Lipids, carbohydrates and micronutrients in the perinatal period. Long chain polyunsaturated fatty acids, myoinositol, vitamin E and polyamines in neonatal nutrition.

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Summary

The Introduction reviews human breast anatomy, biochemistry and physiology of human lactation, and human milk composition with emphasis on lipids and fatty acids (**chapters 1.1 and 1.2**). Present knowledge on perinatal fatty acid transport, parameters of essential fatty acid (EFA) status and the role of long chain polyunsaturated fatty acids (LCPUFA; $\geq C_{20}$) in perinatal growth and development are discussed in the third part of the Introduction (**chapter 1.3**). The scope of the thesis was to contribute to various nutritional aspects in the perinatal period, with emphasis on human milk composition, influence of maternal diet and disease on human milk composition, and EFA and myoinositol in the perinatal period.

Human lactation (**chapter 1.1**) comprises three major processes, i.e. 1) lactogenesis (onset of milk synthesis and secretion), 2) milk ejection and 3) galactopoeisis (continued milk secretion). The underlying mechanisms are hormonally (notably prolactin and oxytocin) and neurologically controlled. Human milk (**chapter 1.2**) volume and macronutrient composition are not constant entities. They are influenced by factors such as gestational age, maternal diet and duration of lactation. Lipids are the most variable milk constituents. They are the main energy source for the newborn, providing 40-50% of total energy. Fatty acids, esterified as triglycerides, are the major lipid components. Adults metabolize the (parent) EFA linoleic and α-linolenic acids by alternating chain-elongation and desaturation, to produce LCPUFA. LCPUFA are structural components of cellular membranes and serve as precursors for eicosanoid synthesis. LCPUFA play important roles in brain development, especially in the last trimester of gestation and first months after birth, when the brain growth spurt takes place. Fetus and newborn are unable to synthesis sufficient LCPUFA to cover their demands. LCPUFA should, therefore, be considered essential in the perinatal period. Low fetal LCPUFA status (chapter 1.3) is related to lower birth weight and duration of gestation, whereas low neonatal LCPUFA status is related to diminished visual acuity. LCPUFA status may be a factor in newborn growth, and cognitive and neurological developments.

Chapter 2 concentrates on lipid and carbohydrate contents of mature human milk as compared with current infant formulas (**2.1**), mature human milk fatty acid composition (**2.2**), and mature human milk polyamine concentrations (**2.3**).

Human milk is the best sole nourishment for term infants in their first months after birth. However, in circumstances where breastfeeding is not possible or undesired, human milk substitutes remain necessary. Current formulas for term infants contain concentrations of macronutrients and some important micronutrients comparable with mature human milk.

There are, however, still marked differences in triglyceride species and micronutrient composition. To establish to what extent current formulas for term infants differ in fatty acid composition, sterols and minor carbohydrates, we investigated their contents in 10 currently available formulas for term babies (**chapter 2.1**). Results were compared with data of 24 hour mature human milk from Dutch women, collected on postnatal days 14.4±3.5 (n=99), 42.1±2.7 (n=99) and 89.2±5.6 (n=25). Milk fatty acids and sterols were analyzed as their methyl esters and trimethylsilyl ethers, respectively, using capillary gas chromatography with flame ionization detection. Milk triglyceride concentration was calculated from its total fatty acid content. For analyses of lactose, monosaccharides and sugaralcohols, milk was deproteinized and further prepurified by solid phase extraction. Carbohydrates were determined as their trimethylsilyl ethers by capillary gas chromatography with flame ionization detection. Formula triglyceride and lactose concentrations were similar to those of human milk. Infant formulas and human milk did not differ in polyunsaturated fatty acid/saturated fatty acid (PUFA/SAFA; P/S) ratio. Formula medium fatty acid (MCFA; C_6-C_{14}) contents were generally higher (6 formulas), whereas LCPUFA were considerably lower or undetectable (all formulas). Formula cholesterol concentrations were 3-35 times lower, whereas phytosterol concentrations were much higher, compared with human milk. Formula glucose (n=7), sorbitol (n=5) and myoinositol (all) concentrations were generally lower, galactose was generally higher (n=9), whereas fucose and erythreitol concentrations were in the lower mean±2SD human milk range. It is concluded that formula and human milk contain similar concentrations of fat and lactose, but that they differ considerably in fatty acid composition and concentrations of sterols, monosaccharides and sugaralcohols. Literature data show that formula MCFA, either derived from coconut oil or semi synthetic medium chain triglycerides, do not augment newborn lipid absorption, probably because of their deviant positions at the triglyceride glycerol moieties. LCPUFA are considered essential in the perinatal period. Addition of LCPUFA to formulas improves LCPUFA status of formula-fed preterm and term infants. Milk cholesterol, phytosterol and P/S ratio influence newborn cholesterol homeostasis. Adequate cholesterol availability is important for newborn brain development. The cholesterol challenge hypothesis postulates that early adaptation to high dietary cholesterol intakes causes lower circulating cholesterol levels in adult life. High circulating myoinositol levels are associated with decreased incidence of bronchopulmonary dysplasia and retinopathy in preterm infants with respiratory distress syndrome. Augmentation of infant formula myoinositol concentration improves newborn myoinositol status. Biological consequences of differences in other minor carbohydrates are as yet unclear.

Chapter 2.2 describes a study on PUFA and LCPUFA in mature human milk. Since LCPUFA are considered essential in the perinatal period, LCPUFA addition to infant formulas, especially those for prematures, is advised. Human milk may serve as reference. Milk fatty acid composition is, however, dependent on maternal diet. We investigated (LC)PUFA balance in 24 hour milk from 99 lactating Dutch women, collected on postnatal days 14 (n=99), 42 (n=99) and 89 (n=25). To estimate the influence of

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maternal diet, food intakes were recorded on postnatal days 12-14, 40-42 and 87-89. The data revealed that linoleic acid (18:2ω6) comprised 85% of daily PUFA intake. Sixty-one percent of the women did not consume fish, resulting in a median (range) daily fish intake of 0 (0-56.8) g. Distributions of milk 20:3ω6 and 20:4ω6 were Gaussian, but those of 20:5ω3 and 22:6ω3 were right-skewed. Milk 20:5ω3 and 22:6ω3 were related. High milk 20:5ω3 (>0.1 g/100 g) and 22:6ω3 (>0.4/100 g) were related to recent fish consumption, rather than to milk 18:3ω3. Milk 20:3ω6 and 20:4ω6 were not related to milk 18:2ω6 either. Literature data show that high formula 18:3ω3/18:2ω6 combined with addition of 20:5ω3 and 22:6ω3 causes retarded growth, probably because of low infant 20:4ω6 status. However, milk from Inuit women and women following fish oil supplementation contains much higher 20:5ω3 and 22:6ω3 contents. The milk of these women may prevent low neonatal 20:4ω6 status due to low-normal 20:4ω6 contents and normal 18:3ω3/18:2ω6 ratios. We conclude that formula 18:3ω3/18:2ω6 and LCPUFA contents have to be balanced to prevent low 20:4ω6 status. From an evolutionary point of view, milk of mothers on diets with high 18:2ω6 and low LCPUFAω3 may not be the ideal reference for balanced addition of LCPUFA to formula.

In **chapter 2.3** we studied longitudinal changes in mature human milk polyamine concentrations. The polyamines putrescine, and especially spermidine and spermine, are essential for growth and development. They derive from *de novo* synthesis, from the diet and from the microbial flora in the gastrointestinal tract. It is suggested that dietary polyamines stimulate gastrointestinal tract proliferation and maturation in newborns. Polyamine concentrations in human milk and formula are highly variable. Putrescine concentrations are comparable, whereas spermidine and spermine concentrations are about 10 times lower in formulas compared with human milk. Twenty-four hour mature milk from Dutch women was collected on days 14 (n=98), 42 (n=97) and 89 (n=25) after delivery. Following a prepurification step to remove milk fat, polyamines were determined by capillary gas chromatography with nitrogen-phosphorous detection. In the observation period, milk putrescine concentration did not change, whereas concentrations of spermidine, spermine and total polyamines decreased. Because of incomplete milk collection, our estimated 24 hour total polyamine milk outputs may be too low. Estimated median newborn 24 hour total polyamine intake from mature milk corresponds with daily requirement for about 0.13-0.20 g mucosal tissue increment. This may be a significant amount, notably when human milk polyamines function as a supplement to local polyamine *de novo* synthesis. Putrescine, spermidine and spermine milk/plasma ratios are estimated to be 16-19, 14-24 and 44-75, respectively. Nutrients with high milk/plasma ratios are usually considered to fulfil biological functions in newborns. Milk polyamines may derive from active uptake from plasma and/or *de novo* synthesis in the mammary gland. Our data suggest that milk polyamines may have importance in newborn nutrition.

Chapter 3 describes the influence of maternal consumption of carbohydrate-rich diets on milk total lipid and phospholipid fatty acid composition (**3.1**) and the effect of maternal

vitamin E supplementation on milk vitamin E concentration (**3.2**). Milk from tightly controlled patients with diabetes mellitus was investigated in **chapter 3.3**.

Chapter 3.1 concentrates on the influence of maternal diets with different carbohydrate contents on incorporation of MCSAFA (6:0-14:0) into milk total lipids and phospholipid subclasses. Milk MCFA are *de novo* synthesized from glucose in the mammary gland. Women living in developing countries produce milk high in MCSAFA contents, due to consumption of traditional carbohydrate-rich foods. We determined fatty acid compositions of total lipids and isolated phospholipid subclasses from milk of women living in Dominica and Belize. The minor milk phospholipid subclasses were separated from the bulk of milk triglycerides by high performance liquid chromatography via a column-switching technique. The total lipid fraction of Dominican milk showed higher relative amounts of MCSAFA and 22:6ω3 and lower amounts of long chain saturated fatty acids (LCSAFA) and monounsaturated fatty acids (MUFA), compared with Belizian milk. Taking both Dominican and Belizian milk data together, there was a positive relationship between the MCSAFA content in total lipids and total phospholipids. Incorporation of MCSAFA in phospholipids occurred at the expense of LCSAFA, MUFA, PUFA and LCPUFA. Previous studies from Western countries revealed low amounts of MCSAFA and high amounts of PUFA and LCPUFA in milk phospholipids. Our data show that carbohydrate-rich diets give rise to incorporation of MCSAFA in phospholipids at the expense of PUFA and LCPUFA. Incorporation of MCFA in all milk phosholipid subclasses suggests that membranes of the lactating cell conserve their function, despite marked changes in fatty acid composition. An alternative explanation is that, in contrast to general believe, milk phospholipids are not derived from structural elements of the endoplasmatic reticulum, Golgi vesicle membrane and apical cell membrane.

Chapter 3.2 describes the short term effect of maternal supplementation with α-tocopherol on mature milk vitamin E concentrations. Since preterm infants are born with inadequate vitamin E stores it is suggested that α-tocopherol supplementation may be beneficial to both formula and human milk fed prematures. Superior α-tocopherol bioavailability from human milk, compared with infant formula, results in a more optimal vitamin E status in breastfed infants. We investigated the influence of a single dose of either 300 or 500 mg *dl*-α-tocopherol acetate on 24 hour vitamin E blood-to-milk transfer kinetics and milk recovery. The day before and after supplementation we collected mature milk from 6 lactating women. To study blood-to-milk α-tocopherol transport mechanism we additionally determined α-tocopherol in plasma, erythrocytes and platelets isolated from 2 lactating women and 4 non-lactating controls subjected to the same protocol. α-Tocopherol equivalents (α-TOC_{eq}), triglycerides (TG), cholesterol (CHOL) and linoleic acid (18:2ω6) were determined. Baseline plasma α-TOC_{eq} and α-TOC_{eq}/TG+CHOL showed, food intake-related, diurnal variation. Following supplementation, plasma α-TOC_{eq}/TG+CHOL showed a, dose-independent, biphasic curve. There were no apparent differences in plasma, erythrocyte and platelet vitamin E kinetics between lactating and non-lactating

subjects. Plasma α-TOC_{eq} did not show a dose-dependent relationship, whereas erythrocyte and platelet α-TOC_{eq}, and milk α-TOC_{eq}/TG and α-TOC_{eq}/18:2ω6 showed gradual, dose-dependent, increases. Twenty-four hour milk recoveries were 0.19 %, dose-independent, and highly variable. Mammary gland vitamin E uptake may comprise several mechanisms. Despite dietary intakes above recommendations for lactating women, presupplementation 24 hour α-TOC_{eq} outputs of 0/6 women and α-TOC_{eq}/18:2ω6 of 3/6 met recommendations for term newborns. In 24 hours following supplementation these criteria were met by 2/6 and 6/6, respectively. Recommended levels of α-TOC_{eq} outputs may only be reached in the second to third day following administration of a single oral dose and these levels can be maintained during continuing supplementation. The data emphasize the important intermediate role of adipose tissue in vitamin E distribution.

Insulin influences transmembrane fluxes of glucose and lipids, and activities of enzymes involved in fatty acid chain-elongation/desaturation. Alterations in milk fatty acid composition of women with insulin-dependent diabetes mellitus may, therefore, be expected. In **chapter 3.3** we investigated macro- and micronutrient compositions of milk from six patients with tightly controlled insulin-dependent diabetes mellitus. Median glycosylated hemoglobin at parturation was 5.2% (range: 4.9-5.3%; reference range: 4.9-6.6%) and 6.1% (range 5.0-6.3%, reference range 5.0-6.4%)] six weeks thereafter. The results were compared with data from five healthy controls. Milk samples were collected halfway through a single nursing at days 3-5 (colostrum), 7, 9, 10 (transitional milk), and 12, 15, 17, 21, 25, 29, 35 (mature milk). We found no abnormalities in macronutrient (triglycerides, lactose, protein), cholesterol, glucose, and myoinositol concentrations, nor in fatty acid composition. High milk glucose, as found in moderately and poorly controlled patients, may be a reflection of mean blood glucose and the magnitude of paracellular transport during the period of milk biosynthesis. From three longitudinally studied patients in our study two showed rather constant ratios between glucose concentrations in milk and capillary blood. The present data show that tight control of blood glucose concentrations prevents the increase of milk glucose concentrations noted during moderate control, and the multitude of milk abnormalities associated with poor control.

Chapter 4 describes studies on the origin of fetal LCPUFA (**4.1**) and parameters of postnatal LCPUFA status (**4.2**). Effects of addition of ribonucleotides to infant formula on newborn LCPUFA status (**4.3**), and different infant formula myoinositol concentrations on newborn myoinositol status (**4.4**) were investigated.

In the study described in **chapter 4.1** we concentrated on the origin of LCPUFA in the newborn. For this, fatty acid compositions of plasma cholesterol esters and triglycerides of 38 singleton deliveries (23-42 weeks), 3 twins (32, 39 and 40 weeks) and their mothers were investigated at birth. Very premature babies had high percentages free cholesterol in plasma, which points at low lecithin:cholesterol acyltransferase activity. LCPUFA were higher, and their precursors (18:2ω6 and 18:3ω3) lower than those in maternal cholesterol

esters and triglycerides. Trapping of LCPUFA by α-fetoprotein in the fetal circulation may be the main underlying cause. There were no gestational age dependent changes in maternal fatty acid compositions. Except for 22:6ω3, 20:2ω6 and 22:5ω6, all LCPUFA in fetal triglycerides increased with advancing gestation. Fetal triglyceride 22:6ω3/22:5ω3 ratio decreased, whereas 22:5ω6/22:4ω6 remained constant. Increasing triglyceride LCPUFA content with advancing gestation suggests partial derivation from Δ6- and Δ5-desaturase maturation in the liver. However, constancy of 22:6ω3 and 22:5ω6/22:4ω6 and decrease of 22:6ω3/22:5ω3 in triglycerides point at low hepatic Δ4-desaturation. On the basis of high postnatal erythrocyte 26:0 levels it is conceivable that low peroxisomal ß-oxidation is the underlying cause. Fetal cholesterol ester and triglyceride 20:3ω9 contents were higher than those of corresponding maternal fractions and did not change with gestation. Transplacental transport of 20:3ω9, followed by fetal conservation and to a lesser degree fetal *de novo* synthesis by 18:1ω9 desaturation/chain elongation, should be considered. Triglyceride 18:2ω6 contents of premature and term babies were linearly related to those of their mothers. Consequently, high 18:2ω6 and low 18:3ω3 intakes by the mother may unfavourably influence fetal capacity to produce 22:6ω3 in the liver, due to competition of parent essential fatty acids for desaturation. Because of low hepatic Δ4-desaturation capacity the influence may, however, be small. Except for cholesterol ester 18:3ω3, the heaviest baby of each twin had the highest EFA content in cholesterol esters and triglycerides. Although the number of twins was small it suggests that low fetal essential fatty acid status is a limiting factor in growth.

LCPUFA contents of circulating lipids are used to monitor critical organ LCPUFA status in the perinatal period. In **chapter 4.2** we studied the possible usefulness of postnatal plasma cholesterol esters and erythrocyte LCPUFA contents as indicators for LCPUFA status. Plasma cholesterol esters and erythrocytes were isolated from seven lactating women and their exclusively breastfed newborns, living on Dominica. In addition, we studied mature milk fatty acid composition. Blood samples were taken from umbilical cord and mother at birth. A sample of breastmilk was collected on day 20-22 postpartum, together with a blood sample from the baby. Our results show that cord blood plasma cholesterol ester and erythrocyte total LCPUFA contents were higher, and linoleic (18:2ω6) and α-linolenic (18:3ω3) acid contents lower, than in corresponding maternal compartments. Preferential LCPUFA transport across the placenta is suggested and may be accomplished by α-fetoprotein. Cord blood erythrocyte LCPUFAω3 content was lower and LCPUFAω6 content higher than in maternal erythrocytes. After birth, feeding with human milk led to a drop in plasma cholesterol ester LCPUFA content, whereas erythrocyte LCPUFA content remained virtually constant. Differences in LCPUFA incorporation efficacy in these compartments may be the underlying cause of the discrepancy. Plasma cholesterol esters are formed by a transfer of a fatty acid from the *sn-2* position of phosphatidylcholine to free cholesterol, catalyzed by lecithin:cholesterol acyltransferase. This enzyme has preference to transfer 18:2ω6. It is conceivable that postnatal decrease in plasma cholesterol ester LCPUFA content is notably caused by the high human milk

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18:2ω6 content. In erythrocytes, the majority of LCPUFA are incorporated into phosphatidylethanolamine and phosphatidylserine during erythropoiesis. Their incorporation is hardly influenced by 18:2ω6 intakes. Therefore, postnatal erythrocyte LCPUFA may reflect LCPUFA status of the bone marrow and may be a more reliable parameter for LCPUFA status than plasma cholesterol ester LCPUFA content. Courses of erythrocyte LCPUFA contents suggest that at birth the newborn has lower LCPUFAω3 status than its mother, and that this does not change during three weeks of exclusive breastfeeding. Whether lower LCPUFAω3 content in newborn erythrocytes reflects lower LCPUFAω3 status in brain, or derives from preferential deposition of these fatty acids in newborn brain (ω3 hierarchy) remains to be established.

Chapter 4.3 describes the effect of ribonucleotide supplementation on LCPUFA status of infant formula fed newborns. In newborns, conversion of 18:2ω6 and 18:3ω3 to LCPUFA is insufficient to prevent postnatal LCPUFA decreases in several body compartments. It has been suggested that supplementation of infant formula with ribonucleotides induces chain elongation and desaturation. We investigated whether ribonucleotide (RN) supplementation of a regular formula for premature infants (PRE; PRE+RN) raises erythrocyte and plasma cholesterol ester LCPUFA of low-birth-weight babies (≤2500 g) to levels encountered in breastfed counterparts. From days 11 to 42, 31 babies received PRE and 37 PRE+RN. Eleven breastfed babies served as reference group. Erythrocyte and cholesterol ester fatty acids were determined on days 11, 21 and 42. There were no differences in erythrocyte and cholesterol ester fatty acid courses of PRE and PRE+RN fed babies. On day 42, formula-fed babies had lower erythrocyte and cholesterol ester LCPUFA of both ω3 and ω6 series, compared with breastfed babies. Subdivision into gestational age and body weight matched subgroups gave similar results. We conclude that ribonucleotide supplementation does not augment erythrocyte and cholesterol ester LCPUFA status of formula-fed babies. LCPUFA supplementation of formulas, notably those for prematures, is indicated.

Myoinositol is a sugaralcohol that either occurs in its free form or as structural component of membrane phospholipids. Free myoinositol may be an osmoregulator. Inositol phospholipids play important roles in eicosanoid synthesis, signal transduction, growth, intracellular Ca^{2+} homeostasis and lipoprotein secretion. After birth, newborns have 3-6 times higher myoinositol levels than adults. Adult levels are reached within 2-6 postnatal months. Breastfed infants show slower myoinositol decreases than formula fed counterparts. It is currently recommended to increase myoinositol concentrations in infant formulas for prematures. In **chapter 4.4** we studied the influence of different myoinositol intakes from infant formulas for prematures on plasma and erythrocyte myoinositol contents on postnatal days 10, 20 and 42. Thirty-four healthy low-birth-weight babies (≤2500 g) received infant formulas with low (PRE, 0.19 mmol/L; n=26) or high (PRE^+, 1.24 mmol/L; n=8) myoinositol concentrations. Eleven breastfed low-birth-weight counterparts (mean: 0.60 mmol myoinositol/L) served as reference group. Plasma and

Summary

erythrocyte myoinositol were low in PRE-fed babies and comparably high in PRE$^+$- and breastfed babies. In the milk myoinositol intake range of PRE and PRE$^+$, plasma and erythrocyte myoinositol exhibited non-linear dose-response relations. In the intake range of mature human milk to PRE$^+$ they reach constant levels. In the 60-360 μmol/L plasma myoinositol range plasma myoinositol determines erythrocyte myoinositol to a large extent. Erythrocyte myoinositol may be regarded as a parameter of cellular myoinositol availability, since erythrocytes contain a similar myoinositol active transport system. High plasma myoinositol in newborns seems, therefore, necessary to accomplish high intracellular myoinositol. Following initiation of postdelivery urinary myoinositol loss the neonate requires high myoinositol intakes to maintain high plasma myoinositol. Data from feeding with PRE show that no compensatory augmentation of *de novo* synthesis or downregulation of myoinositol disposal takes place. Early human milk myoinositol concentration is higher than that in PRE$^+$, and rapidly decreases below PRE$^+$ myoinositol concentration with advancing lactation. PRE$^+$ myoinositol concentration may, therefore, be too low to reach plasma levels similar to breastfed babies in the first postnatal days and may cause higher myoinositol intakes than from human milk when PRE$^+$ feeding is continued for more than six weeks. Preterm babies seem to benefit more from PRE$^+$ than term babies.