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Human milk lipids

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Abstract:

Human milk lipids provide the infant with energy and essential vitamins, polyunsaturated fatty acids, and bioactive components. Adding complex lipids and milk fat globule membranes to vegetable oil based infant formula indicate the potential to enhance infant development and reduce infections. Cholesterol provision with breastfeeding modulates infant sterol metabolism and may induce long-term benefits. Some 98-99 %of milk lipids are comprised by triacylglycerols, whose properties depend on incorporated fatty acids. Attention has been devoted to the the roles of the long-chain polyunsaturated fatty acids docosahexaenoic (DHA) and arachidonic (ARA) acids. Recent studies on gene-diet interaction (Mendelian randomisation) show than breastfeeding providing DHA and ARA improves cognitive development and reduces asthma risk at school age particularly in those children with a genetically determined lower activity of DHA and ARA synthesis. It appears prudent to follow the biological model of human milk in the design of infant formula as far as feasible, unless conclusive evidence for the suitability and safety of other choices is available. The recent European Union legislative stipulation of a high formula DHA content without required ARA deviates from this concept, and such a novl formula composition has not been adequately evaluated. Great future opportunities arise with significant methodological progress for example in lipidomic analyses and their bioinformatic evaluation, which should enhance understanding of the biology of human milk lipids. Such knowledge might lead to improved dietary advice to lactating mothers, as well as to further opportunities to enhance infant formula composition.

Key words: Breastfeeding, milk fat globule membranes, phospholipids, sphngomyelins, gangliosides, arachidonic acid, docosahexaenoic acid,

Lipids are a major source of energy provided with human milk to the infant (1, 2), but also provide essential nutrients such as polyunsaturated fatty acids (PUFA) and lipid soluble vitamins. Many studies have demonstrated important biological effects of the milk lipids provided to the recipient infant, for example on gastrointestinal function, lipid and lipoprotein metabolism, membrane composition and function, infant growth, neurodevelopment and immune function (3).

Human milk lipids provide a major portion of the total energy intake in young infants, with a mean 44 % of the energy supply (4) (Figure 1). The average intake of human milk lipids in fully breastfed infants amounts to 21.42 g/day between birth and 6 months of age (4). This results in an impressive 3.9 kg of human lipid supplied during the first half year of life to fully breastfed infants, equivalent to some 35 000 kcal provided by human milk lipids alone during the first six months of life. While the mean lipid content in human milk is relatively stable during the course of the first months of lactation, there is very wide inter-individual and intra-individual variation of milk fat concentrations (table 2) (4-6). In fact, among the macronutrients in milk, fat shows the most variable concentration. For example, in mature milk samples collected at the infant age of 2 months, we find a coefficient of variation of 37.3 % for milk fat but only of 14.4 % for lactose and 12.9 % for protein (4). Milk fat content tends to increase with longer duration of breastfeeding and varies during the course of a day (1, 6). Milk fat concentration increases with an increasing time interval to the preceding milk expression from the same breast, and it increases with maternal fat deposition in pregnancy indicated by the degree of gestational weight gain (7). Milk fat increases during the course of each breastfeeding meal, with markedly higher milk fat contents in hind milk (at the end of feeding) than in foremilk (at the beginning of the feed) (Figure 2) (8). This may be of biological benefit in that infants will initially get milk rich in the essential water soluble substrates, whereas whose that are hungrier and drink more milk obtain a milk with an increasing fat and energy content to satisfy their caloric needs. Of interest, the increase of milk fat content during the meal is accompanied with a marked increase in the mean size of milk fat globule. Thereby, hindmilk has a higher ratio of triglycerides in the core of the milk fat globule (providing energy) to the surface membranes (rich in phospholipids, complex lipids and essential long-chain polyunsaturated fatty acids, LC-PUFA).

Milk fat globules and complex lipids

Milk can be characterized as an emulsion of milk fat globules in an aqueous liquid. Milk fat globules with markedly variable sizes are formed in the mammary alveolar cells and contain a core of nonpolar lipids comprised primarily of triacylglycerols, with added small amounts of monoglycerides, diglycerides and non-esterified fatty acids. These non-polar lipids are

formed in the endoplasmatic reticulum from fatty acids obtained from the maternal circulation, as well primarily intermediate chain fatty acids with 12 and 14 carbon atoms synthesized from acetyl-CoA. Upon the secretion from the endoplasmatic reticulum of mammary epithelial cells into the cytosol, this triglyceride–rich core is covered by an inner membrane derived from the endoplasmatic reticulum consisting of a monolayer primarily of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cholesterol. When these lipids droplets are further excreted from mammary epithelial cells into the alveolar space, they are covered by a piece of the apical plasma membrane, which results in the addition of another phospholipid bilayer and hence a phospholipid trilayer, and the other components of the mammary epithelial cell membrane such as membrane proteins and glycoproteins (Figure 3). This outer layer of the milk fat globule membrane (MFGM) consists of a bilayer of amphipatic lipids, primarily phosphatidylcholine, sphingomyelin and cholesterol, as well cerebrosides, ganglosides, glycosylated proteins and polypeptides, filaments, mucins, lactadherin, butyrophilin and others, and hence MFGM contain a high density of bioactive components (9).

Phospholipids, plasmalogens and sphingolipids including ceramides and gangliosides provide about 0.2 to 1% of total milk lipids or about 100 to 400 mg/L (2). The concentration of different phospholipids per 100 g milk were reported as 8.5 mg sphingomyelin, 6.8 mg phosphatidylethanolamine, 6.0 mg phosphatidylcholine, 1.4 mg phosphatidylserine and 1.1 mg/100 g for phosphatidylinositol (10). Phospholipids serve structural roles as indispensable components of all plasma membranes of body cells and organelles, and they have an impact on membrane functions and metabolism. Complex lipids also have roles in signal transmission and cell recognition (2, 3). Gangliosides contribute some 10% of brain lipids, with high concentrations in the cerebral cortex.

The biological importance of MFGM is getting increased attention after several controlled trials reported benefits of adding bovine MFGM of complex lipid fractions to infant formula with fat derived predominantly from vegetable oil. A trial on formula enriched with sphingomyelin in preterm infants reported neurobehavioural benefits (11). In a small trial in Indonesia, the addition of a ganglioside-rich bovine milk lipid fraction was reported to improve the hand and eye coordination IQ, performance IQ and total IQ assessed with the Griffiths Mental Developmental Scale at age 24 weeks (12). Another trial providing a milk formula with addition of a similar preparation for 12 weeks enrolled 450 infants aged 8-24 months in India and reported no difference for rotavirus or for all-cause diarrhoea. In a large study that enrolled more than 500 Peruvian infants, MFGM supplemented formula did not affect diarrhea incidence but reduced longitudinal diarrhea prevalence (13). A larger trial that enrolled more than 250 toddlers aged 2.5-6 years in Belgium reported that a milk preparation enriched with a phospholipid-rich lipid fraction resulted in less days with fewer and lower

parental scoring of internal, external and total behavioural problems (14). A further trial enrolled 160 formula fed infants in Sweden as well as a breastfed reference group and evaluated effects of added bovine MFGM, along with reduced formula contents of energy and protein. The MFGM group achieved higher cognition scores in the Bayley test at age one year (Figure 4), and showed a much lower incidence of acute otitis media, and less use of antipyretic drugs (15, 16). These observations lead to the conclusion that MFGM and/or the complex lipids provided with the MFGM fraction may have important biological roles for the development of nervous immune functions.

Cholesterol

Milk fat globule lipids also provide considerable amounts of free and esterified cholesterol, resulting in a total cholesterol content of 90 to 150 mg/L in human milk, in contrast to typically only 0-4 mg/L in infant formula. Cholesterol is an indispensable building block for all cell membranes and is incorporated in considerable amounts into myelin in nervous system during the period of rapid brain growth, and it serves as the substrate for the synthesis of bile acids, lipoproteins, vitamin D, hormones and oxysterols that modulate cholesterol, lipid and glucose homeostasis (3, 9, 17-19). The provision of cholesterol with breastfeeding is associated with higher plasma concentrations of total low density lipoprotein cholesterol in breastfed than in formula fed infants (20). The provision of preformed cholesterol most likely is the cause for the about threefold lower endogenous cholesterol synthesis rate in breastfed than formula fed infants, since the synthesis rate is inversely correlated to the daily cholesterol supply in mg/kg bodyweight (21). In formula fed piglets, dietary cholesterol supply downregulated hepatic hydroxymethyl glutaryl coenzyme A reductase, the rate regulating enzyme for endogenous cholesterol synthesis (22). In human infants aged 4 months, the rate of endogenous cholesterol synthesis also appeared to be regulated by dietary cholesterol supply. Breastfed infants with a high cholesterol and low phytoestrogen intake had the lowest fractional synthesis rate, whole infants receiving cows' milk-based formula with low cholesterol and low phytoestrogen had an intermediate rate, and infants fed soy-based formula with no cholesterol and high phytoestrogen had the highest rate of synthesis (23). When cholesterol was added to soy-based infant formula, the rate of synthesis was changed to similar results as in infants fed cows' milk-based formula, which leads to the conclusion that the amount of dietary cholesterol supply regulates cholesterol synthesis in infants. Lasting effects of early feeding on later cholesterol levels were reported in several studies and reviewed in meta-analyses. A rather modest lowering of total and low density lipoprotein cholesterol was found in adults who had been breastfed in infancy, compared to previously formula fed people, with a greater effect size of exclusive than of partial breastfeeding (24,

25). It was proposed that 30 % of infants are exclusively breastfed resulting in a blood cholesterol reduction in adulthood by 0.15 mmol/L, the population prevalence of cardiovascular disease could be reduced by as much as 5% (25). However, Ip et al noted that the analysis reporting reduced serum lipid levels in adults previously breastfed did not segregate the data according to gender and did not explicitly analyse potential confounders; and they concluded that in view of the limited methodological quality of the meta-analysis the relationship between breastfeeding and adult cholesterol levels cannot be correctly characterized (26). Meta-analyses of available data do not allow definitive conclusions can regarding the relationship between breastfeeding and on all-cause mortality from cardiovascular diseases in adult life, although the confidence limits around the point estimates and the observed between-study heterogeneity do not exclude potential beneficial or adverse cardiovascular effects of breastfeeding (26, 27). Therefore, it appears particularly promising to evaluate the short- and long-term effects of addition of well bioavailable preparations of cholesterol to infant formula in randomized controlled trials, which may shed further light on the potential biological importance of a dietary cholesterol supply in infancy.

Fatty acids provided with milk lipids

Triacylglycerols contribute some 98-99 % of human milk fat. The properties of milk triglycerides are very much influenced by their fatty acid composition. Milk lipids of European women today typically contain some 35-40 % saturated fatty acids, 45-50 % monounsaturated fatty acids and approximately 15 % PUFA (Table 1). The saturated palmitic acid (C16:0) provides approximately 25% of all milk fatty acids and hence the major part of the total saturated fatty acid content. About 70% of human milk palmitic acid is esterified in the middle position (sn-2 position) of triacylglycerols which facilitates absorption. During intestinal digestion, fatty acids in the sn-1 and sn-2 positions are liberated as non-esterified fatty acids by pancreatic lipases. These non-esterified fatty acids are quite well absorbed if they are unsaturated and hence better water soluble. In contrast, liberated long-chain saturated fatty acids such as palmitic acid are poorly water soluble and poorly absorbed, but rather bind to calcium and form calcium soaps that are excreted with stools, thereby reducing both fat and calcium absorption. However, if palmitic acid is esterified in the sn-2 position, as it is predominantly the case in human milk lipids, pancreatic lipolysis yields a palmitoyl-monogylcerol which is well water soluble and well absorbed, thereby reducing fat and calcium malabsorption (28).

The human milk contents of the mono-unsaturated fatty acid oleic acid (C18:1n-9) and of the essential PUFA linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) vary with the maternal dietary intake of these fatty acids. This is illustrated by the approximately 3fold

191 increase of linoleic acid content in mature human milk in the USA since the mid 1940ies,
192 along with the increase of dietary vegetable oil and linoleic acid consumption in the
193 population, whereas alpha-linolenic acid contents have remained rather constant (Figure 5)
194 (29). Thereby the average ratio of the omega-6 linoleic acid to the omega-3 α-linolenic acid in
195 human milk has also increased approximately 3 fold. We studied the transfer of linoleic acid
196 provided to lactating women into their milk using stable isotope labelled fatty acids. An oral
197 dose of 1 mg/kg bodyweight of linoleic acid uniformly labelled with the stable carbon isotope
198 13C was provided repeatedly during the 2nd, 6th, and 12th week of lactation (30). Before
199 and at several times during a 5-day period after tracer intake, samples of breath and milk
200 were collected, the volume of daily milk production was assessed, and dietary nutrient
201 intakes were calculated from prospective dietary protocols. Some 3.5-4.5 % of the ingested
202 linoleic acid was oxidized to CO_2 and exhaled with breath, with no significant differences
203 between the studied time points. Dietary linoleic acid was rapidly transferred into milk, with a
204 peak enrichment reached about 12 hours after intake (Figure 6). Linoleic transfer into milk in
205 unchanged form or as its metabolites did not change during the course of lactation. The data
206 indicate that about 30% of milk linoleic acid is derived directly from dietary intake, whereas
207 about 70% originates from maternal body fat stores. It is tempting to speculate that this
208 largely indirect transfer of dietary linoleic via intermediate body stores may represent a
209 biological benefit to the breastfed infant, since this mechanisms buffers short term variation
210 of maternal dietary supply of the parent essential fatty acid and provides the infant with a
211 relatively stable parent essential fatty acid supply. However, long-term changes in dietary
212 supply will also modify maternal body fat stores and thereby explain the observed marked
213 changes over time (Figure 5). Only about 11% of the milk content of the linoleic acid
214 metabolite dihomo-gamma-linolenic acid (C20:3n-6) in milk originates from direct
215 endogenous conversion of maternal dietary linoleic acid, while of the milk arachidonic acid
216 (C20:4n-6) only 1.2% is directly derived from maternal linoleic acid intake (30).

217

218 **Long-chain polyunsaturated fatty acids**

219 The provision of the long-chain polyunsaturated fatty acid metabolites (LC-PUFA) with milk,
220 in particular of omega-6 arachidonic acid (ARA) and omega-3 docosahexaenoic acid (DHA)
221 has received considerable attention, because many of the biological effects of the essential
222 fatty acids in early life appear to be mediated by LC-PUFA rather than the precursor
223 essential fatty acids. Brenna et al. performed a systematic review on 106 studies of human
224 breast milk worldwide and culled to include only studies that used modern analysis methods
225 capable of making accurate estimates of fatty acid contents, as well as criteria related to the
226 completeness of reporting (31). The final analysis included 65 studies with milk of 2474

women. The authors found a milk ARA content of 0.47+0.13 % (mean±SD, % wt./wt.), whereas milk DHA content was lower at 0.32+0.22 % (31). Higher milk DHA contents were found in coastal populations and those with regular marine food consumption. The greater stability of milk ARA levels with a coefficient of variation (CV) of only 29%, as compared to DHA with a CV of 69%, appears to reflect a greater degree of metabolic regulation of milk ARA content. Stable isotope studies have led us to the conclusion that 90% of human milk AA are not derived directly from absorbed dietary lipids but rather from maternal ARA body stores (32). In contrast, dietary DHA supply is a key determinant of milk DHA content. We showed that the dietary DHA intake is linearly correlated to milk DHA (33) (Figure 6). Breastfeeding women need to achieve a daily DHA intake of at least 200 mg to provide a milk with a DHA content of at least 0.3%, which is required for a fully breastfed infant to obtain the daily supply of about 100 mg DHA/day considered desirable to meet metabolic needs (34). Given that the regulation of human milk ARA and DHA content differs, milk DHA and ARA are not closely correlated (r=0.25, P=0.02) (31), and the ARA/DHA-ratio is not constant. It remains controversial whether the ratio of ARA to DHA in milk - or rather the amounts of DHA and of ARA supplied - are of greater relevance for biological effects in the infant. A balanced supply of both ARA and DHA appears to be relevant for the adequate incorporation of ARA and DHA into the growing brain (35).

In view of the marked accretion of ARA and DHA in the growing brain and the ample experimental evidence of the impact of LC-PUFA on membrane function, eicosanoid and docosanoid formation and the resulting regulation of physiological processes, as well as the development and function of neural and immune tissues, the impact of LC-PUFA provision with human milk and also with infant formula has received considerable interest.

The provision of DHA was shown to enhance the early development of visual acuity. The European Food Safety Authority (EFSA) concluded that a cause and effect relationship has been established between the intake of infant and follow-on formula supplemented with DHA at levels around 0.3% of total fatty acids and visual function at 12 months in formula-fed infants born at term from birth up to 12 months and in breastfed infants after weaning up to 12 months (36). However, some controversy remains with regards to the effects of the supply of preformed LC-PUFA on neurodevelopment of healthy term infants. For example, the authors of a meta-analysis on randomized trials evaluating infant formula with LC-PUFA compared to formula without LC-PUFA concluded that while some studies showed a significant benefit, overall no significant effect was detectable (37, 38). The authors noted the limitation of their conclusions by a large degree of heterogeneity of the included studies, which provided markedly different interventions and also used a variety of very different outcomes and approaches to outcomes assessment. Of importance, the included studies did not adjust for the major impact of genetic variation modulating the rate of endogenous

synthesis of LC-PUFA and related clinical endpoints, in particular variation in the *Fatty Acid Desaturase (FADS)* gene cluster (39, 40). The lack of adjusting for this major modulating confounding factor in the included studies may considerably reduce the sensitivity to detect effects of dietary LC-PUFA effects. The comparison of breastfed infants provided with preformed LC-PUFA with infants fed formula without LC-PUFA in observational studies is also difficult to interpret, because human milk LC-PUFA and particularly DHA supply are closely associated with different dietary and lifestyle choices, including maternal smoking and parental socioeconomic status, which may also influence neurodevelopmental outcomes.

Further insight into PUFA effects are offered by considering the interaction of breastfeeding which always supplies preformed LC-PUFA, and the genetic variation in the *FADS* gene cluster that predicts the enzyme activities of fatty acid desaturases 1 and 2. Gene variants of the *FADS* gene cluster have a major impact on the fatty acid composition of blood, tissues and human milk (39-41). We assessed the single nucleotide polymorphisms in the *FADS* genes along with human milk fatty acid composition in 772 breastfeeding mothers who participated in the prospective Ulm Birth Cohort study both at 1.5 months after infant birth, and also at 6 months postpartum in a subset of 463 mothers who were still breastfeeding at this time (42). At both time points, we found significant associations of *FADS* genotype with milk ARA contents and the ratio of ARA to di-homo-gamma-linolenic acid, indicating that maternal *FADS* genotypes impact on the formation of LC-PUFA provided with breastmilk (42). The variation of *FADS* genotypes was shown to also modulate the interaction of breastfeeding and cognitive development. Genotyping for the rs174575 variant in the *FADS2* gene was performed in 5934 children participating in the ALSPAC study in whom IQ tests had been performed at the age of about 8 years (43). In line with other observational studies, previously breastfed children had higher IQ scores than previously formula fed children, but the relative impact of human milk nutrient supply and of confounding factors associated with breastfeeding cannot be easily deciphered from these observational data alone. Causal inferences on the role of human milk LC-PUFA supply can be drawn from the fact that the beneficial effect of breastfeeding was much more pronounced, with an added advantage of about 4.5 IQ points, in the group of children with a genotype predicting a low ability of LC-PUFA synthesis (43). Replication of these findings was published with the analysis of data from two Spanish birth cohort studies (44). Since the genotype is considered to be distributed in the population at random (“Mendelian randomisation”) and unrelated to the parental decision to breastfeed and to other related lifestyle predictors of IQ at school age, these data provide powerful evidence for causality between early LC-PUFA supply and status during the breastfeeding period and later IQ achievements.

The relevance of LC-PUFA supply for child neurodevelopment was also demonstrated in a randomized clinical trial that enrolled 119 breastfeeding women in Texas, USA (45). The

women were assigned to receive identical capsules containing either a high-DHA algal oil providing approximately 200 mg DHA daily or a vegetable oil without DHA from delivery until 4 months after birth. Provision of DHA to the mother increased DHA in milk by about 70%, and in infant plasma phospholipids by about 20% (45). A the age of 30 months, child psychomotor development was significantly better if mothers had received added DHA during the first 4 months of brestfeeding. At the age of 5 years, there were no differences in visual function, but children whose mothers had received added DHA performed significantly better on the Sustained Attention Subscale of the Leiter International Performance Scale (46.5$\pm$8.9 vs 41.9$\pm$9.3, P<0.008). These results support the conclusion that the DHA supply during early infancy is of importance for specific aspects of neurodevelopment.

Mendelian randomisation also provided strong support for the conclusion that the LC-PUFA supply with breastfeeding is causally linked to protection against later manifestation of bronchial asthma. Many studies have reported a protective effect of breastfeeding on asthma development, even though results are not consistent (26). We evaluated the influence of the *FADS1 FADS2* gene cluster polymorphisms on the association between BF and asthma in 2245 children participating in two prospective German birth cohort studies, the GINI and LISA studies (46). Logistic regression modelling was used to analyse the association between exclusive breastfeeding and doctor's diagnosed asthma occurring up to the age of 10 years, stratified by genotype. In the stratified analyses, heterozygous and homozygous carriers of the minor allele that show a low activity of LC-PUFA synthesis had a much reduced risk for later asthma if they were breastfed for 3 or 4 months and hence were provided with preformed LC-PUFA that can compensate for low endogenous synthesis [adjusted odds ratio between 0.37 (95% CI: 0.18-0.80) and 0.42 (95% CI: 0.20-0.88)]. Interaction terms of breastfeeding with genotype were significant and ranged from -1.17 (P-value: 0.015) to -1.33 (0.0066). Similarly, heterozygous and homozygous carriers of the minor allele who were exclusively breastfed for 5 or 6 months after birth had a reduced risk of asthma [0.32 (0.18-0.57) to 0.47 (0.27-0.81)] in the stratified analyses. In contrast, in individuals carrying the homozygous major allele predicting a greater degree of endogenous LC-PUFA formation, breastfeeding with provision of LC-PUFA showed no significant effect on asthma development. These results of a Mendelian randomisation study demonstrate a lasting causal protection of breastfeeding for at least 3 months against doctor's diagnosed asthma until school age in children with a low rate of LC-PUFA synthesis and a modulating effect of postnatal PUFA status.

A systematic review on human studies on roles of LC-PUFA and an expert workshop that reviewed the information and developed recommendations was recently performed with support from the Early Nutrition Academy (34). It was concluded that breastfeeding women should get $\geq$200 mg DHA/d to achieve a human milk DHA content of at least $\approx$0.3% of fatty

acids. Infant formula for term infants should contain DHA and AA to provide 100 mg DHA/d and 140 mg AA/d, and a supply of 100 mg DHA/d should continue during the second half of infancy. No quantitative advice on AA levels in follow-on formula fed after the introduction of complimentary feeding was provided due to lack of sufficient data and considerable variation in AA amounts provided with complimentary foods.

Should infant formula LC-PUFA composition be guided by human milk composition?

With regards to infant and follow-on formula, the recent revision of the European legislation that came into force in 2016 stipulates that all infant and follow-on formula must contain between 20 and 50 mg DHA/100 kcal (approximately 0.5-1 % of fatty acids), whereas formula without DHA content will not be allowed any more to be placed on the European Union market once this legislation is implemented (47). To the great surprise of many paediatricians and of experts in the field, no requirement for a minimum content of arachidonic acid in infant formula has been defined. This legal regulation is based on advice provided by the European Food Safety Authority that reviewed a variety of aspects and nutrients, including also the LC-PUFA DHA and ARA. In a first report on nutrient requirements and dietary intakes of infants and young children published in 2013, adequate LC-PUFA intakes were defined as 100 mg DHA/day and 140 mg ARA/day from birth to the age of 6 months, while 100 mg DHA/day were considered adequate from 6 to 24 months (48). These conclusions are in line with many other scientific reports, including the recent recommendations of the Early Nutrition Academy supported global expert group that are based on a systematic review of the available scientific evidence (34). In contrast, the subsequently published EFSA report on the compositional requirements of infant and follow-on formula advised that all infant and follow-on formula should contain relatively high amounts of DHA at 20-50 mg/100 kcal, but without the need to provide any preformed ARA (49). This DHA level stipulated by EFSA and the new European legislation is much higher than the about 0.2 to 0.3 % DHA found in most LC-PUFA enriched formulae for term infants currently marketed around the world, which however generally contain also preformed ARA at levels equal to or often 2fold higher than the DHA content. The proposed obligatory inclusion of DHA in all infant and follow-on formulae is welcomed by many scientists and paediatricians in view of the indications for beneficial effects (50), but the advice to provide infant formula from birth that supplies DHA but no ARA has been heavily criticized (51). During pregnancy and infancy, both DHA and ARA are deposited in relatively large amounts in human tissues, including the brain (52, 53). Fetal accretion of both DHA and ARA during pregnancy is facilitated by their active and preferential materno-fetal placental transfer (54). Pregnant women's red blood cell levels of both DHA and ARA were positively associated

with their children's intelligence quotient at school age (55). At birth, higher cord blood contents of both DHA and ARA predicted less later behavioural problems, emotional difficulties, hyperactivity and attention deficit at age 10 years (56). After birth, breastfed infants always get both preformed DHA and ARA, usually with a higher provision of ARA than of DHA (31, 57). DHA along with ARA has been added to infant formulae since the 1980ies in an attempt to approach the nutrient supply and functional effects achieved with breastfeeding (58-60). The global Codex Alimentarius standard on the compositional requirements for infant formula stipulates the optional addition of DHA to infant formula, provided that the ARA content is equal to or higher than the DHA content, thus following the model of typical human milk composition (61).

Infant formula providing both DHA and ARA have been evaluated in many controlled trials in infants (50). In contrast, the proposed composition of term infant formula with up to 1 % DHA and no ARA is a novel approach that has not been systematically tested for it's suitability and safety in healthy infants born at term. ARA is an essential component of all cell membranes. The amount of ARA incorporated into the developing brain during infancy exceeds the deposition of DHA. Although humans can synthesize ARA to some extent from linoleic acid, infants fed formula without pre-formed ARA tend to develop lower ARA levels in blood plasma and erythrocytes than breast-fed infants who receive both DHA and ARA (52, 58, 62). In preterm infants, provision of high amounts of n-3 LC-PUFA without a concomitant supply of ARA has been associated with adverse effects on growth (63, 64). Further concerns regarding the effects of a high supply of DHA without increasing ARA intakes to infants are raised by the findings of a randomized controlled trial assigning term infants to formula providing either no LC-PUFA, or different levels of 0.32, 0.64 and 0.96 % DHA at the same ARA level of 0.64 % (65). The investigators performed developmental testing of the participating children up to the age of 6 years. Positive effects in tests on word production, a card sorting task and an intelligence test were observed with the lower DHA dose. However, performance of children assigned to the highest DHA dose of 0.96 % but with a reduced ratio of dietary ARA to DHA was attenuated in the MBCDI Word Production Test and the Dimensional Change Card Sort Test at the highest DHA level, and it was attenuated at both the two higher DHA levels in the Peabody Picture Vocabulary Test (65). Thus, in contrast to what might have been expected, an increase of formula DHA contents above 0.32% did not further improve or at least stabilize developmental outcomes, but actually had adverse effects which might well be due to the reduced dietary ARA to DHA ratios provided with the higher DHA levels.

The effects of equivalent formulae with similar DHA and ARA contents on brain composition were tested in infant baboons. Brain composition in various regions was analysed. The formula with about 1% DHA induced a trend to lower ARA levels in the retina and all the

eight regions of the brain analysed, with significantly reduced ARA values in the globus pallidus and the superior colliculus, even though the formula contained 0.64% ARA. These observations raise serious concerns that infant formula with high contents of DHA but lack of ARA may induce adverse effects on brain composition and related functional outcomes.

These findings in human infants and in nonhuman primates question the suitability and safety of the compositional requirements stipulated by the new European legislation, i.e. to provide infant formula from birth with up to 1% of fatty acids as DHA without a proportional increase in the intake of ARA. It is generally agreed upon that any major change in infant formula composition should be subjected to a full pre-clinical and clinical evaluation of nutritional adequacy and safety prior to the wide use and marked introduction of such a modified formula (66-71). Therefore, it appears to be inappropriate and premature to market formula for term infants from birth with 20-50 mg/100 kcal DHA without added ARA in the absence of accountable data on the suitability and safety from a thorough clinical evaluation of this novel approach (51).

Conclusions and Perspectives

In addition to meeting the infants needs for energy and essential vitamins and polyunsaturated fatty acids, human milk lipids provide a mixture of milk fat globule membranes, complex lipids and bioactive compounds that may have important biological roles in the breastfed infant, for example with regard to the development of nervous and immune functions. Further studies defining the specific components responsible for such effects and the underlying mechanisms could help to design the best options of nutritional interventions. Methodological progress in the field of metabolomics and lipidomics using liquid chromatography couple with triple mass spectrometry now allows to determine detailed profiles of molecular species of complex lipids in milk as well as in extremely small sample volumes of infant serum or plasma (e.g. 10 microlitres) with high quantitative precision (72-75). Such lipidomic measurements can serve to provide markers for tissue composition (76) and were shown to be associated with important clinical endpoints in children and adults (77-79). It is therefore likely that the use of these sophisticated and detailed analytical methods, if combined with appropriate bioinformatics strategies, provide the opportunity to obtain better insights into the physiological roles of complex lipids in early life, which may lead to further improvements in nutritional strategies. Progress in biotechnology and food technology offers new avenues for preparing lipid components that can more closely mimic the complex lipid body provided with breastfeeding. Careful exploration and evaluation of the short and long term impact in infants could potentially lead to implementation of major improvements for the feeding of infants that cannot be breastfed. Opportunity also exists in improving out

447 understanding on the optimal supply of LC-PUFA in early and later infancy and in the
448 underlying mechanisms and mediators of their effects e.g. on neurodevelopment and
449 behavior, immune-related health outcomes such as allergy and asthma, and pulmonary
450 function.

451

452 **Key messages**

453 • Human milk lipids provide a major portion of the energy supply to breastfed infants,
454 as well as essential vitamins, polyunsaturated fatty acids, complex lipids, and
455 bioactive components
456 • Recent data evaluating the addition of preparations of complex lipids with or without
457 milk fat globule membranes to vegetable oil based infant formula show promising
458 indications for potential improvements of infant development and reduction of
459 infection risk
460 • Analyses of gene-diet interaction following the concept of Mendelian randomisation
461 add to the evidence that the supply of long-chain polyunsaturated fatty acids in
462 infancy is causally related to improving cognitive development and to reducing
463 asthma risk at school age. Current evidence supports the provision of omega-3
464 docosahexaenoic acid (DHA) along with omega-6 arachidonic acid (ARA) with infant
465 formula.
466 • Significant methodological progress both in food technology enabling the provision of
467 new lipid preparations, and in lipidomic analyses, offer major opportunities to explore
468 the biological effects of complex lipids in infancy.

469

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Conflict of interest:

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482 Figure 1: Contribution of macronutrients to total energy intake in breastfed infants aged one
483 month. Drawn from data of (4).

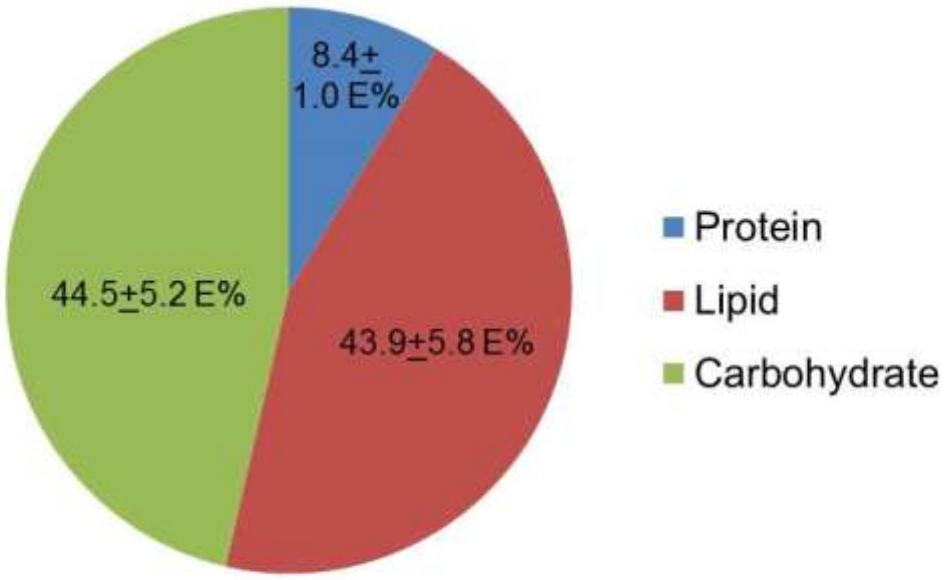

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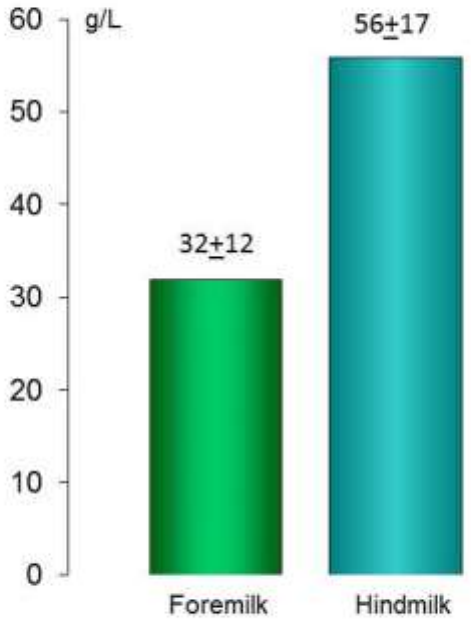
Figure 2: Milk fat concentration in fore- and hindmilk collected before and after breastfeeding of 15 term infants. Drawn from data of (80).



491 Figure 3: Schematic depiction of human milk fat globules

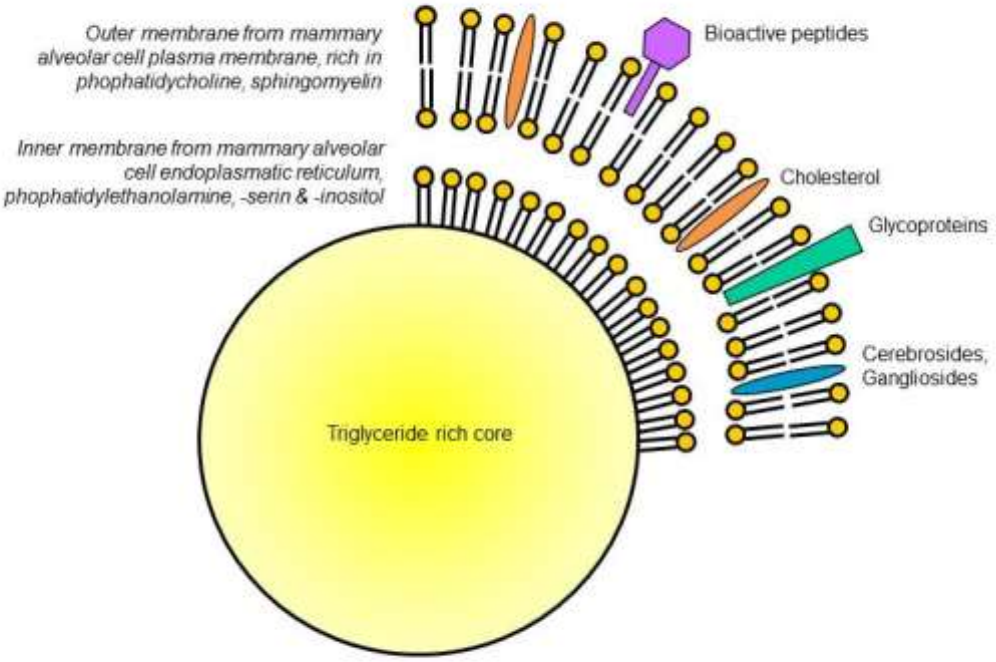


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Figure 4: Infants fed a vegetable oil formula with an added bovine milk fat globule preparation with complex lipids and bioactive proteins showed an improved cognitive outcome at age 1 year that those fed standard formula, and were more similar to the test results in a breastfed reference group. Drawn from data of (16).

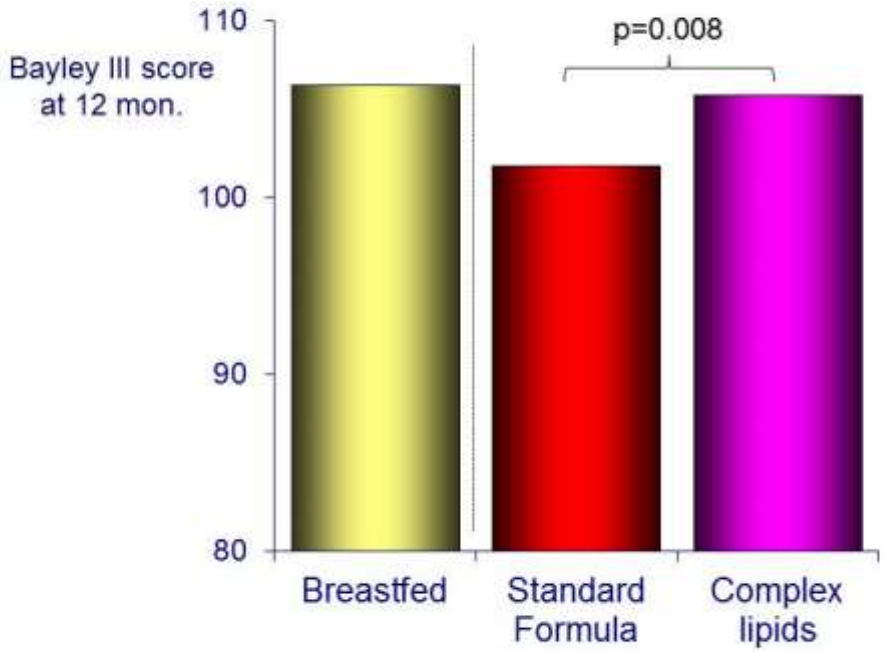
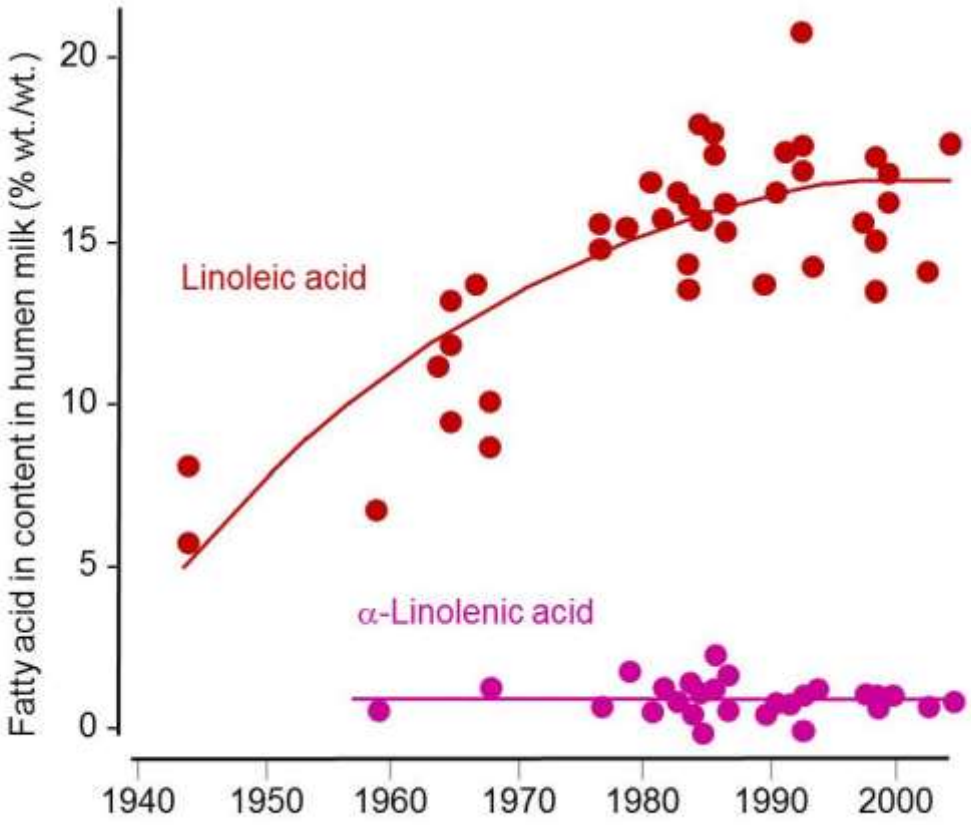
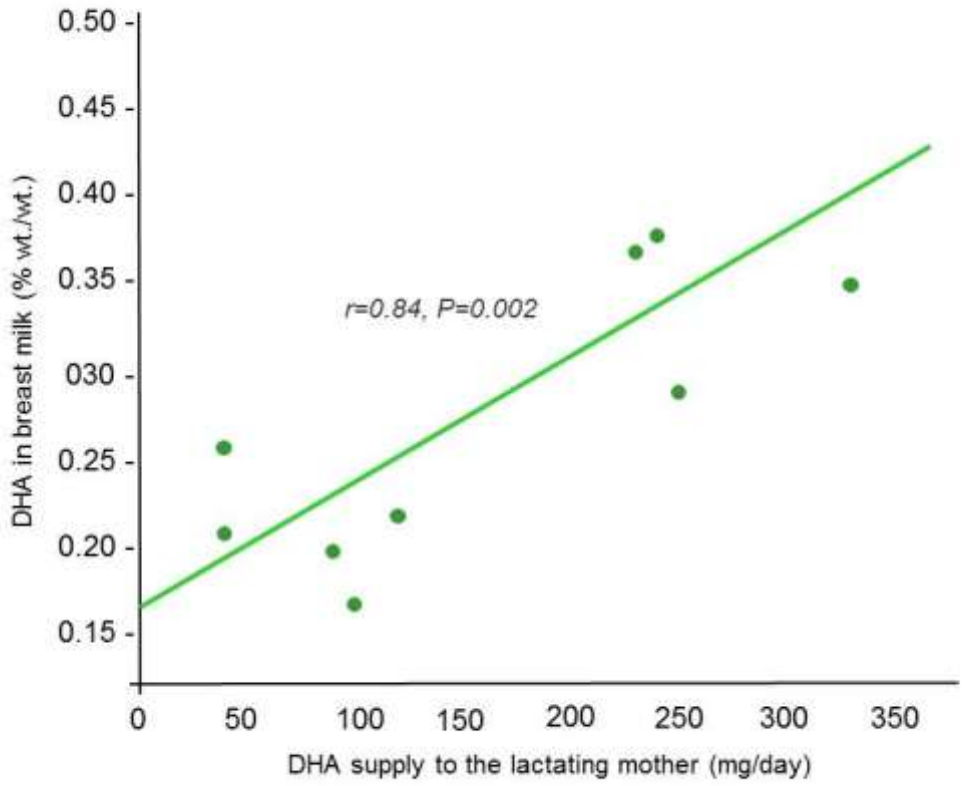


Figure 5: Evolution of the linoleic and alpha-linolenic acid contents in mature human milk in the USA over time. Drawn from data of (29).



503 Figure 6: The DHA supply to the lactating women determines the DHA content in her

504 breastmilk. Drawn from data of (33)



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508 **Table 1:** Longitudinal evolution of human milk constituents in 30 prospectively followed
509 lactating women (mean and standard deviation). The intraclass correlation coefficient that
510 reflects the stability of human milk constituents over time in each woman indicates a very
511 high intra-individual variation for carbohydrates, while stability over time was higher for milk
512 energy, protein and fat content. Among fatty acids, omega-3 FA had the lowest ICC. Modified
513 from (4).

	Age (months)				Intraclass correlation coefficient*	Change in mean over time p value*
	1	2	3	6		
Energy (kcal/100ml)	66.1 (11.1)	68.3 (13.4)	63.0 (10.5)	62.4 (13.3)	0.40	0.065°
Carbohydrates (g/l)	7.28 (1.36)	8.05 (1.15)	7.84 (1.39)	7.96 (1.74)	0.04	0.135
Lactose (g/l)	72.4 (13.5)	80.3 (11.6)	78.0 (13.9)	79.2 (17.3)	0.04	0.129
Galactose (g/l)	0.13 (0.04)	0.11 (0.03)	0.11 (0.04)	0.09 (0.03)	0.26	<0.001
Protein (g/100ml)	1.38 (0.16)	1.16 (0.15)	1.04 (0.13)	0.96 (0.16)	0.43	<0.001
Non-protein nitrogen (g/dl)	0.23 (0.02)	0.20 (0.02)	0.18 (0.02)	0.17 (0.02)	0.35	<0.001
Fat (g/100ml)	3.20 (1.27)	3.16 (1.18)	2.92 (1.23)	2.71 (1.25)	0.40	0.164
Saturated fatty acids[1]	39.0 (5.62)	37.7 (4.38)	37.2 (4.82)	36.8 (4.64)	0.21	0.202
Monounsaturated fatty acids[1]	45.8 (4.62)	46.7 (4.48)	47.0 (4.25)	47.0 (4.26)	0.31	0.517
Polyunsaturated fatty acids (PUFA)[1]	15.2 (4.26)	15.6 (2.95)	15.7 (3.43)	16.3 (4.17)	0.38	0.530
18:2n-6 (linoleic acid)[1]	12.8 (3.88)	13.2 (2.81)	13.5 (3.32)	14.0 (4.08)	0.41	0.435

20:4n-6 (arachidonic acid)[1]	0.51 (0.16)	0.52 (0.13)	0.52 (0.10)	0.52 (0.15)	0.31	0.981
18:3n-3 (α-linolenic acid)[1]	0.62 (0.16)	0.69 (0.18)	0.61 (0.14)	0.67 (0.13)	0.16	0.074
20:5n-3 (EPA)[1]	0.12 (0.03)	0.12 (0.03)	0.10 (0.03)	0.12 (0.05)	0.31	0.090
22:6n-3 (DHA)[1]	0.25 (0.11)	0.24 (0.11)	0.26 (0.09)	0.30 (0.15)	0.21	0.206
n-3 LC-PUFA[1]	0.48 (0.15)	0.48 (0.16)	0.49 (0.13)	0.56 (0.23)	0.17	0.148
n-6 LC-PUFA[1]	1.22 (0.34)	1.22 (0.30)	1.17 (0.20)	1.11 (0.31)	0.34	0.229

[1]% fatty acid of milk total lipids; *based on linear random-effects model with subject as a random effect and month as fixed effect; °linear trend

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516 **Table 2:** Absolute fatty acid supply with human in prospectively followed lactating women
517 (mg/day, mean and standard deviation). Modified from (4).

	Age (months)			
	1	**2**	**3**	**6**
Saturated fatty acids	7420.3 (2425.5)	7911.4 (2398.4)	7344.1 (2390.0)	4205.1 (3107.4)
Monounsaturated fatty acids	8712.8 (2998.6)	9821.8 (3115.3)	9238.6 (2974.8)	5344.3 (3953.1)
Polyunsaturated fatty acids (PUFA)	2851.5 (913.8)	3278.8 (1063.0)	3082.1 (999.4)	1884.8 (1454.4)
18:2n-6 (linoleic acid)	2407.0 (767.2)	2764.9 (915.0)	2635.1 (859.7)	1619.5 (1275.4)
20:4n-6 (arachidonic acid)	95.6 (32.9)	109.6 (38.6)	101.1 (33.1)	58.7 (43.5)
18:3n-3 (α-linolenic acid)	118.8 (47.7)	144.7 (49.0)	118.8 (39.1)	76.8 (58.2)
20:5n-3 (EPA)	22.7 (9.23)	24.2 (7.90)	20.4 (6.45)	14.1 (10.77)
22:6n-3 (DHA[1]	48.5 (25.5)	51.3 (20.2)	50.3 (17.1)	32.7 (23.4)
n-3 LC-PUFA	92.3 (42.9)	101.2 (36.8)	95.0 (30.8)	62.2 (44.1)
n-6 LC-PUFA	228.7 (75.4)	256.9 (86.5)	229.7 (72.7)	126.3 (92.2)
n-3 PUFA	215.9 (85.2)	244.1 (81.6)	209.6 (66.1)	138.9 (99.5)
n-6 PUFA	2635.7 (836.0)	3021.8 (990.9)	2865.0 (927.9)	1745.8 (1362.9)

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