

Nutrition, Growth, and Allergic Diseases among Very Preterm Infants after Hospital Discharge

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PREFACE

This Ph.D. thesis presents results obtained from a randomized controlled trial (RCT) on post discharge nutrition for very preterm infants and was initiated in collaboration with neonatologists from the Neonatal Units at four Paediatric Departments in Denmark. The trial went on from July 2004 – August 2008.

The trial has been carried out at the Departments of Paediatric at Holbaek Hospital, Kolding Hospital, Aarhus University Hospital at Skejby, and at Hans Christian Andersen Children's Hospital at Odense University Hospital in Odense, Denmark.

The present work was conducted during an employment as a research assistant at The Paediatric Research Unit at Hans Christian Andersen Children's Hospital, Odense University Hospital, Denmark, and the Institute of Clinical Research, Faculty of Health Sciences, University of Southern Denmark from 2007 to 2010.

THE 3 ORIGINAL PAPERS ARE:

[1] Zachariassen G, Faerk J, Grytter C, Esberg B, Juvonen P, Halken S: Factors associated with successful establishment of breastfeeding in very preterm infants. *Acta Paediatr* 2010;99:1000-1004.

[2] Zachariassen G, Faerk J, Grytter C, Esberg BH, Hjelmberg J, Mortensen S, Thybo CH, Halken S: Nutrient enrichment of mother's milk and growth of very preterm infants after hospital discharge. *Pediatrics* 2011;127:e995-e1003.

[3] Zachariassen G, Faerk J, Esberg BH, Fenger-Gron J, Mortensen S, Christesen HT, Halken S: Allergic diseases among very preterm infants according to nutrition after hospital discharge. *Pediatr Allergy Immunol* 2011;22:515-520.

As supplemental material, data for two planned manuscripts are presented.

4. Protein-content in human milk from mothers of very preterm infants.

5. Feeding-pattern and -problems among very preterm infants according to nutrition - from hospital discharge until introduction of complementary feeding.

Appendixes are not published with the thesis but are available from the author on request.

ABBREVIATIONS AND DEFINITIONS

- AD: Atopic dermatitis defined as areas of scaly, erythematous, and itchy eczematous rash for at least 3 months
- AGA: Appropriate for gestational age (-2 SDS > BW Z-score < 2 SDS)
- Allergic/atopic symptoms: Bronchial asthma, recurrent wheezing (wheezy bronchitis), atopic dermatitis, allergic rhinoconjunctivitis, allergic urticaria, and/or food allergy
- Allergic disease: Hypersensitivity reaction initiated by immunological mechanism(s), may be IgE-mediated or non-IgE-mediated
- Atopic predisposition: At least one first-degree relative with documented atopic disease
- BUN: Blood-urea nitrogen
- BW: Birth weight in grams
- Catch-up growth: Accelerated rates of growth following a period of growth failure in order to reach the growth reference of normal preterm or term infants
- CA: Corrected age (weeks, months, or year after term)
- CF: Complementary feeding
- CI: Confidence interval
- CMPA: Cow's milk protein allergy defined as proven or likely immunological mediated reaction to cow's milk protein diagnosed by controlled elimination / challenge procedures
- CMP: Cow's milk protein
- DEXA: Dual Energy X-ray Absorptiometry
- GA: Gestational age at birth (weeks and days)
- HbA1C: Glycohemoglobin (haemoglobin A (1C))
- HbF: Haemoglobin F (fetal haemoglobin)
- HC: Head circumference (cm or mm)
- HMA: Human milk analyzer
- HMF: Human milk fortifier
- IgE: Immunoglobulin E
- ITT: Intention to treat
- LBW: Low birth weight (weighing less than 2500g at birth)

- MAP: Mean arterial blood pressure
- Mix: Both breast- and bottle-feeding
- MF: Mature or term formula
- NEC: Necrotising enterocolitis
- NICU: Neonatal intensive care unit
- Non-SGA: BW Z-score > -2 SDS (in this study equivalent to AGA)
- OR: Odds Ratio
- PF: Preterm or premature formula
- PMA: Postmenstrual age (GA + weeks and /or days since birth)
- PP: Per protocol
- RCT: Randomized controlled trial
- REM: Random effect model
- RW: Recurrent wheezing: at least two episodes of wheezing requiring bronchodilator treatment and diagnosed by a physician
- SD: Standard deviation
- SGA: Small for gestational age (BW Z-score \leq -2 SDS according to a reference)
- S-IgE: Allergen Specific Immunoglobulin E
- SPT: Skin prick test
- Term: 280 days PMA
- UHM: Unfortified human milk
- VPI: Very preterm infants (GA \leq 32+0)
- VLBW: Very low birth weight (weighing less than 1500g at birth)
- Z-score: Standard deviation score (SDS). The difference between the actual growth and the expected reference growth divided with one standard deviation (ex.: (BW – reference BW)/1 SD)

1. INTRODUCTION

Despite advances in perinatal care, with improvements in lowering morbidity and mortality and advances in nutrition, growth failure remains a major problem for preterm infants who need neonatal intensive care [4;5]. Once respiratory status is stabilized, nutrition becomes the most urgent challenge facing clinicians caring for high-risk newborns admitted to the neonatal intensive care unit (NICU) [6].

Growth and the accumulation of nutrient reserves are higher during the second and third trimester of pregnancy than at any other time during life, and it is essential to develop strategies to feed preterm infants in order to maintain a goal of normal in utero growth rates. Feeding less will continue to produce growth-restricted infants with limited growth- and developmental capacity particularly of the brain and its many essential functions. Feeding more may result in obesity that can have harmful consequences [7].

Agreement on recommendations about optimal nutrition and growth of very low birth weight (VLBW) preterm infants after hospital discharge has not yet been achieved. Close monitoring of growth after hospital discharge has been recommended, and if the infant is discharged with subnormal weight for age supplementation has been recommended [8;9]. A nutrient enriched formula can be used for non-breastfed very preterm infants, but the nutritional intervention with fortification of mother's milk for breastfed preterm infants after hospital discharge has not yet been proven optimal as a feeding strategy as regards growth and

neurological development. The risk of developing allergic diseases due to exposure to allergens such as cow's milk protein is also unknown among very preterm infants.

Solely breastfeeding of very preterm infants at and beyond hospital discharge is a common practice in Denmark. Therefore, a randomized controlled trial (RCT) to investigate the effect of nutrient enrichment of mother's own milk while breastfeeding after hospital discharge, and at the same time characterizing very preterm infants and their mothers at hospital discharge, and evaluating the incidence of allergic diseases among preterm infants within the first year of life, was planned.

2. BACKGROUND

GROWTH AMONG PRETERM INFANTS

Most VLBW infants are discharged before they reach term postmenstrual age (PMA) and at the time of discharge, many very preterm infants have deficits in accretion of energy, protein, minerals, and other nutrients. Nutrient deficit already in the first weeks of life can be directly related to postnatal growth retardation [5;10], and at hospital discharge VLBW preterm infants have often not achieved the median birth weight of the reference fetus at the same postmenstrual age [4;11], and nutritional support might still be relevant at and beyond hospital discharge.

Preterm infants must achieve catch-up growth in order to attain the growth-parameters of term infants of the same PMA. Some catch-up growth is observed in most VLBW infants, although the rate and time of catch-up differs between studies [12;13]. Low birth weight infants as a group do catch-up, but many remain smaller compared to infants of normal birth weight [14;15]. Infants born small for gestational age (SGA) and VLBW infants show slower rates of catch-up growth compared to appropriate for gestational age (AGA) VLBW infants [12;13;16;17].

Improvement of growth among VLBW preterm infants can be achieved by feeding a nutrient enriched formula during hospitalization and after hospital discharge [18;19]. Human milk has though shown to have many benefits – especially on the IQ among infants born SGA and VLBW preterm infants [20;21]. But human milk requires nutrient fortification to meet the protein and mineral needs of the rapidly growing preterm infant during hospitalization [6].

NUTRITIONAL NEEDS FOR PRETERM INFANTS

Nutritional needs are higher for preterm infants than for term infants because of less stores, altered absorption, and rapid growth rates. Extremely low birth weight infants have greater nutritional needs than VLBW infants, and enterally fed infants have greater nutritional needs than parentally fed infants [6]. Preterm infants lose approximately 10-20% of their birth weight during the first 4-6 days of life with an expectation that the weight will be regained by 2-3 weeks of age. The weight loss results mainly from a concentration in the extracellular fluid compartment and is more pronounced in preterm than in term infants [22].

Nutrition of the very preterm infant therefore is an urgent challenge, and shortly after birth parental nutrition in combination with minimal enteral nutrition, also called hypocaloric priming or trophic feeding, has to be started [23;24]. Immediately after

delivery, the mother of the preterm infant is encouraged to start pumping her breast milk. Preterm infants are routinely tube fed with expressed milk from the mother until they are developmentally and physiologically ready to begin the process of learning to suck, swallow, and breathe in a coordinated fashion [25].

While human milk offers many advantages, the quantity of protein and minerals is inadequate for the growing preterm infants. The protein-content in human milk decreases within the first months after birth for both preterm and term infants [26]. Feeding solely human milk therefore may lead to insufficient intake of protein and energy, and the use of fortified human milk improves adequate growth and satisfies the specific nutritional requirements of preterm infants [27]. Commercial human milk fortifiers (HMF) are available and increase the nutrient density of human milk. Nutrients of particular interest for the preterm infant include e.g. protein, calcium, and phosphorus. Fortified human milk and preterm formulas (PF), when fed adequate volume, will meet most nutritional requirements [6]. Cow's milk based fortifiers have been used for years in many NICU's for nutrient enrichment / fortification of human milk e.g. Enfamil® human milk fortifier was introduced in 1984 and was reformulated with a higher protein-level in 2002 [28]. In general, human milk fortification is continued throughout hospitalization and is easy to administer as long as the infant has a nasal-gastric tube. At the time when the infant is fully breastfed and ready for discharge, human milk fortification becomes a challenge.

BREASTFEEDING PRETERM INFANTS

Breastfeeding-rates of very preterm infants at and beyond hospital discharge are reported to vary considerably in the literature. This could possibly be explained by the duration of maternity leave, mothers opportunities for spending time in the hospital with their infant(s) and different policies for supporting breastfeeding. An American retrospective study from 2008 with a cohort of 361 mother-infant pairs reported that 60% provided expressed milk feeding for their VLBW infants, but only 27% provided direct breastfeeding [29]. In a Swedish study from 2007, 53% of preterm infants (GA < 37 weeks) were exclusively breastfed at discharge from the neonatal unit. The rate of exclusively breastfeeding was though lower among the most immature infants (born with GA < 32 weeks) [30]. A Danish study from 2007 with 77 very preterm infants (GA ≤ 30 weeks) found 62% to be exclusively breastfed, 19% to be bottle-fed, 16% to be both breast- and bottle-fed, and 3% to be bottle-fed and supplemented with parental nutrition at hospital discharge [31]. A study from the Danish national birth cohort reported an overall prevalence of breastfeeding among term and preterm infants to be 88% at 1 month of age and 69% at 4 months of age, while the prevalence among low birth weight (LBW) infants at 4 months of age (chronological) was 56% [32].

Preterm infants are as already described routinely tube fed until they are developmentally and physiologically ready to begin the process of learning to suck, swallow, and breathe in a coordinated fashion which often occurs at 32-35 weeks PMA [25]. One study found that preterm infants allowed early non-nutritive suckling at the breast were able to demonstrate nutritive suckling (≥ 5g milk-volume by test-weighing) as early as 30.6 weeks PMA [33]. The same author found in another study that full breastfeeding was attained at a median age of 35 weeks (range 32 to 38 weeks) among 15 preterm infants [34]. Kangaroo-care dyads have been

found to breastfeed more exclusively and for a longer period compared to less or none skin-to-skin contact [35]. Kangaroo-care with early skin-to-skin contact as early as possible is a routine in Danish neonatal units.

Feeding mother's milk and breastfeeding has many advantages and breastfeeding seems possible to establish among preterm infants, but in order to improve breastfeeding-establishing policies and to identify those who need extra attention, it is important to estimate the breastfeeding rate, and to characterize the mothers and the very preterm infants.

HEALTH EFFECTS ASSOCIATED WITH NUTRITION OF VERY PRETERM INFANTS

In preterm infants, the beneficial effects of human milk generally relate to improvements in host defences, digestion, absorption of nutrients, gastrointestinal function, neuro-developmental outcomes, and maternal psychological well-being [36]. Breastfeeding provides a broad multi-factorial anti-inflammatory defence for the infant [37]. Even donor breast milk is associated with a lower risk of developing necrotising enterocolitis (NEC) compared to formula milk in preterm and low birth weight infants [38]. Breastfed children also have shown significantly higher developmental scores in comparison with formula fed children. The benefit obtained from breastfeeding was most pronounced in children with low birth weight. Also, a significant benefit from breastfeeding on cognitive development was obtained for breastfeeding exposure for more than 8 weeks [21]. Protective factors associated with breast milk probably even supersede the harm associated with smoking while breastfeeding [39].

Feeding solely human milk for very preterm infants during hospitalization will though lead to insufficient intake of protein and energy, and poor postnatal growth impairment – especially of the head, has been shown associated with increased levels of motor and cognitive impairments at 7 years of age [40]. Rates of brain growth are highest in the last part of gestation and the first year of life, which is the critical period of catch-up growth among VLBW infants. In children born at term, IQ scores at 4 years were highest in children whose heads grew most during infancy [41]. A randomized controlled study on high-energy and -protein diet for term and preterm infants with brain injuries showed improved head-growth at 1 year of age among infants fed more than the recommended intake of energy and protein, supporting the hypothesis that growth impairment due to lack of nutrients may decrease postnatal brain growth [42]. Optimal nutrition for achieving catch-up growth during infancy among very preterm infants is therefore important in order to achieve head (brain) growth and decrease the risk of neurological impairment.

Development of allergic diseases among preterm infants

The expression of allergic diseases varies with age, and symptoms may disappear and be replaced by other symptoms. In infancy the main allergic symptoms are atopic dermatitis (AD), gastrointestinal symptoms, and recurrent wheezing (RW), whereas bronchial asthma and allergic rhino-conjunctivitis are the main problems later in childhood. Adverse reactions to food, mainly cow's milk protein (CMP), are most common in the first year of life, whereas allergy to inhalant allergens mostly occurs later. A variety of factors are known to influence the risk of allergic disease, such as atopic predisposition, exposure to allergens (e.g. cow's milk and egg), and environmental factors [43]. It is also well known that

the mode of early feeding influences the risk of food allergy and that breastfeeding compared with cow's milk formula feeding is associated with a lower frequency of AD, RW, and cow's milk protein allergy (CMPA) [43-45]. The incidence of CMPA in infancy seems to be approximately 2 to 3 % in developed countries and the onset of diseases is in most cases closely related to the time of introduction to cow's milk products [46]. A review on breastfeeding and allergic diseases concludes that there are beneficial effects of breastfeeding especially among infants with atopic predisposition. The same review concludes that exposure to small doses of cow's milk during the first days of life appears to increase the risk of CMPA, but does not affect the incidence of allergic diseases later on [47]. Meanwhile, it remains to be established whether fortification of human milk with a cow's milk based fortifier for very preterm infants has any impact on the development of allergic diseases, such as cow's milk protein allergy.

Development of metabolic syndrome

Catch-up growth among preterm infants is often necessary in order to attain the growth-parameters of term infants of the same PMA, but catch-up growth has been suggested to increase the risk of developing "the metabolic syndrome" with obesity, cardiovascular disease, and insulin resistance in adulthood. An animal study have shown a reduced lifespan among male mice after in utero growth restriction and a postnatal period of accelerated growth with an obesity-inducing diet [48]. A study among term infants found an association between increased risk of elevated systolic blood pressure at 7 years of age and catch-up growth with crossing weight-percentiles upward during early childhood, but infants born SGA were though not at risk [49]. Preterm infants assigned to human milk (donor breast milk) have been found to have marked benefits up to 16 years later for all of the major components of the metabolic syndrome (blood pressure, leptin resistance, insulin resistance, and lipid profile) compared to formula fed infants. A positive dose-response association between the proportion of breast milk intake in the total feeding volume and later beneficial effect on blood pressure has been indicated as well [50-52].

There is definitely a dilemma with preterm infants that need sufficient nutrition with especially protein and fatty acids for promoting growth and brain-development on one side, and possible metabolic risks later in life by early induced catch-up growth [53;54]. Based on the current knowledge, dietary restrictions resulting in poorer growth are not recommended for preterm infants and further investigations are of high priority [55].

In order to achieve the goal for optimal growth and development among preterm infants after hospital discharge – without any negative health effects, it is important to investigate whether nutrient enrichment of mother's milk while breastfeeding after hospital discharge is possible, and whether it influences growth, increases the risk of feeding problems, and the development of allergic diseases and metabolic syndrome in later life.

3. THE AIMS OF THE THESIS

This Ph.D. project and thesis is part of a study including later follow-up.

Primary aim:

- For "healthy" very preterm infants, to compare the effect of human milk fortifier, added to mother's own milk while breastfeeding after hospital discharge, versus solely mother's milk given until 4 months corrected age (CA). Primary outcome was growth as measured by length, weight, and head circumference until 1 year CA.

Secondary aims:

- To investigate breastfeeding rates among preterm infants at discharge, and to investigate the duration of breastfeeding, and the possibility of fortifying mother's own milk after hospital discharge while breastfeeding.
- To investigate if there is any relationship between intake of fortifier added to mother's milk and blood-urea nitrogen, serum-phosphorus, and haemoglobin until 4 months CA.
- To describe growth-pattern in general including catch-up growth among preterm infants (both SGA and non-SGA) fed different diets after hospital discharge and until 1 year CA.
- To describe the occurrence of allergic diseases / symptoms in relation to nutrition in very preterm infants during the first year of life.
- To investigate the relationship between type of nutrition and haemoglobin A1C (HbA1C) at 4 months CA.
- To investigate the relationship between type of nutrition and blood pressure during the intervention-period until 4 months CA and at 1 year of age.
- To describe the content of macronutrients in human milk until 4 months CA from mothers who delivered prematurely.
- To investigate eating habits including frequency of meals and possible feeding problems (regurgitation and constipation) according to type of nutrition after hospital discharge.

Further outcomes beyond this thesis:

- To investigate if there are any long term consequences of fortification of mother's milk after hospital discharge on growth (including catch-up), intelligence, and socio-psychological behaviour, and the risk of metabolic disease and allergic diseases at 6 years of age.

HYPOTHESES

- It is possible to fortify mother's milk while breastfeeding after hospital discharge, without any disadvantages or risks of interfering with breastfeeding.
- Growth is increased among "healthy" very preterm infants, who are fed human milk fortifier in combination with mother's milk while breastfeeding, compared to preterm infants who are fed solely mother's milk.
- Growth of preterm infants born small for gestational age differs from preterm infants born appropriate for gestational age.
- Preterm infants achieve catch-up growth during hospitalization and catch-up growth possibly continues within the first year(s) of life.
- There is not an increased incidence of allergic diseases among very preterm infants supplemented with a human milk fortifier or fed a preterm formula, compared to exclusively breastfeeding after hospital discharge.

4. ETHICS

The study was approved July 1st 2004 by the Danish National Committee on Biomedical Research Ethics (J.nr. VF20030208), and handling of data and registrations were approved February 2006 by the Danish Data Protection Agency (J.nr. 2007-41-1349). Informed consent was obtained from parents of the very preterm infants participating in the intervention study after oral and written information.

There are no conflicts of interest. Mead Johnson Nutritionals donated the products used in the intervention study, but the company had no influence on the project, neither on the design nor on the products and methods used.

5. MATERIALS AND METHODS

STUDY-DESIGN AND STUDY-POPULATION IN GENERAL

This Ph.D. thesis is based on a prospective, randomized population-based birth cohort study with consecutive recruitment of newborn infants with a GA $\leq 32+0$ weeks and data-registration performed prospectively at four neonatal units in Denmark (Holbaek Hospital (HH), Kolding Hospital (KH), Hans Christian Andersen Children's Hospital at Odense University Hospital (OUH), and Aarhus University Hospital, Skejby (AUH)). The newborn infants were born and admitted to the neonatal units from July 2004 in Odense, December 2004 in Holbaek, March 2005 in Skejby, and May 2005 in Kolding, and until August 2008 for all units.

Feeding regimens were identical at the four neonatal units with early parenteral nutrition and early trophic feeding. Until PMA of at least 30 weeks, the infants were all fed expressed mother's milk and/or donor breast milk. Fortification with HMF was initiated day 10-14 after birth. Fortification of mother's own expressed milk was done until discharge, but with decreasing amounts during the last week(s) while the infant was improving to suck directly from the breast. If the mothers did not have enough milk of their own, the infants were supplemented with a preterm formula after 30 weeks PMA. The breastfed infants were discharged when sucking full amount direct from the breast and gaining weight. If the mothers decided not to breastfeed or breastfeeding stopped before discharge, the infants were bottle-fed, and they were discharged when bottle-fed without problems. SGA very preterm infants were fed like non-SGA infants, and not routinely given a larger volume or extra calories.

The counselling of the mother to breastfeed her infant(s) was done as a routine in the departments and according to the guidelines from The Danish National Board of Health (2003, 2005, 2006, and 2008) [56]. Kangaroo-care with skin-to-skin contact was established as soon as possible also during ventilator-treatment. As soon as the very preterm infant was stable (respiratory and cardiovascular) skin-to-skin contact was established once a day (2-3 hours) when ventilator-treated and twice a day (2 x 2-3 hours) when treated with continuous positive airway pressure (CPAP). The mothers were all encouraged to start expressing milk as soon as possible after birth and later on to breastfeed if possible and if they wanted to. Nutritive suckling / breastfeeding was initiated on an individual basis at 34-36 weeks PMA where the infant sucked increasing amount of mother's milk directly from the breast. At the same time the amount of mother's milk in the feeding-tube and thereby the amount of fortifier decreased.

Breastfeeding was not possible in some mothers due to breast-surgery, chemotherapy, or other medication contraindicating breastfeeding. There were several available breast pumps at all departments.

Inclusion-criteria:

- GA $\leq 32+0$ weeks
- "Healthy" at time of randomization and not excluded due to diseases or circumstances possibly influencing eating ability and growth.

Exclusion-criteria:

- Death
- Serious congenital or chromosomal anomalies
- Surgery due to necrotising enterocolitis (NEC) or ductus arteriosus persistens (DAP)
- Intraventricular haemorrhage (IVH) III-IV and/or periventricular leucomalacia (PVL)
- Bronchopulmonary dysplasia (BPD)
- Eating disability at 42 weeks PMA, including suspected adverse reactions to the intervention diet
- Mothers with language problems (unable to communicate in Danish or English)
- Severe social problems (mothers placed in institutions, alcohol, or drug abuse)
- Families who moved out of the involved regions

Basic characteristics describing infants and mothers:

- At birth: birth weight (BW), GA, and single birth or multiple births were recorded for each infant. Based on patient records and questionnaires, information on mother's age, education, and smoking habits were obtained. Mother's social group was defined according to The Danish National Centre of Social Research based on education and occupation [57].

PART 1. BREASTFEEDING RATE AT HOSPITAL DISCHARGE

A population based observational part of the study describing breastfeeding rate and possible factors influencing breastfeeding until hospital discharge among very preterm infants and their mothers.

Data-registration was performed consecutively from birth until discharge for all not-excluded very preterm infants (with permission from the Danish Data Protection Agency).

Exclusion was due to diseases or circumstances that would influence the eating- and/or feeding-ability at discharge (see 5.1).

Basic characteristics describing infants and mothers

As described in 5.1.

In addition, PMA and weight at discharge were recorded for each infant

Outcome measures (secondary in the thesis)

At discharge, feeding practice (breastfeeding, bottle-feeding, or combined breast- and bottle-feeding) was recorded for each infant.

PART 2. NUTRIENT ENRICHMENT AND GROWTH AFTER HOSPITAL DISCHARGE

A randomized controlled trial investigating the effect on growth when adding a human milk fortifier to mother's own milk while breastfeeding her very preterm infant(s) after hospital discharge.

Participants

Included were "healthy" very preterm infants whose parents accepted to participate in the intervention study by their signature after both written and oral information within the first two weeks from birth. Exclusion was due to diseases or circumstances that would influence the eating- and/or feeding-ability at discharge (as described in 5.1.) including verified cow's milk allergy diagnosed by controlled elimination/challenge procedures.

Sample size calculation

See statistics.

Intervention

Shortly before hospital discharge, the breastfed infants were randomized to either breastfeeding without supplementation (group A) or intervention with fortification (group B). Five packets of HMF (Enfamil® HM Fortifier, Mead Johnson Nutritional, Evansville IN, USA) (17.5 kcal, 1.375g protein / 5 packets) (composition in details in appendix 1) was added to a small amount (20-50 ml) of mother's expressed milk given in a bottle or with a small cup every day until 4 months CA if possible. Many of the mothers in the intervention group were fortifying a small amount of their own defrosted expressed milk after hospital discharge. Both breastfeeding groups were encouraged to breastfeed as long as possible. The study was not blinded due to the lack of a placebo-product without influence on breastfeeding, nutrition, and growth.

If the infant(s) were bottle-fed at discharge, the infant(s) were fed a preterm formula (PF) (group C) (Enfalac® Premature Formula, Mead Johnson Nutritionals, Nijmegen, Netherlands) (68 kcal, 2g protein, 7.4g carbohydrate, and 3.5g fat / 100 ml) (composition in details in appendix 1) until 4 months CA.

The above products from Mead Johnson Nutritionals were chosen since they were already used and known at the four neonatal units involved in the trial.

Introduction to complementary food was not recommended until 4 months CA for any of the groups. At that time, they were recommended the same complementary feeding as term infants without any special restrictions.

Randomization

Sealed envelopes with randomization numbers made prior to the study-start for each of the four neonatal units involved were used for randomization. Envelopes contained even numbers (assigned to group A) or uneven numbers (assigned to group B). Multiple births were randomized together. Only doctors enrolled participants while both nurses and doctors assigned to the project made the randomization.

In case of change of nutrition after randomization, the infant continued in the study with parents' permission. If breastfeeding was not sufficient (group A and B) within the first month after discharge, the infant was supplemented with or changed to PF. If breastfeeding ceased between 1 and 2 months after discharge the infant was supplemented or changed to preterm or term formula based on an individual assessment made by the physician

involved in the trial. If breastfeeding (group A and B) ceased later than 2 months after discharge the infant was supplemented or changed to term formula.

Basic characteristics of infants and parents

As described in 5.1.

In addition, length and head circumference (HC) were recorded at birth. Data on weight, length, and HC were obtained during hospitalization. Information on mother's previous breastfeeding experience was also obtained by interviews based on questionnaires at the time of randomization.

Primary outcome measures on growth

Infants were seen at the outpatient clinics at term, 2, 4, 6, and 12 months CA, where data on growth such as weight, length (crown-heel), and head-circumference (occipital-frontal) were obtained. During hospitalization, weight was measured on the same weighing machine each time and tape measures were used for length and head circumference. At the outpatient clinics the same weighing machines were used each time, tape measures were used for measuring HC, and infant measuring rods or stadiometers for measuring length (the infant lying until 1 year of age).

Secondary outcome measures

Feeding practice and breastfeeding duration

Data on duration of breastfeeding and feeding practice (breastfeeding with or without fortifier, feeding a preterm or term formula, or complementary feeding) during the intervention period were recorded.

Blood-samples

At randomization, discharge, term, and 4 months CA, blood was drawn to measure serum hemoglobin, serum phosphorus, and blood-urea nitrogen (BUN).

At 4 months CA, blood was also drawn to measure HbA1C. Haemoglobin F (HbF) was also measured.

Blood pressure

At time of randomization, at term, and at 2, 4, 6, and 12 months CA, the infant had their blood pressure measured. The mean blood pressure was recorded. Both doctors and nurses were measuring blood pressure at the departments and at the outpatient clinics.

Dropouts

Data from dropout infants were with the parents' permission used until the date of withdrawal.

PART 3. ALLERGIC DISEASES DURING THE FIRST YEAR OF LIFE

A population based part of the study describing allergic diseases in relation to nutrition in very preterm infants until 1 year of age.

Inclusion and exclusion criteria were as described in part 2, except that infants excluded from the RCT due to verified cow's milk allergy during the intervention period, or parents' decision not to participate in Part 2 due to severe family predisposition to allergic disease, were included in this part of the study, when the parents' accept was obtained.

Basic characteristics describing infants and parents

As described in 5.1.

In addition information on atopic predisposition (at least one first-degree relative with allergic disease), and previous breast-feeding experience were obtained by questionnaire based interviews at the time of randomization.

Outcome measures (secondary in the thesis)

At 4 and 12 months of age a standardized questionnaire based interview about allergic diseases/allergic symptoms such as urticaria, AD, gastrointestinal symptoms (colic, diarrhea, or vomiting without known infection), episodes of RW (diagnosed by a physician) and rhinitis/conjunctivitis, and treatment was performed by a paediatric nurse or doctor. CMPA was proven by controlled elimination/challenge test in a hospital setting. At 4 months CA a blood-sample was drawn for later analysis for specific IgE antibodies (egg-white, milk, peanut, dust mites (pteronyssinus and farinae), dog, cat, grass pollen, and latex) by ImmunoCAP 250 (Pharmacia detection limit 0.35 kIU/L). All blood-samples were analysed at Odense University Hospital. Data on duration of exclusively breastfeeding and introduction of formula and/or complementary food were recorded.

PART 4. MACRONUTRIENTS IN HUMAN MILK

A population based part of the study describing macronutrients in human milk from mothers who delivered prematurely.

Inclusion and exclusion criteria were as described for part 2 (the RCT).

Outcome measures (secondary in the thesis)

Content of protein, fat, lactose and energy in human milk.

Milk samples during hospitalization

The mothers were expressing milk as soon as possible after birth (within hours) using breast pumps available at all departments. The first milk sample for analysis of macronutrients was collected 2 weeks from birth, and then every second week until hospital discharge.

Milk sample collection: Each time the mother expressed milk during 24 hours (4-8 times) 2 ml human milk was stored in the same test-tube and frozen as soon as possible after 24 hours collection.

Milk samples after hospital discharge – at term (40 weeks PMA), 2, and 4 months CA

The mother emptied one breast by hand or with a breast pump once in 24 hours (no specific time during the day) and stored 10 ml of this in a test-tube. It was frozen as soon as possible.

Analysis of human milk samples

The milk-analyses were made using Human Milk Analyzer (HMA) from Miris AB, Sweden. The HMA measurement principle is based on mid-infrared transmission spectroscopy.

HMA was originally developed for the analysis of macronutrients in cow's milk, but has been modified and calibrated for human milk against reference methods for fat, protein, lactose, and total solids [58].

The HMA used for analyzing milk samples in this project was calibrated with reference-milk analysed with the methodologies of Rose-Gottlieb (fat), Kjeldahl (protein), dry-oven (solids / lac-

tose) at a certified laboratory (Fødevarestyrelsen, Region Nord) for official controls of foods under the Danish Ministry of Food, Agriculture, and Fisheries in Aarhus, Denmark.

The energy content in the milk samples was calculated from the individual fat, protein, and lactose values using following equation:

Energy = (9.25 kcal/g x fat) + (4.40 kcal/g x protein) + (3.95 kcal/g x lactose)

All milk samples had been frozen and were analysed according to the guidelines from Miris A/B: defrosted in a refrigerator, heated in warm water until a temperature of 40° Celsius and homogenized before analysis.

PART 5. FEEDING-PATTERN AND -PROBLEMS AFTER HOSPITAL DISCHARGE

A part of the RCT investigating possible feeding-problems, when supplementing mothers own milk with a human milk fortifier, compared to exclusively breastfeeding and formula feeding after hospital discharge.

Included and excluded infants were the same as in Part 2 (the RCT).

Outcome measures (secondary in the thesis)

At time of randomization, at term, 2, and 4 months CA the mothers filled in a questionnaire on:

- Nutrition: human milk with or without fortification, preterm formula, term formula, both human milk and formula, or complementary feeding during the last five days
- Number of meals: how many meals (breast and/or bottle) each day during the last five days
- Regurgitation-frequency: how many times each day during the last five days
- Stool-frequency: how many stools each day during the last five days and use of anti-constipation medicine

If the infant received anti-constipation medicine they had to register if it was Movicol® (Junior) or Lactulose.

6. DATA HANDLING AND STATISTICS (PART 1-5)

Data were analysed using STATA (Statacorp, College Station, TX, USA): version 9.2 in part 1, and version 11.0 in parts 2, 3, 4, and 5. In part 4, Microsoft Excel 2003 (Microsoft, Redmond, WA, USA) has also been used.

IN GENERAL:

Age, gender, and primary outcome measures on growth (weight, length, and head circumference) were transferred to Z-scores or standard deviation scores (SDS) in order to be able to compare nutrition groups containing both genders in parts of the statistical analyses.

PART 1. BREASTFEEDING RATE AT HOSPITAL DISCHARGE

Z-score or standard deviation score (SDS) was calculated as the difference between the actual weight and the expected reference weight divided with 1 standard deviation (SD) (ex.: (BW – reference BW) / 1 SD). The growth reference used for calculating Z-

scores for each gender was according to Marsal [59]. Instead of comparing weight at certain PMA for each gender, the Z-scores were used for comparisons between groups. In this study, the very preterm infants were defined as SGA if weight Z-score at birth was below -2 SDS and large for gestational age if weight Z-score was above +2 SDS.

Group comparisons were conducted with t-test for continuous variables and chi2-test for categorical data or Wilcoxon rank-sum test when data were not parametrically distributed (BW and GA). Logistic regression was used to produce univariate odds ratio (OR) and 95% confidence intervals (CI). Multivariate logistic regression was used to determine which factors (weight Z-score at birth, PMA at discharge, multiple births, young mother, maternal social group, and smoking) were independently associated with breastfeeding at discharge. Excluded from the final model were factors that clinically were more a result rather than a cause of feeding practices such as weight and weight Z-score at discharge, factors strongly correlated to other factors in the model (SGA, BW, and maternal age), and insignificant factors not influencing the final model (gender, duration of hospitalization, and GA).

PART 2. INTERVENTION WITH HUMAN MILK FORTIFIER AFTER HOSPITAL DISCHARGE

Sample-size calculation was made ahead of the study-start based on growth in absolute terms with a standard deviation of 5 g/day, significance at 5%, the lowest weight-difference not to be missed at 2.5 g/day, and power at 90%. The sample-size calculation showed that at least 85 infants were needed in each group.

In order to compare nutrition groups Wilcoxon rank-sum test or t-test were used for continuous variables and chi2-test was used for categorical variables.

To evaluate factors influencing the duration of breastfeeding and introduction to complementary feeding a multiple logistic regression model with clinical relevant variables (nutrition group, mother's age, social group, smoking habit, previous breastfeeding experience, multiple births, gender, GA, and SGA) was used.

Age was calculated and shown as post-menstrual age (PMA) or corrected age (CA):

At birth = GA. At time of randomization = 34 weeks = 238 days. At time of discharge = 36 weeks = 252 days. At term = 40 weeks = 280 days. At 2 months CA = 341 days. At 4 months CA = 402 days. At 6 months CA = 463 days. At 1 year = 12 months CA = 645 days.

Growth converted to Z-scores

Z-scores were calculated for weight, length, and HC as described in chapter 6. Part1. The preterm infants were defined as SGA if weight Z-score was below -2 SDS at birth. In part 2, all Z-scores have been calculated according to Niklasson and Albertsson-Wikland [60]. By linear interpolation weight, length, and HC was estimated at day 238 (randomization of the first infant), 252 (discharge of first infant), 280 (term) days PMA, and at 2, 4, 6, and 12 months CA, and then calculated as Z-scores according to the chosen reference. Mean Z-scores were used to calculate change in Z-score (delta Z-score) from day 238 PMA and until day 252 PMA, 280 PMA, 2, 4, 6, and 12 months CA. Multiple logistic regression was used to evaluate variables (gender, nutrition group, SGA/non-SGA, and multiple/single-birth) influencing delta Z-score.

Growth in absolute terms

Because data consists of repeated observations on the same subject taken over time and in order to exploit the full population sample, random effect models (REM) with intercept and slope random effects [61] have also been used from randomization time of the first infant (238 days PMA) until 12 months CA. According to clinical relevant variables possibly influencing growth and likelihood-ratio-tests comparing random effect models, the following variables were included in the final REM: the age of the infants at the different times of measure, nutrition group, gender, multiple births, SGA, baseline-weight, -length, or -HC (weight, length, or HC at 238 days PMA), and a 4.grade polynomial on time interacting with gender and nutrition group. Weight was transformed by taking the square root. The residuals of the model showed normal distribution. Random intercept and random slope was added in order to account for the unobserved heterogeneity between individuals measured at multiple occasions.

Growth charts on weight, length, and HC from randomization and until 1 year of age were made. These growth charts are based on REM including the age at the different times of measurements, nutrition group, gender, and a 4.grade polynomial on time interacting with gender and nutrition group.

Intention-to-treat and per-protocol

All analyses on growth were calculated as both by intentions to treat (ITT) and treated per protocol (PP).

Outliers

Possible outliers from the dataset did not influence the results from regression-model-analysis on delta Z-scores or random effect model.

Blood-samples and blood pressure

A linear regression model was used for comparing the 3 nutrition groups possibly influencing hemoglobin, s-phosphorus, and BUN. Only infants who were still feed the assigned nutrition at the time of blood-sample were part of this analysis.

A multiple regression model was used to investigate the impact of GA, BW, the time of blood-sampling, and PMA on HbF and HbA1C.

Mean blood pressure was calculated at time of randomization, term, 2, 4, 6, and 12 months CA and analysed both by ITT and treated PP. A multivariate logistic regression analysis with variables possibly influencing blood pressure (nutrition group and gender) was performed.

PART 3. ALLERGIC DISEASES ACCORDING TO NUTRITION UNTIL 1 YEAR OF AGE

The incidence is the percentage of infants with allergic symptoms until 1 year CA. The prevalence is the percentage of infants with allergic symptoms at 12 months CA. The prevalence is corrected for missing data at 12 months CA.

Group comparisons were conducted with univariate analysis: t-test for continuous variables and chi2-test for categorical data or Wilcoxon rank-sum test when data were nonparametric distributed (BW and GA). Logistic regression was used to calculate univariate odds ratio and 95% confidence intervals. Multivariate logistic regression was used to determine which clinical relevant factors (GA, BW, gender, atopic predisposition, nutrition group,

time of introduction to complementary food, mother's age, social group, and parents smoking at home) possibly influenced the development of AD and/or RW before 12 months CA. The pre-term infants were defined as SGA if weight Z-score was below -2 SDS at birth according to a reference [60]. Analyses were performed by ITT and treated PP.

PART 4. NUTRIENT CONTENT IN HUMAN MILK FROM MOTHERS WHO DELIVERED PREMATURELY

Comparison of protein-content in human milk was conducted with a t-test.

PART 5. POSSIBLE FEEDING PROBLEMS ACCORDING TO NUTRITION AFTER HOSPITAL DISCHARGE

Logistic regression was used to determine the type of nutrition (unfortified mother's / human milk (UHM), HMF, PF, mature formula (MF), both breast- and bottle-feeding (Mix), and complementary feeding (CF)) possibly influencing the number of meals each day, regurgitation, and/or use of anti-constipation medicine.

7. RESULTS

A total number of 633 infants born with a GA $\leq 32+0$ from July 2004 until August 2008 were recorded (GA 23+0 – 32+0 weeks and BW 428g – 2255g). Characteristics are shown in Table 1.

PART 1. FACTORS ASSOCIATED WITH SUCCESSFUL ESTABLISHMENT OF BREASTFEEDING

Of 633 eligible very preterm infants and their mothers, 155 infants were excluded (24% of initial cohort) as shown in Table 2.

The study-population consisted of 478 infants (GA 24+1 to 32+0 and BW 520g-2255g) distributed within the four neonatal units: Holbaek hospital: 76, Kolding hospital: 103, Hans Christian Andersen Children's Hospital at Odense University Hospital: 179, and Skejby hospital at Aarhus University Hospital: 120. There were 224 girls and 254 boys. A total of 180 infants (38%) were multiple births (166 twins, 11 triplets, and 3 quadruplets) and 113 infants (24%) were born SGA. No infants were large for gestational age making AGA and non-SGA identical groups. At discharge mean PMA was 37+2 (SD = 12 days), mean weight was 2634g (SD = 406g), 285 (60%) infants were exclusively breastfed, 167 (35%) were bottle-fed, and 26 (5%) were both breast- and bottle-fed.

The following analyses are based on two feeding groups according to whether the infant was 1) exclusively breastfed, n=285 (60%), or 2) not exclusively breastfed, n=193 (40%) at discharge. Results are shown in Table 3.

For the final analysis, complete dataset were available on 409 very preterm infants and their mothers (86% of study-cohort (478) and 65% of initial cohort (633)). Information on mother's age was obtained among mothers of 474 infants. Information on smoking was obtained from mothers of 436 infants and 19% of 436 mothers smoked during pregnancy and lactation. Information on mother's social group was obtained from mothers of 423 infants and the mothers were divided into 5 different social groups: 1=high social group (12%), 2 (28%), 3 (6%), 4 (39%), and 5=low social group (15%).

Table 1. Distribution and characteristics of infants at the four neonatal units and in total.

Very preterm infants (VPI)	Holbaek hospital	Kolding hospital	HCA CH OUH	AUH Skejby	Total
Initial registration	95	98	249	191	633
Transferred to (-) or received (+) from another hospital	(*)	+33	-15+2	-20	0
Participants part 1 on breastfeeding at discharge.					
Excluded	19	28	57	51	155
Study-cohort	76	103	179	120	478
Single birth (n) (%)	53 (70)	60 (58)	102 (57)	83 (69)	298 (62)
Breastfeeding at discharge (n) (%)	47 (62)	54 (52)	106 (59)	78 (65)	285 (60)
Bottle-feeding at discharge (n) (%)	28 (37)	43 (42)	67 (37)	29 (24)	167 (35)
Breast- & bottle-feeding at disch. (n) (%)	1 (1)	6 (6)	6 (3)	13 (11)	26 (5)
Participants part 2 on nutrient enrichment and growth (ITT).					
Excluded	19	28	59	51	157
Refused to participate	31	37	33	55	156
Study-cohort	45	66	144	65	320
Single birth (n) (%)	34 (76)	39 (59)	81 (56)	46 (71)	200 (63)
Randomized to no fortifier (gr. A) (n) (%)	12 (27)	21 (32)	40 (28)	29 (45)	102 (32)
Randomized to fortifier (gr. B) (n) (%)	14 (31)	21 (32)	49 (34)	21 (32)	105 (33)
Bottle-feeding (gr. C) (n) (%)	19 (42)	24 (36)	55 (38)	15 (23)	113 (35)
Blood-samples (n)	342	484	1389	965	3180
VPI who delivered blood-sample (n)	45	65	144	64	318
Participants part 3 on allergic diseases					
Excluded	19	26	57	51	153
Refused to participate	31	37	33	55	156
Initial Study-cohort (ITT)	45	68	146	65	324
Withdrawals	2	11	16	8	37
Lack of data before 1 year CA	1	1	1	1	4
Final Study-cohort	42	56	129	56	283
For Specific IgE-analyses	13	33	84	33	163
Participants part 4 on macronutrients in human milk					
Milk samples					736
Number of mothers					214
Participants part 5 on feeding problems					
Registrations on feedings	79	15	402	133	769
Number of VPI	34	60	136	56	286
Follow-up at 6 years (#)	43	55	127	52	277

(*) The youngest VPI according to GA were born at Department of Neonatology, Rigshospitalet in Copenhagen and transferred to Holbaek after birth. (#) VPI in the study by March 2010 - VPI that will be invited to the 6-year follow-up. Parents of these VPI received a letter in December 2009 – January 2010 with information on the project until December 2009, test-results if blood was drawn for IgE-analyses, and information about the follow-up.

Table 2. Excluded very preterm infants in part 1.

Cause of exclusion	Number (% of total population)
Death	34 (5)
Surgery due to Necrotising Enterocolitis (NEC) (n=30) or due to other gastrointestinal problems (n=2)	32 (5)
Mb cordis incl. Ductus Arteriosus Persistens (DAP)	12 (2)
Intraventricular Haemorrhage (IVH) (grade III or IV), Periventricular Leucomalacia (PVL), or Hydrocephalus	24 (4)
Malformations, incl. Downs Syndrome (n=4)	9 (1)
Bronchopulmonary Dysplasia (BPD) (Oxygen dependent at discharge)	9 (1)
Language problems (Mothers who were not able to communicate in Danish or English)	12 (2)
Social problems (Mothers with cancer, drug-abuse, or placed at an institution)	9 (1)
Eating disability (1 still tube-fed at discharge)	4 (1)
Moved before discharge (6 out of the country, 2 to another region in DK, 2 born in other countries)	10 (2)
Total	155 (24)

Mean GA 27+6 (23+0 – 32+0). Mean BW 1062g (428g – 2121g). Twins 32%.

In the multivariate logistic regression, a higher rate of breastfeeding at discharge was found among mothers of high social group ($p=0.000$) and mothers who did not smoke ($p=0.003$). There was a strong correlation between smoking and low social group with a mean social group 4.07 ± 1.02 and 3.00 ± 1.30 among mothers who smoke and mothers who did not smoke respectively ($p=0.000$). A higher rate of single birth infants tended to be breastfed at discharge though not significantly ($p=0.09$). A lower rate of young mothers (<25 years) were breastfeeding at discharge ($p=0.007$ univariate), though not significantly in the final model ($p=0.28$) but young age was also correlated with low social group ($p=0.000$).

Previous breastfeeding experience among mothers of 299 infants did not have any significant influence on breastfeeding.

Low weight Z-score at birth tended to be negatively correlated to breastfeeding at discharge ($p=0.02$ univariate), though not significant in the final model ($p=0.09$). Less SGA infants were exclusively breastfed at discharge (21.1% vs. 27.5% not exclusively breastfed), but not significantly ($p=0.11$ univariate). Weight Z-score at discharge seemed lower among exclusively breastfed infants though not significant ($p=0.06$ univariate). Change in weight Z-score from birth to discharge was 0.34 SDS (-1.36 to -1.02) among not exclusively breastfed and -0.10 SDS (-1.09 to -1.19) among exclusively breastfed infants ($p=0.000$ univariate).

The not exclusively breastfed group consisted of 167 solely formula fed and 26 formula and breastfed (combined) very preterm infants. Change in weight Z-score from birth to discharge was 0.41 (SD = 0.76) in the solely formula fed group compared to -0.15 (SD = 0.72) in the combined group and -0.10 (SD = 0.88) in the exclusively breastfed group.

Length and head circumference (HC) was not obtained on the exact day of discharge among all infants why it was not possible to calculate change in Z-score on length and HC from birth to discharge.

Including only single birth infants ($n=253$) in the final model the results did not change comparing exclusively breastfed with not exclusively breastfed infants. There was no significant differences except that maternal social group ($p=0.02$) and smoking ($p=0.01$) were still negatively correlated to breastfeeding at discharge. Change in weight Z-score among single birth was 0.28 SDS (formula fed) and -0.10 SDS (exclusively breastfed) ($p=0.000$ univariate).

Main results on factors associated with breastfeeding very pre-term infants at hospital discharge

In 478 very preterm infants 60% were exclusively breastfed, 35% were exclusively bottle-fed, and 5% were both breast- and bottle-fed at discharge.

Mothers in a high social group ($p=0.000$) and “not smoking” ($p=0.003$) were significantly more often exclusively breastfeeding their preterm infant(s) at discharge.

Factors like low weight Z-score at birth, multiple births, and young mothers below 25 years were negatively correlated to exclusively breastfeeding at discharge.

Infant age at discharge and duration of hospitalization did not influence breastfeeding at discharge.

PART 2. NUTRIENT ENRICHMENT OF HUMAN MILK AND GROWTH OF VERY PRETERM INFANTS

Before randomization the same number ($n=155$) of infants were excluded initially as in part 1. In addition, two very preterm infants were excluded after randomization due to eating disability and severe cerebral palsy in one case, and severe social problems, PVL and severe cerebral palsy in another case (Figure 1). The final study-cohort consisted of 320 (51%) very preterm infants, as parents of 156 (25%) refused to participate in the RCT and a total of 157 (25%) were excluded.

The excluded infants had a lower GA and BW whereas those with parents who refused to participate had a higher GA and BW compared to the study-cohort ($p \leq 0.002$). Compared with mothers who refused to participate, mothers in the study-cohort were older (30.8 years vs 29.3 years, $p=0.003$) and more often breastfeeding (65% vs 50%, $p=0.002$) (Table 4).

The total number of infants in the study-cohort (ITT analysis) was 320. The number that completed in their assigned nutrition groups was 283 (88%) at term, 211 (66%) at 2 months and 108 (34%) at 4 months (Figure 1). Due to parents choices many changes of nutrition between 2 and 4 months within all 3 nutrition groups were performed with 211 infants completing in their assigned nutrition groups until 2 months (for PP-analysis). The gender distribution among these 211 infants is boys/girls: Group A 38/35, B 26/25, and C 54/33. ITT- and PP analyses were at 6 months based on 303 and 206 infants, and at 12 months based on 277 and 188 infants respectively.

Characteristics of the infants and their mothers in the nutrition groups are presented in Table 5. At discharge only one infant was fed both bottle and breast, but this infant was one of the infants excluded after randomization due to severe cerebral palsy and eating problems. Sixty-five percent (207/320) infants were exclusively breastfed (group A and B) and 35% (113/320) were bottle-fed with a preterm formula (group C) at discharge.

Table 3. Characteristics associated with nutrition at discharge among 478 very preterm infants in the study-cohort recruited from 4 neonatal units in Denmark from July 2004 – August 2008.

Characteristics	Exclusively bottle-fed with formula (n=167) ^a	Combined formula and breast-feeding (n=26) ^a	Exclusively breastfeeding at discharge?			Univariate		Final model (n=409) (86% of study-cohort, 65% of initial cohort)	
			No ("") (n=193)	Yes (n=285)	p-value *	Odds Ratio (95% CI)		p-value	
Preterm Infants									
Gender (% male)	57.5	42.3	55.4	51.6	0.41	0.86 (0.59-1.24)	–	–	–
Birth weight (median) (g)	1270	1433	1285	1350	0.07	1.05 (1.00-1.11) #	–	–	–
GA at birth (median) (wk)	29.6	31.2	29.7	30.3	0.11	1.04 (0.94-1.15)	–	–	–
Weight Z-score at birth ± 1 SD (mean) (SDS)	-1.35 \pm 1.30	-1.47 \pm 0.88	-1.36 \pm 1.25	-1.09 \pm 1.24	0.02	1.19 (1.03-1.38)	1.17 (0.98-1.40)	0.09	0.09
SGA (weight Z-score < -2 SDS) (%) in group)	27.5	26.9	27.5	21.1	0.11	0.70 (0.46-1.08)	–	–	–
Weight at discharge ± 1 SD (mean) (g)	2684 \pm 403	2486 \pm 351	2655 \pm 401	2620 \pm 409	0.35	–	–	–	–
PMA at discharge ± 1 SD (mean) (wk)	37.2 \pm 1.7	37.5 \pm 1.3	37.3 \pm 1.7	37.4 \pm 1.7	0.39	1.05 (0.94-1.17)	1.08 (0.94-1.23)	0.28	0.28
Weight Z-score at discharge ± 1 SD (mean) (SDS)	-0.93 \pm 1.03	-1.61 \pm 0.70	-1.02 \pm 1.02	-1.19 \pm 0.94	0.06	–	–	–	–
Change in weight Z-score, birth to discharge ± 1 SD (SDS)	0.41 \pm 0.76	-0.15 \pm 0.72	0.34 \pm 0.77	-0.10 \pm 0.88	0.000	0.53 (0.42-0.67)	–	–	–
Hospitalized ± 1 SD (mean) (days)	54.3 \pm 17.0	47.9 \pm 13.9	53.5 \pm 17.0	53.6 \pm 21.5	0.93	1.00 (0.99-1.01)	–	–	–
Multiple births (%) multiple)	37.1	61.5	40.4	35.8	0.31	0.82 (0.56-1.20)	0.67 (0.43-1.06)	0.09	0.09
Mothers									
Maternal age ± 1 SD (mean) (y) (n=474)	29.8 \pm 5.9	29.3 \pm 3.8	29.7 \pm 5.7	30.7 \pm 4.5	0.06	1.04 (1.00-1.08)	–	–	–
Young mother (%) <25y) (n=474)	19.8	0.0	17.1	8.8	0.007	0.47 (0.27-0.81)	0.70 (0.37-1.33)	0.28	0.28
Maternal social group ± 1 SD (mean) (1=high, 2, 3, 4, 5=low) (n=423)	3.7 \pm 1.2	3.1 \pm 1.4	3.6 \pm 1.3	2.9 \pm 1.3	0.000	0.65 (0.55-0.77)	0.71 (0.59-0.86)	0.000	0.000
Smoking (%) (n=436)	33.3	10.0	30.6	11.3	0.000	0.29 (0.17-0.48)	0.43 (0.25-0.76)	0.003	0.003

*Wilcoxon rank-sum test if median, t-test if continuous variables or chi2-test if categorical variables. # Odds Ratio per 100g.

Duration of breastfeeding

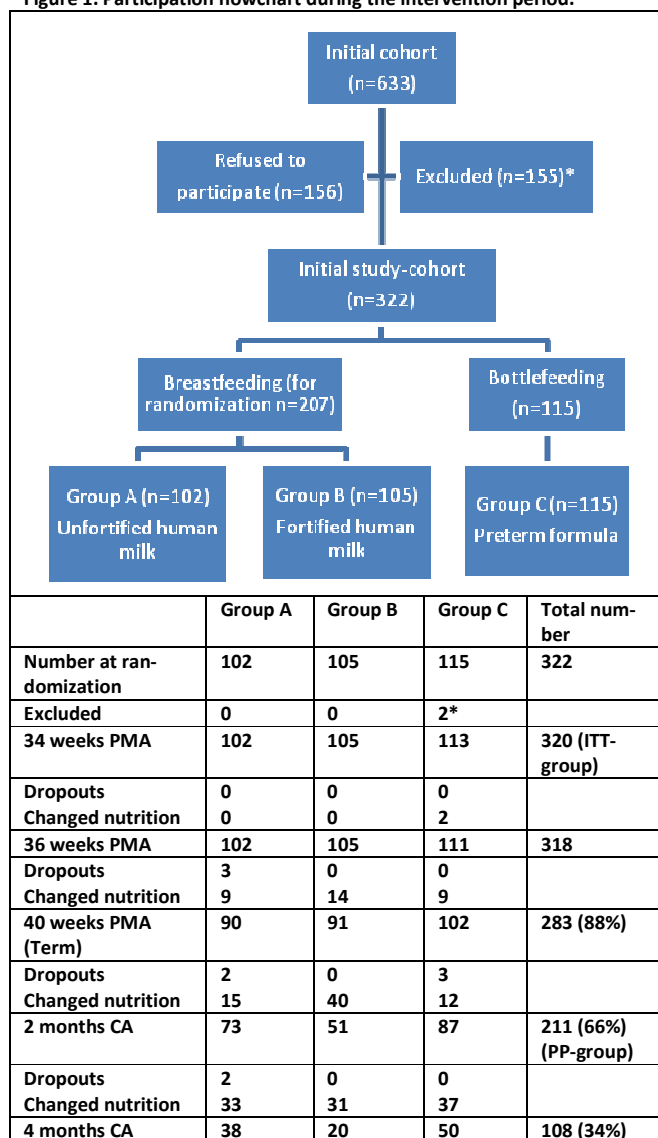
Duration of breastfeeding was not influenced by fortification. Mothers of multiple births stopped breastfeeding earlier than single birth infants (p=0.000), and mothers in the lowest social group (p=0.02) and younger mothers (p=0.03) also discontinued breastfeeding earlier. Mean duration of breastfeeding was 11.8 \pm 7.7 weeks in group A and 10.6 \pm 7.5 weeks after term in group B (no significant difference) (Figure 2). Mean age for introduction of complementary food was 16.4 \pm 3.9 weeks (min 6.0 – max 27.4 weeks), 18.3 \pm 4.4 weeks (min 8.4 – max 39.7 weeks), and 17.0 \pm 3.4 weeks (min 7.1 – max 24.3 weeks) after term in group A, B, and C respectively. Older mothers (p=0.000) and group B compared to group A (p=0.002) introduced complementary food significantly later.

Overall growth (using Z-scores and growth in absolute terms)

All very preterm infants, no matter the nutrition group, showed a nadir in weight, length, and HC Z-score at 34 weeks PMA. Mean Z-scores however, did never drop below normal range (-2 SDS). All weight, length, and HC Z-scores increased irrespective of the nutrition group after 34 weeks PMA, but tended to decrease after 6 months CA to a mean weight Z-score: -0.69 (ITT) and -0.75 (PP), mean length Z-score: -0.05 (ITT) and -0.14 (PP), and mean HC Z-score: +0.10 (ITT) and +0.06 (PP) at 12 months CA (Figure 3, Figure 4 and appendix 2).

Boys compared to girls showed significant higher weight, length, and HC within all 3 nutrition groups from term (280 days PMA) until 1 year CA (645 days PMA) using random effect models (REM). Results are shown in tables in appendix 2 (ITT and PP), and growth charts based on ITT-analysis are shown in appendix 3.

Figure 1. Participation flowchart during the intervention period.



*Exclusion as described in Table 2 with the addition of two infants (1 due to periventricular leucomalacia and 1 due to eating disability).

These growth-charts were made without using e.g. growth base-line-values.

Growth according to nutrition (using Z-scores and growth in absolute terms)

ITT: Infants in group C increased significantly more in length and weight Z-score compared to both breastfed groups (A and B) (Figure 3). Among boys, length Z-score increased significantly more in group C compared to both group A and B (2 and 4 months), while girls increased significantly more in length Z-score comparing group C with A (2 and 4 months), but not with B (Figure 5).

In addition, REM showed weight (2 – 6 months) and HC (term – 4 months) to be significantly higher among girls in group B compared to A (Table 6).

PP: Compared to A and B, group C increased significantly more in weight Z-score (term – 4 months) and length Z-score (2 and 4 months) (Figure 4). Girls in group C increased significantly more in weight Z-score compared to both A and B (term) and increased significantly more in length Z-score compared to A but not to B (2 – 6 months) (Figure 6).

In addition, REM showed length and HC to be significantly higher among girls in group B compared to A (2 – 4 months) (Table 6).

Catch-up growth (Z-scores)

In our study, non-SGA infants seemed to have achieved catch-up on HC at discharge, on weight at 2 months CA, and on length at 4 months CA., while SGA infants showed rapid catch-up growth on HC until term, on weight until 4 months, and length-growth even continued until 1 year CA (details in Figure 7, Figure 8 and in appendix 4).

Subgroup analyses (Z-scores)

Growth during hospitalization

Growth (weight, length, and HC Z-scores) during hospitalization among infants in the RCT. Weight Z-score increased from birth to discharge (day 252 PMA) in group C and decreased in A and B (comparable to results in part 1 with measurements on the exact day of discharge). Length Z-scores decreased while HC Z-score increased within all 3 nutrition-groups from birth to discharge (day 252 PMA). Results are shown in appendix 4.

Table 4. Characteristics of initial cohort, excluded, refusals, and study-cohort.

Infants	Initial Cohort (n = 633)	Ex-cluded (n = 157)	Refusal to partici-pate (n = 156)	Study-Cohort (SC) (n = 320)	Refu-sals versus SC (p-value)
GA at birth (median) (min-max) (days)	208 (161-224)	195 (161-224)	214 (175-224)	208.5 (169-224)	0.002
BW (median) (min-max) (g)	1256 (428-2255)	1014 (428-2121)	1436 (520-2220)	1271 (535-2255)	0.001
Boys	330/631	78/155	77/156	175/320	ns
Weight Z-score at birth $\pm 1SD$ (mean) (SDS)	-1.08 \pm 1.22	-1.15 \pm 1.40	-0.96 \pm 1.23	-1.10 \pm 1.11	ns
SGA at birth	136/624	39/148	29/156	68/320	ns
Multiple births	229/633	49/157	60/156	120/320	ns
PMA at discharge $\pm 1SD$ (mean) (days)	–	–	262 \pm 12	261 \pm 12	ns
Weight Z-score at discharge $\pm 1SD$ (mean) (SDS)	–	–	-1.29 \pm 1.14	-1.11 \pm 0.92	ns
Mothers					
Breastfeeding at discharge	–	–	78/156	207/320	0.002 *
Mothers age $\pm 1SD$ (mean) (years)	–	–	29.3 \pm 5.1	30.8 \pm 4.9	0.003 *
Social group $\pm 1SD$ (mean) (1=high and 5=low)	–	–	3.29 \pm 1.31	3.13 \pm 1.31	ns
Smoking	–	–	20/117	61/317	ns

*Also significant ($p<0.05$) in a multiple logistic regression model (GA, BW, weight Z-score at birth, multiple births, mother's age, smoking habit, social group, and breastfeeding at discharge)

Table 5. Characteristics of the nutrition groups.

Infants	A (n = 102)	B (n = 105)	A vs. B (p-value)	A and B (n = 207)	C (n = 113)	A and B vs. C (p-value)
GA at birth (median) (min-max) (days)	208.5 (169-224)	212 (171-224)	ns	210 (169-224)	207 (176-224)	ns
BW (median) (min-max) (g)	1260 (548-2255)	1320 (535-2100)	ns	1287 (535-2255)	1233 (612-2140)	ns
Weight Z-score at birth ± 1 SD (mean) (SDS)	-1.02 \pm 1.16	-1.03 \pm 1.05	ns	-1.02 \pm 1.10	-1.23 \pm 1.13	ns
SGA	20/102	21/105	ns	41/207	27/113	ns
Boys	58/102	52/105	ns	110/207	65/113	ns
SGA boys	11/20	10/21	ns	21/41	13/27	ns
Multiple births	27/102	42/105	0.04	69/207	51/113	0.04#
Baseline weight at day 238 PMA ± 1 SD (mean) (g)	Girls 1882 \pm 293 Boys 1964 \pm 287	Girls 1865 \pm 268 Boys 1988 \pm 268	ns ns	Girls 1872 \pm 278 Boys 1975 \pm 277	Girls 1895 \pm 358 Boys 1996 \pm 280	ns ns
Baseline Length at day 238 PMA ± 1 SD (mean) (cm)	Girls 43.9 \pm 2.5 Boys 44.2 \pm 2.5	Girls 43.8 \pm 2.1 Boys 44.9 \pm 2.0	ns ns	Girls 43.9 \pm 2.3 Boys 44.5 \pm 2.3	Girls 43.5 \pm 2.5 Boys 44.0 \pm 2.2	ns ns
Baseline HC at day 238 PMA ± 1 SD (mean) (cm)	Girls 30.8 \pm 1.4 Boys 30.9 \pm 1.3	Girls 30.4 \pm 1.1 Boys 31.2 \pm 1.2	ns ns	Girls 30.6 \pm 1.2 Boys 31.1 \pm 1.2	Girls 30.6 \pm 1.4 Boys 31.1 \pm 1.3	ns ns
PMA at discharge ± 1 SD (mean) (days)	264 \pm 15	260 \pm 10	0.04	262 \pm 13	259 \pm 10	ns
Weight Z-score at discharge ± 1 SD (mean) (SDS)	-1.22 \pm 0.94	-1.22 \pm 0.88	ns	-1.22 \pm 0.91	-0.92 \pm 0.92	0.006#
Mothers						
Mother's age ± 1 SD (mean) (years)	30.9 \pm 4.5	31.0 \pm 4.3	ns	31.0 \pm 4.4	30.4 \pm 5.8	ns
Social group ± 1 SD (mean) (1=high and 5=low)	2.88 \pm 1.27	2.89 \pm 1.30	ns	2.88 \pm 1.28	3.59 \pm 1.25	0.000#
Smoking	13/99	10/105	ns	23/204	38/113	0.000#
Previous breast-feeding experience	35/93	33/95	ns	68/188	43/109	ns

Wilcoxon rank-sum test or t-test if continuous variables and chi2-test if categorical variables. #Also significant ($p<0.05$) in a multiple logistic regression model (variables: GA, BW, weight Z-score at birth, weight Z-score at discharge, multiple births, mother's age, smoking habit, social group, and previous breastfeeding experience).

Number of infants with Z-scores < -2 SDS at birth and during the first year of life

The number of infants with Z-scores < -2 SDS on weight, length, and HC increased from birth until 34 weeks PMA and then decreased mainly until term on weight, but after term and until 4 months on length. After term the number of infants with Z-scores < -2 SDS on both weight and length seemed to decrease more among infants supplemented with fortifier or fed a preterm formula – though not significant. Details are shown in appendix 4.

ITT subgroup analysis on infants with subnormal weight

SGA infants (n=68) compared to non-SGA increased significantly more in length Z-score during the entire study period with no significant difference comparing nutrition groups (Figure 7 and Figure 8). SGA boys increased significantly more in weight Z-score until 2 months compared to girls, while SGA girls increased significantly more in HC Z-score compared to boys until 6 months (both $p<0.05$).

Among 53 infants with subnormal weight at discharge, group C increased significantly more in delta weight Z-score compared to A and B until 2 months CA, but with no significant difference at 12 months CA.

Results on serum chemical determinations according to nutrition groups

Blood-samples from 265 infants at time of randomization, 177 at discharge, 60 at term, and 65 at 4 months CA (fed either A, B, or C

at the time of blood-sample) showed significant higher levels of BUN in group C compared to both A and B at all 4 periods ($p<0.05$). Infants in group B had a significant higher level of BUN and s-phosphorus compared to group A at 4 months CA (Table 7). Results on HbA1C and HbF are shown in appendix 5.

Results on blood pressure

Procedures and equipment measuring blood pressure turned out to be different at the four departments. Results on blood pressure are shown in appendix 6.

Main results on nutrient enrichment

At discharge 65% were breastfed (n=207) and 35% were bottle-fed with preterm formula (n=113). Comparing the breastfeeding-groups (A) mothers milk without (n=102) and (B) mothers milk with fortification (n=105), no significant difference in mean duration of breastfeeding after term (11.8 and 10.6 weeks respectively) was found.

Compared to group A and B, infants in C increased significantly more in weight Z-score until term and length Z-score until 6 months CA. No significant difference was found in weight, length, or head-circumference at 12 months CA between group A and B. At 12 months CA using REM, boys in group C were significantly longer and heavier compared to A and B, while girls in C were longer and heavier compared to A only. By PP analysis, girls in group B compared to A were longer and had larger HC at 2 and 4 months CA ($p<0.05$).

Higher protein-intakes were related to higher blood protein-levels.

Figure 2. Kaplan Meier plots illustrating duration of breastfeeding among very preterm infants (group A: Mothers milk / Unfortified Human Milk (UHM) and B: Mothers milk supplemented with Human Milk Fortifier (HMF)) for all breastfed very preterm infants and single birth.

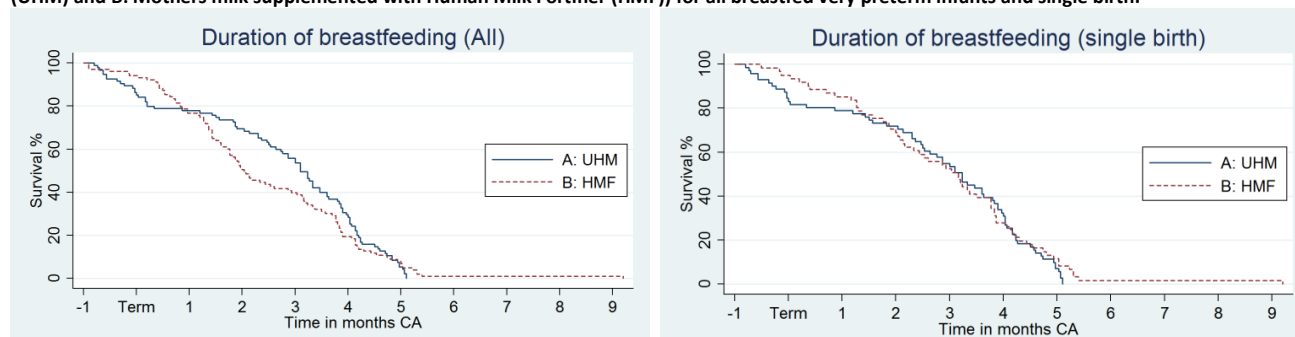


Figure 3. (ITT) Z-scores from birth to 12 months CA (both gender) according to nutrition-groups (mean Z-score \pm 1SD) and change in Z-score (delta Z-score from 34 weeks PMA). Significant difference in delta Z-score ($p < 0.05$) comparing nutrition-groups shown as \bullet C > A and \blacktriangle C > B.

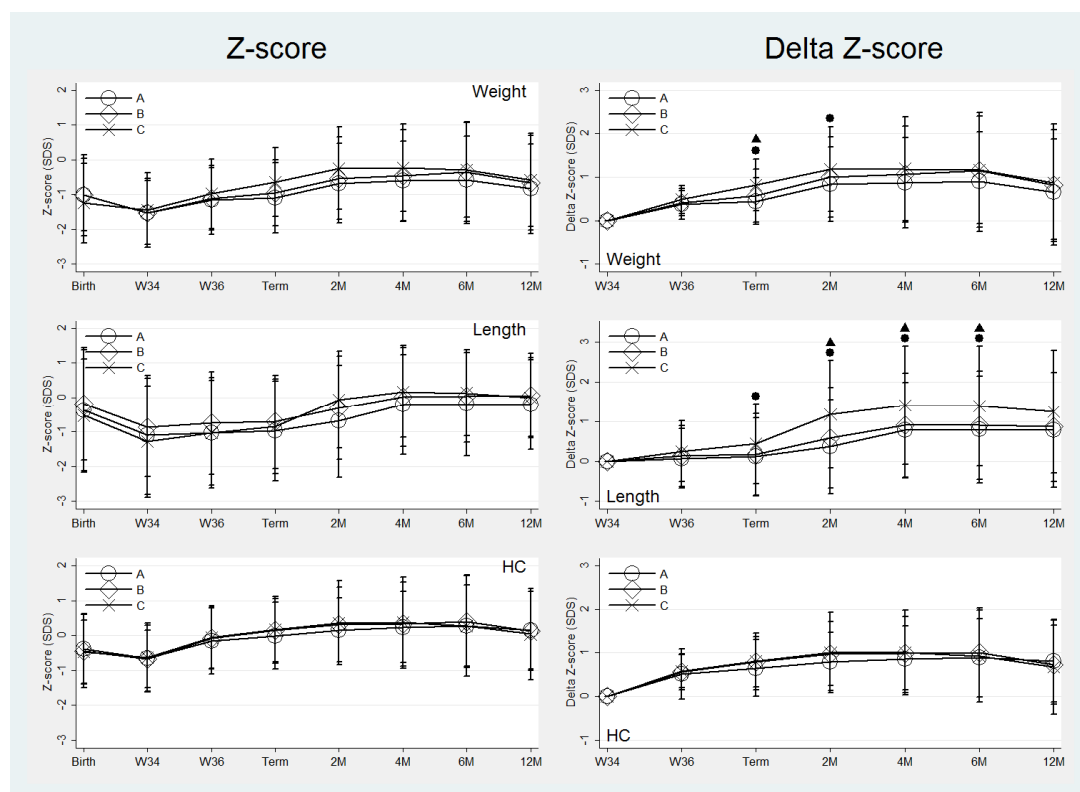


Figure 4. (PP) Z-scores from birth to 12 months CA (both gender) according to nutrition-groups (mean Z-score $\pm 1SD$) and change in Z-score (delta Z-score from 34 weeks PMA). Significant difference in delta Z-score ($p < 0.05$) comparing nutrition-groups shown as \bullet C > A and \blacktriangle C > B.

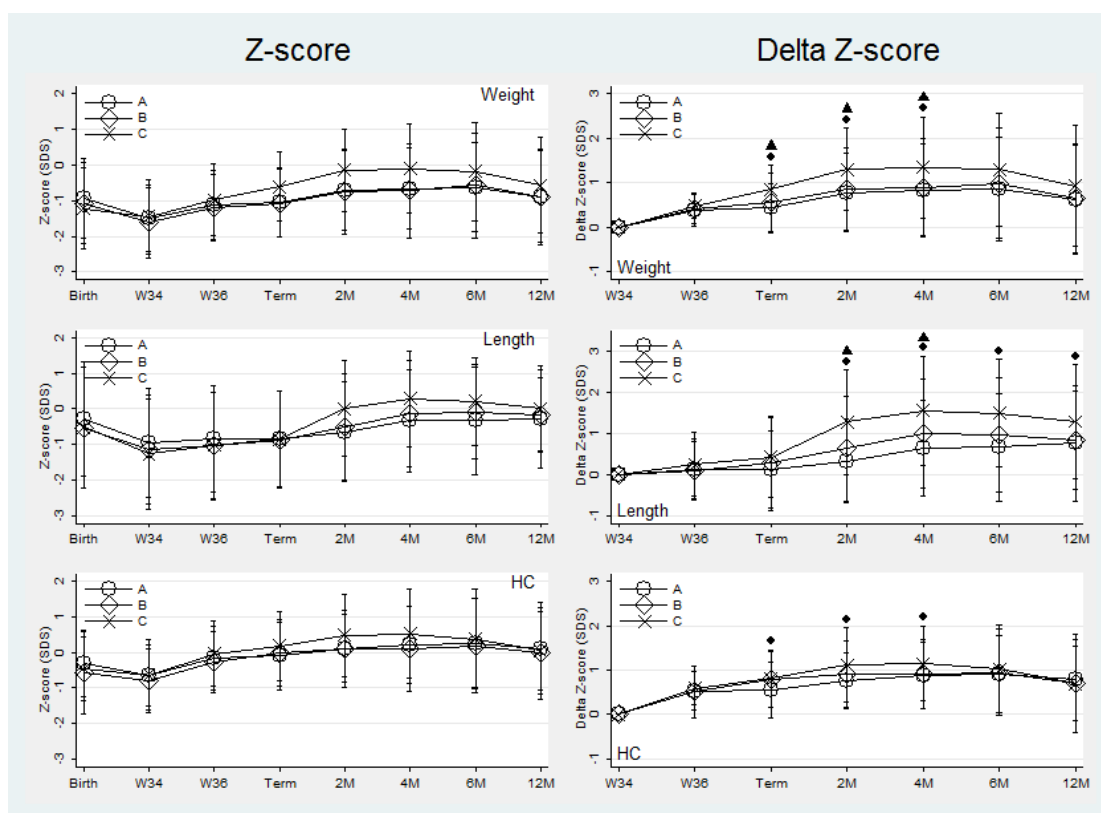


Figure 5. (ITT) Change in Z-score (delta Z-score from 34 weeks PMA) according to gender. Significant difference in delta Z-score ($p < 0.05$) comparing nutrition-groups shown as \bullet C > A and \blacktriangle C > B.

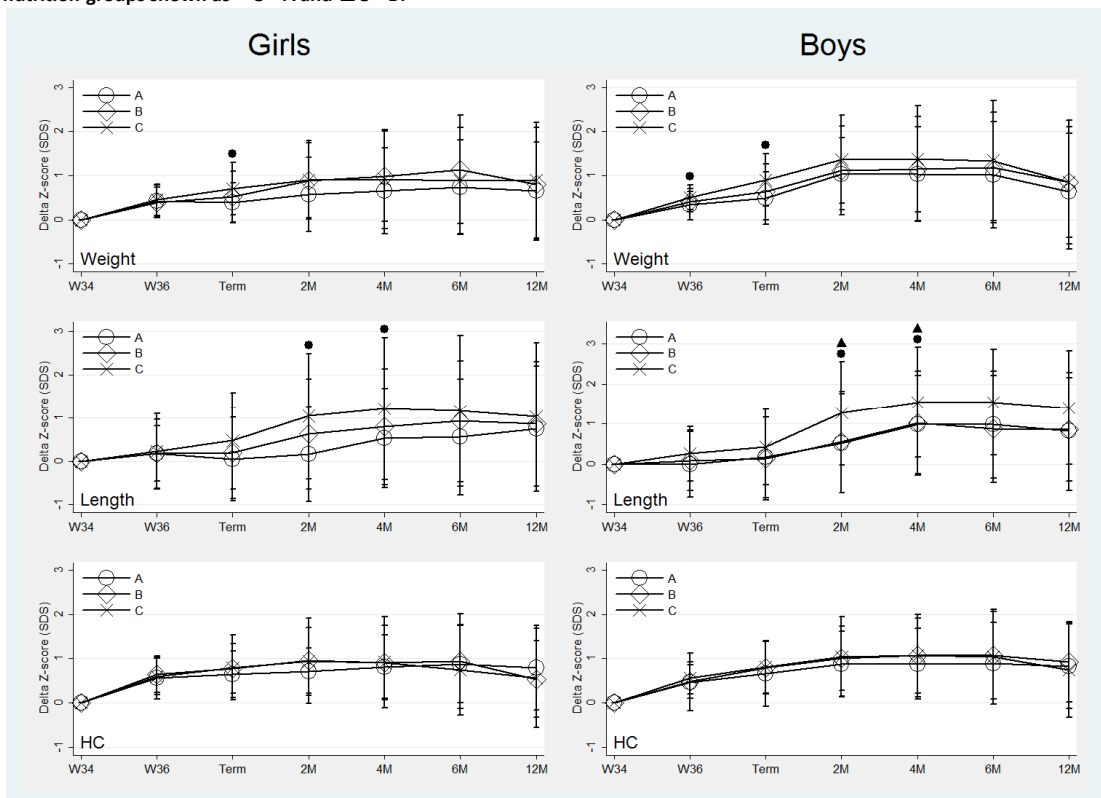


Figure 6. (PP) Change in Z-score (delta Z-score from 34 weeks PMA) according to gender. Significant difference in delta Z-score ($p < 0.05$) comparing nutrition-groups shown as ● C > A and ▲ C > B.

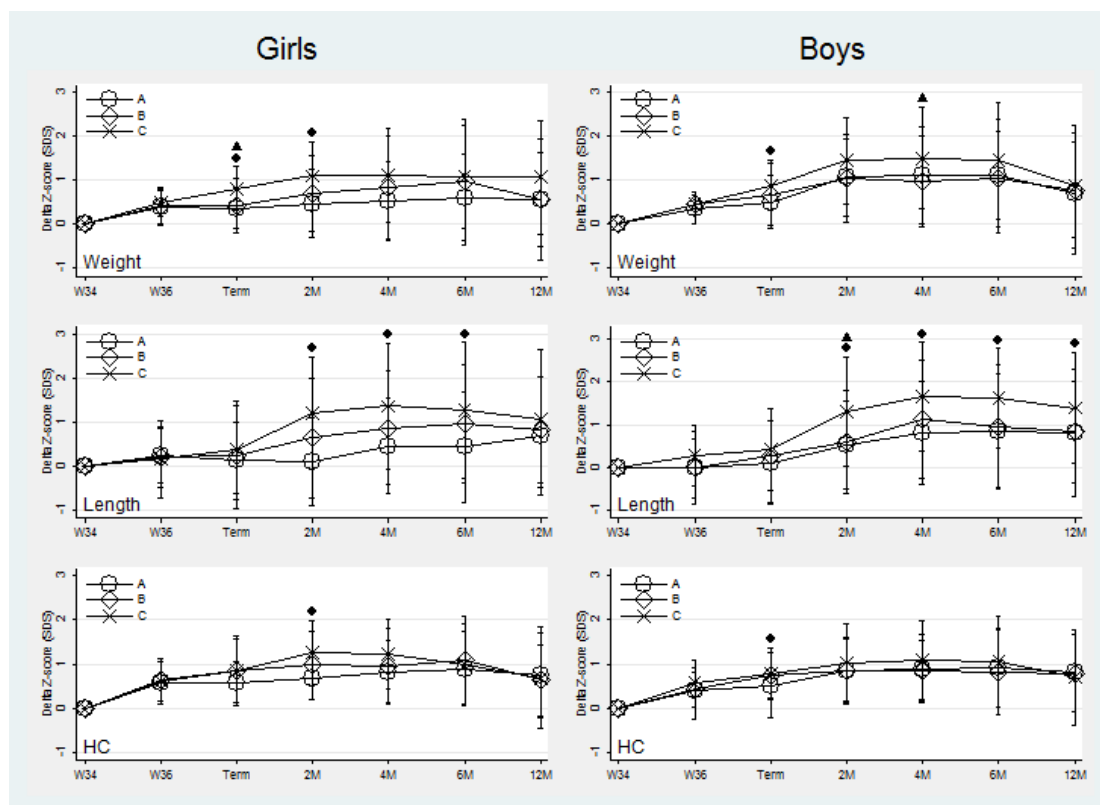


Figure 7. (ITT) Anthropometric data as Z-scores for AGA (=non-SGA) and SGA on weight, length, and HC (both gender).

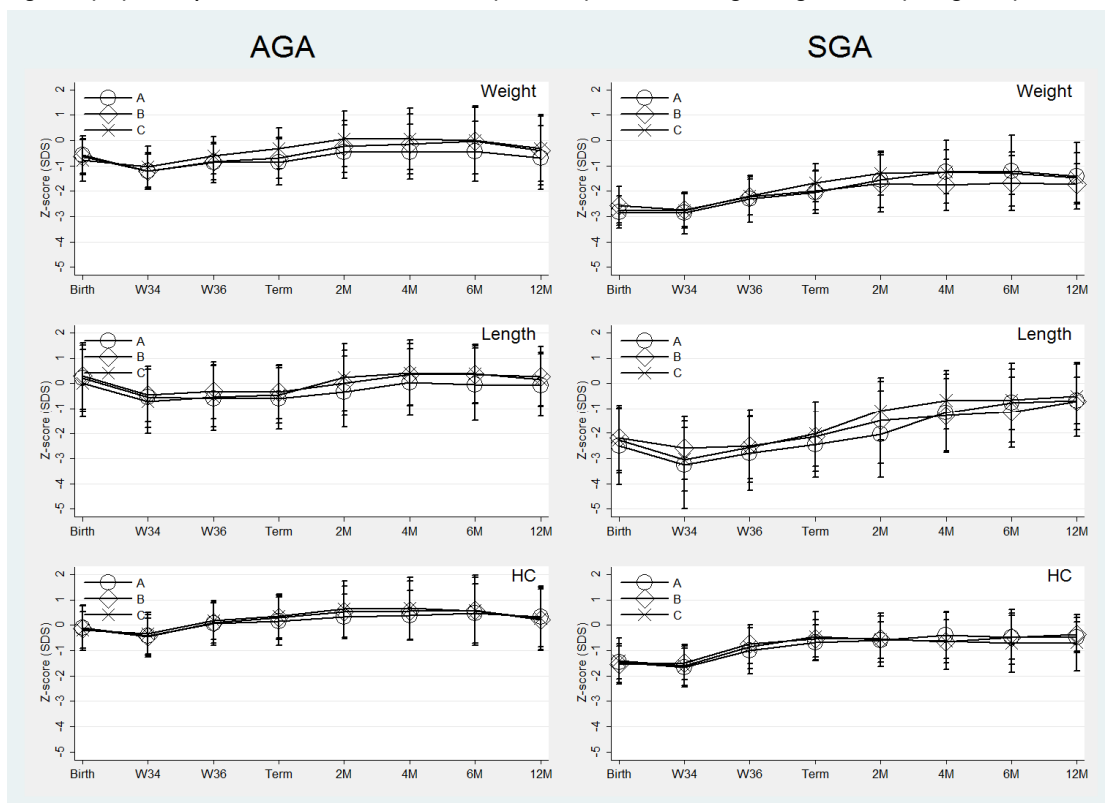


Figure 8. (PP) Anthropometric data as Z-scores for AGA (=non-SGA) and SGA on weight, length, and HC (both gender).

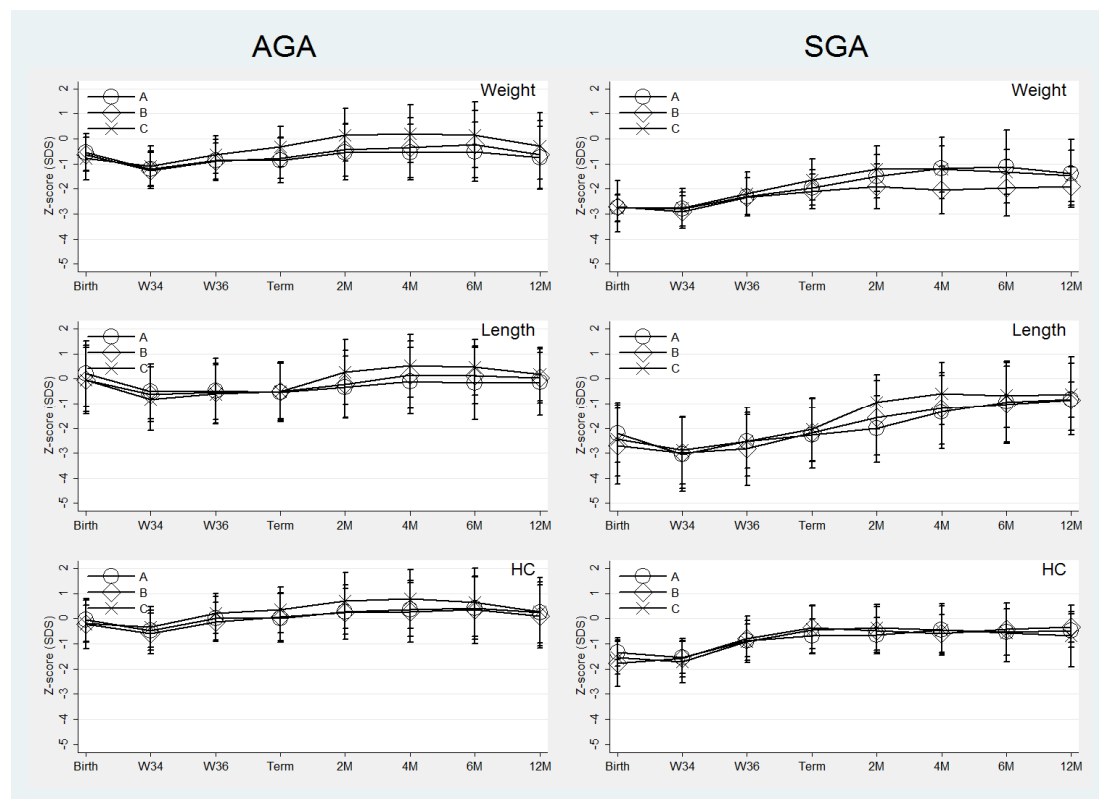


Table 6. Main results on growth according to nutrition and gender using REM with significant difference ($p < 0.05$) (ITT and PP). See appendix 2 for details.

	By ITT (at n months CA)	Treated PP (at n months CA)
Weight		
Girls	B > A at 2, 4, and 6 C > A at 6 and 12	C > A at 4, 6, and 12 C > B at 12
Boys	C > A at term, 2, 4, 6, and 12 C > B at 4, 6, and 12	C > A at 2, 4, and 6 C > B at 2, 4, 6, and 12
Length		
Girls	C > A at 2, 4, 6, and 12	B > A at 2 and 4 ($p=0.059$ at 6) C > A at 2, 4, 6, and 12 C > B at 12
Boys	C > A at term, 2, 4, 6, and 12 C > B at 2, 4, 6, and 12	C > A at ($p=0.053$ at term), 2, 4, 6, and 12 C > B at ($p=0.053$ at 2) 4, 6, and 12
HC		
Girls	B > A at term, 2, and 4 C > A at term, 2, and 4	B > A at 2 and 4 C > A at term, 2, 4, and 6
Boys	No significant difference	No significant difference

Table 7. Mean blood-urea nitrogen (BUN), s-phosphorus, and hemoglobin (whole blood). Type of nutrition (A, B, or C) on the day of blood-sample was known. Conversion factors from [62].

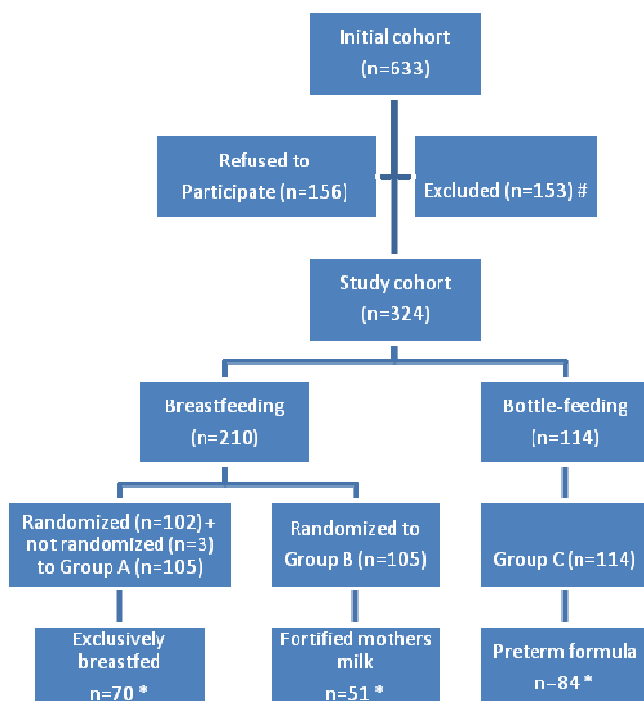
	Nutrition group	Week 34-35	Week 36-38	Week 39-40	4 months CA
BUN $\pm 1SD$ (mean) (mmol/L) 1 mmol/L = 2.80 mg/dL	Numbers of VPI	265	177	60	65
	A	1.7 \pm 0.6	1.5 \pm 0.4	1.5 \pm 0.8	2.2 \pm 0.9
	B	1.8 \pm 0.7	1.8 \pm 0.7	1.9 \pm 1.0	3.1 \pm 1.2 *
	C	2.1 \pm 0.9 #x	2.5 \pm 0.9 #x	3.3 \pm 1.3 #x	4.2 \pm 0.7 #x
S-phosphorus $\pm 1SD$ (mean) (mmol/L) 1 mmol/L = 3.10 mg/dL	Numbers of VPI	260	167	56	64
	A	2.09 \pm 0.22	2.02 \pm 0.23	1.87 \pm 0.33	1.74 \pm 0.44
	B	2.10 \pm 0.27	2.09 \pm 0.22	1.96 \pm 0.27	1.96 \pm 0.19 *
	C	2.16 \pm 0.24	2.17 \pm 0.22 x	2.17 \pm 0.23 #x	2.05 \pm 0.11 x
Hemoglobin $\pm 1SD$ (mean) (mmol/L) 1 mmol/L = 1.61 mg/dL	Numbers of VPI	207	161	60	61
	A	6.6 \pm 1.2	6.4 \pm 0.8	6.2 \pm 0.7	7.3 \pm 0.6
	B	6.8 \pm 1.2	6.4 \pm 0.8	6.1 \pm 0.6	7.5 \pm 0.6
	C	6.3 \pm 0.9 &	6.1 \pm 0.9	5.9 \pm 0.6	7.2 \pm 0.6

Significant difference ($p < 0.05$) with higher levels of BUN comparing group C with A (x) and B (#), group B with higher levels of BUN and s-phosphorus compared to A (*) at 4 months CA, and group C with lower levels of hemoglobin compared to B at time of randomization (&). Very preterm infants (VPI).

PART 3. ALLERGIC DISEASES DURING THE FIRST YEAR OF LIFE

The study-cohort consisted of 324 (51%) very preterm infants, as parents of 156 (25%) refused to participate and 153 (24%) were excluded (Figure 9).

Figure 9. Participation flowchart. Treated PP (*) (completed in assigned nutrition-group until 2 months CA) 205/211 and by ITT 283/324 remained at 1 year of age.



(#) Exclusion: Death (n=34), serious congenital or chromosomal anomalies (n=9), surgery due to necrotizing enterocolitis (n=32), persistent ductus arteriosus (n=12), intraventricular haemorrhage III-IV and/or periventricular leucomalacia (n=25), bronchopulmonary dysplasia (n=9), eating disability at 42 weeks PMA (n=1), severe language problems (n=12), social problems (n=9), or moved (n=10).

Parents of 3 infants chose not to participate in the RCT due to severe atopic predisposition (and were categorized as excluded in the RCT). None of them received human milk fortifier during their hospitalizations: A couple of twins (two boys) (GA 31+4) were exclusively breastfed until 6 months CA. No allergic symptoms were reported within the first year CA.

A girl (GA 32+0) was exclusively breastfed until 4 months CA. At 9 months CA the mother reported that the girl had an episode with rhinitis and conjunctivitis.

One boy (GA 29+3) with AD from 3 months CA was suspected of CMPA after hospital discharge (but was for other reasons excluded in the RCT). He received HMF added to mother's own milk or donor milk during hospitalization and was bottle-fed with preterm formula after hospital discharge. A skin prick test (SPT) to milk was positive (4 mm), while specific (Immunoglobulin E) IgE was < 0.35 kIU/L to milk and egg respectively. Controlled elimination/challenge procedure with milk was negative and thus the suspicion of CMPA was not confirmed. There was no clinical suspicion on allergy to egg.

Characteristics of participating infants and parents are shown in Table 8.

Table 8. Characteristics of 324 preterm infants and their mothers.

Preterm infants	
GA at birth (median) (min-max) (weeks+days)	29+6 (24+1 – 32+0)
BW (median) (min-max) (g)	1283 (535 – 2255g)
SGA (weight Z-score < -2 SDS)	68/324
Boys	178/324
Multiple births	122/324
Predisposition to allergic disease Breastfed (A+B) vs. Formula fed (C)	63/253 46/171 (26%) vs. 17/82 (21%)
Introduction to complementary food $\pm 1SD$ (mean) (weeks after term CA)	17.3 \pm 4.0 weeks (after term)
Mothers	
Mother's age $\pm 1SD$ (mean) (years)	30.7 \pm 4.9
Social group $\pm 1SD$ (mean) (1=high, 2, 3, 4, and 5=low)	3.14 \pm 1.31
Parental smoking (one or two parents) smoking at home	116/319 (36%)

Maternal smoking	61/319 (19%)
Paternal smoking	94/294 (32%)

Information on allergic symptoms was obtained in 90% (290/324) infants. Due to withdrawal of 7 infants before 12 months CA there were 283 infants remaining at 12 months CA (ITT-analysis). An overall incidence and prevalence (corrected for missing data from 4 VPI) of allergic symptoms at 12 months CA among those 283 infants is shown in Table 9.

Table 9. Incidence and prevalence of allergic symptoms within and at 12 months corrected age (CA) and mean age of onset of allergic symptoms among 283 very preterm infants.

Allergic symptoms	Number of cases	Incidence until 12 months CA (%)	Median corrected age of onset of symptoms (min-max)	Prevalence (%) of symptoms at 12 months CA (\$)
Urticaria	7	2.5	7.2 (0.8 – 9.3)	0.0
Atopic dermatitis	51	18.0	6.1 (-2.6 – 11.9)	12.1
Treated with steroids	8	2.8	6.7 (0.2 – 11.9)	–
Treatment unknown	43	15.2	6.1 (-2.6 – 11.9)	–
Gastrointestinal symptoms	38	13.4	1.0 (-3.3 – 6.8)	1.4
Recurrent wheezing	111	39.2	7.1 (-0.4 – 12.0)	32.7
Treated with oral/inhaled bronchodilators (*)	7 (oral) 23 (inh.)	10.6	8.0 (1.9 – 11.9)	–
Treated with inhaled glucocorticosteroids (#) (&)	31	11.0	6.1 (-0.4 – 11.1)	–
Treatment unknown	50	17.7	7.3 (-0.2 – 12.0)	–
Rhinitis ⌘	27	9.5	2.1 (-2.7 – 11.8)	3.7
Conjunctivitis ⌘	10	3.5	5.8 (-0.2 – 11.1)	1.8
One or more of the above symptoms	159	56.2	–	41.8

Treatment with: (*) beta-2-agonist oral or inhaled, (#) inhaled glucocorticosteroids, (&) 2 infants were also treated with leukotrienreceptor-antagonists. (⌘) One child was treated with oral antihistamine (Claritin®). (\$) corrected for missing data from 4 infants at 12 months CA.

Information on atopic predisposition was obtained from 78% (253/324) with 25% (63/253) predisposed to allergic diseases. Among breastfed infants (group A (21/86) + B (25/85)) 26% (46/171) were predisposed to allergic disease(s) compared to 21% (17/82) in the formula fed (group C) with no significant difference between groups.

The number of infants who completed in their assigned nutrition groups until 2 months CA was 205 (63%) with 199 (61%) remaining at 12 months CA (PP-group).

Comparing nutrition groups (A, B, and C, or just A and B) no difference was found regarding development of atopic dermatitis

(AD) or recurrent wheezing (RW) during the first year of life, whereas atopic predisposition and gender were associated with an increased risk of developing AD and/or RW using both univariate and multiple regression models. Infants with mothers in low social groups had a lower risk of developing AD in the univariate analysis ($p=0.01$), but not significant in the final model (ITT). The risk of developing AD increased with increasing age of the mother in the univariate analysis ($p=0.03$) (PP), but was not significant in the final model. Early introduction to complementary feeding was associated with an increased risk of developing RW in both univariate ($p=0.02$) and the final model ($p=0.006$) (PP). All results are shown in Table 10. There was no significant association between parental smoking and the development of RW or AD.

Specific IgE were analysed in blood-samples from 51% (163/320) infants at 4 months CA (Table 11). In two infants, specific IgE to cow's milk was detected: In a girl (GA 31+0), specific IgE (milk) was 0.38 kIU/L. She received HMF added to donor milk and mother's milk during hospitalization. She was exclusively breast-fed at discharge and supplemented with a term formula and complementary food shortly before 4 months CA. She had two episodes with viral induced wheezing treated with bronchodilator at 9 and 11 months CA while no other allergic symptoms were reported. In a boy (GA 29+4), specific IgE (milk) was 0.36 kIU/L. He also received HMF added to human milk during hospitalization. He was bottle-fed with preterm formula from hospital discharge until 4 months CA. He did not show any allergic symptoms until 1 year CA.

Table 10. Results on specific IgE-analyses to different allergens.

Specific IgE (n=161)	Median (kIU/L) (min-max)	Number > 0.35 kIU/L
Egg-white (n=161)	0.03 (0.02-0.14)	0
Milk (n=161)	0.03 (0.02-0.38)	2 *
Peanut (n=160)	0.00 (0.00-0.03)	0
Dust mite: Pteronyssinus n=157	0.01 (0.00-0.32)	0
Dust mite: Farinae n=153	0.00 (0.00-0.27)	0
Dog (n=149)	0.01 (0.00-0.16)	0
Cat (n=151)	0.00 (0.00-0.08)	0
Grass pollen (n=160)	0.00 (0.00-0.07)	0
Latex (n=159)	0.05 (0.03-0.14)	0

* a) 0.38 kIU/L and b) 0.36 kIU/L.

Main results on allergic diseases

The incidence during and prevalence at 12 months CA of RW was 39.2% and 32.7%, while AD was 18.0% and 12.1% respectively. Predisposition to allergic disease increased the risk of developing AD ($p=0.04$) (OR 2.6 (95% CI 1.0 – 6.4)) (PP) and the risk of developing RW ($p=0.02$) (OR 2.7 (95% CI 1.2 – 6.3)) (PP). Boys compared to girls had an increased risk of developing RW ($p=0.003$) (OR 3.1 (95% CI 1.5 – 6.5)) (PP). No difference was found between nutrition groups. None developed food allergy.

Table 11. Significant associations with development of atopic dermatitis and recurrent wheezing.

By ITT			Univariate (*)		Final model	
			p-value	Odds Ratio (95% CI)	p-value	
Atopic dermatitis (AD)						
Group A, B, and C	+AD	-AD			(n=219)	
Atopic predisposition (n=223)	16/41	37/182	0.013	2.5 (1.2-5.2)	2.3 (1.1-5.0)	0.03
Maternal social group $\pm 1SD$ (mean) (1=high, 2, 3, 4, 5=low)	2.69 \pm 1.32 (n=51)	3.21 \pm 1.30 (n=230)	0.011	0.7 (0.6-0.9)	–	ns
Group A and B					(n=153)	
Maternal social group $\pm 1SD$ (mean) (1=high, 2, 3, 4, 5=low)	2.39 \pm 1.15 (n=36)	2.99 \pm 1.30 (n=156)	0.013	0.7 (0.5-0.9)	–	ns
Recurrent wheezing (RW)						
Group A, B, and C	+RW	-RW			(n=219)	
Boys (Both gender=283)	76/111	80/172	0.000	2.5 (1.5-4.1)	3.3 (1.8-6.1)	0.000
Group A and B					(n=153)	
Boys (Both gender=194)	50/73	51/121	0.000	3.0 (1.6-5.5)	4.1 (1.9-8.6)	0.000
Treated PP						
Atopic dermatitis (AD)						
Group A, B, and C	+AD	-AD			(total n=155)	
Atopic predisposition (n=159)	12/30	28/129	0.041	2.4 (1.0-5.6)	2.6 (1.0-6.4)	0.043
Group A and B					(total n=95)	
Mothers age $\pm 1SD$ (mean) (year)	32.9 \pm 4.6 (n=23)	30.6 \pm 4.2 (n=95)	0.027	1.1 (1.0-1.3)	–	ns
Recurrent wheezing (RW)						
Group A, B, and C	+RW	-RW			(total n=155)	
Atopic predisposition (n=159)	19/55	21/104	0.049	2.1 (1.0-4.3)	2.7 (1.2-6.3)	0.016
Boys (Both gender=199)	49/72	61/127	0.007	2.3 (1.3-4.2)	3.1 (1.5-6.5)	0.003
Group A and B					(total n=95)	
Boys (Both gender=118)	26/39	34/79	0.017	2.6 (1.2-5.9)	3.6 (1.2-10.4)	0.018
Introduction to CF $\pm 1SD$ (mean time since term CA) (weeks)	16.0 \pm 3.5 (n=39)	17.8 \pm 3.7 (n=79)	0.020	0.98 (0.97-1.0)	0.97 (0.95-0.99)	0.006

(*) Wilcoxon rank-sum test if median, t-test if continuous variables or chi2-test if categorical variables. CF=complementary feeding. n=total number of very preterm infants in the model.

PART 4. MACRONUTRIENTS IN HUMAN MILK FROM MOTHERS WHO DELIVERED PREMATURELY

A number of 214 mothers delivered 736 human milk samples (from 2 weeks after birth until 4 months CA).

Main results on macronutrients in human milk

Macronutrients in human milk showed decreasing content of protein, but stable contents of fat and lactose from birth to 4 months CA (Figure 10). Energy in human milk is shown in Figure 11.

Figure 10. Content of lactose, fat, and protein in human milk

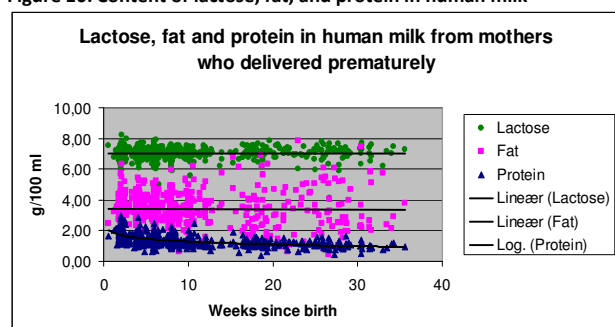
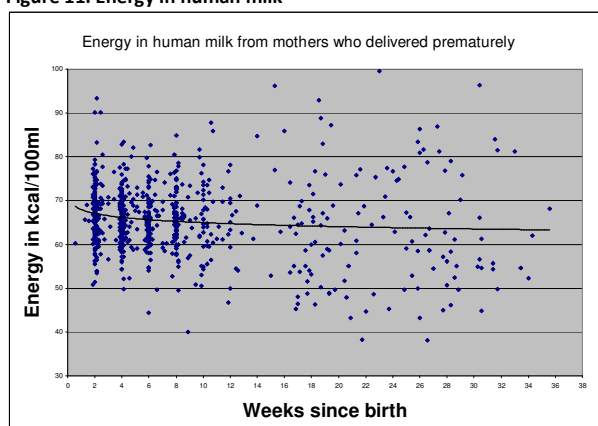


Table 12. Mean protein-content and SD in human milk samples

Weeks after birth	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	32
Number of milk Samples	155	175	110	90	48	21	3	12	29	16	11	11	18	17	9	5	4
Protein-conc. (mean) (g/100ml)	1.8	1.5	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.1	1.2	1.0	1.2	1.0	1.0	0.9	1.0
1 SD	0.4	0.3	0.3	0.3	0.3	0.2	0.2	0.4	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.1	0.2

Figure 11. Energy in human milk



Using t-test comparing protein-content in human milk at different times, there was a significant difference between the following weeks:

Four weeks: Human milk had lower content of protein compared to 2 weeks ($p=0.000$).

Six weeks: Human milk had lower content of protein compared to 4 weeks ($p=0.024$).

Eight weeks: Human milk had lower content of protein compared to 6 weeks ($p=0.036$).

Protein-contents in human milk samples from mothers of very preterm infants are shown in Figure 12, Figure 13, and Table 12.

Figure 12. Protein-content in human milk from mothers of very preterm infants.

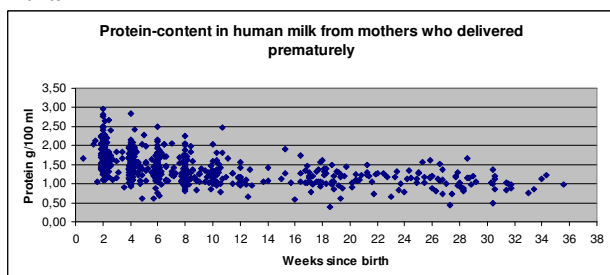
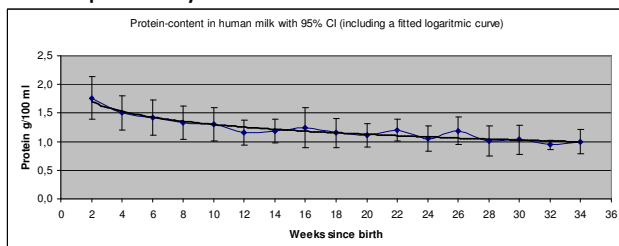


Figure 13. Mean protein-content in human milk from mothers who delivered prematurely



PART 5. EATING HABITS AND FEEDING PROBLEMS: MEALS, REGURGITATION, AND USE OF ANTI-CONSTIPATION MEDICINE
A total number of 769 questionnaires based on 286 (89% of study-cohort) infants were completed.

Main results on eating habits and possible feeding problems

Both breastfeeding groups (A and B) were fed more meals each day compared to formula fed groups (preterm and term formula). Results on number of meals each day are illustrated in Figure 14 and Table 13.

Many episodes of regurgitation were reported especially around 2 months CA with 74.0% among all very preterm infants. No significant difference was found on regurgitation comparing the breastfed groups. Results on regurgitation frequency each day is illustrated in Figure 15.

Infants fed formula (group C and mature formula (MF)) were more often treated with anti-constipation medicine compared with both breastfed groups (A and B). At term, group B received more anti-constipation medicine compared to group A. Results on use of anti-constipation medicine are illustrated in Figure 16 and Table 14.

Number of meals each day.

Questionnaires with information on "number of meals each day" (751/769=98%):

235 answered questions about meals each day in the period from week 34-37 PMA.

115 answered questions about meals each day in the period from week 38-44 PMA.

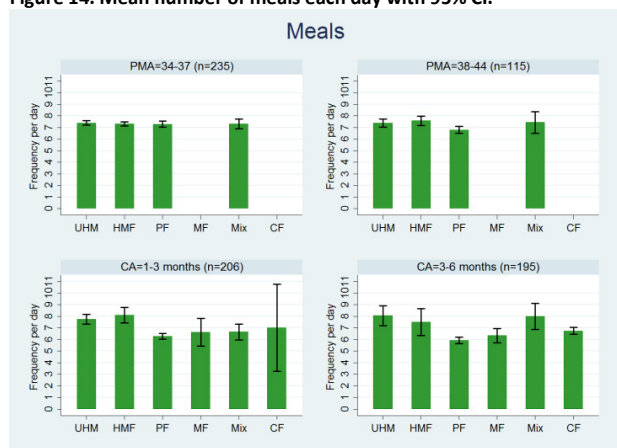
206 answered questions about meals each day in the period from 1-3 months CA.

195 answered questions about meals each day in the period from 3-6 months CA.

Table 13. Significant difference in number of meals each day comparing nutrition groups.

	Comparing groups	Coefficient	p-value	95% CI
34-37 weeks PMA	–	–	Ns	–
38-44 weeks PMA	UHM vs. PF	0.58	0.032	0.05-1.10
	HMF vs. PF	0.78	0.001	0.31-1.25
1-3 months CA	UHM vs. PF	1.47	0.000	0.96-1.98
	UHM vs. MF	1.11	0.024	0.15-2.07
	UHM vs. Mix	1.10	0.000	0.37-1.82
	HMF vs. PF	1.83	0.000	1.21-2.45
	HMF vs. MF	1.47	0.005	0.44-2.49
	HMF vs. Mix	1.45	0.001	0.64-2.26
3-6 months CA	UHM vs. PF	2.12	0.000	1.42-2.82
	UHM vs. MF	1.72	0.001	0.74-2.70
	UHM vs. CF	1.32	0.000	0.64-2.00
	HMF vs. PF	1.57	0.002	0.58-2.55

Figure 14. Mean number of meals each day with 95% CI.



UHM=unfortified human milk

HMF=human milk fortification added to mother's own milk

PF=preterm formula

MF=mature formula

Mix= both breast and bottle

CF=complementary feeding

Both breastfeeding groups (A=UHM and B=HMF) were fed more meals each day compared to the three formula groups (C=PF, MF, and Mix) and complementary-fed (CF) group.

Regurgitation among very preterm infants each day according to nutrition.

Questionnaires with information on "regurgitation each day" (730/769=95%)

228 answered questions about regurgitation each day in the 1.st period from week 34-37 PMA.

113 answered questions about regurgitation each day in the 2.nd period from week 38-44 PMA.

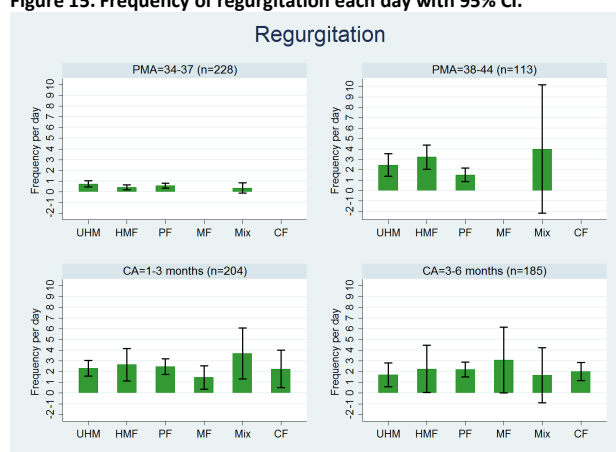
204 answered questions about regurgitation each day in the 3.rd period from 1-3 months CA.

185 answered questions about regurgitation each day in the 4.th period from 3-6 months CA.

No significant difference on regurgitation between the groups in the first period.

No significant difference between the breastfeeding groups in the second period, but the infants in the fortified group (HMF) had more regurgitation compared to the infants fed PF (p=0.034) (1.72 95% CI 3.30 – 0.14) but not compared to infants fed unfortified human milk (UHM). No significant difference was found on regurgitation comparing groups in the third or fourth period. Infants with reported regurgitation in the first period was 33.7%, in the second period was 65.5%, in the third period 74.0%, and in the fourth period was 62.2%.

Figure 15. Frequency of regurgitation each day with 95% CI.



Use of anti-constipation medicine.

Questionnaires with information on "use of anti-constipation medicine" (742/769=96%)

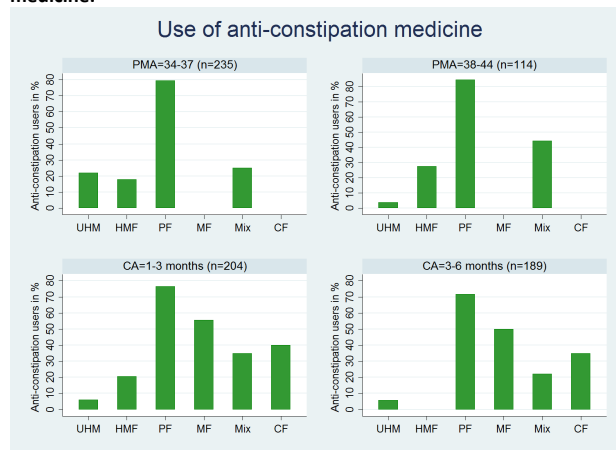
235 answered questions about medicine each day in the period from week 34-37 PMA.

114 answered questions about medicine each day in the period from week 38-44 PMA.

204 answered questions about medicine each day in the period from 1-3 months CA.

189 answered questions about medicine each day in the period from 3-6 months CA.

Figure 16. Percentage of very preterm infants using anti-constipation medicine.



38.7% of the infants received anti-constipation medicine in the period from 34-37 weeks.

43.0% of the infants received anti-constipation medicine in the period from 38-44 weeks.

45.1% of the infants received anti-constipation medicine in the period from 1-3 months CA.

42.9% of the infants received anti-constipation medicine in the period from 3-6 months CA.

Table 14. Significant difference in use of anti-constipation medicine comparing nutrition groups.

	Comparing groups	Coefficient	p-value	95% CI
34-37 weeks PMA	PF vs. UHM	0.57	0.000	0.44-0.71
	PF vs. HMF	0.62	0.000	0.48-0.75
38-44 weeks PMA	HMF vs. UHM	0.24	0.016	0.04-0.43
	PF vs. UHM	0.81	0.000	0.61-1.00
	Mix vs. UHM	0.41	0.007	0.11-0.70
	PF vs. HMF	0.57	0.000	0.40-0.74
1-3 months CA	PF vs. UHM	0.70	0.000	0.56-0.83
	MF vs. UHM	0.50	0.001	0.21-0.78
	Mix vs. UHM	0.29	0.005	0.09-0.49
	PF vs. HMF	0.56	0.000	0.39-0.72
	MF vs. HMF	0.35	0.025	0.04-0.65
3-6 months CA	PF vs. UHM	0.66	0.000	0.42-0.90
	MF vs. UHM	0.44	0.010	0.11-0.77
	CF vs. UHM	0.29	0.016	0.06-0.53
	PF vs. HMF	0.71	0.000	0.38-1.05
	MF vs. HMF	0.50	0.015	0.10-0.90
	CF vs. HMF	0.35	0.036	0.02-0.68

In general infants fed formula (C=PF and MF) were more often treated with anti-constipation medicine compared with both breastfed groups (A=UHM and B=HMF). At term, group B (HMF) received more anti-constipation medicine compared to group A (UHM).

8. GENERAL DISCUSSION

STUDY-DESIGN

The findings presented in this thesis originate from an intervention study on nutrition of very preterm infants after hospital discharge. The study includes a prospective and consecutive registration of very preterm infants from four neonatal units creating a population based birth cohort of very preterm infants followed until 1 year CA. Our study consisted of an observational part from birth to shortly before planned hospital discharge, followed by an interventional part. The intervention study was a randomized controlled trial with randomization shortly before hospital discharge in order to test if fortification of mother's own milk while breastfeeding was possible, and to test the effect of fortification of mother's own milk on growth among very preterm infants, compared to feeding solely mother's milk after hospital discharge. In our randomized trial, we compared a treatment group assigned to fortification of mother's milk with a control group not receiving fortification of mother's milk. A third nutrition group of infants fed a preterm formula was created without randomization. It is not possible to randomize to breastfeeding versus bottle-feeding. Therefore, based on the observational part of the study, we have characterized the breastfed compared to the bottle-fed infants and their mothers.

The primary aim of this study was to investigate the effect of human milk fortifier, added to mother's own milk while breastfeeding, on growth. The study was randomized but not blinded due to the lack of a placebo-product without influence on breastfeeding, nutrition, and growth.

The most important advantage of a randomized trial is that selection bias will be eliminated by balancing both known and unknown factors influencing the outcome of the treatment.

Of the 633 eligible infants in our study, 157 were excluded due to death or diseases influencing eating ability and/or growth. The excluded group consisted of the youngest (GA) and smallest (BW) (including SGA) very preterm infants. Among parents of 156 infants who refused to participate in the intervention study, the mothers were younger and more often bottle-feeding their infants at discharge. Among 320 infants in the intervention study

mothers of the bottle-fed infants more often had "multiple births", were more often smokers, and belonged to lower social groups compared to mothers of breastfed infants.

More girls and more multiple births were randomized to the fortification-group possibly influencing the outcome. Optimally randomization should have been done also according to e.g. gender and multiple versus single birth in order not to have groups with uneven distribution of gender and multiple birth infants. Meanwhile, as the study was carried out at 4 centers, it was considered impossible to include these two parameters in the randomization process.

In our study, 25% were excluded and parents of further 25% refused to participate. Other studies dealing with preterm infants have shown similar problems with exclusion and refusals. In a Canadian study, 36% were excluded and 37% refused to participate [63]. The parents often have experienced their infants critically ill and they can not manage also to participate in a study. In our study, dropouts / change of nutrition were as expected highest in the intervention-group (B) due to extra workload with expressing milk and fortification of mother's milk.

Breastfeeding should be provided as long as mutually desired by mother and infant / child according to ESPGHAN Commentary on Breastfeeding [64]. In our study, the intervention was planned until 4 months CA, but in both breastfed groups (A and B) breastfeeding stopped between 2 and 4 months CA in most cases, making intervention with fortification further on to be difficult. The "oldest" infants in the study were born with a GA of 32+0 weeks and at the age of 2 months CA their mothers (if nursing / breastfeeding) have been expressing milk or breastfeeding for 4 months already. Therefore, it is a challenge to maintain an increasing amount of breast milk for the preterm infant several months after birth and especially when nursing multiple births. Due to some "changes of nutrition" during the intervention period both ITT and PP analyses were made. Overall, participation and compliance was high in our intervention study, and with the above considerations in mind, we believe that our results can be transferred to other populations of very preterm infants. Care must be taken not to extrapolate the results of the RCT to patients excluded from the study. It would have been interesting to investigate the effect of fortification of human milk among the sick preterm infants who were excluded, but this was not the aim of the study.

GROWTH OF PRETERM INFANTS

It is a general problem to establish adequate growth in very preterm infants. It takes time to establish adequate dietary intakes in the immature infant, and infants often become malnourished during the initial hospitalization [5]. A common goal for optimal nutrition of very preterm infants is important, and accurate and reproducible outcome measures and references for growth (weight, length, and head circumference) are important for consensus, in order to discuss the extent of nutritional supply during and after hospitalization.

Assessment of growth

Provision of energy and nutrients at levels to support optimal growth and development is the goal of nutritional support, but to demonstrate growth pattern among VLBW infants, monitoring of growth is important. Body weight comprises the total mass of infant's lean tissue, fat, and extracellular and intracellular fluid

compartments. Weight gain or loss, therefore reflects changes in body-composition [22]. Body-weight can be measured more accurately and reproducibly than linear growth even though change in linear growth is generally regarded as the best measure of assessing adequacy of dietary intake [22;65]. It is difficult to measure total length of a sick preterm infant especially in the incubator. Therefore, measurement of knee-heel length has been advocated, but data suggests that these measurements are neither an accurate nor a more sensitive indicator of total linear growth in preterm infants [65]. In our study, the infants had their crown-heel length measured with a tape measure during hospitalization, while an "infant measuring rod" / a stadiometer was used at and beyond hospital discharge.

Anthropometric data on weight, length, and HC has been obtained during hospitalization for all infants in the study. Weight was measured several times every week while length and HC was registered less often. Especially the registration of HC at birth and length at the day of discharge were missing. The main aim of the study and this thesis was to investigate nutrition and growth after hospital discharge for which reason not all anthropometric data during hospitalization have been presented.

Growth references

In order to discuss optimal growth and catch up growth, a reference describing growth is needed. Growth references for preterm infants from birth, through discharge, term, and during the first year of life are few, making it difficult to describe "optimal" growth of very preterm infants. Growth references can be "descriptive", depicting how children actually grow or "prescriptive", describing how children should optimally grow [66]. Some preterm growth references (descriptive) are based on in-utero-measurements (ultrasound) [59] and some are based on measurements at birth [60]. Growth references used for preterm infants from 40 weeks PMA can be based on mature or preterm infants, breastfed or formula fed infants, infants from same part of the world or pooled data from different parts of the world, like the WHO growth references [67]. In our intervention study, we chose a descriptive reference based on measurements of weight, length, and head-circumference among Swedish infants from preterm and term birth to 24 months CA [60] as they probably reflect the growth of Danish preterm infants well. The integration of a term-born growth curve until 24 months could explain why Z-scores in our nutrition groups tended to decrease among most infants from 6 to 12 months CA. Another explanation could be that low birth weight infants as a group has been shown at greater risk to remain smaller than normal birth weight peers throughout the years of growth until young adulthood, with the extremely low birth weight infants at greatest risk [66]. A third explanation could be that the growth-pattern of both non-SGA and SGA preterm infants is different as compared to mature infants no matter the nutrition.

As different growth references vary in population and statistics, it is important to specify which growth reference has been used in a certain study. The new WHO growth references have been criticized because the references for both girls and boys are heavier as compared to the infant references used in the UK and by the US Centre for Disease Control and Prevention [68-70]. On the contrary, we found the WHO growth references to be a little lighter especially at term compared to the Swedish population-based reference.

Catch-up growth and small for age

In the past, catch-up growth has been advised, especially for SGA infants because early enhanced nutritional intake in VLBW infants, leading to catch-up, has shown to be associated with better long-term neurodevelopmental outcome [66;71]. However, because almost all preterm infants loose percentiles after birth, catch-up growth has been advised for basically all preterm infants [72].

To achieve the goal for catch-up growth, special formulas, taking the nutritional requirements of preterm infants into consideration, has been developed and evaluated in studies comparing preterm formula or post-discharge formula with a term formula and/or human milk [19;73-76]. These studies have demonstrated that a significant proportion of LBW infants, regardless of how they were fed post-discharge, did catch-up, although not completely. The advantage of nutrient enrichment though seems to appear early (within 1-2 months post-term), suggesting that there is a finite period during which catch-up in response to higher nutrient intakes is most likely [11]. In our study, the infants fed a preterm formula achieved catch-up at an earlier age compared to both breastfed groups. Non-SGA infants in our study seemed to have achieved catch-up on HC at discharge, on weight at 2 months CA, and on length at 4 months CA.

A study on growth among children born with a GA < 32 weeks found that SGA infants with rapid initial growth (during the first 3 months) already attained normal height for target height at 2 years of age while those with slow initial growth still showed persisting stunting at the age of 10 years [13]. SGA infants in our study had greater catch-up growth compared to non-SGA infants during the study-period, but with no significant difference comparing nutrition groups. All SGA infants showed rapid catch-up growth on HC until term, on weight until 4 months, and length-growth even continued until 1 year CA.

Both SGA and non-SGA infants in our study did achieve some catch-up growth during hospitalization but did not fully reach the chosen growth reference of the fetus / preterm infant with the same PMA before hospital discharge but seemed to be achieved before 1 year of age on all growth parameters. The group of infants with subnormal weight at discharge increased significantly more in weight when fed PF compared to both breastfed groups. The two sub-groups of infants with subnormal weight (at birth and/or at discharge) seemed to have a more rapid catch-up growth for a longer period compared to infants with "non-subnormal" weight, but the groups were however small, resulting in lower statistical power allowing no firm conclusion or recommendation.

NUTRITIONAL REQUIREMENTS AND RECOMMENDATIONS FOR PRETERM INFANTS

Inadequate nutrition has also been proven to increase the risk of neurodevelopment impairment and bone-disease among preterm infants [77-79] as well as growth failure. Suboptimal nutrition or even malnutrition has been supposed to affect structural and functional development of the nervous system in the preterm brain, possibly affecting long term development of neurological functions [80]. Preterm infants with "failure to thrive", especially SGA infants, had the lowest cognitive scores, significantly lower than both the non-SGA and SGA infants with normal postnatal growth [40;66;79].

In addition, inadequate nutrient intakes of calcium, phosphorus, and vitamin D, in combination with e.g. a prolonged period of

parental nutrition, increase the risk of reduction in bone mineral content known as “osteopenia of prematurity” [81]. Substrate supply is therefore very important in very preterm infants in order to improve body and organ growth in general and brain growth in particular. Early initiation with adequate amount of calories and amino-acids in e.g. parental nutrition is recommended for the very preterm infant [82;83]. Enteral feeding, particularly with breast milk, may be started within the first few days of life [6;84] and also timely increased. Human milk has many advantages and is the preferred feeding for term infants [64]. As a source of nutrients for preterm infants, human milk is however, not sufficient in the usual feeding volumes. Human milk therefore needs fortification with proteins and minerals in order to meet the needs of the growing preterm infant [6;85] and may not meet the nutritional requirements of the growing preterm infant after hospital discharge either. The protein-content in human milk from mothers who delivered prematurely has been shown to decrease significantly within weeks and months after birth [26]. Several weeks after birth, at the mean time of discharge \approx 37 weeks PMA, the protein-content of mother’s milk had decreased to a level equivalent to human milk 4-8 weeks after birth from mothers who delivered at term [26]. We found the same significant decrease in protein-content in mother’s milk, from 2-8 weeks after birth in milk samples from mothers in our study. At the time of discharge, usually 2-4 weeks before term, the protein-content in mother’s milk was low and did not meet the needs of a growing preterm infant.

In order to meet the optimal nutritional requirements of the growing preterm infant, several studies have been published and the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) has recently (2010) published recommendations on enteral nutrient supply for preterm infants up to a weight of approximately 1800g with e.g. recommended protein-levels, calcium-, and phosphorus-levels [83].

Preterm formulas have been developed to meet the nutritional needs for preterm infants.

To meet the recommendations when feeding human milk, fortification can be done by different methods, either a “standard fortification” using the same standard amount of fortification based on the assumption of a standard composition of human milk, or an “individualized fortification”. The latter can be done with “adjustable fortification” based on the infants metabolic response evaluated by BUN-levels, or a “targeted fortification” based on analysis of human milk [85-87]. These recommendations on fortification of human milk are made for and are very useful for tube-fed infants during hospitalization, but have not yet been tried or verified as useful for breastfed very preterm infants when practicing breastfeeding directly from the breast after hospital discharge.

We found that formula fed infants increased more in growth compared to breastfed infants in weight Z-score from birth to discharge (in part 1 on the exact day of discharge, and in part 2 at day 252 = 36 weeks PMA. This can be explained by: 1. Breastfed infants losing weight during the period practicing breastfeeding, since they are changed to be fed on demand and not on schedule. 2. Formula fed infants received constantly all the nutrients they need because they did not have to practice breastfeeding. 3. The breastfed infants supplemented with HMF did not receive enough fortification during hospitalization – and especially not during the breastfeeding establishing period.

A comparison of compositions of mother’s milk and products for feeding preterm infants during hospitalization and after hospital discharge is shown in appendix 1. The “Premature Formula” used in our trial has almost the same composition as the two different “Discharge Formulas” for preterm infants with more energy, protein, calcium, and phosphorus compared to both human milk 8 weeks after preterm birth and a term formula. Adding only 5 packets of HMF to mother’s milk does not provide as much energy and protein as the formulas. This probably explains some of the differences on growth in the RCT with increased growth in the formula group compared to both breastfed groups.

BREASTFEEDING PRETERM INFANTS AT DISCHARGE

In our prospective observational part of the study, 60% of 478 very preterm infants were exclusively breastfed at discharge and the corresponding number in the intervention study was 65% of 320 infants. All but one of the infants fed both mother’s milk and formula at discharge refused to participate in the RCT. According to the literature, breastfeeding rates of preterm infants are reported to vary considerably. The definition of breastfeeding, also vary in the literature, with breastfeeding defined as “exclusively breastfeeding” or “breastfeeding including supplementation with a formula” (mixed feeding). A study from 2010 [88] describes variations in breastfeeding-rates in Europe with very low rates of exclusively breastfeeding and higher rates of mixed feeding. The highest breastfeeding rate was in the UK with 29% exclusively breastfeeding and 6% mixed feeding making 65% to be formula fed [88]. In a study from 2002 with 119 mothers of single birth VLBW infants, it was found that 73% intended to breastfeed but only 34% (25% of total cohort) continued lactation beyond 40 weeks PMA. Mothers who continued breastfeeding were older, more often married, and had a higher level of education compared to those who discontinued lactation. Significant factors influencing lactation beyond 40 weeks PMA included start of milk expression before 6 hours post-delivery, expressing milk more than 5 times a day, and kangaroo care [89]. An Italian study from 2008 found the highest probability of initiating and maintaining breastfeeding among infants with mothers aged between 27 and 34 years, with high educational levels, non-smokers, and with previous breastfeeding experience [90].

Very few studies describe the duration of breastfeeding among term and preterm infants. In our observational study and in the RCT, 60% and 65% of the infants were directly breastfed at discharge respectively. At the time of discharge, the infants had received human milk for an average of 8 weeks. In comparison, a Scandinavian study from 1996 found a breastfeeding rate among term infants 6 weeks post partum to be 77% [91].

Previous studies has shown a negative association of maternal smoking on breastfeeding initiation and duration [92-94]. In our study, with 19% of the mothers of very preterm infants being smokers, we found a negative correlation between maternal smoking and breastfeeding. Mothers in low social groups, were also negatively correlated to breastfeeding at discharge. Smoking and mothers in low social groups were strongly correlated, but both variables were significant in the multivariate logistic regression model. The number of smoking mothers apparently has decreased during the past 2 decades in Denmark. A study from 1996 on term infants found 20% smoking mothers [91], and another study from 2006 found 26% smoking mothers during preg-

nancy [94], while a study from 1991 reported 38% mothers smoking in pregnancy and 42% smoking during lactation period [95]. It has been shown that mothers perceived that a strong risk of harming the baby was posed by smoking while breastfeeding and that they received little encouragement to continue breastfeeding because of inability to stop smoking [96]. This indicates a need for more consistency in promoting breastfeeding in spite of smoking post partum.

Studies have shown positive associations between breastfeeding and cognitive development also within the low birth weight group [20;21;97]. Therefore, breastfeeding is also recommended for the low birth weight infants.

NUTRIENT ENRICHMENT OF MOTHER'S MILK FOR PRETERM INFANTS AFTER HOSPITAL DISCHARGE

To our knowledge, only one other group has studied fortification of breast milk for very preterm infants after discharge. A Canadian pilot-study followed growth until 1 year of age of human milk fed very preterm infants who were randomized to fortification of expressed mother's milk (half of their feedings) or not for 12 weeks after hospital discharge [63;98]. The infants that received fortification remained longer and had a greater whole-body mineral content (both significant) until 12 months of age [98]. The infants in the Canadian study were not direct breastfed and were fed a higher amount of fortification (supplied roughly half the volume of human milk as nutrient enriched feedings (4 packets / 100 ml)) compared to the infants in our study. We chose to supplement with only 5 packets of fortifier each day (~1 packet / 100 ml) because of the risk of interfering with breastfeeding if using higher amounts. The duration of breastfeeding was not influenced by fortification of human milk in these amounts, showing that this fortification was practically possible, but we do not know if it would be so with higher amounts of fortification.

In our study, infants fed PF achieved weight and length catch-up growth earlier compared to infants fed mother's milk with or without fortification, probably due to feeding more protein as shown by elevated BUN-levels indicating elevated protein-metabolism. BUN-values has previously been shown as indicators of protein-metabolism [99] and even used as a method to fortify mother's milk for infants during hospitalization (adjustable fortification) [86]. Agreement on optimal BUN-values for optimal growth among very preterm infants has not yet been achieved. Hall has recommended a BUN-value to be > 0.8 mmol/l (> 5 mg/dl) among preterm infants post-discharge, otherwise intervention is suggested [100]. The PF fed infants in our study show BUN-values almost identical with PF-fed infants in a similar study on post-discharge nutrition comparing formulas for preterm infants [76]. The difference between infants fed PF and breast milk could possibly be explained by feeding an increasing amount of formula / day (and thereby protein), while the breastfed infants received the same amount of fortifier and maybe even the same amount of human milk each day during the intervention period. The latter suggestion is based on studies on mature breastfed infants that has shown an increase in milk volume until 1 month CA, while after 1 month the mean day-to-day variability within subjects is fairly constant, but between subjects the volume differs between 600 and 900 ml [101;102]. The exact volume intake within our three nutrition groups was not registered. Compared to infants fed unfortified mother's milk, those who completed fortification until 4 months CA had higher s-phosphorus-

and BUN-values. Though this was not associated with increased weight gain, it may reflect a better growth potential.

Preterm girls seemed to benefit from fortification of mother's milk after hospital discharge compared to girls fed solely mother's milk. Fortification in the amount given in our study did not affect growth significantly at 1 year CA for both genders. Fortification though improved weight at 2-6 months CA and HC at term - 4 months CA (ITT) and length- and HC-growth at 2 - 4 months CA (PP) significantly among girls using REM. Studies based on body composition of preterm infants have described boys to be programmed to grow faster and accrete more lean mass compared to girls. At 12 months CA boys were greater on lean mass, bone area, and bone mineral mass compared to girls [103]. It has been suggested that preterm boys fed a high amount of protein seemed to benefit more from this diet [104]. Looking at data on growth between 12 weeks and 18 months using standardized Z-scores, the same author (as the two previous mentioned studies) found that catch-up on length was apparent in preterm girls and not boys, suggesting that dietary intake during this period more adequately met the needs of girls than of boys [18]. Our results may reflect that boys and girls are programmed differently according to growth and may have different nutritional needs, though a type 1 error should always be considered as a possibility. Further studies on growth according to gender differences are needed.

METABOLIC SYNDROME – IS THERE A RISK?

Catch up growth may however, not be the only goal for feeding strategies of feeding preterm infants. Enhanced nutrition may increase the risk of metabolic and cardiovascular disease later in life [50;51;105]. The definition of "the metabolic syndrome" and the metabolic abnormalities possibly affecting the development of this syndrome with increased risk of diabetes and cardiovascular disease is though not standardized in the literature [106;107]. The mechanism(s) tying together anthropometric, physiological, and biochemical abnormalities are not completely understood and studies have to be carefully read and interpreted. Based on epidemiological evidence and among predominantly term born populations, it has been hypothesized that the adaptations in the fetus due to e.g. undernourishment induce alterations in metabolism, hormonal output, and cardiac output, which result in central obesity, diabetes, and cardiovascular disease in middle age. Subjects with rapid catch-up growth are hypothesized at greatest risk for these consequences [53;108]. However, as described, catch-up growth among preterm infants also have beneficial effects. Rapid growth of preterm infants during infancy, especially among those who failed to grow adequately in-utero or during the neonatal period, is regarded as a sign of good health and the resolution of chronic complications of prematurity. Especially catch-up of head size is associated with beneficial effects on cognitive development [65]. This is however, a great dilemma in nutrition of preterm infants, with VLBW infant's demonstrating atypical low weight gain in the early years of life having a higher probability of less than optimal cognitive development over time, while those with excessive weight gain have a greater likelihood of later childhood and adult obesity, cardiovascular disease, and diabetes [66].

The protective effects of breastfeeding of mature infants regarding cardiovascular disease may be explained, at least partly, by the lower rates of weight gain, which may be related to differences in substrate intakes [109]. Our study showed the same

growth pattern with lower rates of weight- and length-gain (PP and ITT) among breastfed compared to PF fed infants. SGA infants though showed no significant difference on growth comparing nutrition groups while they showed more rapid catch-up on growth during the entire study-period compared to non-SGA infants.

It is still unknown if there is a risk for preterm infants of developing metabolic syndrome secondary to metabolic / nutritional events early in life (programming). This risk has been described small compared with the contribution from other risk factors, such as parental size, weight as an adolescent, and various life-style factors such as physical activity [110;111].

The balance between optimized growth and neurological outcomes without sacrificing metabolic and cardiovascular health among preterm infants still needs to be investigated. One study have examined preterm infants and reference infants at 35 and 36 weeks GA using DEXA, and found preterm infants to have a reduced fat-free mass and an increased global and central fat mass, that has been suggested to increase the risk of the metabolic syndrome [112]. In a further follow-up, our infants will be tested on neurological development and growth including body-composition measured using Dual Energy X-ray Absorptiometry (DEXA).

We also wanted to measure HbA1C at 4 months CA and blood pressure among very preterm infants during the study-period and to correlate these measures to later outcomes. Measuring HbA1C though turned out to be difficult due to unexpected constantly elevated levels of HbF at 4 months CA among preterm infants. Results are shown in appendix 5 (have been presented as a poster at The EAP-meeting in 2008). The method for measuring blood pressure without standardized equipment and procedures for VLBW infants turned out to be unreliable and evaluated to be invalid. The results are shown in appendix 6. Blood pressure will be measured in the follow-up study at 6 years of age with standardized equipment and procedures.

ALLERGIC SYMPTOMS AND FEEDING PROBLEMS

Allergic symptoms among infants in their first year of life are often unspecific and only seldom represent a true allergic disease. The most common allergic symptoms are atopic dermatitis (AD) and recurrent wheezing (RW). When these conditions are caused by allergy, it will most often be a food allergy e.g. to milk or egg in this age group. We found an overall incidence of allergic symptoms among infants within 12 months CA to be 56.2% while the prevalence at 1 year CA was 41.8%. Predisposition to allergic disease and being a boy increased the risk of developing AD and RW within the first 12 months CA. Information on predisposition was obtained by interviews at the time of randomization. Parents of 3 infants chose not to participate in the RCT due to severe atopic predisposition. These infants were all breastfed and because they were assigned to solely breastfeeding without randomization they can possibly bias the results on allergic disease. A larger number of infants participating without randomization could possibly lead to "reverse causality" with predisposed infants in one of the nutrition groups leading to an incorrect conclusion on nutrition and allergic diseases.

Nutrition with or without CMP after hospital discharge was not associated with the development of any allergic symptoms.

Studies describing allergic symptoms and studies on dietary prevention among preterm infants are very few. By the age of 18

months CA, Lucas et al found an overall incidence of one or more allergic symptoms among 777 preterm infants (BW <1850g) to be 44% (wheezing 22.5% and eczema / AD 19.4%). They found atopic predisposition, maternal smoking, vaginal delivery, and duration of ventilation to be associated with wheezing, and multiple births to be associated with atopic dermatitis [113]. The same study-population was used for two randomized trials (A: banked donor milk vs. preterm formula and B: term vs. preterm formula) and it was found that CMP-based formula was not associated with an overall increased risk of developing allergic diseases, but in the subgroup with atopic predisposition, early exposure to CMP increased the risk of developing allergic diseases – especially AD and CMPA [114].

The allergic symptoms urticaria, rhinitis, and conjunctivitis are unspecific and difficult to interpret in this age group, also among preterm infants. In two children suspected of these symptoms no sensitization was found by SPT and specific IgE-analysis.

The main allergic symptoms reported in our study are RW and AD. Our results are based on standardized questionnaire based interviews performed by the doctors and/or nurses when the infants attended the outpatient clinics for follow-up examination at 4 and 12 months CA.

Recurrent wheezing

Chronic or recurrent respiratory morbidity has been described as common following preterm birth, particular if complicated by broncho-pulmonary dysplasia (BPD) [115;116]. In our study, infants with BPD have been excluded from the RCT. A recent, but solely register- and population-based study on "administrative claims data", found that preterm infants (born ≤32 weeks) had a higher prevalence of persisting asthma later in childhood (11.7%) compared with term births (8%) (OR 1.51 (95% CI 1.40-1.63)) [117]. A study from New Zealand based on parental reports at 12 months of age found an incidence of RW among very preterm infants (<33 weeks) to be 14.5% and among term infants to be 3%. They found significant risk factors to be parental history of asthma, maternal smoking, siblings at home, neonatal oxygen supplementation at 28, 36, and 40 weeks of gestation [118]. Another study found risk factors for developing asthma to be chronic lung disease, neonatal mechanical ventilation, corticosteroids, and a higher childhood body mass index, while being a SGA girl and septic post birth to be protective factors among former preterm infants at 8 years of age [119]. A Swedish birth cohort study (on preterm and term infants) found a cumulative incidence on recurrent wheezing (doctors diagnosed or any wheezing) to be 27% up to two years of age [120]. The incidence and prevalence of RW up to one year of age among "healthy very preterm infants" were 39.2% and 32.7% respectively in our study, indicating that preterm birth might increase the risk of RW even though preterm infants with BPD were excluded from our study. It is well recognized that in infants most cases of "acute expiratory wheeze" is associated with viral infections [121]. In our study, we did not routinely register concurrent viral infections as a possible cause of wheezing episodes, but it was probably a major cause of RW, and preterm infants may have an increased risk of RW due to viral infections and prematurity in combination. Parental smoking is also known as a risk factor of developing RW in infancy and early childhood [122]. In our study, parental smoking was though not significantly associated with an increased risk

of developing RW. Later follow-up must reveal risk factors and the risk of bronchial asthma in this population.

Atopic dermatitis

A population based birth cohort study among term infants in Denmark found an incidence of AD to be 11% [123] and the cumulative 1-year prevalence to be 8.2% [124]. An association between AD and being a boy and predisposed to atopy (maternal) was found [123]. In a large Norwegian prospective cohort study, based on clinical examinations if parents reported allergic symptoms, was found a prevalence of AD at 1 year of life to be 13% among preterm infants (GA 28-35 weeks) and 10% among term infants [125]. A much higher prevalence of AD at 12 months post-term of 35.8% was found in a British prospective study among preterm infants (GA \leq 37+0), but in that study the diagnosis was based on questionnaires and home-visits by midwives only [126]. Our study is also a population based birth cohort of preterm infants, and the incidence and prevalence of AD at 1 year in our study was 18% and 12.1% respectively, and seems to correlate very well with the results of the Norwegian study.

Feeding problems and gastrointestinal symptoms

In our study most of the gastrointestinal symptoms were reported early and mainly during the intervention period. Regurgitation of gastric content is common in preterm infants due to relatively large milk intake, horizontal posture, and immature lower sphincter function, so gastro-oesophageal reflux is a common symptom in very preterm infants and has been shown in some cases to trigger apnoea [127]. Gastroesophageal reflux has been described as a clinical symptom responding to anti-reflux treatment [128]. The infants in our study were not investigated by gastric pH-measurements and possible symptoms during hospitalization were not recorded, but at randomization, at term, and at 2 and 4 months CA registration of regurgitation was obtained. Supplementing with a cow's milk based protein fortifier to mother's own milk did not increase regurgitation compared to solely human milk fed infants in our study.

One study on primarily preterm infants and infant stools (the "Amsterdam" stool form scale) has described the amount of stool produced by formula fed infants to be significantly larger compared to breastfed infants [129]. In our study, we did not look at stools but registered the use of anti-constipation medicine, and preterm formula fed infants did receive anti-constipation medicine significantly more often during the entire study-period compared to both breastfed groups, while infants supplemented with HMF were treated with anti-constipation medicine more often compared to exclusively breastfed infants during a period around term PMA. Overall, we could not find any significant feeding problems comparing fortification of mother's milk with solely mother's milk.

In our study, gastrointestinal symptoms were reported with an incidence of 13.4% with a prevalence of 1.4% at 1 year of age. Symptoms from the gastrointestinal tract are unspecific and are rarely due to allergic reactions. A Norwegian study [130] among preterm and term infants diagnosed 27 (9 preterm) of 555 with adverse reactions to cow's milk such as pain behavior, gastrointestinal symptoms (excessive vomiting or diarrhea), respiratory symptoms, and AD. Twenty six of these infants were diagnosed by elimination/challenge tests (all but one was exclusively breast-

fed). Non-IgE-mediated reactions were the most frequent and only one child with AD as the presenting symptom had a positive SPT as well as elevated IgE-level to cow's milk. At 1 year of age 13 were tolerant to cow's milk. No difference was found between preterm and term infants. Adverse reactions to cow's milk (especially gastrointestinal symptoms) can be difficult to interpret and are possibly over diagnosed. In our study, 38 of 283 reported gastrointestinal symptoms, but none of these infants were suspected to have CMPA and did not have an elimination/challenge test performed.

Sensitization and development of allergic disease

The development and phenotypic expression of allergic disease depends on a complex interaction between genetic and several environmental exposure to food and inhalant allergens, and non-specific adjuvant factors (e.g. tobacco smoke, air pollution, and infections) [43]. Sensitization is an IgE response to a foreign antigen (allergen) as measured by in vitro IgE determination or SPT. At 4 months CA only 51% infants in our study had blood drawn for specific IgE-analysis, because many parents did not want their infant to experience a veno-puncture. A Norwegian study found one child with elevated IgE-levels to both egg and milk, and one child with only elevated IgE-levels to egg [130]. In our study, one child was suspicious of CMPA due to AD and a positive SPT, but CMPA was not confirmed by elimination/challenge test, and further two infants (2/163) had positive IgE-reactions to cow's milk but none of them were suspected to have CMPA.

Breastfeeding possibly influences the occurrence of asthma and allergic disease through different mechanisms. The infant gut is immature, and may poorly eliminate multiple allergens or large quantities of allergens that can react with the immune system. Human milk contains substances that may influence the micro flora in the gut and possibly protect the intestinal mucosa. Cytokines / growth factors in human milk may also promote the maturation of gastrointestinal mucosa restricting the penetration of antigenic material and contribute to the anti-inflammatory effect of human milk [131]. Especially preterm infants with an even more immature intestine have been suggested to have an increased risk of absorption of food allergens and development of food allergy (such as CMPA). In the literature, 3 cases of preterm infants, who presented signs suggestive of sensitization to CMP after supplementation of their mother's milk with a human milk fortifier, have been reported. The diagnoses were based on clinical response to elimination of the allergen from the diet only [132]. In our study, 321 very preterm infants received a cow's milk based human milk fortifier during hospitalization, and only one infant was suspected to have CMPA after hospital discharge while he was fed a cow's milk based preterm formula but the diagnosis was not confirmed by elimination challenge test. Human milk, only supplemented with a small amount of HMF, might be protective to the immature gut. Another protective factor might be that the very preterm infants in our study received solely human milk for at least 10 days before HMF was started. Multicomponent fortification of human milk for preterm infants is associated with improvements in growth, and support the use of fortification as a common practice in neonatal intensive care units [133;134]. In our study, fortification was used also after hospital discharge and was not associated with an increased risk of developing allergic diseases among very preterm infants during the first year of life.

9. CONCLUSIONS AND FUTURE PERSPECTIVES

Although still controversial, the goal for nutrition of the preterm infant could be to supply nutrients to achieve the rate of growth and a body-composition, which would equal that of a normal fetus of the same postmenstrual age, and from term to equal the breastfed term infant of the same corrected age, and through the first year of life.

In very preterm infants, breastfeeding is recommended, but supplementation of mother's milk with protein is necessary in order to achieve optimal growth during hospitalization. After hospital discharge, optimal nutrition and supplementation is still unknown and for discussion.

We found that breastfeeding could be established in 60% of the "healthy" very preterm infants at discharge. However, mothers belonging to lower social groups and mothers who are smokers are less often breastfeeding their infants. An active nutrition- and feeding-policy during hospitalization is necessary in order to establish breastfeeding with a special attend on multiple births, infants with low weight for age, mothers of lower social groups, and smokers who want to breastfeed their very preterm infant(s) at and beyond hospital discharge.

Fortification of mother's milk, while exclusively breastfeeding after hospital discharge, was demonstrated to be possible and did not interfere with the duration of breastfeeding in our study. Preterm girls seemed to benefit temporarily from nutritional supply with fortification of mother's milk after hospital discharge compared to girls fed solely mother's milk. Fortification in the amount given in this study did not affect growth significantly at 1 year of age.

In our study, catch-up tended to be achieved early in HC (between discharge and term) and weight (between 2 and 4 months CA) but later in length (between 4 to 12 months CA) indicating a period after term for catch-up growth on weight and length. SGA infants showed greater catch-up growth compared to non-SGA infants during the study-period, but no significant difference on growth comparing nutrition groups. All SGA infants showed catch-up growth especially on weight until 4 months after term, and length growth even seemed to continue until 1 year CA.

Feeding problems such as regurgitation due to fortification of mother's milk were not increased compared to exclusively breastfed infants. Treatment with anti-constipation medicine was primarily seen among preterm formula fed infants compared to both breastfeeding groups during the intervention period.

Cow's milk based fortification or preterm formula for preterm infants after hospital discharge did not increase the risk of developing allergic symptoms in this population. Allergy to cow's milk protein was not found among any of the infants that received a cow's milk based human milk fortifier during and after hospitalization. It may be of importance that HMF was not introduced until day 10-14, and that all the infants were fed only human milk until then. Predisposition to allergic disease and being a boy increased the risk of developing both atopic dermatitis and recurrent wheezing. Compared to other studies of preterm infants a high incidence and prevalence of recurrent wheezing were found, while atopic dermatitis was found to have an incidence and prevalence comparable to other studies.

Much is still unknown with regard to nutrition of preterm infants. Recent studies have shown hormones in human milk to be involved in energy balance regulation, possibly having a role in the regulation of growth and development in the neonatal period and in infancy. The long term consequence of these hormones (e.g. leptin, ghrelin, adiponectin, resistin, and obestatin) among both term and preterm infants especially on the development of the metabolic syndrome is unknown and need further investigation [135-137]. Another growing research area is the regulation of the growth hormone (GH), insulin growth factor-1 (IGF1), and insulin which is different in fetal life compared to childhood and adulthood, and might be influenced and regulated differently in low birth weight infants with a possible association to long-term endocrine programming effects [138;139].

The content of protein in human milk is declining within weeks after birth, and the amount of protein will be inadequate in order to meet the nutritional needs of the growing preterm infant. Multi-component cow's milk based fortification of human milk for preterm infants is associated with improvements in growth, which supports the use of fortification as a common practice in neonatal intensive care units [133;134]. A human-milk based fortifier has in one study been shown to reduce the risk of necrotizing enterocolitis among preterm infants [140], but similar studies and studies on long term health effects - such as reducing the possible risk factors like cow's milk protein allergy also needs to be evaluated.

Breastfeeding preterm infants instead of feeding a term formula should be promoted in all countries. After hospital discharge, a larger amount of fortifier added to mother's milk (fresh or defrosted) may be possible while breastfeeding and should be considered if supplementation with extra protein is needed. In countries where human milk fortifiers are not available, breastfeeding can be supplemented with a nutrient enriched formula, if available, in order to better meet the nutritional needs of the growing preterm infant, but studies on the effect of such a feeding strategy have still not been performed.

Close monitoring of growth during hospitalization and after hospital discharge is important in order to identify the infants with subnormal weight for age, infants that might be at risk of neuro-development impairment and bone-diseases. If catch-up growth on length and head circumference has been achieved before hospital discharge, supplementation to improve growth might not be necessary, though it is still unknown if supplementation after discharge improves neuro-developmental outcome. Both "healthy" and "sick" very preterm infants, who have not achieved catch-up on length and head circumference at discharge, should be supplemented with a fortifier added to mother's milk or a nutrient enriched (preterm or post discharge) formula after hospital discharge in order to improve growth and neuro-developmental outcome. A gradual return to normal for all growth variables while avoiding excessive weight gain should be the goal for nutrition of very preterm infants during and after hospitalization.

Later follow-up of the infants in our study (at 6 years of age) has been planned and will show whether fortification of human milk after hospital discharge improves neuro-developmental outcome or has other health effects such as early signs of metabolic syndrome and possible allergic diseases among the very preterm infants in our study.

SUMMARY

The aims of this Ph.D. thesis were: 1. Primarily to investigate the effect, of adding human milk fortifier to mother's milk while breastfeeding very preterm infants after hospital discharge, on growth until 1 year corrected age (CA) 2. Secondly to describe breastfeeding rate and factors associated with breastfeeding among very preterm infants at hospital discharge. 3. To describe possible feeding-problems during the intervention-period, and allergic diseases during the first year of life, among very preterm infants related to their nutrition after hospital discharge. 4. To describe the content of macronutrients in human milk from mothers delivering very preterm.

This Ph.D. thesis is based on a prospective, randomized, and controlled interventional birth cohort study. A total of 633 very preterm infants with a gestational age (GA) $\leq 32+0$ weeks were recruited consecutively from July 2004 until August 2008 of whom 157 were excluded due to diseases or circumstances influencing nutrition. Further 156 refused participation in the interventional part of the study, but data on breastfeeding, weight, and some epidemiological data until discharge were available. Results on breastfeeding rate at discharge were therefore based on data from 478 infants, and parents of 320 infants accepted participation in the intervention study. Of these 320 infants, 207 were exclusively breastfed and they were shortly before hospital discharge randomized to either breastfeeding without (group A) or with fortification (group B) until 4 months CA. Infants (n=113) who were bottle-fed at discharge (group C) were given a preterm formula (PF) until 4 months CA. Infants were examined at the outpatient clinics at term, and at 2, 4, 6, and 12 months CA, where parameters on growth, allergic diseases, possible feeding problems, blood-samples, and milk samples were obtained. Data on duration of exclusively breastfeeding and time of introduction to formula and/or complementary food were also recorded.

Among the 478 infants 60% (n=285) were exclusively breastfed, 35% (n=167) were exclusively bottle-fed, and 5% (n=26) were both breast- and bottle-fed at discharge. Compared to mothers in lower social groups and mothers who smoked, mothers in higher social groups and "non-smokers" were significantly ($p=0.000$ and $p=0.003$ respectively) more often breastfeeding their very preterm infants at discharge. Single birth infants tended more often to be breastfed ($p=0.09$). Infant age at discharge and duration of hospitalization did not influence breastfeeding at discharge. Increase in weight Z-score from birth to discharge was largest in the bottle-fed group compared to the breastfed group ($p=0.000$), probably due to feeding practice the last week(s) of hospitalization.

In the intervention study, 207 exclusively breastfed very preterm infants were randomized to group A (n=102) and B (n=105) respectively. The duration of breastfeeding was not influenced by fortification of mother's milk after hospital discharge. There was no significant difference on growth comparing group A and B at 12 months CA. Both boys and girls in group C achieved catch-up in weight and length earlier as compared to group A and B. Per protocol (PP) analysis showed that girls, but not boys, were longer and had a larger head circumference but were not heavier in group B (n=51) compared to group A (n=73) at 2 and 4 months CA ($p<0.05$). Protein-concentration in mothers' milk declined significantly from 2 weeks (1.8 g/100 ml) to 6 weeks after birth (1.4 g/100 ml) and declined further to 1.2 g/100 ml 12 weeks after birth.

The incidence and the prevalence at 12 months CA of recurrent wheezing was 39.2% and 32.7% respectively, while atopic dermatitis was 18.0% and 12.1% respectively. Predisposition to allergic disease increased the risk of developing atopic dermatitis ($p=0.04$) (OR 2.6 (95% CI 1.0 – 6.4)), and the risk of developing recurrent wheezing ($p=0.02$) (OR 2.7 (95% CI 1.2 – 6.3)). Boys had an increased risk of developing recurrent wheezing ($p=0.003$) (OR 3.1 (95% CI 1.5 – 6.5)).

In conclusion breastfeeding can successfully be established in very preterm infants. Fortification of human milk after hospital discharge while breastfeeding was possible without influencing the duration of breastfeeding. Fortification in the amount given in this study did, however, not affect growth significantly at 1 year of age. An increased amount of protein was correlated with increased BUN-values indicating a better growth potential. Fortification of mother's milk or preterm formula was not associated with an increased risk of developing allergic diseases. Future follow-up of this cohort investigating e.g. growth, allergic diseases, and neuropsychological development is planned at 6 years of age. The definition of optimal growth and nutrition of preterm infants is though still a question for debate and further investigations are needed.

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