</td>
<td>0.177</td>
<td>15.8</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td>0.286</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.2</td>
<td>0.320</td>
<td>0.160</td>
</tr>
<tr>
<td>GA at measurement</td>
<td>135.4</td>
<td>1.374</td>
<td>138.3</td>
</tr>
<tr>
<td>Small for GA</td>
<td>-890.0</td>
<td>-898.4</td>
<td>-849.0</td>
</tr>
<tr>
<td>Constant</td>
<td>3240.1</td>
<td>3241.6</td>
<td>3233.2</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>-0.02</td>
<td>-0.15</td>
<td>0.096</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.003</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>GA at measurement</td>
<td>0.9</td>
<td>0.93</td>
<td>0.93</td>
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<tr>
<td>Small for GA</td>
<td>-4.1</td>
<td>-4.2</td>
<td>-4.3</td>
</tr>
<tr>
<td>Constant</td>
<td>48.5</td>
<td>48.5</td>
<td>48.5</td>
</tr>
<tr>
<td><strong>BMC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>-1.21</td>
<td>-1.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.65</td>
<td>0.183</td>
<td>0.56</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.005</td>
<td>0.006</td>
<td>0.009</td>
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<tr>
<td>GA at measurement</td>
<td>2.99</td>
<td>3.03</td>
<td>2.98</td>
</tr>
<tr>
<td>SGA</td>
<td>-11.5</td>
<td>-12.1</td>
<td>-10.5</td>
</tr>
<tr>
<td>Constant</td>
<td>54.2</td>
<td>54.3</td>
<td>54.0</td>
</tr>
<tr>
<td><strong>BMD</strong></td>
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<td></td>
<td></td>
</tr>
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<td>Calcium</td>
<td>-0.001</td>
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<td>0.132</td>
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<tr>
<td>Phosphorus</td>
<td>0.0001</td>
<td>0.915</td>
<td>-0.001</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
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<td>0.001</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.0001</td>
<td>0.337</td>
<td>0.354</td>
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<tr>
<td>GA at measurement</td>
<td>0.003</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>Small for GA</td>
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<td>-0.093</td>
</tr>
<tr>
<td>Constant</td>
<td>0.163</td>
<td>0.163</td>
<td>0.163</td>
</tr>
</tbody>
</table>

BMC: bone mineral content (gram); BMD: bone mineral density (gram/cm²)—the nutritional variables reflect the intake of the first 10 days; Ca: calcium intake (mmol/kg per 10 days); P: phosphorus intake (mmol/kg per 10 days); protein intake: (gram/kg per 10 days); GA at measurement: gestational age at time of measurement; Small for GA: small for gestational age; all nutritional variables were adjusted for birth weight, GA at measurement and Small for GA. The constant term in the models represents the expected value for Group C, for infants born appropriate for GA, and who received the mean intake of nutrients. Bold p-Values reflect a significant association between one of the investigated nutrients and outcome.
CHAPTER 8 GROWTH AND BONE MINERALIZATION OF PRETERM INFANTS

Discussion

This randomized cohort study evaluated the effect of different amounts of Ca and P intakes, during the first 10 days of life, on growth and bone mineralization of very preterm infants. The early stopping of patient inclusions led to lower numbers than the originally anticipated 65 infants per group. Thus, the study may be underpowered to answer the research questions. This study found no differences in weight, length, bone mineral content (BMC) and bone mineral density (BMD) between three different intake groups of very and extremely preterm infants at term corrected age. The regression analysis further showed that group assignment was not associated with the studied outcome measures; however, we found significantly positive associations between P intake and length, as well as the amount of human milk intake and BMC. In contrast, Ca intake was associated with a decrease in BMC, which further decreased after the addition of P and protein in the analysis.

Bone mineralization, at term corrected age, in relation to enteral nutrition of preterm infants has been evaluated in only a few studies during the last three decades. Studies often had an observational design and evaluated more stable infants at a higher gestational age than nowadays treated. (1,2,4,24–31) Eleven studies investigated the effect of either human milk, fortified human milk or various compositions of preterm formulas in randomized studies, leading to varying results. (32–42) Only two studies found an increase in BMC, according to gestational age changes, in combination with high amounts of minerals in preterm formula, while others found the highest weight gain and BMC specifically with preterm formula. (2,23,34,35,37,42,43)

Since timing, amount of fortification or composition of formulas differed in all studies as well as the method and timing of scanning (single photon absorptiometry versus dual X-ray absorptiometry), it is difficult to compare these results to our findings. The largest double-blinded randomized study, performed by Faerk et al., did not find an effect of human milk fortification or preterm formula on BMC, compared to unfortified human milk. (39) However, infants fed preterm formula had significantly higher weights at TCA and the amount of supplemented phosphorus was significantly associated with weight at TCA. (39) All infants achieved a BMC below that of healthy term born infants. (31,39) This negative result may be explained by a relatively late timing of fortification, at a mean age of 15 days, and a low amount of fortification of human milk; which was below the ESPGHAN (European Society for Paediatric Gastroenterology Hepatology and Nutrition) recommendation for enteral intakes of Ca and P. (44) In comparison, we could not demonstrate an association between P, Ca and protein intakes and weight in this study; nevertheless, we found that P and the amount of human milk were positively associated with study outcomes. The differences in outcomes could be explained with the fact that our infants received amounts of minerals within the ESPGHAN recommendations.

According to the nutritional protocol of our hospital, the full recommended intake (including parenteral and enteral intakes) was provided, as soon as possible after birth, aiming at a postnatal growth and bone mineralization comparable to development in utero and to limit a postnatal nutritional deficit, as described in several studies. (45–47) This included parenteral mineral supplementation directly after birth, early fortification of human milk and additional supplementation of minerals, based on biochemical parameters. Group C, following the institutional protocol, received a significant higher amount of Ca and P during the first 10 days, compared to Groups A and B, who received no enteral fortifications until day 10. The highest weight gain and bone mineralization could be expected in group C. However, the outcomes of group C compared to Groups A and B were not different, and the total group in comparison to the reference population was still growth retarded. Probably, this may be explained by the short intervention period, with only a few days of significantly different enteral intake. Further, this study included relatively more immature and sicker infants, compared to infants in the previously mentioned trials, probably indicating even higher requirements of minerals for very and extremely preterm infants than currently recommended.

The effect of Ca and P intakes on outcomes seemed contradictory. Calcium intake was associated with a significant negative effect on BMC and non-significant negative effects on all other outcomes, while phosphorus intake had a significant positive effect on length, and a non-significant positive effect on BMC. In general, the effect sizes were small, and BMC was the only outcome that indicated a positive interaction between Ca and P. Again, any interpretation should be performed with caution, since the results may be distorted by the small number of patients investigated with DXA scans. However, an explanation for this phenomenon may be that both minerals are closely related in the formation of bone and shortage of one item may influence the effect of the other mineral. Based on our previous study, we have strong indications that the supply of at least phosphorus was insufficient. For the same cohort of infants evaluated in this study, we reported changes in biochemical parameters for calcium and phosphorus homeostasis, in relation to nutritional intake. (18) Despite a high intake of P, serum P concentrations remained low in all three groups. It was demonstrated that serum P concentrations were significantly associated with amino acid intake, indicating that phosphorus was preferably used for cell metabolism instead of bone mineralization. Hypophosphatemia, in relation to high amino acid intake, has previously been reported in preterm infants and currently is recognized as ‘Placental Incompletely Restored Feeding (PI-Refeeding) syndrome’, caused by an imbalanced nutritional intake of amino acids and phosphorus. (18,48,49) Furthermore, we demonstrated that low gestational age was associated with higher renal excretion of phosphorus, irrespective of nutritional intake. Thus, considering the results of both studies, we speculate that, despite a high intake of minerals in Group C, a high cell
metabolism and renal phosphorus wasting prevented adequate availability of phosphorus for adequate bone mineralization and consecutively prevented adequate use of calcium for bone mineralization. Again, this indicated that mineral requirements, to achieve bone mineralization equivalent to term born infants, for the most immature infants, may be higher than currently recommended. (13,31)

The method of administration of minerals may have affected the outcomes between groups as well as the results of the regression analysis. As demonstrated in Table A2, the groups differed significantly in the amount of enteral supplementation of minerals, but all groups received more than 50% of the total intake as parenteral supplement. Parenteral nutrients are directly available for metabolism, while supply by the enteral route is also determined by the amount of intestinal absorption. (10,50)

The parenteral supplement in our study may have compensated for the low enteral intakes in Groups A and B and may have ameliorated differences in outcomes and the analyses regarding the effects of nutritional intake. In comparison, the study of Faerk et al. did not provide any information on parenteral supplements, although the intervention period (start of supplementation) only started at a mean of 15 ± 7 days. (39) Nowadays, the clinical practice for nutritional support of preterm infants is to provide full parenteral nutrition, including mineral supplementation, shortly after birth. Therefore, the provision of parenteral nutrition in the study of Faerk cannot be excluded and one may speculate that the comparatively positive outcomes of infants who received unfortified human milk in this study could partly be explained by parenteral supplementation of nutrients.

Independent of group assignment, the percentage of MOM was very high in this study. In the studies mentioned previously, which found an improvement in bone mineralization, this was overall related to the use of preterm formula with a high amount of minerals. We did not include a group with exclusively preterm formula, because our general practice is to provide preferably the mother’s own milk. On the other hand, a positive effect of human milk on bone development has previously been reported. (51,52) The positive effect of the amount of human milk on BMC in this study supports our assumption that even for very and extreme preterm infants, it should be possible to achieve adequate bone mineralization at term corrected age, in combination with human milk. Furthermore, this study confirmed earlier findings, that early fortification was well-tolerated. (53) However, this study also demonstrated, that current concepts of mineral supplementation and fortification of human milk are insufficient and need to be further evaluated, while recommendations probably need to be adapted.

This study had several limitations. Firstly, this study did not evaluate the maternal vitamin D status, nor were vitamin D concentrations determined in the participating infants. Epidemiological studies have shown a high prevalence of suboptimal vitamin D concentrations in pregnant and lactating women, even in countries where adequate exposure to sunlight and sufficient dietary intakes may be expected. (54,55) The foetus relies on maternal vitamin D stores, and thus postnatal vitamin D insufficiency may have impaired intestinal calcium absorption directly after birth. However, parenteral vitamin D supplementation was started directly after birth in all infants and enteral supplementation within the first week of life, following the recommendations for very low birth weight infants of the ESPGHAN 2010. (13) Recently, it was demonstrated that doses of vitamin D, as provided in this study, led to sufficient vitamin D concentrations in very low birth weight infants, in the postnatal period, at 4 weeks of age. (60) The small differences in start day of enteral vitamin D supplementation between Groups A/B and C cannot explain the differences found between the groups. Furthermore, all infants received the same amount of parenteral intake, with relatively high amounts of minerals. Based on blood and urine analyses, it was permitted to provide extra parenteral mineral supplementation during the intervention period. The protocol for parenteral nutrition has been the standard of care at our department for many years and we felt it would be unethical to withhold the standard of care to high-risk patients. There was room for a more individualized treatment in relation to enteral supplementation. This possibly has ameliorated the differences between the three groups and subsequently decreased the size of any effect of enteral supplementation. On the other hand, the study protocol reflected current generally accepted clinical practice, with a combined parenteral and enteral nutritional intake. We would like to note that none of our patients developed signs of rickets or fractures and that at follow up, around term age, our patients had achieved a median BMC comparable to 38–39 weeks gestation, according to Lapillone et al. (31) Up to this date, most studies have been limited to the evaluation of enteral intake. We suggest that further improvements in the combined nutritional supply of parenteral and enteral intake in the early postnatal period are possible and that future trials should include both routes of administration, starting directly after birth. A limitation of our study was the fact that we were unable to include the intended number of patients. This may, on the one hand, have led to overestimation of observed effects; on the other hand, there is a chance of having missed certain effects. It should be noted that we only included three nutrients for the regression analysis, while enteral nutrition, especially human milk, is a complex emulsion and the composition and interactions between various factors that may have impacted on the digestion and absorption of nutrients were not included in the analysis and thus may have been missed.

Despite these limitations, we decided to present our data because there are limited studies available investigating bone mineralization in preterm infants at term age, specifically for the group of very and extremely low gestational age. The specific nutritional needs of this high-risk group need further evaluation. Often studies remain of a small size because it is difficult to include sufficient patients within a reasonable timeframe. The strength of this study is the randomized design and that reporting of
data occurred accordingly to the recently proposed standardization of nutrition and growth outcomes. (57) This offers the opportunity to combine our data with studies of a comparable setting, which may add to the significance of the overall results.

Conclusions

On the basis of this study, there is no evidence that early high mineral intake through early fortification of human milk further improves bone mineralization in combination with high parenteral mineral intake. Mineral intake, according to current recommendations for preterm infants, seems to provide an insufficient amount of minerals to achieve bone mineral content, comparable to term born newborns. The positive effect of human milk on bone mineral content needs to be further evaluated.

Acknowledgments

All authors gratefully thank Wendy R.J.C. Jansen, research nurse of the Pediatric Drug Research Center Radboudumc, and Laure J.M.L. Vorstenbosch, medical student, for the dedicated support in patient recruitment and data collection, as well as Dr. Timothy A.J. Antonius, neonatologist at Radboudumc, for the development of the algorithm to calculate the nutritional intakes.

Appendix A

Table A1 Standard parenteral nutritional intake

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid mL/kg/day</td>
<td>8</td>
<td>100</td>
<td>125</td>
<td>150</td>
</tr>
<tr>
<td>Carbohydrates grams/kg/day</td>
<td>8</td>
<td>9.6</td>
<td>11.7</td>
<td>13.8</td>
</tr>
<tr>
<td>Amino acids grams/kg/day</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3</td>
</tr>
<tr>
<td>Lipids grams/kg/day</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Energy Quotient Kcal/kg/day</td>
<td>44</td>
<td>62</td>
<td>82</td>
<td>94</td>
</tr>
<tr>
<td>Calcium mmol/kg/day</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3.00</td>
</tr>
<tr>
<td>Phosphorus mmol/kg/day</td>
<td>0.48</td>
<td>0.96</td>
<td>1.44</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Parenteral nutritional intake based on standardized parenteral solutions [35,53]. For infants below 1000 g, amino acids were additionally added, according to current recommendations [12]. Amino acid solution: Primene (Baxter, the Netherlands); lipid emulsion including vitamins: Clinoleic (20%; Baxter, The Netherlands) or SMOFlipid 20% (Fresenius Kabi; The Netherlands).

Table A2 Route of supplementation

<table>
<thead>
<tr>
<th>Distribution of Mineral Intake</th>
<th>Group A (n = 34)</th>
<th>Group B (n = 29)</th>
<th>Group C (n = 30)</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>Calcium, mmol/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15.0 (13.8, 17.5)</td>
<td>15.9 (14.1, 19.2)</td>
<td>22.0 (19.3, 25.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Enteral</td>
<td>3.9 (2.4, 4.8)</td>
<td>6.0 (2.9, 7.5)</td>
<td>10.3 (4.9, 14.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Enteral supplement</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Parenteral</td>
<td>12.1 (9.4, 15.1)</td>
<td>10.9 (8.2, 12.5)</td>
<td>12.2 (9.4, 15.2)</td>
<td>0.309</td>
</tr>
<tr>
<td>Parenteral supplement</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Phosphorus, mmol/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16.4 (14.5, 18.5)</td>
<td>16.3 (15.1, 19.1)</td>
<td>21.6 (19.1, 24.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Enteral</td>
<td>3.0 (1.7, 3.7)</td>
<td>4.1 (2.5, 5.4)</td>
<td>9.4 (3.6, 14.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Enteral supplement</td>
<td>0.0 (0.0, 0.3)</td>
<td>0</td>
<td>0.0 (0.0, 3.6)</td>
<td>0.063</td>
</tr>
<tr>
<td>Parenteral</td>
<td>12.5 (9.8, 16.3)</td>
<td>11.8 (9.2, 14.9)</td>
<td>11.9 (9.0, 16.8)</td>
<td>0.524</td>
</tr>
<tr>
<td>Parenteral supplement</td>
<td>3.3 (1.5, 6.1)</td>
<td>3.1 (1.2, 5.1)</td>
<td>2.1 (0.0, 3.6)</td>
<td>0.498</td>
</tr>
</tbody>
</table>

All values are median (IQR). All intake is presented as the sum of the intake during the first 10 days. Missing values: Group A (n:2); Group B (n:4); Group C (n:3).
CHAPTER 8 GROWTH AND BONE MinerAlIZATION OF PRETERM INFANTS

References


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Viola Christmann

Bone Reports 2018;8:38-45
Abstract

Background
Preterm infants are at risk of impaired bone health in later life. Dual-energy X-ray absorptiometry-scan (DXA) is the gold standard to determine bone mineralization. Phalangeal quantitative ultrasound (pQUS) is an alternative technique that is inexpensive, easy to use and radiation-free. The aim of this study was to investigate whether both techniques reveal equivalent results.

Materials and Methods
Sixty former preterm infants (31 boys; 29 girls) received a DXA and pQUS at age 9 to 10 years. DXA measured bone mineral content (BMC) and bone mineral density (BMD) for total body and lumbar spine (L1-4), while pQUS measured the amplitude dependent speed of sound (AD-SoS) and bone transit time (BTT) at metacarpals II-IV providing continuous values and Z-scores based on age and sex. Four statistical methods evaluated the association between both techniques: Pearson’s correlation coefficients, partial correlation coefficients adjusted for gestational age, height and BMI, Bland-Altman analysis and cross tabulation.

Results
Both techniques showed a statistically significant weak correlation for continuous values as well as Z-scores (0.291-0.462, p<0.05). Boys had significant and relatively high correlations (0.468-0.585, p<0.05). In comparison, the correlations for girls were not significant. Correlation coefficients further decreased while calculating the partial correlations. The Bland-Altman plots showed poor agreement. Sensitivity ranged from 33% to 92% and specificity from 16% to 68%. Positive and negative predictive values ranged from 4% to 38% and 82% to 97%, respectively.

Conclusions
We found statistically significant weak correlations and poor agreement between DXA and pQUS measurements. DXA is not equivalent to pQUS and therefore not replaceable by this technique in former preterm born children at the age of 9 to 10 years.

Introduction
Bone development is one of the key processes during fetal, neonatal and infant development. Mineralization of bone mainly starts during the third trimester of pregnancy based on active placental transfer of calcium and phosphorus to the fetus. Up to 80% of the body calcium of a term infant is accrued during the last trimester. Preterm infants miss out the active fetal bone development and therefore are at risk of reduced bone mineralization and development of osteopenia. Inadequate bone mineralization is seen as a risk factor for the development of osteoporosis in later life, which is an important cause of morbidity and mortality in elderly people and a considerable factor of healthcare expenditure. The peak bone mass is attained before skeletal maturity. Any factor that influences the acquisition of peak bone mass may represent a mechanism to affect later osteoporosis risk. The evaluation of bone development in preterm born children is relevant for the determination of the individual health risk as well as the evaluation of medical treatment that aimed at improvement in bone development.

Currently, there are two techniques available to determine bone mineralization, either dual-energy X-ray absorptiometry-scan (DXA) or quantitative ultrasound (QUS). DXA is the most commonly used technique for assessing bone mineralization in children and adolescents. Although DXA is a non-invasive and standardized method, it is not available for all medical centers and it uses a low amount of radiation. In recent years, QUS has been proposed as an alternative method to replace DXA for the evaluation of bone status, especially since it is relatively inexpensive, fast, easy to use, portable and radiation-free.

Studies investigating the association between the measurements of DXA and QUS revealed inconsistent results. While a number of studies showed a significant positive correlation between DXA and QUS, others found a discrepancy between the measurements of the two methods. This could be a result of the different QUS measurement sites or different patient categories investigated. Only a limited number of studies used the phalangeal QUS and only one study looked at the specific group of former preterm born children.

The aim of this study was to investigate whether the measurements of dual-energy X-ray absorptiometry scan (DXA) and phalangeal quantitative ultrasound (pQUS) performed in preterm born children aging from 9 to 10 years reveal comparable results. We hypothesized that both techniques were equivalent in diagnosing the state of bone mineralization. Equivalent results would mean that the pQUS could replace the DXA for evaluating bone mineralization as a diagnostic tool.
CHAPTER 9  DIAGNOSING BONE MINERALIZATION IN PRETERM BORN CHILDREN

Materials and Methods

Study design
This study was a cross-sectional study using the data collection of the study “Long-term follow up of growth and bone mineralization of former preterm infants” (FoBoMin). This study was approved by the Ethics committee (CMO nr 2013/594) of the Radboud University Medical Center. Informed consent was obtained from all parents after approval by the local ethics committee.

Study population and procedure
The study included 60 former preterm infants at the age of 9 to 10 years. All subjects participated in the FoBoMin-study. This long-term follow-up study evaluated two cohorts of very preterm infants with a birth weight below 1500 grams and gestational age less than 34 weeks. The cohorts differed by nutritional intake during the first two weeks of life. The second cohort received higher intake of protein, energy as well as calcium and phosphate. This was associated with improved weight gain during the early postnatal period. The aim of the FoBoMin-study was to compare long-term growth and bone mineralization in relation to early nutritional intake in preterm born children at age 9 to 10 years. All participants of the studies were evaluated by DXA and pQUS. The measurements were performed on the same day for the individual participant. Four statistical methods were used to compare both methods.

Measurement instruments and variables
Bone mineralization of the total body and lumbar spine (L1-L4) was determined using the QDR Discovery A S/N 85606 (Hologic, Inc., USA). According to the International Society for Clinical Densitometry (ISCD), the lumbar spine (L1-L4) and whole body scan are the preferred skeletal sites for measurement in children. The measurements of the DXA were analyzed using the APEX-system software version 13.3. The DXA uses a low dose of radiation depending on measurement site. The effective dose, reflecting the real radiation risk for children of 10 years old, for the whole body is 4.8 μSv and for the lumbar spine 7.1 μSv. According to the ‘Rijksinstituut voor Volkgezondheid en Milieu’ (RIVM) the yearly averaged ambient dose equivalent rate for the NMR station in the area of Nijmegen is 74 nSv/h, resulting in a daily exposure in Nijmegen of 1.78 μSv. Therefore, the radiation dose of DXA can be regarded as very low and is negligible. Results of the DXA were expressed as Bone Mineral Content (BMC; g), Bone Mineral Density (BMD; g/cm²), and Z-scores, representing the number of standard deviations above or below the mean for the patients’ sex and age. The Z-scores were calculated by the DXA software on the basis of reference values for sex and age obtained from a large U.S. population provided by the manufacturer. The Z-scores of the whole body were calculated using the reference data of the National Health and Nutrition Examination Survey (NHANES, 2008) (39), while lumbar spine Z-scores were based on the reference data of the Bone Mineral Density in Childhood Study (BMDCS). A Z-score less than or equal to -2.0 SD is considered to indicate ‘low bone mineral status’. (36)

The quantitative ultrasound (pQUS) was performed on the second to the fifth metacarpals of the phalangeal bones using a DBM Sonic Bone Profiler (IGEA, Carpi, Italy). The mean value of the measurements per person was calculated. The transmitter of the pQUS generated a sound frequency of 1.25 MHz. This technique measured the amplitude dependent speed of sound (AD-SoS) and bone transit time (BTT), which were both expressed in continuous values and in Z-scores. The AD-SoS (m/s) was the ultrasound velocity inside the finger and was derived from the measurement of the time interval between emission and reception of the ultrasound signal, considering the first signal with a minimum amplitude of 2 mV at the receiver probe. The BTT (μsec) reflected the bone characteristics without the interference of the soft tissue by calculating the difference between transmission time in soft tissue and bone and transmission time in soft tissue. The Z-scores were determined on the basis of the reference values related to sex and age (AD-SoS Z score (age); BTT- Z-score (age)) or sex and height (AD-SoS Z score (height); BTT Z-score (height)). The Z-scores were obtained from a large Italian population provided by the manufacturer.

Additionally, age, sex, gestational age at birth, weight, height, BMI and pubertal development were recorded. Weight (kg) was measured using an electronic digital scale (SECA MOD701) to the nearest 0.1 kg. Height (cm) was determined using a vertical stadiometer (SECA MOD240) to the nearest 0.1 cm. Body mass index (BMI; kg/ m²) was calculated by dividing weight (kg) by the square of height (m²). Pubertal development was self-assessed from pictures showing the different Tanner stages.

The children were asked to indicate which picture most resembled their current appearance.

Statistical analysis
The statistical analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS Inc., Chicago, IL, USA, version 22.0). All results were expressed as mean ± SD. Four statistical methods were used for the analysis of the association between pQUS and DXA. First, the Pearson’s correlation coefficients (r) were calculated for evaluation of the correlation between continuous values as well as the Z-scores of DXA and pQUS. The correlation coefficients were determined for every outcome for the total group as well as for boys and girls separately. Secondly, the partial correlation coefficients were determined to correct for possible confounders on the original correlation between DXA and pQUS. Possible confounders of bone development, such as age, sex, gestational age, weight, height, BMI and Tanner stages at follow-up were included in the analysis. Only three of these confounders, namely gestational
age, height and BMI were used to calculate partial correlation coefficients, because of the limited number of participants in this study. The three confounders were chosen based on calculating whether they correlated significantly with DXA and pQUS measurements. Thirdly, a Bland-Altman analysis was performed to evaluate the agreement between both techniques using the Z-scores of either DXA and pQUS. Plots were created with the mean of two Z-scores within the same subject resulting from the two techniques on the horizontal axis and the difference of the Z-scores on the vertical axis. Finally, a cross tabulation was performed and the sensitivity, specificity as well as positive and negative predictive values were calculated, where DXA was considered as the gold standard. In agreement with the ISCD, a DXA Z-score less than or equal to -2.0 SDS should be considered as low bone mineralization. (36) A Z-score between -1.0 and -2.0 was considered as reduced (43) and a Z-score above -1.0 SDS is normal. For the current study a cut-off value of -1.0 SDS was used for the assessment of low or normal bone mineralization, in the absence of participants with a Z-score less than -2.0. A two tailed p-value of < 0.05 was considered statistically significant.

Results

Patient characteristics

The baseline characteristics of the participants (total group as well as boys and girls separately) are presented in Table 1. Anthropometric characteristics, gestational age at birth and pQUS measurements at follow-up were comparable between boys and girls. No statistically significant differences were found.

Correlation

Table 2 presents the correlation coefficients of DXA and pQUS measurements for the continuous values. The correlation coefficients between the DXA and both pQUS measurements (BTT; AD-SoS) showed statistical significance, though the r value was low. The correlation coefficients between DXA and BTT were higher, ranging from 0.341 to 0.462 (p<0.05), compared to correlation coefficients between DXA and AD-SoS, ranging from 0.291 to 0.345 (p<0.05). In comparison, boys showed a statistically significant and slightly higher correlation, which was not found for girls (boys: 0.468-0.585, p<0.05 versus girls: 0.008-0.335, p>0.05). Nevertheless, the differences found between boys and girls, calculated with the Fisher’s r-to-Z transformation, were not statistically significant, except for lumbar spine BMD and AD-SoS (p=0.039).

Table 3 presents the correlation coefficients for the Z-scores. AD-SoS Z-score (age) and BTT Z-score (age) showed a statistically significant but weak correlation with DXA Z-scores (0.327-0.401, p<0.05). The correlation coefficients for BTT Z-scores (age) were higher than those for AD-SoS Z-scores (age). Since the Z-scores (height) showed no statistically significant correlation coefficients, they were not further evaluated. In comparison, the Z-scores (age) of boys showed a statistically significant correlation coefficient in contrast to girls (boys: 0.436-0.520,p<0.05 versus girls: -0.026-0.274, p>0.05). In general, the difference found between boys and girls, calculated with the Fisher’s r-to-Z transformation, was not statistically significant, except for lumbar spine Z-score and AD-SoS Z-score (age) (p=0.027).

As an example, Figure 1 illustrates an overlay scatterplot of the correlation coefficients between the AD-SoS Z-score (age) and the whole body Z-score for boys and girls. No statistically significant differences were found.
and girls. Other pQUS and DXA measurements revealed comparable scatterplots.

**Partial correlation**

Table 4 presents the original correlation coefficients and the partial correlation coefficients adjusted for gestational age, height and BMI for the continuous values and the Z-scores (age). The adjustment for the three confounders induced a further decrease of the correlation coefficients. On average, the remaining coefficients, although significant, were very weak.
Agreement

The agreement was considered for all parameters. Only the Bland-Altman plot of the whole body Z-score and the AD-SoS Z-score (age) will be presented, because the other plots showed comparable results (Figure 2). The agreement between the two techniques was low, based on the following results. First, the mean difference between the Z-scores as presented in figure 2 was 2.73 and thereby significantly different from zero determined using a paired T-test (p-value: 0.011). Thereby, the 95%-limits of agreement in this figure had a large interval between -0.54 and 6.00.

Lastly, the plot showed that the differences of the two Z-scores (whole body Z-score minus AD-SoS Z-score (age)) were negatively dependent on the mean. This means that the difference between DXA and pQUS Z-scores increased with a lower mean Z-score, leading to an increasing disagreement between the two techniques while assessing bone mineralization for lower Z-scores.

Table 4 Correlation coefficients and partial correlation coefficients (adjusted for gestational age, height and BMI) of continuous variables and Z-scores of DXA and pQUS

<table>
<thead>
<tr>
<th></th>
<th>AD-SoS BTT</th>
<th>AD-SoS Z-score (age)</th>
<th>BTT Z-score (age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body BMC</td>
<td>Correlation coefficient (r)</td>
<td>0.325</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>0.215</td>
<td>0.267</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>0.004</td>
<td>0.000</td>
</tr>
<tr>
<td>Whole body BMD</td>
<td>Correlation coefficient (r)</td>
<td>0.313</td>
<td>0.393</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>0.220</td>
<td>0.236</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>0.012</td>
<td>0.009</td>
</tr>
<tr>
<td>Lumbar spine BMC</td>
<td>Correlation coefficient (r)</td>
<td>0.291</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>0.191</td>
<td>0.241</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>0.024</td>
<td>0.005</td>
</tr>
<tr>
<td>Lumbar spine BMD</td>
<td>Correlation coefficient (r)</td>
<td>0.345</td>
<td>0.341</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>0.280</td>
<td>0.249</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>0.012</td>
<td>0.031</td>
</tr>
<tr>
<td>Whole body Z-score</td>
<td>Correlation coefficient (r)</td>
<td>-</td>
<td>0.327</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>-</td>
<td>0.238</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>-</td>
<td>0.012</td>
</tr>
<tr>
<td>Lumbar spine Z-score</td>
<td>Correlation coefficient (r)</td>
<td>-</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td>Partial correlation coefficient</td>
<td>-</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>P-value partial coefficient</td>
<td>-</td>
<td>0.019</td>
</tr>
</tbody>
</table>

AD-SoS, amplitude-dependent speed of sound; BTT, bone transit time; BMC, bone mineral content; BMD, bone mineral density; Z-score (age), Z-score adjusted for sex and age.

Figure 2 Bland-Altman plot

The Bland-Altman plot presents the agreement between whole body Z-score measured by dual energy X-ray absorptiometry and AD-SoS Z-score (age) measured by phalangeal quantitative ultrasound. The middle horizontal line represents the mean difference of the Z-score; the upper and lower horizontal lines represent the plus and minus limits of agreement.
Cross tabulation

Cross tabulation was performed for all combinations of pQUS and DXA measurements. The number of pQUS measurements with a Z-score below -1.0 SDS was higher compared to DXA. The sensitivity for all measurements ranged from 33% to 92%. The specificity ranged from 16% to 68%. The positive and negative predictive values ranged from 4% to 38% and 82% to 97%, respectively.

As an example, Table 5 shows a cross table for the BTT Z-score (age) and the lumbar spine Z-score. This table revealed the best agreement of all measurements, but the specificity, sensitivity, positive predictive value and negative predictive value in general, were low. The sensitivity and specificity for BTT Z-score (age) in comparison with lumbar spine Z-score were 69% and 68%, respectively, and for the positive and negative predictive value this was 38% and 89%, respectively. Overall, we found a large discrepancy between the two methods for discriminating a patient with a normal or reduced bone mineralization.

Discussion

This study evaluated two different diagnostic techniques for bone development in former preterm born children, who are at risk for impaired bone mineralization. Four statistical tests showed that the results of dual-energy X-ray absorptiometry scan (DXA) and phalangeal quantitative ultrasound (pQUS) had a significant weak correlation that further decreased after adjustment for confounders. In addition, there was a low agreement between the two techniques and a discrepancy in differentiating the same children with normal or reduced bone mineralization.

The correlation coefficients were calculated for the continuous values as well as for the standard deviation scores (Z-scores) based on reference data. According to Baroncelli et al (9) the Z-score is the more appropriate value to express bone mineralization in children. The DXA Z-scores were available adjusted for sex and age, while the pQUS presented two types of Z-scores, either adjusted for sex and age (Z-score (age)) or sex and height (Z-score (height)). In our study the continuous values and Z-scores (age) showed statistically significant but weak correlations with DXA measurements, whereas correlation coefficients between DXA Z-scores and pQUS Z-scores (height) revealed to be non-significant. This is in accordance with the reference data provided by Barkmann et al (44), who found that the QUS signals correlated less with height compared to age. Therefore the Z-scores (height) were left out for further analysis.

Our analysis showed different results for boys and girls. Only boys had statistically significant correlations for continuous values as well as Z-scores when comparing pQUS and DXA. This is in agreement with the study of Halaba et al (28), who found a significant correlation between QUS and DXA in boys (0.40-0.47, p=0.000) and no correlation in girls. They evaluated 150 healthy Caucasian patients aged from 14 to 19 years. According to Halaba et al, the gender-related bone differences could be related to puberty development and influence of bone size as a result of earlier skeletal maturation in girls compared to boys. Our children had a lower age range and were mainly prepubertal. We do not have an explanation for this phenomenon and therefore suggest that these gender differences should be further investigated.

The association between QUS and DXA has previously been evaluated in a number of studies (10, 12-33, 45, 46) Table 6 presents an overview of these studies. The results are inconsistent and difficult to compare to our study, partly because a number of studies investigated different populations or used different measurement sites of QUS. Seven studies used the same equipment as we did, comparing pQUS to DXA. (14, 16, 18, 22, 23, 28, 46) The correlation coefficients between pQUS and DXA found by Pluskiewicz et al (28) and Di Mase et al (14) are in agreement with our calculations (0.45-0.56 and 0.42-0.52, respectively). As mentioned above, Halaba et al (28) found positive correlations only in boys using continuous values, comparable to our study. In contrast to our results, Halaba et al found no correlation for both sexes using the Z-scores. Nevertheless, in agreement with our analyses these studies found comparable poor correlation coefficients and thereby questioning the equivalence of pQUS and DXA. In contrast, two other studies found relatively stronger correlations for the continuous values (0.59-0.74, p<0.05), but no significant correlations while comparing Z-scores, except for the AD-SoS Z-score and whole body Z-score which had a poor significant correlation coefficient (0.31, p<0.02). (16, 22) These five studies evaluated children and adults at an age ranging from 4-27 years, while Catalano et al (23, 46) evaluated postmenopausal women. The younger and smaller age range in our group could be an explanation for the different results we found.

Furthermore, Table 6 gives an overview of the authors’ conclusion per study, showing different interpretations for in general a relatively low correlation between
### Table 6: Literature study of studies investigating the association between the measurements of DXA and QUS

<table>
<thead>
<tr>
<th>Author (yr)</th>
<th>QUS</th>
<th>Age (yr)</th>
<th>Sample size</th>
<th>Morbidity</th>
<th>Statistical method</th>
<th>Outcome</th>
<th>Pearson's correlation coefficients (r)</th>
<th>Authors’ conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluskiewicz (2002) (18)</td>
<td>Phalanges</td>
<td>9 - 23</td>
<td>30</td>
<td>ESRF</td>
<td>LCC, SCC</td>
<td>AD-SoS</td>
<td>LS/TB BMD, Z-score</td>
<td>0.45-0.56</td>
</tr>
<tr>
<td>Di Mase (2012) (14)</td>
<td>Phalanges</td>
<td>4 - 25</td>
<td>28</td>
<td>Idiopathic SH</td>
<td>PCC, MRA</td>
<td>AD-SoS, BTT, Z-scores</td>
<td>LS BMD, Z-score</td>
<td>0.42-0.52</td>
</tr>
<tr>
<td>Catalano (2013) (46)</td>
<td>Phalanges</td>
<td>43 - 78</td>
<td>80</td>
<td>Postmenopause</td>
<td>SCC</td>
<td>AD-SoS, BTT, UBPI, T-scores</td>
<td>FN BMD, T-score, LS BMD, T-score</td>
<td>FN T-score: 0.61, LS T-score: 0.55</td>
</tr>
<tr>
<td>Gonçalves (2014) (16)</td>
<td>Phalanges</td>
<td>6 - 27</td>
<td>70</td>
<td>21-OHD</td>
<td>PCC, SCC, CT, ROC</td>
<td>AD-SoS, BTT, UBPI, Z-scores</td>
<td>LS/TB BMD, Z-scores</td>
<td>0.59-0.72, Z-scores: no correlation</td>
</tr>
<tr>
<td>Bąk-Drabik (2016) (22)</td>
<td>Phalanges</td>
<td>10 - 15</td>
<td>51</td>
<td>IBD</td>
<td>SCC, MRA</td>
<td>AD-SoS, Z-score</td>
<td>LS/FB BMD, Z-scores</td>
<td>Continuous: 0.69-0.74, TB Z-scores: 0.31, LS Z-scores: NS</td>
</tr>
<tr>
<td>Catalano (2017) (23)</td>
<td>Phalanges</td>
<td>60 - 70</td>
<td>60</td>
<td>Postmenopause, BC</td>
<td>SCC, MRA</td>
<td>AD-SoS, BTT, UBPI</td>
<td>LS/FN BMD</td>
<td>AD-SoS/FN BMD: 0.32</td>
</tr>
<tr>
<td>Olszniski (2015) (24)</td>
<td>Radius/tibia/ phalanges</td>
<td>30 - 97</td>
<td>4123</td>
<td>Healthy</td>
<td>PCC</td>
<td>SOS</td>
<td>FN, hip, LS BMD</td>
<td>0.21-0.29</td>
</tr>
<tr>
<td>Hartman (2004) (32)</td>
<td>Radius/tibia</td>
<td>5 - 18</td>
<td>41</td>
<td>Celiac disease</td>
<td>LRA, MRA</td>
<td>SOS, Z-score</td>
<td>LS BMD, Z-score</td>
<td>0.3</td>
</tr>
<tr>
<td>Zuckerman-Levin (2007) (25)</td>
<td>Radius/tibia</td>
<td>11 - 39</td>
<td>27</td>
<td>Turner's syndrome</td>
<td>PCC</td>
<td>SOS, Z-score</td>
<td>LS/FN/TB BMD, Z-scores</td>
<td>0.46-0.63</td>
</tr>
<tr>
<td>Gianni (2008) (10)</td>
<td>Radius/tibia</td>
<td>4 - 6</td>
<td>45</td>
<td>Ex-preterm</td>
<td>PCC</td>
<td>SOS, Z-score</td>
<td>LS BMD, Z-score</td>
<td>NS</td>
</tr>
<tr>
<td>Mora (2009) (26)</td>
<td>Radius/tibia</td>
<td>4 - 22</td>
<td>88</td>
<td>HIV-infection</td>
<td>MRA, Y1 test</td>
<td>SOS, Z-score</td>
<td>LS/TB BMD/BMC, Z-scores</td>
<td>0.58-0.66</td>
</tr>
<tr>
<td>Christoforidis (2010) (30)</td>
<td>Radius/tibia</td>
<td>4 - 18</td>
<td>27</td>
<td>Hemosphiola A</td>
<td>PCC, CX</td>
<td>SOS, Z-score</td>
<td>LS BMD, Z-score</td>
<td>Tibia: NS, Radius: -0.019</td>
</tr>
<tr>
<td>Christoforidis (2011) (31)</td>
<td>Radius/tibia</td>
<td>2 - 16</td>
<td>20</td>
<td>CKD</td>
<td>PCC, SCC, MRA</td>
<td>SOS, Z-score</td>
<td>LS BMD, Z-scores</td>
<td>Tibia: 0.129, Radius: 0.022</td>
</tr>
<tr>
<td>Williams (2012) (33)</td>
<td>Radius/tibia</td>
<td>5 - 20</td>
<td>621</td>
<td>Healthy, CF, obese</td>
<td>CT, BAA</td>
<td>SOS, Z-score</td>
<td>LS BMD, BMAD, Z-scores</td>
<td>-</td>
</tr>
<tr>
<td>De Schepper (2012) (45)</td>
<td>Radius</td>
<td>12 - 38</td>
<td>64</td>
<td>CF</td>
<td>LRA, MRA</td>
<td>SOS, Z-score</td>
<td>TB BMC/BMD, Z-scores</td>
<td>0.39-0.44, Z-scores: NS</td>
</tr>
<tr>
<td>Chong (2015) (29)</td>
<td>Radius</td>
<td>7 - 11</td>
<td>134</td>
<td>Healthy</td>
<td>CT, BAA, ROC</td>
<td>SOS, Z-score</td>
<td>TB BMC/BMD, Z-score</td>
<td>-</td>
</tr>
<tr>
<td>Van Rijn (2000) (13)</td>
<td>Tibia</td>
<td>7 - 23</td>
<td>146</td>
<td>Healthy</td>
<td>MRA</td>
<td>SOS</td>
<td>LS/TB BMD, BMAD</td>
<td>0.63-0.81</td>
</tr>
<tr>
<td>Tuna (2008) (12)</td>
<td>Tibia</td>
<td>40 - 72</td>
<td>200</td>
<td>Postmenopause</td>
<td>LRA, SCC, CT, ROC</td>
<td>SOS</td>
<td>LS/FN BMD</td>
<td>0.29-0.36</td>
</tr>
<tr>
<td>Sundberg (1998) (20)</td>
<td>Calcaneus</td>
<td>11 - 16</td>
<td>280</td>
<td>Healthy</td>
<td>PCC</td>
<td>SOS, BUA, SI</td>
<td>TB, LS, FN BMD</td>
<td>0.44-0.70</td>
</tr>
<tr>
<td>Falcini (2000) (15)</td>
<td>Calcaneus</td>
<td>6 - 18</td>
<td>53</td>
<td>Juvenile arthritis</td>
<td>PCC, LRA</td>
<td>BUA, Z-score</td>
<td>LS BMD, Z-score</td>
<td>0.83</td>
</tr>
<tr>
<td>Sani (2011) (19)</td>
<td>Calcaneus</td>
<td>6 - 17</td>
<td>8</td>
<td>Thalassemia</td>
<td>PCC</td>
<td>SOS, BUA</td>
<td>TB/LS BMD</td>
<td>BUA: 0.492-0.507, SOS: NS</td>
</tr>
<tr>
<td>Xu (2014) (21)</td>
<td>Calcaneus</td>
<td>5 - 19</td>
<td>392</td>
<td>Healthy</td>
<td>PCC</td>
<td>SOS, BUA, SI</td>
<td>TB BMC/BMD</td>
<td>0.690-0.693</td>
</tr>
<tr>
<td>Weeks (2016) (27)</td>
<td>Calcaneus</td>
<td>4 - 18</td>
<td>389</td>
<td>Healthy</td>
<td>PCC, LRA, MRA</td>
<td>BUA</td>
<td>TB/LS/FN BMD, BMC</td>
<td>0.47-0.56</td>
</tr>
</tbody>
</table>

Numbers in brackets behind the author names reflect the reference. Morbidity: ESRF, End-stage renal failure; Idiopathic SH, Idiopathic subclinical hypothyroidism; BC, breast cancer; CRD, Chronic rheumatic disease; IBD, Inflammatory bowel disease; CF, Cystic fibrosis; 21-OHD, 21-hydroxylase deficiency; CKD, Chronic kidney disease. Statistical methods: PCC, Pearson's correlation coefficient; LCC, linear correlation coefficient; SCC, Spearman's correlation coefficient; CT, cross tabulation; ROC, receiver operating characteristic analysis; LRA, linear regression analysis; MRA, multiple regression analysis; BAA, Bland-Altman analysis; CT, Cohen x analysis. Outcome: LS, lumbar spine; TB, total body; FN, femoral neck; AD-SoS, amplitude-dependent speed of sound; SOS, speed of sound; BTT, bone transit time; UBPI, ultrasound bone profile index; BUA, broadband ultrasound attenuation; SI, stiffness index; BMC, bone mineral content; BMD, bone mineral density; BMAD, bone mineral apparent density; NS, not significant; F, female; M, male. Author's conclusion: +, good association; +/-, moderate association ; -, weak/poor association.
QUS and DXA. An advantage of our study was that, besides the small range of age of the participants, more statistical tests were used to compare both techniques, leading to a more reliable overall conclusion.

The partial correlation coefficient was used to evaluate the effect of possible confounders on the original correlation. To our knowledge only a few studies that evaluated the association between pQUS and DXA looked at the influence of possible confounding factors with regard to correlation coefficients. Halaba et al (28) and Di Mase et al (14) performed a multiple linear regression analysis to evaluate the effect of anthropometric characteristics on DEXA or QUS measurements using confounders such as age, sex, weight, height and BMI.

Only four studies used other statistical tests such as the Bland-Altman analysis (29, 33) and cross tabulation (12, 16, 29, 33). Although these studies looked at tibial and radial QUS, their conclusions are consistent with our findings of poor agreement between the two techniques and a large discrepancy in differentiating the same children as having normal or reduced bone mineralization.

The overall absence of an association between DXA and pQUS for continuous values and Z-scores could be explained by the fact that measurements were influenced by different bone composition and bone mineralization at different sites. In addition, children may be in different growth phases and some bones may grow faster than others, which potentially could have an effect on the bone development and thereby may have affected the results. Quantitative ultrasound can be applied on various parts of the extremities, such as the phalanx, radius, tibia and calcaneus. Recent studies suggest that the phalanges may be the most appropriate measurement site, because this site is sensitive to changes in bone status (47-49).

For the assessment of pQUS we chose the DXA scan as the golden standard for comparison. Quantitative Computed Tomography (QCT), or peripheral QCT may have been good alternatives and may have provided more accurate results and additional information on bone strength. However, QCT uses a relatively high radiation dose, especially for young children in research settings, and for both methods normative data for a pediatric population are lacking. DXA has been recommended as an appropriate method for clinical densitometry of infants and young children by the International Society for Clinical Densitometry (ISCD) in 2013. The advantage of QUS is the ability to easily repeat measurements, giving the opportunity to follow the development of bone over time, especially under circumstances were diagnostic tools as DXA are not possible or available. Our results were limited to a single measurement per child. Therefore intra-individual repeated measurements with QUS should be further evaluated with regard to the reliability to predict the intra-individual development of bone over a longer time period.

Our study had several limitations. The number of patients was limited. Firstly, this limited the number of confounders we were able to use for calculation of the partial correlation coefficient. Secondly, low bone mineralization with a significant Z-score below 2.0 SD was only found in 2 out of 60 children for lumbar spine measurements, and none for the whole body measurements. To increase the number of children with deviant bone mineralization we chose a cut-off value of -1.0 SDS for determination of normal or low bone mineralization, in contrast to general practice. This is in accordance with the study of Gianni et al, who evaluated the prevalence of Z-scores < -1.0 SDS for tibial or radial QUS (10), because the QUS values were higher in comparison with DXA. According to the literature a similar cut-off value can be used for AD-SoS and BTT (14, 41).

In general, the Z-scores were based on reference data derived from different populations. The reference data of the DXA Z-scores were based on an U.S. population, while the reference data for the pQUS Z-scores were recorded from an Italian population. It is not known whether the populations are comparable to our population and theoretically this might explain some of our differences.

Conclusion

This study demonstrated a weak association between DXA and pQUS measurements, established from a statistically significant weak correlation, a poor agreement and a discrepancy in differentiating the same children with normal or reduced bone mineralization. Therefore, pQUS measurements are not equivalent to DXA for the evaluation of bone health and cannot replace the DXA in former preterm born children at the age of 9 to 10 years.
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PART 4

General Discussion and Summary
Discussion and future perspectives
General discussion

As described in chapter 1 (Part 1), preterm birth is a critical event for the infant, with major impact on health directly after birth, but also with high risk of unfavorable long-term development. The survival of preterm infants has increased significantly during the last decennia, consequently it is important to further improve postnatal treatment to achieve the best possible development. During the third trimester, the fetus exhibits an exponential development of all organ systems leading to optimal growth, neurodevelopment and bone development. Ideally, after birth, the treatment of preterm infants should aim to continue the intra-uterine development, to achieve growth and functional development comparable to healthy term born infants.

Nutrition in the early postnatal period has been recognized as a key determinant for neonatal outcomes. For this thesis we evaluated nutritional interventions in very low birth weight infants, comprising the group of very, and extreme preterm infants, with emphasis on the early postnatal period because this has been shown to be a critical period in the transition from constant rich flow of nutrient supply in healthy pregnancies, through the umbilical cord to enteral and parenteral supply of nutrients with risk of under- or overnutrition and consecutively impaired development.

The aim of the thesis was to study effects of nutritional interventions on growth, development and bone composition in very low birth weight infants.

Part 2 of this thesis presents the results of clinical studies on the effect of amino acid intake on growth and neurodevelopment, while the studies presented in Part 3 assessed the calcium and phosphorus metabolism as determinant for bone mineralization and growth.

Amino acids and postnatal growth

Chapter 1 described that the preterm born infant has amino acid requirements comparable to the fetal requirements. Fetal amino acid concentrations are higher than maternal concentrations, mostly obtained by production within the placenta and based on an active, carrier-mediated transport. Quantitative balance studies showed that amino acid uptake exceeded the amount necessary for protein accretion, indicating that the human fetus oxidizes amino acids to generate energy. Fetal amino acid requirements are high to provide sufficient substrate to enable a high protein synthesis rate with adequate growth during early gestation. Animal studies have shown that protein deficits in fetuses lead to growth restriction, but also adversely affect organ development such as that of the kidney, pancreas and brain, thereby inducing changes with long-term consequences such as hypertension and diabetes and functional outcome.
Chapter 3 compared a new feeding regimen with higher amounts of amino acids in parenteral nutrition, according to international recommendations, in combination with the intention of rapid increment of enteral feeding (Cohort 2), to the previous standard nutritional protocol with lower amounts of amino acids and energy (Cohort 1).\(^{14, 13-15}\) Very low birth weight infants were included prospectively during two consecutive years. We demonstrated that the new parenteral nutrition provided a significant higher amount of amino acids during the first week of life and this was associated with a significant higher weight gain in appropriate for gestational age born infants until week 5 postnatally. This short-term improvement was in agreement with the retrospective cohort study by Berry et al (1997) who determined that amino acid intake in the first two weeks of life in very low birth weight infants was an independent positive prognostic determinant of growth.\(^{16}\) Olsen et al compared more than 500 preterm infants below 30 weeks gestational age from 6 different neonatal intensive care units.\(^{17}\) They found that, even after adjustment for a number of clinical factors, differences in weight gain between units could be explained by the variation in nutritional intake. Altogether, these results indicate that the administration of a sufficient amount of amino acids, not only can reverse a negative nitrogen balance in preterm infants, as has been described by a number of studies, but also has an effect on growth.\(^{18-24}\) However, a further finding of our cohort study was that despite the higher weight gain rates from the second week onwards, all infants still demonstrated a steady decrease in standard deviation score (SDS) for weight that resulted in a mean loss of 2 SDS for both cohorts around term corrected age, indicating that our nutritional improvement still was insufficient. Infants in both cohorts developed cumulative deficits in protein and energy intake relative to the daily requirements, that were not compensated during the observational period of five weeks. Embleton et al. demonstrated that postnatal growth retardation in preterm infants was associated with a nutritional intake of protein and energy below the current recommendation during the first 2 weeks of life.\(^{25}\)

The insufficient nutritional intake as seen in our cohort, despite improvement of parenteral intake, can be explained by an insufficient (i.e. not meeting the requirements) amount of protein and energy provided with enteral feeding. According to our revised nutritional protocol full enteral feeding was achieved significantly earlier and consecutively the duration of parenteral nutrition was significantly shorter. Despite early fortification of human milk, Cohort 2 achieved the recommended intake of protein only at median day 10 (min-max: 4–35 days) while 16% of the cohort never reached a recommended enteral protein intake of 3.8 gram/kg/day. More than 90% of infants in both cohorts received mother’s own milk (MOM). The exact composition of MOM was not known and therefore exact protein intake could not be calculated, a limitation of our study. As elaborated in chapter 1 the composition of human milk is dependent on the gestational age of the infant and the duration of lactation. During the last 30 years several studies have tried to define reference values for the composition of human milk. Most of them evaluated human milk provided by mothers of more mature preterm infants than nowadays treated and most of them analysed only a small number of samples and at different times.\(^{26-32}\) Furthermore the method of protein determination may influence the results.\(^{41}\) This may explain the great diversity of data concerning the composition of human milk. For the study presented in chapter 3 we used the reference of Anderson et al since this has frequently been cited as reference for the composition of human milk and therefore facilitates the comparison with other publications.\(^{33}\) Anderson et al. evaluated, besides term and donor milk, human milk from mothers of 14 preterm infants born between 28 and 36 weeks GA. Samples were taken at day 3, 7 and 14 after birth. Anderson et al also recognized, as many other studies, a decreasing amount of protein during the lactation period with an assumed protein content of 1.4 g/dl after two weeks.\(^{34}\) In contrast, more recent data presented by Bauer et al investigated a large sample (102) of mothers of preterm infants with a gestational age between 23 and 33 weeks who were followed according to a standardized procedure for 8 weeks.\(^{35}\) Both studies used 24 h samples. Analyses of milk samples performed by Bauer et al during the first postnatal week revealed a mean protein content of 2.8 – 2.2 g/dl depending on the gestational age and a decrease in protein content during the lactation period with a mean protein concentration of 2.1 g/dl for preterm infants after eight weeks. To rule out an underestimation of nutritional intake, the intake for both cohorts was also calculated using the data provided by Bauer et al. with a varying protein intake between 2.5 and 2.0 gram. This resulted in higher nutritional intakes and shorter time to achieve the recommended intake, however for both cohorts the cumulative deficits were not resolved by the end of the observational period of five weeks. Therefore the conclusion was that optimal nutritional intake, as actually recommended, cannot be achieved with currently available standard human milk fortifiers. We suggest that standardized human milk fortifiers should provide a higher amount of protein, or otherwise that protein content of human milk should be adapted to the individual requirements of the infant based on individual analyses of MOM.

In contrast to our findings two recent observational studies, evaluating comparable cohorts of preterm infants, demonstrated that it was possible to prevent cumulative nutritional deficits in the early postnatal period and that this was associated with less postnatal growth restriction.\(^{33-35}\) To understand the differences in outcomes compared to our results, it is necessary to look in detail at differences between the nutritional protocols as well as the interpretation of the data. Senterre et al evaluated a cohort of 102 preterm infants with birth weight below 1250 grams. The first study was a description of weight changes until discharge in relation to nutritional intake with a sub-categorization of infants into appropriate (AGA) and small (SGA) for gestational age.\(^{33}\) The second study evaluated 84 infants of the same cohort with a
subdivision of infants according to gestational age at birth below 28 weeks or between 28 and 30 weeks. (54) Infants in this study experienced a negligible cumulative protein deficit during the first two weeks and had the maximum decrease of 0.9 SDS for weight by the second week that improved to a loss around -0.5 SDS for weight after six postnatal weeks. Infants of this study received significantly higher protein intakes during the first month than infants of studies that previously related postnatal nutritional intake to postnatal growth retardation. (25, 56, 57)

Maas et al compared two historical cohorts, evaluating an enhanced volume intervention exclusively in extremely preterm infants. (58) Both cohorts had a decrease of –0.15 SDS for weight. Recently Maas et al compared a comparable cohort study evaluating the same nutritional intervention exclusively in extremely preterm infants. (58) All of these studies have in common that the first week nutritional intake affected growth at discharge, and differences in outcome, among other things, compared to our results, can be explained by differences in nutritional protocols.

In comparison to the studies presented by Senterre et al., our cohorts started enteral nutrition on the first day of life (mean 0.96 ± 0.39 vs. 0.91 ± 0.22 days), while for the total Senterre cohort enteral feeding was introduced at a mean of 3.9 ± 4.2 and 2.9 ± 4.2 days for AGA and SGA infants respectively. Our infants received parenteral nutrition (PN) for a mean duration of 12.8 ± 3.8 vs. 10.6 ± 3.6 days, while full enteral feeds were achieved by a mean of 13.6 ± 3.4 vs. 11.6 ± 3.6 days. According to Senterre, PN was discontinued at a mean age of 28.6 ± 18.8 and 21.3 ± 15.1 days. (53) In the second study evaluating infants born below 30 weeks’ gestation introduction of enteral feeding occurred even later and duration of PN was even longer. (54) Therefore the higher nutritional intake in the Senterre cohort was predominantly achieved by PN. Twelve infants of the Senterre group (about 10 % of the total study group) received individualized fortification after human milk analysis. It remains unclear how these analyses were used in the calculation of the nutritional intake. One may speculate that this may have had a substantial effect (in combination with continuing PN intake) on the improved weight gain of their patients compared to our patients. Maas et al., in comparison to the cohorts studied in this thesis, provided a higher amount of protein and energy with human milk fortifier (protein: 1.1 – 1.6 vs. 0.8 g/dl; energy: 15 - 20 vs. 15 kcal/dl). (55, 58) In this way, we conclude that higher supplementation of protein is needed with early increment of enteral feeding to prevent postnatal growth retardation. Finally, the differences in nutritional management used in all the above mentioned studies, reveal the essential scientific question concerning optimal feeding for preterm infants. It is not known what the optimal way of feeding is, either by parenteral or enteral route. As explained in the introduction, both ways have their (dis)-advantages. Most studies demonstrate a positive effect of human milk as stimulation of gastrointestinal maturation, support of infant host defence, improved neurodevelopment, as well as protection against necrotizing enterocolitis. (59, 60)

Therefore we suggest that improvements of enteral feeding strategies with human milk need to be further investigated.

A second aspect of differences in the results among the above mentioned studies is based on the fact that AGA and SGA infants seem to have a different growth pattern. We performed sub-group analyses for AGA and SGA infants of both cohorts. Nutritional intake was not different between the sub-groups. The sub-groups of AGA infants for different gestational ages within a cohort showed no differences in growth pattern, while SGA infants, comprising 16 and 18 percent of both cohorts, showed the highest weight gain and a lower decrease in SDS for weight until term corrected age (TCA) compared to AGA infants. (chapter 3, figure 2) A comparable growth pattern was seen for SGA infants (20%) in the Senterre study, even ending up with a positive change in SDS for weight at discharge. (53) For the second study, Senterre et al included SGA in the subdivided cohorts according to gestational age, thus combining AGA and SGA infants in one group. This may have had a positive effect on the changes in SDS score for the total subgroup. The historical cohorts evaluated by Maas et al comprised a comparable number of SGA infants, but sub-group analyses where not performed. (55, 58) In addition to the retrospective studies, Maas et al presented a small randomized trial that evaluated postnatal growth in relation to a further increment of protein in human milk fortifiers. (61) In this study a higher increase of protein did not further enhance the growth, while the lower protein intake group seemed to have none significant better weight changes. Nearly 40 percent of this group were classified as SGA. We speculate that in this randomized trial, the relatively lower protein intake for SGA infants may have been sufficient for an optimal catch-up growth and thereby may have biased differences resulting from nutritional intake. Appropriate for GA infants have demonstrated a normal growth potential until birth, therefore this sub-group of preterm infants should serve as a reference for the evaluation of optimal postnatal growth, while sub-group analysis of SGA infants is necessary as well, to define optimal growth in this group without increased risk of long-term negative consequences. (62) We conclude that AGA and SGA preterm infants have different protein requirements for optimal growth. Specific nutritional needs of SGA preterm infants are currently not known. We suggest that nutritional intervention studies, trying to specify the requirements of sub groups of AGA and SGA preterm infants, are needed.

Chapter 4 presents the results of the long-term follow up of the same cohorts, evaluating changes in length and BMI. We were unable to demonstrate an effect of the first week nutritional intake on growth at age of nine to 10 years. Both cohorts were already able to catch up in length to standard deviation scores (SDS) within the range for the Dutch reference population and slightly lower BMI SDS by six months of corrected age with no further significant increment at the age of nine to 10 years. The
subgroup of SGA children needed more time, but showed a full catch-up in length at age nine to 10. Our findings are in contrast with a number of studies, among which the Dutch nationwide prospective study POPS, that followed growth development of very and extreme preterm infants. [63-68] These follow-up studies showed that weight and length until young childhood and early adulthood of AGA born children who developed an extra-uterine growth retardation remained small, and SGA children as well showed persistent stunting. Fast postnatal weight gain was associated with increase in BMI at young adulthood, thus a risk factor for development of metabolic syndrome. [69] Children evaluated for these studies were born between the 70ies and 90ies of the last century and outcome was not related to nutritional intake. We speculate that the differences in outcome compared to our cohort may reflect in general, improvement of neonatal intensive care treatment, but also improvement in nutritional practices. However, despite the fact that growth of children in our cohorts approached the range of the reference population, AGA children remained below their initial birth SDS, indicating that further improvement of the nutritional protocol is needed.

Amino acids and neurodevelopment
Chapter 5 describes the evaluation of infants of the above mentioned cohorts at corrected age of 24 months with emphasis on neurodevelopmental outcome, postnatal weight gain and head circumference in relation to the first week nutritional intake of protein and energy. Specifically boys who had received more protein and energy demonstrated a short-term improvement in postnatal weight gain, while catch-up growth in head circumference seemed to be improved as well. We found differences in the developmental scores between boys and girls.

The Bayley Scales of Infant Development (BSID II) were used for assessment of the cognitive (MDI) as well as motor (PDI) development. [70] Adequate testing requires on the one hand specifically trained and experienced psychologists and physical therapists and on the other hand the persisting cooperation of a small child. Thus, failure to reveal a test score can either be the result of lack of cooperation of an otherwise normal child or lack of ability to pay attention or capability to fulfill the tasks, both indicating a subnormal development. For 10 infants, comprising 18% of cohort 1, we were unable to define a MDI score. Based on written observations of the investigators (in addition to some available scoring results) and the parents’ concerns about child behavior we decided to categorize these infants into the group of sub-normal development (MDI/PDI < 85) and consequently were able to include them in the logistic regression. Since the continuous scores were not available, these infants are missing in the linear regression analysis. Since all 10 children derived from the cohort with lower nutritional intake, there is a chance of underestimation of the effect of nutritional intake on the continuous scores. However, in clinical practice and for the individual child, the categorization into normal or sub-normal development may be more relevant, thus we suggest that the logistic regression analysis in our study represents the more relevant results.

Postnatal weight gain and head growth have been positively associated with mental and psychomotor development. [71-76] Improvement in head growth has specifically been associated with higher amino acids intake. [64, 77, 78] However, until now it has been difficult to prove the relationship between higher amino acid intake and improvement of neurodevelopment. There is no consensus on the positive effect of amino acids on neurological outcome. [79, 80] The comparison of different studies is hampered by diversity of nutritional interventions and various neurodevelopmental outcome measures. In contrast to most other studies we were able to directly relate the psychomotor indices to the individual nutritional intake of the first week. [79]

Various multi-center follow-up studies have reported impaired functional outcome and a higher incidence of disabilities in preterm compared to term born children. [81-88] Numerous risk factors are known to negatively affect neurodevelopment among which perinatal morbidities. For our cohorts the diagnosis and treatment of patent ductus arteriosus (PDA) was, together with male sex, the greatest negative determinant of neurodevelopment. The association of PDA with impaired neurodevelopment has been a subject of a number of epidemiological studies. [89-91] While a validated explanation for the association currently is missing, none of the studies accounted for the nutritional intake of infants with PDA. A conservative measure of treatment of a PDA is fluid restriction and cautious enteral feeding, often at the expense of the daily required amount of nutritional intake. In our study group, infants with PDA received statistically significant less protein and energy during the first two weeks compared to infants without PDA. We speculate that not the PDA itself, but undernutrition in a period of rapid brain growth is an important determinant of the impaired outcome. The same may hold true for other perinatal diseases such as sepsis or necrotizing enterocolitis that in clinical practice often lead to a reduction in nutritional intake despite high nutritional needs. We suggest that evaluation of outcome in relation to prematurity related diseases should take into account the individual nutritional intake.

Besides perinatal conditions, gender has been reported to be an important determinant for outcome after preterm birth. In general, long-term follow-up evaluations of preterm infants have shown that boys are more at risk for unfavorable outcome than girls. [64, 92-94] Hintz et al. evaluated risk factors for adverse neurodevelopmental outcomes of preterm born children and found male gender to be an independent risk factor for severe impairment of psychomotor development at two years of age. [95] In accordance with these findings, boys in our cohorts had lower developmental scores than girls. However, the first week increase in protein and energy intake was associated with an increase in PDI score and significantly increased
the chance of having a normal PDI, specifically in boys. For girls higher protein and energy intake of the first week significantly increased the chance of normal MDI. Several nutritional intervention studies already indicated that boys may take more advantage of improvements of nutritional intake than girls. Lucas et al found that preterm boys fed preterm formula after discharge had a greater head circumference compared to preterm boys fed term formula, although this was not related to improvement in developmental scores. Poindexter found that a higher intake of parenteral amino acids during the first postnatal week resulted in significantly less impaired growth of head circumference in male infants at 18 months corrected age. Frondas-Chauty et al evaluated a French cohort of very preterm infants and found an association between in-hospital growth velocity and neurological outcome at 2 years of corrected age. The association between early growth and neurodevelopment was significantly stronger for boys than for girls, even after adjustment for a great number of different determinants of neurodevelopment. This study did not include nutritional management in the statistical analyses, therefore a suggestion of gender specific benefits in relation to nutritional interventions in the early postnatal period based on the results of this study remains speculative. Van den Akker et al. assessed very preterm infants at the corrected age of two years after participation in a randomized trial that provided either parenteral glucose or glucose in combination with amino acids during the first three days after birth. In this study boys had significantly more often a normal neurodevelopmental outcome if they had received amino acids from birth onwards. While girls tend to have a more favorable outcome compared to boys, girls who participated in this study showed no improvement in relation to the intervention.

All of these studies indicate that specific nutritional interventions may be advantageous for male infants, especially when they are compared with groups that are underfed. The physiology of gender-specific nutritional requirements is not well understood yet. Boys and girls grow differently and experience a different metabolic and endocrine environment. Male vulnerability has been related to faster growth and hence greater substrate demands compared to females, different exposure to sex steroids, impaired adaptive response to stress, as well as altered speed in development and consecutively metabolic bone disease (MBD). Studies tried to define nutritional requirements, but it is often uncertain whether the nutritional intake of Ca and P provided to preterm infants is sufficient and is actually used for bone mineralization. In daily clinical practice it is impossible to directly evaluate the effect of mineral intake on bone development, therefore biochemical parameters of the Ca-P homeostasis in blood and urine are frequently used to evaluate nutritional intake.

Chapter 6 presents an observational descriptive study of Ca and P metabolism in the cohort of preterm infants that received improved parenteral mineral intake. Changes in biochemical parameters in urine and blood during the first five weeks after birth are described. Mineral imbalances as hypercalcemia, hypophosphatemia, hypercalciuria and high tubular phosphate reabsorption during the first week after birth were observed as well as an increasing alkaline phosphatase (ALP) and hyperphosphaturia in the following period, indicating insufficient supply of phosphorus as well as unfavorable ratios of calcium and phosphorus supplementation. Infants in this cohort received amounts of Ca and P within the recommendations of the guidelines our study had an increased chance of normal development with higher intake of protein and energy. Our results indicate that both female as well as male infants are vulnerable to nutritional deficits, but with different needs. Furthermore our study comprised a short-term intervention with a positive effect on weight, as well as on neurodevelopment. In agreement with our study, Belfort et al found in a cohort of former preterm infants, that early linear growth was associated with better cognition, but also with an increased risk of overweight or obesity later in childhood. In summary, these findings indicate that short-term nutritional interventions can effect outcome positively in male as well as in female preterm infants, but the optimal nutrient composition, the amount and timing of nutritional intake, to achieve further improvement are currently unknown. There may be a critical window for nutritional interventions to achieve optimal neurodevelopment without increasing the risk for obesity. We suggest that further research is needed to evaluate gender specific nutritional requirements in preterm infants, while studies in general should take into account differences in outcome between boys and girls and therefore need to be adequately powered to detect a gender effect. Future studies should investigate the effect of gender specific nutritional protocols on growth and neurodevelopment.

Calcium and phosphorus metabolism in the early postnatal period of preterm infants

Nutritional intake of calcium (Ca) and phosphorus (P) in general, aims to provide adequate amounts of minerals to achieve optimal bone mineralization, specifically in preterm infants who have the highest requirements during the immediate postnatal period. Inadequate intake of Ca and P increases the risk for impaired bone development and consecutively metabolic bone disease (MBD). Studies tried to define nutritional requirements, but it is often uncertain whether the nutritional intake of Ca and P provided to preterm infants is sufficient and is actually used for bone mineralization. In daily clinical practice it is impossible to directly evaluate the effect of mineral intake on bone development, therefore biochemical parameters of the Ca-P homeostasis in blood and urine are frequently used to evaluate nutritional intake.
for parenteral and enteral intake for very low birth weight infants presented by the ESPGHAN. A number of studies demonstrated that adding Ca and P to the PN solution caused a positive mineral balance and increasing the amount of Ca and P increased the retention of both minerals. None of these studies provided amounts that would equal the fetal accretion rate, probably because of limited solubility of parenteral fluids. The use of sodium-glycero-phosphate enabled us to obtain a stable PN solution with the recommended amount of Ca and P. However, the mineral disturbances as seen in this study indicate that the composition of minerals may not have been adequate. The PN used for this study contained Ca and P with a molar ratio of Ca:P of 1.56:1. All of the above mentioned studies used varying ratios (0.9:1, 1.3:1, 1.7:1, 2:1). The Ca:P ratio for PN as recommended by the ESPGHAN varies between 1.2–1.7:1 and is based on the fetal mineral accretion ratio (1.7:1) and the fixed ratio for hydroxyapatite (1.67:1). In contrast, the American Society for Clinical Nutrition and Tsang et al. recommended ratios between 1:1 and 1:3:1, thus comparatively higher intakes of P. Since the PN used in our study provided an amount and ratio of Ca and P according to ESPGHAN recommendations, the findings of this study indicate that the European recommendation needs to be adapted to higher P intake, thus lower ratios. Our results are in agreement with a recently published study by Mulla et al. who compared two PN regimens with different Ca:P ratios. Reverting the molar ratio of Ca:P in PN from 1:3-1:5:1 to 1:0:1 was associated with a lower incidence and severity of hypophosphatemia and hypercalcemia. Higher requirements of P may be explained by the fact that besides being an essential mineral for bone mineralization, P also plays an important role in energy metabolism and cellular functions. In addition, the higher amino acid intakes provided, resulting in higher protein synthesis rates, may increase the need for additional P. We suggest that a ratio as recommended by the American Society for Clinical Nutrition is more appropriate.

Minerals provided by PN are directly available for metabolism in contrast to the dependency on intestinal absorption of enteraly supplemented nutrients. Consecutively enteral daily requirements are assumed to be higher than parenteral needs and molar Ca:P ratios are assumed to be different. Balance studies estimated a significant variability of intestinal absorption of Ca (40 - 70 %) and P (60 - 95%) among preterm infants. The efficacy of recommended Ca:P ratios for enteral intake has not been evaluated systematically. Only a few studies, mostly dating back to more than two decennia ago, looked at different mineral intakes in either human milk or preterm formula. Most studies evaluated small numbers of infants, either at term age or higher gestational age than currently cared for in neonatal intensive care units. These studies showed varying results, but mostly found that higher intake was related to improved bone mineralization. However, nowadays in clinical practice, very low birth weight infants rarely receive exclusively either parenteral or enteral nutrition in the direct postnatal period. Parenteral nutrition is started directly after birth, while enteral intake starts with small amounts several hours after birth with gradual daily increase. Therefore, we suggest that future guidelines should provide recommendations for a combined parenteral and enteral intake. Furthermore, taking into account the variability of intestinal absorption, we speculate that it may be difficult to guarantee sufficient intake even while providing amounts of minerals in adequate ratios according to recommendations. Subsequently, we suggest that supply of a calculated mineral intake may not guarantee adequate real intake and therefore monitoring of mineral homeostasis could be a tool to adapt nutritional supply to guarantee that Ca and P are sufficiently supplemented to support cell metabolism as well as bone mineralization. Thus we suggest that guidelines for nutritional intake of Ca and P could be improved by not only recommend a certain amount of minerals, but also define a target range of biochemical parameters that will guarantee optimal bone mineralization. However, neither the optimal amount of minerals, nor target ranges for biochemical parameters, that may indicate sufficient bone mineralization in VLBW infants, are currently known.

Pohlandt et al. recommended to use a ‘small surplus’ of phosphorus and calcium in spot urine as indicator for sufficient supplementation of minerals necessary for optimal bone mineralization. Hypophosphatemia in our cohort was treated with additional P supplementation. As demonstrated in the sub-analysis, we observed urinary excretion of P above the recommended surplus, independent of additional supplementation, in combination with low serum P and an increasing Alkaline Phosphatase (ALP). Therefore based on the results in chapter 6 we question the reliability of the ‘small surplus’ of P and Ca in spot urine as instrument to guide optimal mineral supplementation.

The evaluation of Ca–P homeostasis generally occurs under the assumption that biochemical parameters within the normal range will lead to optimal bone mineralization and is standard of care in many neonatal units as elaborated in chapter 7. However, the appropriateness of either parameter, frequency of measurements or reference values in relation to bone development have yet not been defined and there is lack of consensus and variation in practices regarding screening methods for metabolic bone disease. Currently, it has not been proven whether biochemical parameters are able to indicate sufficiency of nutritional intake. Chapter 7 presents the attempt to understand changes in biochemical markers in relation to nutritional intake. We performed an observational study in very low birth weight infants of initially three randomized groups that received different amounts of enteral Ca and P intake during the first 10 days of life. We found no differences between the groups. However the linear regression mixed model analyses showed that Ca intake significantly was associated with an increase in urinary excretion of Ca, renal tubular P reabsorption and a decrease in ALP, while P intake was significantly associated with
increase in serum P concentration, urinary excretion of P and ALP. Furthermore, higher protein intake was associated with decrease in serum P concentration, while low gestational age and male gender were associated with increased renal excretion of P.

This study not only confirmed but also quantified the increased needs of P in combination with high intake of amino acids.\(^{150, 151}\) We calculated that 1 mmol/kg/day of P was needed for every extra gram/kg/day of protein to keep the serum phosphorus concentration unchanged. We also confirmed the finding of our previous study concerning high renal excretion of P, irrespective of low serum P concentrations and independent of P intake. In agreement with the regression analysis, a sub-analysis, not presented in chapter 7, revealed that high renal excretion mostly was found in infants with a gestational age below 28 weeks. Low tubular reabsorption of P has been related to immaturity of the kidneys.\(^{152}\) Current recommendations for nutritional intake do not take into account differences in renal function based on gestational age. Minerals lost through renal excretion will not be available, neither for cell metabolism nor bone mineralization and therefore this loss should be compensated. Standardized repeated measurements, as used in our clinical practice, enabled us to individually adjust mineral supply. We used an increasing concentration of ALP as marker for P insufficiency and the necessity to increase P supplementation. This policy is in agreement with our study results. Despite high P intake we recognized an increasing ALP concentration, probably related to an increasing intake of protein.

The exact role of ALP in bone mineralization has not completely been elucidated, but experimental data based on studies using chicken and rat embryonic bones indicate a catalyzing activity linked to the initial mineralization process.\(^{153-160}\) ALP is a tissue-nonspecific membrane-bound metalloenzyme that is present in high amounts in matrix vesicles of bone tissue. In developing bone the matrix vesicles are released from chondro- and osteoblasts and deposited within the newly formed osteoid under the mineral facing surfaces of osteoblasts. Matrix vesicles are the initial site of calcification in cartilage and bone. The initial mineralization occurs in a biphasic pattern. The first phase is under tight control regulated by enzymes within the matrix vesicle, while the second phase mainly takes place in the extracellular matrix. Studies showed that ALP is needed to initiate calcification, while P is needed to activate the enzyme function of ALP. Phosphate molecules are brought into the matrix vesicle by ALP, while the intravesicular Ca concentration is increased by its affinity for lipids and Ca-binding proteins of the interior vesicle membrane. This results in deposition of CaPO_4 and initial crystal forming. Crystals built within the matrix vesicles finally perforate the vesicle membranes and then serve as templates for further crystal proliferation. Mineral particles keep growing and ultimately fill the extracellular matrix. It is not known whether these experimental results may be extrapolated to the human situation. An increase in ALP has been associated with growth in pubertal children as well as accelerated growth in height after growth hormone therapy.\(^{161}\) In

preterm infants high serum ALP levels usually have been associated with impaired bone mineralization as a result of insufficient supply of mineral intake. In contrast to the above mentioned pubertal children, preterm infants often reach serum levels far above the normal range. We reported increases in ALP in Chapter 6 and Chapter 7 in very low birth weight infants during the early postnatal period despite relatively high mineral intake. Taking into account the results of the experimental studies described above, we propose a ‘two step’ explanation for increases in ALP seen in the early postnatal period. The initial small increase may indicate activation of bone mineralization, comparable to changes as seen in children with growth spurt. This may be seen as a positive phenomenon. Further increase in ALP may occur after perforation of matrix vesicles without full activation of ALP by P, then entering the extracellular fluid and increasing the serum concentration. This probably may indicate insufficiency of P supply. In case of the study presented in chapter 7 and as described above, the intake of protein was significantly associated with a decrease in serum P concentration, while protein further was associated with a significant increase in ALP. All together this may be interpreted as a relatively insufficiency of P for bone mineralization. Thus, we regard ALP as a useful indicator for mineral supply for the benefit of bone mineralization in the early postnatal period.

The statement that ALP is useful for the evaluation of bone mineralization is controversial.\(^ {147, 148, 162, 163}\) Lucas et al. found an association between high ALP and impaired growth in length in childhood, whereas Tinnion and Embleton stated that serum P, Ca and ALP may be normal, while the bone actually is osteopenic.\(^{147, 162}\) They also found ALP not useful to predict the need to start treatment for MBD or predict MBD at discharge. The systematic review by Visser et al., analyzing the validity of biochemical markers in metabolic bone disease in preterm infants, found conflicting results concerning a correlation between high serum ALP and MBD.\(^ {148}\) All of these studies looked at biochemical parameters as markers for MBD and in case of ALP either looked at an incidental measurement or the maximum concentration. We used standardized repeated measurements of biochemical parameters to optimize nutritional supply of minerals with the aim to achieve bone mineralization comparable to term born infants. None of our patients developed signs of MBD, whereas a recent cohort study reported an incidence of MBD in preterm infants below 1000 grams of 31 % of which 34 % developed spontaneous fractures.\(^ {164}\) According to our opinion the provision of adequate intake of Ca and P from the first week of life onwards can prevent MBD even in the smallest infants, but monitoring of intake is necessary to account for increased requirements of P in combination with high amino acid intakes and renal (or fecal) losses related to immaturity. We conclude that changes in biochemical parameters are associated with nutritional intake.

Careful follow up of biochemical parameters has also been proposed in recent studies evaluating mineral homeostasis in relation to parenteral and enteral nutrition.
in preterm infants. However, none of the studies, including our own, has sufficiently defined target values of biochemical parameters to aim for. Research is needed to provide a concept of target values for biochemical parameters to guarantee sufficient intake of Ca and P. As an example we suggest that urinary P excretion should be further studied to define an upper range of mineral excretion to enable the differentiation between immature tubular reabsorption or over dosage of mineral supply. Under the assumption that protein and mineral intake is provided according to the recommendations, urinary excretion of P in combination with a low concentration of ALP may indicate sufficiency and thereby define the upper range of P excretion. Additional studies are needed to define this range with special attention to differences in gestational ages.

Growth and bone mineralization of preterm infants

Chapter 4 evaluates the long-term follow-up of growth and bone mineralization of children at the age of nine to 10 years who previously participated in the observational cohorts 1 and 2. We hypothesized that improved nutritional intake would lead to improved length and bone mineralization. The outcome in length has been discussed under section ‘amino acids and growth’. The mean standard deviation score (SDS) for bone mass measured for whole body (WB) and lumbar spine (LS) was within the normal range for the reference population, while bone mass seemed not to be related to the nutritional intake of the first two weeks. As previously discussed, our studies revealed that the improved nutritional protocol did not prevent nutritional deficits and infants demonstrated a severe extra-uterine growth retardation at term corrected age (TCA). However, at nine to 10 years, the bone mass SDS was within the normal range of the reference population for both cohorts. Several studies indicated a positive effect of human milk on bone mineralization despite low mineral content. More than 90% of all infants in both cohorts received MOM. For cohort 1 the use of MOM may have compensated for the low mineral intake. Furthermore this study indicates that preterm infants may have the capability for catch-up in bone mineralization. Several follow-up studies reported catch-up in bone mineralization in former preterm infants over the years. In contrast, a recent Chinese cohort study evaluated more than 2000 term and preterm newborn infants, including 149 extremely preterm infants, repeatedly during the first 12 months after birth. During this first year all preterm infants remained significantly smaller in weight and length and bone mineral density compared to the reference of term born infants, with the group of extremely preterm infants having the lowest scores during the whole period. In this study the preterm infants demonstrated a significant higher increase in bone mineral density compared to the reference, even after adjustment for well-known determinants of growth, but did not achieve a full catch-up. We speculate that despite the capability to catch-up in bone mineralization, special attention is needed for nutritional intake even in the post-discharge period, especially in relation to severe postnatal growth retardation. Van de Lagemaat et al. demonstrated that a mineral-enriched post discharge formula compared to standard formula was associated with improved gain in bone mineral content until six months of corrected age.

Chapter 8 describes a randomized study in very and extreme preterm infants who were randomly assigned to one of three groups that differed by enteral Ca and P intake until day 10. The aim of the study was to evaluate bone mineralization and growth at term corrected age (TCA). We hypothesized that higher intake would lead to improved growth and bone mineralization at TCA. In contrast to our assumption the outcomes for bone mineral content (BMC), bone mineral density (BMD), weight and length were similar across the groups. The nutritional intake of Ca, P and protein was not associated with an effect on weight. P intake was associated with a small positive effect on length, whereas Ca intake was associated with a decrease in BMC. The volume of human milk intake during the first 10 days was significant positively associated with BMC. The effect sizes found in this study were small. While one would expect that nutritional intake of Ca and P would influence each other, testing of the interaction of both minerals was negative for all outcomes except for BMC. Any interpretation based on the results of this study should be performed with caution since the results may be distorted by the small amount of patients investigated with DXA scans. A limitation of this study was that we were unable to include the intended number of patients and that a considerable number of disapprovals of dual-energy x-ray absorptiometry (DXA) scans led to low numbers of scans per group. This may have led to overestimation of observed effects, as well as perhaps having missed certain effects. However, since there is a limited number of studies with a randomized design evaluating this subject in very, and extremely preterm infants, it may be considered that this study provides an important contribution to the research.

In agreement with the study protocol the groups differed significantly in the amount of Ca and P intake, based on the enteral supplementation. However all groups received more than 50% of the total intake as parentreral supplement according to the PN protocol. Further, based on blood and urine analyses additional parentreral mineral supplementation was permitted since we felt it would be unethical to withhold standard of care to high risk patients. Parentreral nutrients are directly available for metabolism, while intake by the enteral route is also determined by the amount of intestinal absorption. This possibly has ameliorated the differences between the three groups and possibly decreased the size of any effect of Ca and P intake.

At term corrected age anthropometric data were available for all 93 surviving infants. This study was performed about 8 years after the first observational cohort study and patients included had lower gestational age and birth weight. While infants of Cohort 1 and 2 developed a decrease of about 2 SDS for weight by the time they
reached TCA, participants of the more recent study had a decrease in SDS between -0.75 and -0.41 for weight and -0.69 and -0.33 for length. We speculate that the smaller decrease in SDS will enable these infants to achieve a full catch up to birth SDS, in contrast to the previous cohorts.

The lower decrease in SDS until TCA may be interpreted as an improvement in nutritional management. However, the nutritional protocol basically remained unchanged, using the same standard PN solution, preterm formula and human milk fortifier. The only additional product that was used was an enteral supplement of protein. Practically, this improvement may also be related to more adherence to the protocol, availability of daily calculated nutritional intake in combination with consequent registration of growth into digital growth curves which were used to adapt the nutritional intake in case of impaired growth. Further improvement probably could be achieved by individual fortification of human milk based on determination of the actual nutritional content of mother’s own milk. (172, 173)

In agreement with the anthropometric results bone mineralization was comparable between the groups. As elaborated above, this may be a result of the relatively high amount of minerals provided by PN, but also the short intervention period. Independent of group assignment, the percentage of MOM was very high in this study. Previous studies that evaluated bone mineralization in relation to enteral feeding found the highest weight gain and BMC with preterm formula. (174, 175) We did not include a group with exclusively preterm formula because our general practice is to provide preferably mother’s own milk. A positive effect of human milk on bone mineralization has previously been reported and is confirmed by our analyses. (176, 177) This may support our assumption that even for very, and extreme preterm infants it should be possible to achieve adequate bone mineralization at TCA in combination with human milk. According to the references for body composition in appropriate and small for gestational age infants as presented by Lapillonne et al. the participants of our study achieved a median BMC comparable to 38-39 weeks gestation at term corrected age. (178) Taking into account the results of our long-term follow up of the previous cohort study, we speculate that infants who participated in the last study gained sufficient weight, length and bone mineralization during the early postnatal period to achieve a full catch up growth to birth SDS during early childhood.

**Diagnostic tools and bone mineralization**

Metabolic bone disease (MBD) often is defined as decreased bone mineral content relative to the expected level of mineralization of an infant of a comparable size in combination with either biochemical or radiographic changes. (179) Impaired bone mineralization is asymptomatic in most infants, therefore screening seems necessary for a timely diagnosis or prevention of MBD. Currently there is no consensus on diagnosis, treatment or timing of initiation of treatment. This is partly based on the lack of evidence based parameters to evaluate bone status. (147-148) Besides biochemical parameters as discussed in the previous section, screening methods currently used also include imaging and quantitative ultrasound. While radiographic signs of impaired bone mineralization or fractures are usually late signs, they are also not quantifiable. Dual-energy-x-ray absorptiometry (DXA) currently is recognized as the gold standard and regarded as a sensitive method to detect and quantify small changes in body composition and specifically bone mass. (180) Both methods use radiation and are not suitable for daily screening, while also the availability of a DXA scan is limited. Quantitative ultrasound (QUS) has been proposed as an easy to use, portable, and radiation free alternative. The method is based on the idea that changes in ultrasound are related to changes in bone mineral density and bone structure. (181)

Chapter 9 presents a statistical assessment of DXA and QUS results collected during the long-term follow up study of former preterm infants at nine to 10 years of age. We evaluated whether both techniques are equivalent in diagnosing the state of bone mineralization. Equivalent results would allow to replace DXA by QUS as diagnostic tool for the evaluation of bone mineralization and would provide a method to evaluate changes of bone mineralization in daily clinical practice. In contrast to our expectations we found statistically significant weak correlations and poor agreement between the methods and a discrepancy in differentiating the same children with normal or reduced bone mineralization. Thus according to our analysis we cannot recommend to replace DXA scans by QUS for the evaluation of bone mineralization. However, reference values and standard deviation scores for both methods were derived from different countries, thus different populations. (182-184) Different pubertal stages at different ages, depending on the population cannot be excluded. This may have affected our results. Furthermore we evaluated a single measurement and did not investigate whether QUS may be useful to evaluate changes over time, irrespective of any comparison with DXA. Two studies performed repeated measurements in preterm infants during hospital stay and analyzed a possible association with markers of bone health and severe illness. (185, 186) Fewtrell et al. found the QUS signal to be positively associated with gestational age, but markers of impaired bone mineralization and severe illness were not related to changes in QUS signal, while Betto et al. found significant weak correlations between the QUS signal and anthropometric data as well as a number of biochemical markers of bone mineralization. Both studies looked at incidental measurements with intervals of several weeks and thereby may have missed certain effects. Standardized repeated measurements in the early postnatal period in relation to nutritional interventions have yet not been performed. We would like to note that despite the fact that most children in this study achieved growth and bone mineralization within the range of the reference population, this was still below their initial birth SDS. Therefore, improvement in postnatal growth development for preterm infants is still needed. This means that growth and bone mineralization need
to be evaluated in follow up and pQUS may serve as a simple, non-invasive, cheap tool. Despite the lack of agreement between DXA and QUS, we suggest that the usefulness of QUS for direct clinical evaluation in very preterm infants should be further evaluated since this method provides the possibility to observe changes in bone development in high risk infants directly from birth onwards, while a negative impact for the infant is low. Ideally, repetitive measurements in the early postnatal period may be used to evaluate the adequacy of nutritional interventions and consecutively limit blood and urine sampling. However, before the implementation of QUS as diagnostic tool is possible, further research is needed to better understand the nature of changes of ultrasound in growing bone of very preterm infants.

**Future perspectives**

Our studies comprised interventions in preterm infants during the first days after birth. We demonstrated that small changes in nutritional supply during this period had impact on later development. However, many issues concerning optimal nutritional management in preterm infants still remain unclear and need further investigations. This has been discussed throughout this chapter. In this paragraph we will summarize our suggestions for future research.

The nutritional management presented in this thesis focused among other things on standardized nutritional care. We discussed the positive effects. On the other hand, the requirements of preterm infants are neither ‘standard’ nor ‘equal’ for the individual infant. Extremely preterm infants have the highest nutritional needs that have to be provided in combination with the smallest volumes, while maturation and growth during the postnatal period will change the requirements and in case of use of human milk the protein content is variable and usually unknown. We demonstrated that adaptation of the nutritional intake according to the individual needs cannot be provided using current available multi-component human milk fortifiers. Therefore further improvements in nutritional care should investigate ‘standardized individualized’ supply of nutrients for enteral as well as parenteral nutrition. Further research should determine whether individualized enterally protein supplementation based on regular milk analyses of mother’s own milk leads to further improvement in growth and neurodevelopment. While the use of parenteral standardized multi-component solutions has the advantage of a well-balanced intake of nutrients and increases patient safety, these solutions are often unable to provide optimal intake with low volumes as required for extremely low birth weight infants. Concepts of adequate parenteral nutrition specifically for infants with a birth weight below 1000 grams need to be developed.

We demonstrated a positive effect of early nutritional intake on neurodevelopmental outcome for boys and girls, as well as for appropriate and small for gestational age infants. However, we also showed that nutritional needs seem to be different. We speculate that male infants have higher nutritional needs than female infants. Our studies indicated that improvements were mainly associated with protein intake. We suggest that at first, research should define the specific requirements of boys as well as for girls, which should include the effect of other nutrients. A next step would be to prove that a specified nutritional protocol for boys will lead to neurodevelopmental outcomes comparable to girls.

We also found an improved response to nutritional interventions in the subgroup of SGA children compared to AGA children indicating that SGA infants have lower protein requirements than AGA infants. Further studies should try to specify the nutritional requirements of SGA born infants aiming at nutritional intakes that allow optimal growth, while avoiding accelerated growth with high risk of developing obesity, diabetes and cardiovascular disease. Studies should look for and try to define the critical window for ‘healthy catch up growth’.

We suggested that insufficient nutritional intake could be a cause of impaired neurodevelopmental outcome in relation to prematurity related diseases. Further studies are needed to evaluate whether adequate nutritional intake in combination with morbidities such as patent ductus arteriosus, sepsis, necrotizing enterocolitis and chronic lung disease will improve outcome. In addition, specific nutritional requirements in relation to certain diseases have yet not be defined.

Part 3 of this thesis evaluated Ca–P homeostasis and bone mineralization. Current recommendations for nutritional intake need to be adapted taking into account different requirements according to gestational age. The aim of adequate supplementation of Ca and P should not be the prevention of metabolic bone disease but the development of adequate bone mineralization comparable to term born infants. The efficacy of newly developed recommendations should be evaluated in long-term follow up that evaluates bone mineralization as well as side effects such as nephrocalcinosis. Non-invasive screening tools that reflect changes in bone mineralization during the early postnatal period need to be developed. We suggest to search for target ranges of biochemical parameters that indicate adequate nutritional intake, taking into account immature renal tubular reabsorption. Further study is needed to prove whether repeated measurements of quantitative ultrasound may serve as a non-invasive tool for early changes in skeletal development.

As mentioned in Chapter 1 bone mineralization or skeletal development is not only dependent on nutritional intake, but also driven by functional requirements. Active muscle contractions increase bone strength, while lack of mechanical challenge will decrease the need for bone formation. (187, 188) The studies presented in this thesis focused on nutritional intake, while nowadays standard care of preterm infants
does not stimulate active movements or muscle contractions comparable to the fetal situation. We speculate that ‘developmental care based physical training’ in combination with adequate intake will further improve the early postnatal development of bone mineralization.

References


Summary

Part 1
Chapter 1
Chapter 1 provides an introduction on the subject of this thesis, namely the effects of nutritional intake in the early postnatal period of very low birth weight (VLBW) infants on growth, neurodevelopment and bone mineralization. We describe the consequences of preterm birth as a critical event for the infant, with a major impact on health directly after birth, but also a high risk of unfavorable long-term development. The current knowledge of the role of nutrients for the preterm infant is described in detail, along with their requirements and the way to supply nutrition. Nutrition in the early postnatal period has been recognized as a key determinant for neonatal outcomes. While the aim of nutritional support for preterm infants is to achieve a postnatal growth rate similar to fetal growth combined with satisfactory functional development, comparable to term-born infants, preterm infants are known to experience growth retardation and impaired executive functioning. Nutritional intake has been associated with growth, neurodevelopment and bone mineralization. A description of the current knowledge of these three outcome parameters with specific attention being paid to nutritional interventions is provided.

Chapter 2
This chapter gives an outline of the thesis. For this thesis we evaluated nutritional interventions in VLBW infants, comprising the group of very and extreme preterm infants. Emphasis was placed on the early postnatal period because in healthy pregnancies, this has been shown to be a critical period in the transition from a constant rich flow of nutrient supply through the umbilical cord to an enteral and parenteral supply of nutrients with a risk of under- or overnutrition and consequently impaired development. The aim was to gain more knowledge concerning the efficacy of current recommendations for the nutrient supply for VLBW infants. Research questions focused on the effect of early postnatal amino acid intake on growth and neurodevelopment and calcium and phosphorus metabolism as determinants of bone development.

Part 2
Chapter 3
This chapter evaluates whether increasing the amount of amino acids and energy administered in parenteral nutrition combined with a rapid increase of enteral feeding would improve postnatal growth in preterm infants. The observational study analysed two consecutive year-cohorts of preterm infants admitted to the neonatal intensive care unit of the Radboudumc. A new feeding regimen that provided a higher amount
of amino acids with parenteral nutrition, according to international recommenda-
tions, in combination with the intention of a rapid increase of enteral feeding (Cohort 2) was compared with the previous standard nutritional protocol with a lower amount of amino acids and energy (Cohort 1). The new parenteral nutrition was shown to provide a significantly higher amount of amino acids during the first week of life. This was associated with a significantly greater weight gain in appropriate for gestational age born infants (AGA) until week 5 postnatally. Small for gestational age (SGA) infants showed a greater weight gain compared with AGA infants, but growth in head circumference failed to improve. A further finding of this study was that, despite the positive weight gain from the second week onwards, all infants demonstrated a steady decrease in standard deviation score (SDS) for weight, which resulted in a mean loss of 2 SDS for both cohorts around the term-corrected age. We concluded that improved parenteral intake may lead to improved short-term postnatal weight gain, but a fast increase in enteral nutrition, specifically using fortified human milk, failed to prevent nutritional deficits.

Chapter 4

In this chapter we describe the results of the long-term evaluation of the previously studied former preterm children at the age of 9 - 10 years. The aim of the study was to investigate whether increased nutritional supplementation of calcium, phosphate and protein in VLBW infants during the first 14 days after birth was associated with any improvement in length and bone development. The children who were born at gestational age below 32 weeks or had a birth weight below 1500 grams were invited for additional follow-up at 9 - 10 years of age. Anthropometric data were collected during standard follow-up visits until five years of age and additionally at 9 - 10 years, including measurements of bone mineral content, bone mineral density of the whole body and lumbar spine determined by dual-energy X-ray absorptiometry. Long-term growth trajectories of both cohorts were evaluated separately for participants born appropriate (AGA) and small for gestational age (SGA), stratified by gender. Multivariate linear regression was used to examine the effect of nutritional intake and clinical covariates on length and bone mineralization. Both cohorts caught up in length to SDS within the normal range by 6 months. Bone mineral content and density at 9 - 10 years were within the normal range and did not differ between the cohorts. SGA children caught up in length at 5 years, with bone mineralization at 9 - 10 years being comparable to AGA children. There was no evidence of an association between early nutritional intake and bone mineralization at 9 - 10 years. We concluded that children born as AGA or SGA preterm infants are able to catch up in length after the postnatal period, and achieve a normal length and bone mineralization at age nine - ten years. An improvement of calcium and phosphate intake during the first 14 days after birth was not associated with an improvement in length and bone development.

Chapter 5

The study described in this chapter assessed whether an increased amino acid and energy intake in preterm infants during the first week of life is associated with improved neurodevelopment at the corrected age (CA) of 24 months. The previously described cohorts were evaluated. Neurodevelopment was determined using the Bayley Scales of Infant Development, 2nd Edition at 24 months CA. Linear and logistic regression analyses analyzed the association between nutritional intake and neurodevelopment. We found that Cohort 2 received significantly higher nutritional intake during the first week compared to Cohort 1, and weight gain until week 5 was significantly higher, especially in boys. The mean Mental Developmental Index (MDI) scores did not differ between the groups, but Cohort 2 was associated with an increased chance of having an MDI ≥ 85, with an odds ratio of 6.4 and 95% confidence interval (CI) of 1.5-27.4. For all girls, this was associated with a higher protein intake (5.3, 1.2-23.3). For boys, the chance of having a Psychomotor Developmental Index ≥ 85 was positively associated with increased protein intake. We concluded that higher nutritional intake was associated with different improvements in growth and neurodevelopment in boys and girls.

A sub-analysis of our data showed that besides male sex, a patent ductus arteriosus was found to be the greatest negative determinant of neurodevelopment. Infants with patent ductus arteriosus received significantly less protein and energy intake during the first two weeks. Fluid restriction and cautious increase of enteral feeding are generally accepted measures of conservative treatment of patent ductus arteriosus. In many cases this is at the expense of the daily required nutritional intake. We speculate that the impaired outcome in relation to patent ductus arteriosus could be the consequence of undernutrition in a period of rapid brain growth and suggest that neurodevelopmental outcome of prematurity-related clinical conditions should also be evaluated in relation to nutritional intake.

Part 3

Chapter 6

This chapter describes an observational study that analyzed the postnatal calcium (Ca) and phosphorus (P) metabolism in preterm infants who were included in cohort 2. During a 5-week period, serum concentrations and the urinary excretion of calcium and phosphorus (Ca/P) were recorded and related to the intake of minerals and vitamin D. The recommended intake of minerals was achieved by day 5 and for vitamin D, by 4 weeks. Infants developed hypercalcaemia, hypercalciuria, and hypophosphataemia during the first postnatal week, leading to additional P supplementation in more than half of the infants. The renal tubular reabsorption of P was > 95% until day 9 but decreased to < 70% after the second week. Alkaline phosphatase was normal at birth, increased to a maximum of 450 IU/L by day 14, and persisted above
the normal range for the remaining period. The conclusion of this study was that parenteral intake of P appeared to be too low, leading to mineral imbalances in the early postnatal period, and vitamin D intake was also below recommendations.

Chapter 7
The study described in this chapter used data collected for the Early Supplementation Study (ESS), which was part of the ‘Early Nutrition Study’ (ENS), a multi-center, double-blind, randomized trial that evaluated the effects of human milk on survival and infections in preterm infants. The primary objective of the ESS was bone mineralization in preterm infants in relation to different mineral supplementation. The aim of this study was to evaluate changes in the biochemical parameters of the Ca-P homeostasis in blood and urine in relation to different nutritional intakes during the first 14 days of life in VLBW infants. Our hypothesis was that the nutritional intake of Ca and P would have an effect on the biochemical parameters of the Ca-P homeostasis. For this study we compared two groups of very and extremely low birth weight infants that differed by nutritional intake of Ca and P (High versus Low) during the first 14 days after birth. Biochemical parameters such as serum (s)/urinary (u) Ca and P; alkaline phosphatase (ALP); tubular reabsorption of P (TrP); and urinary ratios for Ca/creatinine and P/creatinine were determined on postnatal days 1, 3, 5, 7, 10, and 14. The daily median concentrations of the biochemical parameters did not differ between the groups, but the analyses showed that Ca intake was significantly associated with an increase in uCa and TrP and a decrease in ALP. Phosphorus intake was significantly associated with increase in sP, uP, uP/creat ratio and ALP and a decrease in TrP (p < 0.00). Protein intake was associated with a decrease in sP, while low gestational age and male gender were associated with a significantly increased renal excretion of P. Standardized repeated measurements showed that the biochemical parameters were affected by nutritional intake, gestational age and gender.

Chapter 8
This chapter presents the results of a randomized study that evaluated the effect of different enteral intakes of Ca and P on bone mineralization and growth at term corrected age in very and extremely preterm infants. Infants (n=109) with birth weight below 1500 grams were randomly assigned to one of three groups that differed by the nutritional protocols delivered until day 10: Group A - mother’s own milk (MOM) and donor milk (unfortified); Group B - MOM (unfortified) and preterm formula; Group C - MOM (start fortification > 50 ml/d) and preterm formula. The early stop of patient inclusions led to lower numbers than the originally anticipated 65 infants per group. Thus, the study may be underpowered and unable to answer the research questions. This study found no difference in weight, length, bone mineral content (BMC) and bone mineral density (BMD) between three different intake groups of very and extremely preterm infants at term corrected age. The regression analysis further showed that group assignment was not associated with the studied outcome measures; however, we found significant positive associations between P intake and length, as well as the amount of human milk intake and BMC. In contrast, Ca intake was associated with a decrease in BMC that decreased further after the addition of P and protein in the analysis. On the basis of this study, there is no evidence that early high mineral intake through early fortification of human milk improves bone mineralization in combination with high parenteral mineral intake. The positive effect of human milk on bone mineral content needs to be evaluated further.

Chapter 9
The study described in chapter 9 is a comparison of two diagnostic tools for the evaluation of bone mineralization. The study assessed whether Phalangeal Quantitative Ultrasound (pQUS) was equivalent to the golden standard dual-energy x-ray absorptiometry scan (DXA). Phalangeal Quantitative Ultrasound (pQUS) is an alternative technique that is inexpensive, easy to use and radiation-free. Sixty former preterm infants were investigated when aged between 9 and 10 years old using both methods on the same day. DXA measured bone mineral content (BMC) and bone mineral density (BMD) for total body and lumbar spine (L1-4), while pQUS measured the amplitude-dependent speed of sound (AD-SoS) and bone transit time (BTT) at metacarpals II-IV, providing continuous values and Z-scores based on age and sex. Four statistical methods evaluated the association between both techniques: Pearson’s correlation coefficients; partial correlation coefficients adjusted for gestational age, height and BMI; Bland-Altman analysis; and cross-tabulation. Both techniques showed a statistically significant weak correlation for continuous values as well as Z-scores (0.291-0.462, p<0.05). The correlation coefficients decreased further after adjustment for confounders. The Bland-Altman plots showed poor agreement, while sensitivity and specificity had wide ranges. According to these results, pQUS measurements are not equivalent to DXA for the evaluation of bone health and cannot replace the DXA in former preterm children at the age of 9-10 years.

Chapter 10
The findings of the above-mentioned studies are discussed in the context of the relevant literature. In addition, suggestions for future research are provided. The main findings from the studies presented in this thesis:

- Increase in protein and energy intake during the first postnatal week improves postnatal weight gain in very preterm infants.
- The standardly available multicomponent fortification of human milk is insufficient to prevent postnatal growth retardation in very preterm infants.
Deel 1
Hoofdstuk 1
Hoofdstuk 1 geeft een inleiding in het onderwerp van dit proefschrift, namelijk het effect van voeding in de vroege fase na de geboorte van zeer kleine, te vroeg geboren kinderen. Een vroeggeboorte is vaak geassocieerd met negatieve gevolgen voor de gezondheid, zowel direct na de geboorte als in de latere ontwikkeling. De huidige kennis over de rol van voedingsstoffen voor te vroeg geboren, de voedingsbehoefte en de toedieningsvormen wordt uitvoerig beschreven. Tegenwoordig wordt erkend dat de voeding die in de vroege fase na de geboorte toegediend wordt, een belangrijke factor voor de ontwikkeling is, ook op lange termijn. In algemene zin is het streven om na de geboorte met behulp van voeding, een groei te bereiken die vergelijkbaar is met die van kinderen die à term geboren worden. Het blijkt echter, dat te vroeg geboren kinderen vaak kleiner blijven en minder goed functioneren dan hun op termijn geboren leeftijdsgenootjes. Voeding wordt over het algemeen in verband gebracht met groei, neurologische ontwikkeling en botmineralisatie. In dit hoofdstuk wordt de huidige kennis ten aanzien van voedingsinterventies in relatie tot deze drie uitkomstmatten beschreven.

Hoofdstuk 2
Dit hoofdstuk beschrijft de indeling van het proefschrift, waarin voedingsinterventies in de vroege fase na de geboorte bij zeer kleine (< 1500 gram) en extreem kleine (< 1000 gram), te vroeg geboren kinderen worden bestudeerd. In deze fase vindt de overgang plaats van een constante rijke toevoer van voedingsstoffen via de navelstreng naar opname van voeding via het onrijpe maagdarmstelsel of kunstmatige voeding via een infuus. Hierbij bestaat de kans op zowel onder- als overvoeding en vervolgens een beperkte ontwikkeling. Het doel van dit proefschrift is om de effectiviteit van de huidige aanbevelingen ten aanzien van toediening van voeding aan te vroeg geboren kinderen te onderzoeken. De onderzoeksvragen zijn toegespitst op het effect van aminozuren op de groei en de neurologische ontwikkeling, en op de invloed van de calcium-fosfaathuishouding op de botontwikkeling.

Deel 2
Hoofdstuk 3
In hoofdstuk 3 wordt onderzocht of een verhoogde toediening van aminozuren en energie in de parenterale voeding, in combinatie met sneller ophogen van enterale voeding, tot een verbeterde groei in de eerste fase na de geboorte leidt. Voor deze observationele studie werden gegevens verzameld van twee achtereenvolgende
Hoofdstuk 3

In de studie die in dit hoofdstuk beschreven wordt, werd onderzocht of een hogere toediening van aminozuren en energie gedurende de eerste levensweek (Cohort 2) tot een verbeterde neurologische ontwikkeling op de gecorrigeerde leeftijd van 24 maanden leidde. De ontwikkeling werd bepaald met behulp van de ‘Bayley Scales of Infant Development- 2nd Edition’ bij 24 maanden. Het verband tussen de voedingsinname en de scores werd geanalyseerd met behulp van lineaire en logistische regressie. Uit de analyse bleek dat Cohort 2 in vergelijking met Cohort 1 significant meer eiwitten en calorieën toegediend kreeg gedurende de eerste week en dat dit vooral voor jongens tot een significant grotere gewichtstoename leidde. De gemiddelde mentale scores (MDI) verschilden niet tussen de groepen, maar kinderen in Cohort 2 hadden een significant hogere kans op een normale verstandelijke ontwikkeling (MDI ≥ 85), een 95% betrouwbaarheidsinterval van 1,5-27,4. Bij meisjes werd een hogere eiwitinname in verband gebracht met een hogere MDI (5,3; 1,2-23,3), terwijl de hogere eiwitinname bij jongens de kans op een normale motorische ontwikkeling (MDI ≥ 85) significant verhoogde. De conclusie uit dit onderzoek was dat een verhoging van de voedingsinname verschillende effecten had op groei en neurologische ontwikkeling bij jongens en meisjes.

Een deenalyse liet zien dat mannelijk geslacht en een persisterende ductus arteriosus (PDA) de belangrijkste factoren waren voor een beperkte neurologische ontwikkeling. Te vroeg geboren kinderen die in onze studie een PDA hadden, kregen significant minder eiwitten en calorieën toegediend gedurende de eerste twee levensweken. Vochtbeperking en voorzichtige toediening van voeding hoort vaak bij de behandeling van een PDA. In veel gevallen gaat dit ten koste van de dagelijkse voedingsbehoefte. Wij suggereren dat de slechte neurologische ontwikkeling die vaak gezien wordt bij kinderen met PDA, niet gebaseerd is op de PDA maar het gevolg is van ondervoeding in een periode van snelle hersengroei. Wij stellen dat in de beoordeling van de neurologische ontwikkeling bij andere aan vroeggeboorte gerelateerde ziekten ook rekening gehouden moet worden met de voedingsinname.

Deel 3

Hoofdstuk 4

Hoofdstuk 4 beschrijft de langetermijnontwikkeling van de eerder onderzochte groep die vroeg geboren kinderen op de leeftijd van 9 tot 10 jaar. Het doel van de studie was te onderzoeken of een hogere toediening van calcium en fosfaat in de eerste twee weken na de geboorte geassocieerd was met een betere lengtegroei en botontwikkeling. Kinderen die voor de 32e zwangerschapsweek geboren waren van minder dan 1500 gram wogen bij de geboorte, werden rond de leeftijd van 9 -10 jaar uitgenodigd voor een additioneel vervolgonderzoek. Groeigewichten van de schizophrenen van Cohort 2. Gedurende de periode van de eerste vijf levensweken werden de concentraties calcium en fosfaat (Ca/P) in serum en urine geregistreerd en gerelateerd.
aan de inname van mineralen en vitamine D. De aanbevolen hoeveelheid mineralen werd vanaf dag 5 toegediend, terwijl dit voor vitamine D pas na 4 weken bereikt werd. In de eerste levensweek werden hypercalciëmie, hypercalcurie en hypofosfatemie gezien, waarvoor meer dan de helft van de kinderen extra fosfaat toegediend kreeg. De renale terugresorptie van fosfaat was aanvankelijk zeer hoog (>95%) en daalde tot minder dan 70% na de tweede week. De alkalische fosfatase was normaal ten tijde van de geboorte, steeg tot een gemiddeld maximum van 450 IE/l op dag 14 en bleef daarna boven de normaalwaarde gedurende de observationele periode. De conclusie uit deze studie was dat de toediening van fosfaat in de voeding te laag was waardoor in de vroege postnatale periode een ontregeling van mineralen ontstond. Verder was de toediening van vitamine D lager dan de aanbevolen dosis.

**Hoofdstuk 7**

De studie die in hoofdstuk 7 gepresenteerd wordt, maakte gebruik van de data-verzameling van de ‘Early Supplementation Study’ (ESS), die deel uitmaakte van de ‘Early Nutrition Study’ (ENS), een dubbelblinde, gerandomiseerde studie die het effect van moedermelk op de overleving en het ontstaan van infecties bij zeer kleine vroege geboren onderzocht. Het belangrijkste doel van de ESS was het onderzoek naar de botmineralisatie bij te vroeg geboren in relatie tot verschillende hoeveelheden toegediend calcium (Ca) en fosfaat (P). Het doel van de studie die in hoofdstuk 7 beschreven wordt, was om veranderingen in biochemische parameters van de Ca/P-huishouding in bloed en urine te onderzoeken in relatie tot de voeding die gedurende de eerste 14 dagen toegediend werd. Onze hypothese was dat de inname van Ca en P een effect zou hebben op de biochemische parameters van het Ca/P-metabolisme.

In deze studie werden twee groepen extreem kleine en zeer kleine prematures die gedurende de eerste 14 dagen verschillende hoeveelheden calcium en fosfaat toegediend kregen (hoog, laag), met elkaar vergeleken. Biochemische parameters zoals serum/urine-Ca, P, alkalische fosfatase (ALP), renale terugresorptie P en de urine-creatineratio van Ca en P werden op dag 1, 3, 5, 7, 10 en 14 na de geboorte bepaald. De dagelijkse mediane concentraties van de biochemische parameters waren eigenlijk gelijk voor alle drie groepen van zeer kleine en extreem kleine prematures rond de gecorrigeerde à terme-leeftijd. De analyse toonde verder dat in elke van de groepen niet geassocieerd was met een van de uitkomsten, maar dat er een positief verband was tussen de inname van P en de lengtegroei, en tussen de hoeveelheid toegediende moedermelk en het botmineraalgehalte. Op basis van deze studie is er geen bewijs dat vroeg verrijkings van moedermelk in combinatie met hoge parenterale toediening van mineralen de botmineralisatie verbetert. Het positieve effect van moedermelk op de botmineralisatie zou verder onderzocht moeten worden.

**Hoofdstuk 8**

Dit hoofdstuk presenteert de resultaten van een studie die het effect onderzocht van verschillende hoeveelheden Ca en P in de voeding op de botmineralisatie en groei rond de gecorrigeerde uitgerekende leeftijd bij zeer kleine en extreem kleine prematures. Hiervoor werden 109 kinderen met een geboortegewicht onder de 1500 gram na loting in een van drie groepen ingedeeld. Deze groepen verschilden van elkaar ten aanzien van het voedingsprotocol gedurende de eerste 10 levensdagen. Groep A: moedermelk (MOM) of donormelk (niet verrijkt), Groep B: MOM (niet verrijkt) of kunstvoeding voor prematures, Groep C: MOM (verrijkt vanaf 50 ml/dag) en kunstvoeding voor prematures. Door een voortijdige stop van de patiëntencuratie werd het oorspronkelijk beoogde aantal kinderen per groep niet bereikt. Om deze reden heeft de studie waarschijnlijk onvoldoende power om alle onderzoeksvragen te kunnen beantwoorden. Op basis van het studieprotocol had groep C een significant hogere inname aan Ca en P gedurende de eerste 10 dagen. In deze studie werd geen verschil gevonden in gewicht, lengte, en botmineraalgehalte en -dichtheid tussen de drie groepen van zeer kleine en extreem kleine prematures rond de gecorrigeerde à terme-leeftijd. De analyse toonde verder dat in elke van de groepen niet geassocieerd was met een van de uitkomsten, maar dat er een positief verband was tussen de inname van P en de lengtegroei en dat de hoeveelheid toegediende moedermelk in combinatie met hoge parenterale toediening van mineralen de botmineralisatie verbetert. Het positieve effect van moedermelk op de botmineralisatie zou verder onderzocht moeten worden.

**Hoofdstuk 9**

In hoofdstuk 9 wordt een studie beschreven die twee diagnostische methoden voor de beoordeling van de botmineralisatie vergelijkt. De studie onderzocht van ultraschallongonderzoek (botecho) aan de vinger gelijkwaardig is aan de gouden standaard, namelijk dual-energy X-ray absorptiometrie-scan (DXA). De botecho is goedkope, eenvoudig in gebruik en stralingsvrij. Zestig te vroeg geboren kinderen werden onderzocht op de leeftijd van 9 tot 10 jaar, waarvoor bij ieder kind beide onderzoeken op de zelfde dag verricht werden. DXA bepaalde het gehalte en de dichtheid van het bot van het hele lichaam en de lumbale wervelkolom en met de botecho werd de snelheid van geluid en de duur van het geluid door bot gemeten. Voor alle metingen werden continue waarden en Z-scores verkregen. Voor de vergelijkingen werden vier statistische methoden gebruikt: Pearson’s correlatie, partiële correlatie aangepast aan zwangerschapsduur, lengte en BMI, een Bland-Altman plot en kruistabellen. Beide technieken lieten een statistisch significant zwakke correlatie zien, die verder daalde na correctie voor mogelijke verstoringen variabelen. Tevens liet de Bland-Altman plot zwakke overeenkomsten zien, terwijl de sensitiviteit en specificiteit van een brede marge
hadden. Op basis van deze resultaten kan geconcludeerd worden dat de botecho niet gelijkwaardig is aan de DXA-scan voor de evaluatie van de botontwikkeling en daarmee de DXA-scan niet kan vervangen voor onderzoek bij prematures op de leeftijd van 9 tot 10 jaar.

Hoofdstuk 10
In dit hoofdstuk worden de bevindingen van bovengenoemde studies besproken in samenhang met de relevante literatuur. Daarnaast worden suggesties gegeven voor verder onderzoek.

De belangrijkste bevindingen van dit proefschrift zijn:

• Een verhoging van de eiwit- en calorie-inname gedurende de eerste week na de geboorte verbetert de gewichtstoename na de geboorte bij zeer kleine vroeggeboren kinderen.
• Met standaard multi-component moedermelkverrijker kan een postnatale groeiretdatie niet worden voorkomen.
• De voedingsinname tijdens de eerste levensweek heeft verschillende uitwerkingen op de neurologische ontwikkeling van jongens en meisjes op de leeftijd van 24 maanden.
• Vroeggeboren kinderen met een te laag geboortegewicht hebben een andere eiwitbehoefte dan zij met een normaal gewicht bij de geboorte om een optimale groei te bereiken.
• Vroeggeboren kinderen zijn in staat om een lengte en botmineralisatie te bereiken die vergelijkbaar zijn met de referentiebevolking maar blijven onder de initiële standaard deviatie scores ten tijde van de geboorte.
• Huidige aanbevelingen voor de toediening van calcium en fosfaat aan vroeggeboren zijn ontoereikend om een stabiele calcium-fosfaathuishouding te krijgen.
• De calcium-fosfaathuishouding wordt beïnvloed door inname van mineralen met de voeding, de eiwittoediening en de gestatieleeftijd van het kind.
• Verbetering van de botmineralisatie in de vroege fase na een vroeggeboorte zou verder onderzocht moeten worden.
• De botecho kan de dual-energy x-ray absorptiometriescan niet vervangen voor de beoordeling van de botmineralisatie.
Zusammenfassung

Teil 1
Kapitel 1

Kapitel 2

Teil 2
Kapitel 3
Kapitel 3 untersucht ob, mit einer höheren Zufuhr an Aminosäuren und Energie in der parenteralen Infusion, die Gewichtszunahme in den ersten Wochen nach der Geburt

Kapitel 4

Kapitel 5

Eine Teilanalyse zeigte, dass männliches Geschlecht und ein persistierend Ductus Arteriosus (PDA) die wichtigsten Faktoren für eine ungünstige neurologische Entwicklung waren. Frühgeborene aus unserer Studie, die einen PDA hatten, erhielten während der ersten zwei Lebenswochen signifikant weniger Eiweiß und Kalorien. Eine Beschränkung der Flüssigkeitszufuhr und zurückhaltende Steigerung der enteralen Nahrung werden häufig zur Behandlung eines PDA angewandt. In vielen Fällen führt dies zu einer geringeren Einnahme von Nahrungsstoffen. Wir schlagen vor, dass eine ungünstige neurologische Entwicklung, so wie sie bei Kindern mit PDA häufig gesehen wird, nicht verursacht wird durch einen PDA, sondern die Folge ist...
einer Unterernährung zum Zeitpunkt einer schnellen Entwicklung des Gehirns. Wir schlagen weiterhin vor, dass bei der Beurteilung der neurologischen Entwicklung im Zusammenhang mit anderen mit Frühgeburtlichkeit assoziierten Erkrankungen, die Nahrungseinnahme als Faktor mit einbezo gen werden sollte.

**Teil 3**

**Kapitel 6**


**Kapitel 7**


**Kapitel 8**

mineralisation verbessert. Die positive Auswirkung der Muttermilch auf die Entwicklung der Knochenmineralisation sollte weiter untersucht werden.

Kapitel 9

Kapitel 10
In diesem Kapitel werden die Erkenntnisse der oben beschriebenen Studien besprochen im Zusammenhang mit der relevanten wissenschaftlichen Literatur. Weiterhin werden Vorschläge gemacht für zukünftige Studien.

Die wichtigsten Erkenntnisse der hier vorgestellten Arbeiten sind:

- Eine Erhöhung der Einnahme an Eiweiß und Kalorien in der ersten Lebenswoche verbessert bei sehr untergewichtigen Frühgeborenen die Gewichtszunahme nach der Geburt.
- Mit einem Standard Multi-Komponenten Supplement für Frauenmilch kann bei sehr untergewichtigen Frühgeborenen eine Wachstums retardierung nach der Geburt nicht verhindert werden.
- Die Einnahme von untergewichtigen Frühgeborenen mit einem normalen Gewicht bei der Geburt, einen anderen Eiweißbedarf um ein optimales Wachstum zu erreichen.
- Ehemalige Frühgeborene sind in der Lage eine Körperlänge und Knochenmineralisation zu erreichen, die der der Referenzpopulation entspricht, aber bleiben unterhalb der Standardabweichung zum Zeitpunkt der Geburt.
- Heutige Empfehlungen für Frühgeborene zum Bedarf an Kalzium und Phosphat sind unzureichend um einen stabilen Kalzium-Phosphathaushalt zu erreichen.
- Der Kalzium-Phosphat Metabolismus wird beeinflusst durch die Einnahme an Mineralien, Eiweiß, und dem Gestationsalter des Kindes.
- Zur Verbesserung der Knochenmineralisation in der frühen Phase nach einer Frühgeburt sind weitere Studien nötig.
- Der Knochenschall kann den Dual-Energy X-Ray Absorptiometry Scan zur Beurteilung der Knochenmineralisation nicht ersetzen.
PART 5

Abbreviations
Author's affiliations
List of publications
Abbreviations

AAP: American Association of Pediatrics
AD-SoS: Amplitude dependent speed of sound
AGA: Appropriate for gestational age
ALP: Alkaline phosphatase
BMC: Bone mineral content
BMD: Bone mineral density
BMI: Body mass index
BSDI-II: Bayley Scales of Infant Development – Second Edition
BTT: Bone transit time
BW: Birth weight
Ca: Calcium
CA: Corrected age
CLD: Chronic lung disease
Creat: Creatinine
CVC: Central venous catheter
DEXA: Dual energy X-ray absorptiometry
ELBW: Extremely low birth weight
ESPGHAN: European Society of Paediaetric Gastroenterology Hepatology and Nutrition
GA: Gestational age
GI symptoms: Gastrointestinal symptoms
HC: Head circumference
HMF: Human milk fortifier
IQR: Inter quartile range
IRDS: Infant respiratory distress syndrome
ISCD: International Society of Clinical Densitometry
IVH: Intraventricular hemorrhage
LBW: Low birth weight
LS: Lumbar spine scan
MDI: Mental developmental index
MOM: Mother's own milk
MV: Mechanical ventilation
N-CPAP: Nasal continuous positive pressure support
NEC: Necrotizing enterocolitis
P: Phosphorus
PDA: Patent ductus arteriosus
PDI: Psychomotor developmental index
PN: Parenteral nutrition
pQUS: Phalangeal quantitative ultrasound
QUS: Quantitative ultrasound
ROP: Retinopathy of prematurity
sCa: Serum calcium
sP: Serum phosphorus
SDS: Standard deviation score
SGA: Small for gestational age
SOS: Speed of sound
TCA: Term corrected age
TrP: Tubular reabsorption of phosphorus
uCa: Urinary excretion of calcium
uP: Urinary excretion of phosphorus
VLBW infant: Very low birth weight infant
WB: Whole body scan
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Dankwoord

Curriculum Vitae

RIHS PHD portfolio
Dankwoord

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Prof. Dr. H.N. Lafeber. Beste Harrie, als voorzitter van de werkgroep voeding voor pasgeborene heb jij misschien even aan mijn wat ‘stellige, kritische houding’ moeten wennen, waardoor mogelijk aanvankelijk sommige uitspraken van mij tot misverstanden geleid hebben. Van mijn kant was het altijd goed bedoeld en had dit misschien meer met een wat ‘Duits communicatie probleem’ en enige mate van fanatisme te maken. Belangrijker is dat ik mij altijd door jou gewaardeerd en gesteund heb gevoeld. Dus, daarom hier ook nog een keer dank voor het feit dat ik mijn manuscript tijdens jouw vakantie in Twente mocht afleveren en wij gezellig de middag konden doorkletsen.

Leden van de werkgroep voeding. Onze gezamenlijke activiteiten gedurende de laatste jaren hebben er in hoge mate toe bijgedragen dat mijn belangstelling voor voeding voor prematures is gestegen en er uiteindelijk toe geleid heeft om er meer werk van te maken. Het afgelopen jaar ben ik meer af- dan aanwezig geweest. Ik hoop vanaf nu weer een actieve bijdrage te kunnen leveren.

De basis voor alle studies, die in dit proefschrift gepubliceerd zijn, is gelegd door het werk van maar liefst 9 studenten die in hun vrije tijd met heel veel geduld en nauwkeurigheid alle statussen door gewerkt, IC-lijsten ontwijkt en alles in grote databestanden ingevoerd en uitgebreide intake berekeningen verricht hebben. Marjolein Engelkes, jij hebt als eerste in je eentje een opstap gemaakt om een complete jaargang uit te pluizen. Omdat jij dit werk ook wilde gebruiken voor je wetenschappelijke stage, was dat in feite het moment voor mij om na te denken wat er eigenlijk met al die gegevens moest gebeuren en welke vraagstellingen belangrijk waren. Het heeft jouw een presentatie op de NVK opgeleverd en ondertussen zijn de gegevens gebruikt voor publicaties. Vervolgens waren er opnieuw drie 2ejaars studenten die actief wilden kennis maken met de kindergeneeskunde, Reina Visser, Anne de Grauw en Mariëlle Schelle. Reina heeft naaflap van haar stage ook nog de lange termijn groeigegevens opgezet en voor alle omliggende ziekenhuizen. Dit gaat de gegevens konden in meerdere publicaties gebruikt worden. Anne ontdekte als eerste dat er ondanks een hogere toediening aan calcium en fosfaat het metabolisme nog steeds niet in orde was en dit was aanleiding voor verdere studies. Ondertussen hebben jullie allen een eigen carrière in de kindergeneeskunde opgebouwd en ben ik trots aan het begin hiervan met jullie te hebben kunnen samen werken. De gegevens voor de ENS-ESS studie zijn door Michelle Körnmann, Charlotte Gradussen en Laure Vorstenbosch ingevoerd. Charlotte heeft daarna met een indrukwekkende statistische analyse de relatie tussen voedingsintake en biochemische parameter aangetoond en deze resultaten zijn ondertussen ook gepubliceerd. Michelene heeft na haar wetenschappelijke stage de hele analyse met alle kinderen uit de studie overgedaan. De resultaten waren niet zo als wij gehoopt hadden maar het manuscript was wel binnen een maand geaccepteerd. Inge Vermeer en Carmen Lageweg hebben beiden mijn collega Mayke van der Putten ondersteund om de lange termijn gegevens in kaart te brengen en de kinderen uit de cohorten 2004, 2005 op oudere leeftijd nog een keer te onderzoeken. De resultaten hiervan zijn onlangs gepubliceerd. Carmen heeft een mooie presentatie tijdens het NVK congres kunnen geven over de uitkomsten van haar stage project en dit is in een manuscript omgezet dat ondertussen eveneens geaccepteerd is. Ik ben jullie dankbaar voor jullie inzet en wens jullie veel succes voor de toekomst.
Mayke van der Putten. Beste Mayke, ondertussen collega neonatoloog in het Maastricht UMC, met jouw fellow onderzoek heb jij mijn grote wens om de kinderen uit 2004 en 2005 nog een keer terug te zien voor een lange termijn follow up gerealiseerd. Dank voor de opzet, planning en realisatie van dit onderzoek, dat met twee publicaties uiteindelijk bekroond is.

Buiten de afdeling neonatologie kon ik rekenen op medewerking van alle kanten. De apotheek: Lang geleden (2003, 2004) is er veel werk van gemaakt om op mijn aanvraag een nieuwe samenstelling voor de parenterale voeding samen te stellen. Drs. Nicole Vink- van Kimmenade en Dr. Anna de Goede hartelijk dank voor jullie constructieve samenwerking sindsdien. De afdeling Nucleaire Geneeskunde: Prof. M. Gotthardt. Beste Martin, hartelijk dank voor je ongecompliceerde en constructieve medewerking bij twee projecten. Daarnaast was het opmerkelijk hoe gastvrij en vriendelijk wij door alle medewerkers werden ontvangen, op een afdeling die zeker niet gewend is om met kleine pasgeboren kinderen om te gaan. Hartelijk dank Marjo van den Ven voor de technische ondersteuning bij de DXA scans en de constructieve medewerking en het geduld als kinderen niet stil wilden liggen tijdens het onderzoek.

De Follow up Poli: naar alle bij de neonatale follow up betrokken personen zou ik graag mijn dank willen uitspreken voor de medewerking aan mijn projecten. Ik ben mij ervan bewust dat dit voor iedereen extra werk opgeleverd heeft zowel voor de kinderen als ook de psychologen, het secretariaat en de medewerking van jullie goede assistent. Hartelijk dank voor jullie toegewijde medewerking aan deze studie.

De afdeling Biostatistiek en de opbouw van de database voor de ESS. Veel succes met jullie toegewijde medewerking aan deze studie.

Lieve collega’s neonatologen. De afgelopen jaren hebben jullie mij veel ruimte gegeven om mijn ‘eigen ding’ te doen en vooral de afgelopen maanden heel veel vrijgespeeld om dit boekje af te kunnen maken. Beste Djien, Arno, Willem, Sabine, Katerina, Tim, René, Marjie, Maresa, Robin, Mathijs, Elske en Joanne, voor mij betekent samenwerking met jullie, dat ten aanzien van het spierpunt ‘hemo-dynamiek’ binnen onze groep vooral het woord ‘dynamiek’ van toepassing is, namelijk een flexibele en vooral heel collegiaal team. Heel bijzonder om hierop te kunnen vertrouwen. Veel dank voor jullie bijdrage aan de inclusie van patiënten voor de ENS-ESS. Naar de follow up dokters: dank voor het invullen van extra-listjes voor de ENS-ESS patiënten en Katerina, dank voor het realiseren van extra poli-capaciteit voor de ENS-2 jaar follow up en dank aan Tim die met het opschrijven van het algemene extra de voedingsintake een analyse van de gegevens pas mogelijk gemaakt heeft.

Team Perinatologie: Eigenlijk was de ENS-ESS studie een eerste proeve hoe wij als afdeling neonatologie en verloskunde samen kunnen werken - en dat ging heel goed! De lactatiekundigen Vera van Haaren, Jessie Cremer en Kim van de Water hebben er met hun voorlichting aan ouders en verpleegkundigen voor gezorgd dat een ongekend hoog aantal moeders succesvol moedermelk aan hun te vroeg geboren kinderen kon geven terwijl de kraamverpleegkundigen moeders ondersteunden om het ‘witte goud’ zo snel mogelijk bij de neonatologie af te kunnen leveren. Voor de neo-verpleegkundigen betekende de studie dat zij in plaats van zo maar een voedingspuntje pakken eerst naar het randomisatieschema moesten kijken en vooral alle intakes en de groei heel goed bij moesten houden, dus nog meer administratie. Iedereen heeft enthousiast meegewerkt, arts-assistenten, verpleegkundigen, zorg-hulpene, en secretarissen, te veel om alle namen te noemen. Apart wil ik nog wel de dames van de voedingskeuken noemen: Yolanda Dekker-Peters, Silvia Hokus, Jet Huijding-van Buren, Sonja Janssen, Heidi Lamers-Bosman, Leona Paas en Carlijn Thijssen en Ricky van Zijljen-van den Broek. Terwijl jullie zeker niet aangenomen zijn voor wetenschappelijk onderzoek, waren jullie wel de spil van de studie, gezien het feit dat jullie de enige waren die wisten welke voeding een patiënt zou moeten krijgen en daarmee het studieresultaat ook van jullie betrouwbare werkwijze afhankelijk was. Jullie enthousiasme was geweldig. Heel veel dank voor jullie toegejuwde medewerking aan deze studie.

Danken aan alle praktijkdeuren, de gang van zaken onder controle houden, afspraken met ouders en poli maakte, monsters verzamelde, de administratie hielp bijhield en de studenten gesuperviseerd heeft. Beste Wendy, heel veel dank voor je onmisbare inzet voor de ENS-ESS. Alles wat er gebeurde, leidde uiteindelijk tot de publicaties. Beste Wendy, veel dank voor de manier waarop jullie medewerking aan deze studie mij bleek te bevorderen, de afdeling Biostatistiek en de opbouw van de database voor de ESS. Veel succes met jullie toegewijde medewerking aan deze studie.

Dear Laura, while it was meant to be a five minute consultation, our first meeting led to quite sophisticated high level statistical analyses with finally two very easily accepted papers. Thank you for your dedicated cooperation, meaning that you performed most of the analyses in your free-time, your critical comments on my statements and your final language-checks. I would like to say that your efforts improved my knowledge in a ‘statistically significant’ way during this last year for which I am very grateful to you.


Liefste Peter. Aan het einde van deze opsomming sta jij als de voor mij meest vertrouwde persoon. Vanaf mijn komst naar Nederland ben jij degene die mij het meest support, het leren van de Nederlandse taal, bijles voor alle vakken in het eerste jaar (in feite heb jij wel de propedeuse geneeskunde gehaald), met humor tegen twijfels en frustraties ingaan. Jij bent mijn baken. Zo goed als 40 jaar zijn wij een goed team en ieder jaar wordt leuker en nu helemaal........
Curriculum Vitae

Viola Christmann was born on the 10th of September 1956 in Hamburg, Germany. She received her secondary school diploma at the Gymnasium Willhöden in Hamburg, Germany in 1975. Until October 1977 she worked as a certified nurse assistant at the DRK Hospital and Marien - Hospital in Hamburg, Germany. She obtained her medical degree at the University of Amsterdam in 1987. For her research internship she spent 2 months at the Children’s Hospital of Cairo University, Egypt, investigating ‘Oral rehydration in paediatric patients with dehydration’ (Supervisor Prof. dr. H.S.A. Heymans). She started as a voluntary resident in November 1987 at the Kinderklinik of the Johannes Gutenberg - Universität Mainz, Germany (Prof. dr. J. Spranger) and started there her paediatric training in June 1988. From April 1992 onwards she continued her training at the Paediatric Department of the St Antonius Hospital in Kleve, Germany, (dr. H. Schumacher) to be able to live together with her husband. She was registered as ‘Facharzt für Kinder und Jugendheilkunde’ in December 1993 and worked as pediatrician consultant at the St. Antonius Hospital until September 1995 when she started a fellowship neonatology at the Department of Neonatology of the Radboudumc in Nijmegen (Prof. dr. M van de Bor). She was registered as Neonatologist in October 1998 and became a member of staff at the Department of Neonatology in March 1999. Since then she remained to work at the Department of Neonatology of the Amalia Children’s Hospital of the Radboudumc. While her initial research interests were focused on the transitional circulation of the neonate, specifically the patent ductus arteriosus, she was assigned to take care of the nutritional protocol of the department. As a result of these activities she participates in the Dutch working group on neonatal and infant nutrition and finally, this lead to the studies presented in this thesis. Viola is happily married to Peter van der Molen since May 1988 and mother of two wonderful children Lennaert (1993) and Wieke (1994).
**PHD PORTFOLIO**

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**Paediatrics**  
**Radboud Institute for Health Sciences**  
**22-02-2011 – 25-01-2018**

**Promotor(s):**  
Prof. dr. C Noordam, Prof. dr. JB van Goudoever.  
**Co-promotor:**  
Dr AFJ van Heijst.

### TRAINING ACTIVITIES

**a) Courses & Workshops**
- Course Clinical Epidemiology and Statistics  
  1997  
- Course Laboratory Animal Science  
  1998  
- BROK certificate plus GCP examen  
  2008  
- BROK certificate renewal  
  2013  
- BROK certificate renewal  
  2017  
- Symposium Dutch Donormilk Bank; Amsterdam  
  2011  
- International Conference on Nutrition and Growth; Paris  
  2012  
- Vlaams-Nederlands Neonatologendag;  
  2012  
- Ipokrates seminar Nutrition of the newborn infant  
  2013  
- Recent Advances in Neonatal Medicine; Würzburg  
  2014  
- 48th ESPGHAN meeting Amsterdam  
  2015  
- XXV European Congress of Perinatal Medicine; Maastricht  
  2016  
- Jahrestagung GNPI; Dresden  
  2017  
- Recent Advances in Neonatal Medicine; Würzburg  
  2017

**b) Seminars & lectures**
- PHBO Cursus Diëtisten HAN;  
  2008  
- Voeding voor Pre- en dysmature vroegegeborenen  
  2009  
- Symposium Kinderlogopedie-Nijmegen Oral Feeding; (Chair)  
  2009  
- Periodieke Conferentie Nijmegen  
  2009  
- Na een toereikende Vitamine D inname  
  2010  
- Nutricia Workshop voor Kinder- en Jeugdartsen  
  2010  
- Hoe worden kleine eters groot?  
  2010  
- Sectievergadering neonatologie  
  Inadequate Vitamine D intake bij vroeg geboren  
  gedurende de eerste 5 weken  
  2010

**c) Symposia & congresses**
- NVK Symposium: Voeding voor Prematuren; Utrecht  
  2012  
- ESPGHAN-Neomune symposium; Amsterdam  
  2015  
- Early nutritional interventions in preterm infants –  
  small changes and long-term consequences  
  2015  
- Vlaams-Nederlands Neonatologendag; Nijmegen  
  2016  
- Effect of Ca & P suppl. on biochemical parameter of the  
  Ca-P homeostasis in preterm infants  
  2016  
- ESPR; Genève  
  Calcium-Phosphorus homeostasis in relation to nutritional  
  intake in very low birth weight infants  
  2016
- Symposium LNF; Leiden 2017 0.5
  Early postnatal nutrition - different effect for boys and girls at two years

Poster presentation
- PAS; Toronto 2007 0.25
  Early Enteral Feeding in Preterm Infants Improves Postnatal Growth
- NVK; Congres Veldhoven (oral) 2008 0.25
  Postnatale vochthuishouding in relatie tot Natriumintake
- PAS; Baltimore 2009 0.25
  Impact of postnatal sodium intake on fluid balance and clinical outcome in preterm infants
  Improving postnatal calcium and phosphorus intake in preterm infants
- ESPR; Hamburg (oral) 2009 0.25
  Protein content of enteral feeding is insufficient for optimal postnatal growth in preterm infants
- ESPR; Copenhagen 2010 0.25
  Improved catch up growth in male preterm infants with increased early protein and mineral intake
- PAS; Denver 2011 0.25
  Only male AGA preterm infants demonstrate improved development at 2 years after early increase of protein intake
  Hypernatremia in the first week of life is not a risk factor for impaired neurodevelopmental outcome of preterm infants

d) Other
Reviewer for: 2002-2018 3
Acta Paediatrica, Pediatric Research, Archives Diseases of Childhood, Journal of Pediatric Gastroenterology & Nutrition, Nutrients, Bone

TEACHING ACTIVITIES

e) Lecturing
Medical science (old curriculum)
- Blok leeftijdswaarschuwings pathologie 2002-2006 2
- Blok kindervoorziening en volwassenziekte Tot 2016 3
- Blok KMR1: Voeding bij gezondheid en ziekte 0.1
Biomedical science
- Blok Determinant 1: Nutrition Tot 2010 0.2

f) Supervision of internships
- M. Engelkes; medical student Radboudumc 2006 1
- R. Visser; medical student Radboudumc 2008 1
- A.M. de Graauw; medical student Radboudumc 2008 1
- M. Schelle; medical student Radboudumc 2008 1
- C.J.W. Gradussen; medical student Radboudumc 2015 3
- M.N. Körnmann; medical student Radboudumc 2015 1
- C.M.T. Lagerweg; medical student Radboudumc 2016 1

TOTAL 37.95