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BIOACTIVE COMPOUNDS IN HUMAN MILK AND INTESTINAL HEALTH AND MATURITY IN PRETERM NEWBORN: AN OVERVIEW

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Abstract

Premature births are increasing worldwide (about 15 millions per year) due to several reasons (an advanced maternal age, fertility treatments, stress, smoking, nutritional deficiencies) and lead to a high societal overall cost. Among neonatal care procedures, the clinical nutrition practices are essential to promote the development and to minimize the sequelae. Premature newborns are at major risk of death by infections due to the immaturity of their intestine. Human milk provides not only nutrients but also a plethora of biologically active components that are tailored to contribute to the development of the intestinal tract early in postnatal life. Among them, some bioactive molecules exhibit trophic effects (LC-PUFA, sphingomyelin, IGF-I and IGF-II, EGF, insulin, leptin, adiponectin, lactoferrin, lactadherin, probiotics, prebiotics, miRNA) or are part of the intestinal cell membranes (PUFA, LC-PUFA, phospholipids, sphingolipids, cholesterol), others educate the intestine for innate microbial recognition (sCD14, sTLR-2, miRNA), many of them display direct fighting against pathogens (some fatty acids and monoglycerides, some phospholipids and sphingolipids, BSSL, insulin, lactoferrin, sIgAs, MUC-1, lactadherin, probiotics, prebiotics), or contribute to establish the gut microbiota (LC-PUFA, lactoferrin, probiotics, prebiotics). A synergetic action exists between several bioactive molecules. All together these precious agents regulate the maturation of the intestinal mucosal barrier, and might program early in postnatal life the future adult intestinal health. This review lists the main bioactive compounds and addresses their plausible roles and mechanisms of action.

Key words: Human milk, phospholipids, sphingolipids, cholesterol, DHA, sphingomyelinase, sCD14, milk growth factors, milk hormones, lactoferrin, MUC-1, lactadherin, prebiotics, probiotics, miRNA, premature newborn, intestine, necrotizing enterocolitis, enteral nutrition, immunological programming.

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INTRODUCTION

According to a recent study, approximately 15 millions births worldwide were premature in 2010 (birth between 22-<37 weeks of gestation as defined by WHO) representing about 11% of total births, of which about 5% concerned extremely preterm births (<28 weeks), 10.5% very preterm newborns (28-<32 weeks), and 84.5% moderate or late preterm (32-<37 weeks) (27). An advanced maternal age, fertility treatments, stress, smoking, and some nutritional deficiencies such as in n-3 polyunsaturated fatty acids (especially docosahexaenoic acid, DHA) are some of the main causes involved (78,105,164,196). Prematurity is one of the main causes of neonatal mortality and morbidity, and the overall societal cost is assumed to be at least 50,000 dollars per year per infant born preterm in USA (27,108,126). The early postnatal life is characterised by wide immaturity of biological functions whose stages of maturation depend on the time of in-utero development. Even for a term birth, and so much more in newborns born prematurely, the intestinal tract is functionally immature and non immunocompetent. The development of the intestine is of primary importance as it is involved in digestion/absorption steps essential for growth, in hosting the gut microbiota for terrestrial survival, and in developing appropriate host immune response to infection in early life.

Just after birth, the gate-keeper function of the intestinal epithelium depends on the quick implementation of different events such as intestinal epithelial cells proliferation and maturation with well-developed tight junction for gut closure (decrease of permeability of the intestinal epithelium), maturation of Peyer's patches (especially induction of T cells and dendritic cells) as well as acquirement of Toll-Like Receptors tolerance (TLR 4 and TLR2, for pathogens recognition) both for operational innate immunity (33,144,234). The time needed to set-up the gut closure and the different processes involved in the protection against pathogens should be short enough for preventing dramatic complications such as septic shock and necrotizing enterocolitis (NEC), a form of intestinal inflammation associated with high mortality, with severe tissue injuries, in premature newborns. This will also impact on programming a future good health of the intestine, but also of other organs, given that dysregulation in these early events, through several bacterial infections, may lead to profound pathophysiological effects such as myocardial dysfunction, acute respiratory failure, and renal failure (29,91). All these phenomena highly depend on the feeding type of the premature infants. Indeed, human milk-fed premature neonates, with continous or bolus feeding, have a more rapid maturation of the intestinal epithelium during the first month of life leading to a quicker gut closure as measured by urinary lactulose/mannitol ratio (45,218). Furthermore, the onset of sepsis and infection as well as of NEC is more reduced in preterm infants fed human milk than preterm infants fed formulas (32,97,146, 207). Therefore, in accordance to the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN), the World Health Organization (WHO) and the

American Academy of Pediatrics (13), fresh own mother's milk is the first choice in preterm infant feeding meaning that strong efforts should be made to promote lactation. When own mother's milk is not available, donor's human milk from established milk-banks following specific safety guidelines is the recommended alternative, the last option being the use of preterm formula.

Because survival and subsequent quality of life in premature infants are largely related to an adequate early nutrition, some specific procedures have been set-up in neonatal intensive care units. Parenteral nutrition is mostly used to provide nutrients immediately after birth but total parenteral nutrition is clearly highly associated with sepsis (37,223). On the other hand, an early full enteral nutrition is not possible because of intestinal digestive immaturity (37,52). A practice of minimal enteral feedings (20 ml milk/kg body weight) instituted at the very first days of life was reported to reduce the incidence of NEC (20). Furthermore, meta-analyses showed that in parenterally-fed infants, the initiation of minimal enteral feedings by 5 days of age was beneficial for regaining weight, for reaching full enteral feedings more rapidly and for hospital discharge (73). In the light of all these data, the nutrition support protocol, generally applied in neonatal units for very premature infants, is the use of parenteral nutrition in parallel to a digestive stimulating phase (or trophic phase) started as soon as possible within the first 5 days of life, with small volumes of human milk from own mother or from milk-bank; the starting volumes are of 10-20 ml/kg/d with an increase of 10-20 ml/kg/d until reaching 60-100 ml/kg/d, then an increase of 20 ml/kg/d until reaching the target enteral volume and the stop of parenteral nutrition (86,96,206). This target enteral feeding volume in a "stable" preterm infant varies from 140-160 ml/kg/d to 161-180 ml/kg/d depending on the country (20,86,124). The milk volume of minimal enteral feeding at start and the further increases of volume as well as the administration schedule (continous feeding of bolus each 2-4 hours) will be defined in accordance with the digestive tolerance and the weight of the premature newborn (96).

In order to give the best chances to premature newborns to develop optimally and to become healthy adults, it is important to understand what are the major bioactive compounds provided by human milk, as well as their minimal effective amounts to better target the intestinal health. The delivery of the right amounts of these bioactive compounds will pursue the programmation, installed by the amniotic fluid within the intrauterine life (234). As milk composition (within a mother and between mothers) is varying a lot, this knowledge can be useful in specific milk fortifiers design, in most appropriate donor's milk selection, and in fully well-adapted preterm formulas development. For this purpose, this review focuses on the bioactive components of human milk that can promote intestinal development and health, and addresses their underlying mechanisms of action.

LIPID-BASED BIOACTIVE COMPOUNDS

Fatty acids

Total lipid content is between 2.0 and 4.2 g per 100 ml in human colostrum, then increases in transitional milk up to 2.5 and 6.0%, and varies from 1.2 to 5.7% in mature milk (87). About 95-99% of total lipids are triglycerides that contain medium chain fatty acids (C8 to C12), long chain fatty acid (C14 to C18) and very long chain fatty acids (C20 to C22) at very variable levels among mothers and depending on whether it is colostrum, transitional or mature milk (87,170,224) (Table 1). Saturated fatty acids account for 36 to 48% of total fatty acids, monounsaturated for 36 to 45%, and polyunsaturated (PUFA) for 12 to 22%. Among these PUFA, human milk is an important source of essential fatty acids i.e. linoleic acid (C18:2 n-6, LA) and α-linolenic acid (C18:3 n-3, ALA), that are precursors of the n-6 and n-3 long chain (LC)-PUFA families, respectively. Human milk is also one of the rare milk of mammals capable of providing indispensable LC-PUFA for the newborn general development, i.e. arachidonic acid (C20:4 n-6, AA) and docosahexaenoic acid (C22:6 n-3, DHA). This has led to the statement of nutritional recommendations for PUFA fatty acid composition in infant formulas in some countries. Updated recommendations especially for preterm newborns in Europe compared to USA are listed in Table 2 (2,8,123).

Fatty acids can act on newborn intestinal health in many different ways. Firstly, some of the fatty acids (caprylic C8:0, lauric C12:0, myristic C14:0, oleic C18:1 n-9, linoleic C18:2 n-6) released from the triglycerides as free fatty acids or as *sn*-2 monoglycerides during the digestion steps, gastric then duodenal, are known to exert anti-microbial activity and to protect the digestive tract against infections (100,106,116,121). These observations were made mainly through *in vitro* studies with purified lipids (100,121) or with human milk or infant formula digested by lipases (100,106), or through *ex-vivo* studies (human milk collected from the stomach of tube-fed babies) (100,116). The degree of protection against infection by these lipids depends on their chain length, their level of unsaturation, the isomers form (*cis* active, *trans* inactive), and their levels generated during digestion (100). Human milk lipids are more highly digested than lipids from formula in premature newborns especially in the stomach (9) suggesting a greater protection from enteral pathogens in premature newborns receiving human milk. But it is possible to increase the antimicrobial properties of infant formulas by adding free fatty acids and *sn*-2 monoglycerides, especially with chain length varying from 8 to 12 carbons as shown *in vitro* (117). The two main mechanisms of action involved are i) the potent detergent effect of these products of lipolysis with considerable lytic activity against enveloped viruses, bacteria and protozoa resulting in the disruption of the lipid bilayer of their plasma membrane and in the inactivation of the micro-organisms (100,116), ii) the inhibition, in a dose-dependent manner, of the replication of the virus within the host cell, by disturbing the assembly of viral components and maturation, and of the release of infectious virus due to a decreased insertion of viral G and M proteins into the plasma membrane (109). Secondly, PUFA (LA and ALA) play a key role in the intestine being precursors of LC-PUFA that are themselves important constituents of cell membrane phospholipid for optimal fluidity and functioning. For instance, an early deficiency in n-6 and n-3 PUFA was shown to lead to a dramatic drop in LC-PUFA in cell membrane phospholipids altering intestinal cells differentiation in post-weanling rats (4). LC-PUFA are involved also in the production of mediators of the inflammatory process for host defense such as eicosanoids (prostaglandins, prostacyclins, thromboxanes, leu-

Table 1. Recent data on main fatty acid composition of human milk in some European countries (France, Spain, Sweden) [1]

Fatty acids	Colostrum			Transitional Milk			Mature Milk		
Ref and n	(87, n = 5)	(170, n = 23)	(224, n = 16)	(87, n = 7)	(170, n = 23)	(224, n = 14)	(87, n = 16)	(170, n = 23)	(224, n = 19)
					% total fatty acids				
Saturated									
Σ SFA	36.2±1.8	35.1±3.3	47.7±0.13	43.1±9.7	39.5±4.6	45.5±0.2	45.4±7.4	38.5±5.4	45.2±0.13
Σ 8:0 /12:0[2]	2.13±1.01	1.48±0.56	-	5.28±3.82	5.16±1.85	-	6.59±4.05	5.07±1.34	-
14:0	4.61±1.11	3.69±0.84	7.85±0.06	6.81±3.26	5.28±1.96	7.05±0.09	8.45±3.39	4.98±1.74	6.92±0.06
16:0	23.60±0.62	22.71±2.08	29.20±0.09	24.43±2.10	21.70±1.66	25.70±0.09	23.09±4.22	21.26±2.79	26.4±0.08
18:0	5.73±1.45	6.21±1.24	6.40±0.25	6.46±0.43	6.45±0.98	6.61±0.03	7.20±1.47	6.39±0.93	6.36±0.18
Monounsaturated									
Σ MUFA	44.6±3.8	43.4±3.8	39.1±0.1	36.4±6.6	41.68±5.21	40.1±0.1	35.9±6.6	42.92±5.34	41.3±0.1
16:1	3.43±0.77	2.04±0.46	-	2.55±0.94	2.25±0.55	-	2.02±0.63	2.38±0.70	-
18:1	40.98±3.22	39.67±3.99	35.70±0.02*	33.77±5.60	37.84±5.15	37.30±0.09*	33.81±6.70	39.11±5.39	37.30±0.47*
24:1 n-9	0.22±0.04	0.32±0.12	0.16±0.00	0.11±0.10	0.08±0.02	0.08±0.00	0.06±0.17	0.05±0.02	0.04±0.00
Polyunsaturated									
Σ PUFA	11.7±3.0	21.5±4.2	13.2±0.1	15.1 ± 5.2	18.8±4.2	14.4±0.1	15.4 ± 5.0	18.5±5.5	13.5±0.1
Σ n-6	9.9±3.0	19.9±4.1	11.3±0.1	12.3±6.0	17.6±4.2	12.4±0.1	11.2±2.6	17.3±5.3	11.6±0.1
18:2 n-6	9.09±3.04	15.95±4.07	9.99±0.11	12.0 ± 4.84	15.17±4.03	11.40±0.09	12.85 ± 4.17	15.24±5.22	10.70±0.06
20:4 n-6	0.71±0.03	0.92±0.19	0.49±0.01	0.67 ± 0.30	0.62±0.11	0.43±0.00	0.44 ± 0.17	0.49±0.08	0.33±0.02
Σ n-3	1.17±0.05	1.37±0.36	1.91±0.03	1.45±0.46	1.16±0.29	1.95±0.02	1.11±0.45	1.18±0.57	1.95±0.02
18:3 n-3	0.42±0.08	0.38±0.17	1.25±0.02	0.51 ± 0.17	0.53±0.18	1.39±0.02	0.95 ± 0.64	0.60±0.25	1.47±0.02
20:5 n-3	0.07±0.01	0.07±0.03	0.10±0.00	0.12 ± 0.07	0.06±0.04	0.11±0.00	0.11 ± 0.17	0.08±0.02	0.11±0.01
22:6 n-3	0.47±0.09	0.55±0.18	0.56±0.01	0.60 ± 0.24	0.41±0.15	0.46±0.00	0.38 ± 0.33	0.35±0.15	0.37±0.01
LA/ALA	21.3±2.4	45.2±13.1	8.0**	26.0 ± 13.3	30.7±10.5	8.2**	16.8 ± 7.1	27.0±8.7	7.3**
AA/DHA	1.5±0.4	1.8±0.6	0.9**	1.1 ± 0.5	1.7±0.6	0.9**	4.5 ± 8.4	1.8±0.7	0.9**
Total n-6/n-3	8.4±3.0	15.4±4.6	6.4±0.02	9.0 ± 3.7	16.1±5.4	6.3±0.02	11.2 ± 6.2	16.5±6.0	5.3±0.02

[1] Values are means ± SD for ref 87 and 170, or ± SEM for ref 224, and are expressed as wt % of total fatty acids. Milk samples are from non-obese mothers who had delivered only full-term newborn (170,224) or term and preterm newborn (87). Mature milk was collected at one month of lactation, and all samples are representative of a whole production (87,170) or represent hind milk (224). AA, arachidonic acid (C20:4 n-6); ALA, α-linolenic acid (C18:3 n-3); DHA, docosahexaenoic acid (C22:6 n-3); LA, linoleic acid (C18:2 n-6); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

[2] Sum of 8:0, 10:0 and 12:0.

* 18:1 n-9 only, sum of 18:1 n-9 + 18:1 n-7 for others.

** Calculated from published means

"-": not indicated in the article cited

Table 2. Updated recommendations for enteral LC-PUFA in preterm infants by Committees in Nutrition

Fatty acids	LA	ALA	LA/ALA	AA	DHA	AA/DHA	EPA	References
ANSES 2011*								(8)
% total fatty acids	6-7	1-1.5	4-6	0.5	0.32	1.5	≤DHA	
% total energy	2.7	0.45		0.2	0.14			
mg/100 Kcal	300	50		22-23	15-16			
ESPGHAN 2010								(2)
% total fatty acids	6.4-25	0.9	5-15	0.3-0.7	0.2-0.5	1-2	≤30% of DHA	
% total energy	3.2-12.6	≥ 0.45		0.15-0.35	0.1-0.24			
mg/100 Kcal	350-1400	≥ 50		16-39	11-27			
mg/kg/d	385-1540	≥ 55		18-42	12-30			
LSRO 2005								(123)
% total fatty acids	8-25	1.75-4	6-16	-	-	-	-	
mg/kg/d	420-1700	90-270		-	-	-	-	

* For a milk or substitute providing 70 kcal and 3.4 g lipids for 100 ml final; "-": no recommendation (citation: "However, LSRO did not find sufficient evidence to support a required minimum concentration of DHA, AA and EPA"). Values for LA/ALA and AA/DHA are ratios. ANSES, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'environnement et du travail (French Agency of Food Safety); ESPGHAN, European Society for Pediatric Gastroenterology Hepatology and Nutrition; LSRO, Life Sciences Research Office for the U.S. Food and Drug Administration (FDA).

kotrienes, lipoxins- from AA, di-homo-gamma-linolenic acid (DGLA), and EPA) or docosanoids (resolvins and the docosatrienes namelly maresins and protectins- from EPA, n-3 DPA (docosapentaenoic acid) and DHA) (157,210,211) (Figure 1). Among these lipid mediators some play a dual-acting anti-inflammatory and pro-resolving role in all mucosal tissues, including the gastrointestinal tract, such as lipoxins (LXA_4, LXB_4 from AA), resolvins (RvE1-3 from EPA, RvD1-6 from DHA, RvD1,2,$5_{n\text{-}3\ DPA}$ from n-3 DPA), protectins (PD1 from DHA, PD1,$2_{n\text{-}3\ DPA}$ from n-3 DPA), and maresins (MaR1 from DHA, MaR1-$3_{n\text{-}3\ DPA}$ from n-3 DPA) (56,211). However, even if the enzymes (desaturases and elongases) necessary to biosynthesize LC-PUFA, especially AA and DHA from dietary LA and ALA, respectively, are available in newborn (42,230), the requirement in these two LC-PUFA for optimal development is so important, and even more in premature newborn, that it cannot be fully covered if preformed AA and DHA are not provided directly (optimal ratio AA/DHA between 1/1-1.3/1). In addition, due to a strong competition between LA and ALA for elongation and desaturation, a balanced LC-PUFA biosynthesis of both families is only possible for a dietary LA/ALA ratio of 2/1-4/1 (26). This ratio is often much higher in human milk due to low levels of ALA (less than 0.9-4% of total fatty acids compared to recommendations for enteral formula) (87,170) (Table 1), that will lead to a biosynthesis in favour of LC-PUFA n-6 to the detriment of LC-PUFA n-3. A balance between LC-PUFA n-6 and LC-PUFA n-3 is necessary for a good maturation and functioning of intestinal cells in general, and for modulation of immune and inflammatory responses more specifically. Indeed, a formula containing PUFA (11.3% LA and 1.29% ALA, as % of total fatty acids) and enriched in n-6 and n-3 LC-PUFA (0.35% AA and 0.26% DHA, as % of total fatty acids), compared to the formula providing only PUFA, enhanced the recovery of damaged small intestine in neonatal piglets by promoting cell growth and a higher maturation rate, by normalizing the lipid and fatty acid composition of jejunum and reducing the histological alteration (143). Furthermore, some LC-PUFA, especially AA, EPA and DHA enhance intestinal barrier integrity quickly (determined by transepithelial resistance) by improving resistance and limitating the interleukin IL-4 mediated permeability as shown via *in vitro* studies conducted on human intestinal epithelial cell monolayers (T84) (239). These effects are dependent on a good lipolysis rate of triglycerides providing these LC-PUFA, and are associated with the incorporation of these efficient LC-PUFA into the cellular membrane phospholipids. In addition, AA and DHA contribute to the development and the function of the gastrointestinal mucosal immune system (especially T cells) enhancing the ability of the premature infants to fight against pathogens (58). Indeed, a proliferation and maturation of memory T cells and an increase in cytokines production by peripheral lymphocytes (especially IL-10) was shown at the first 42 days of life in the neonate fed human milk or formula enriched in AA (0.6%) and DHA (0.4%) compared to standard premature infant formula containing no LC-PUFA (82). Furthermore, a balanced LC-PUFA supplementation via infant formula (34 mg AA and 23 mg DHA / 100 ml, AA/DHA of 1.5/1.0) reduced the incidence of intestinal necrosis in a rat model of NEC, mainly by reducing the metabolism of platelet-activating factor (PAF) (down regulation of PAF production by decrease in phospholipase A2-II synthesis, and of PAF receptor synthesis) thus moderating the inflammatory responses mediated by PAF responsible of the pathophysiology of NEC, and leading to a decreased plasma endotoxemia (40).

The main current problem nowadays is the levels of n-3 PUFA in human milk, which leads to debates on the optimal levels, especially for DHA. From the data collected of a total of 84 studies (2974 subjects), the mean (± SD) of DHA level in human milk obtained after term delivery was 0.32 ± 0.22% of total fatty acids (median: 0.26%) while average AA concentrations, 0.47 ± 0.13% (median: 0.46%), ranged much less compared to DHA (34). It is thought that milk from mothers who delivered prematurely has higher levels of DHA and AA in order to better match the requirements of the premature newborn, but only two studies on six confirmed this point for colostrum and only one of these two for mature milk (28,170). The ranges of DHA and AA average levels in preterm mature milk were

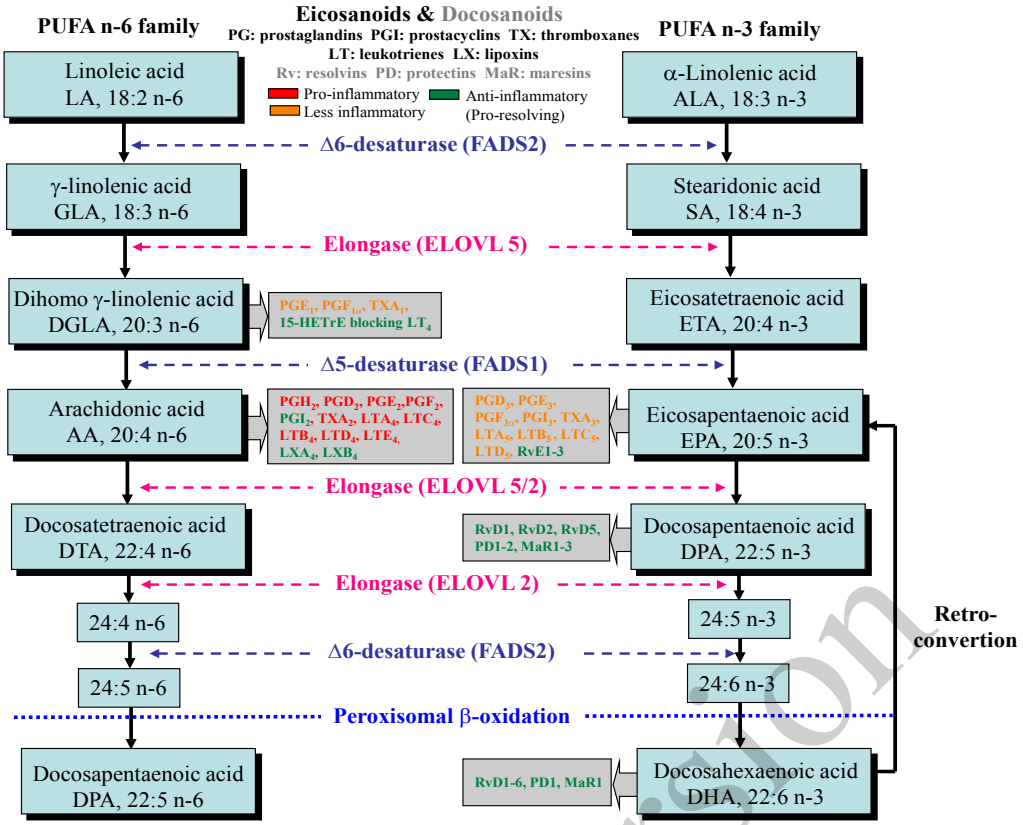


Figure 1. Schema of LC-PUFA biosynthesis in humans and related lipid mediators.
ELOVL: Elongation of very long chain fatty acids genes family coding for elongases; **FADS:** Fatty acid desaturase genes family coding for desaturases.

reported to be 0.22-0.55% and 0.44-0.69% of total fatty acids, respectively (131). DHA level in human milk is rather linked to the amount of DHA consumed by the mothers (14,34,87) whereas AA level is mostly associated to the maternal adipose tissue (storage of AA, or of LA that will be converted to AA) (14,34), and both are dependent on maternal polymorphisms of FADSs genes (132,169,241). Pooled milk from donors, provided by lactarium, are often administrated to premature neonates when own mother's milk is not available, and their levels of DHA can be either similar to own mother's milk (mean ± SEM: 0.33 ± 0.09 versus 0.39 ± 0.23% of total fatty acids, respectively) (86,131) or even much lower (31) according to the donor's milk arbitrarily chosen (Table 3). Considering the average level of DHA in milk (about 0.4% DHA) together with the tolerable volumes of milk administrated by enteral route (10 to 160 ml/kg/d) during the first month of life (86), and the poor to null supply of PUFA via the parenteral route, the high needs in DHA of premature newborn (about 55 to 70 mg/kg/d) (115,131) can not be reached during the early period of life (86,87,130). While the need for n-6 PUFA could be easier reached (552 mg/kg/d i.e. 150 ml milk/kg/d at 3.7% lipids and at least 10% total fatty acids as LA + AA), the need for DHA (i.e. 55-67 mg/kg/d) will be less easily reachable if the milk does not contain 1.2% of total fatty acids as DHA (given a 150 ml milk/kg/d and 3.7% total lipids in milk).

Human milk contains also branched chain fatty acids (BCFA), characterised by one or more methyl branches mostly at the ultimate or penultimate carbon (mainly *iso* C14:0, *anteiso* C15:0, *iso* C16:0, *anteiso* C17:0), that account for about 0.74 or 0.84% of total fatty acids in colostrum or mature milk, respectively (90), i.e. representing an amount of about 52 mg/d for 170 ml of milk in premature infants. It was recently reported that oral administration of BCFA in artificially fed neonatal rat pups model (enriched formula, about 170 mg BCFA/day) reduced by 56% the incidence of NEC through incorporation into ileal phospholipids compared to control (no enriched formula), by promoting the establishment of commensal BCFA-containing bacteria (*B. subtilis*), and by increasing the level of anti-inflammatory cytokine interleukin-10 (197). But interestingly, the dam-fed rat pups developed no episode of NEC, presumably because of all the other bioactive molecules present in milk.

Table 3. Recent data on main fatty acid composition of human milk from lactarium[1]

Fatty acids	Mature donor milk
	% of total fatty acids
Σ Saturated Fatty Acids	47.27 ± 1.47
Σ Monounsaturated Fatty Acids	37.84 ± 1.02
Σ Polyunsaturated Fatty Acids	13.59 ± 0.83
Σ PUFA n-6	12.32 ± 0.77
18:2 n-6 (LA)	11.27 ± 0.72
20:4 n-6 (AA)	0.40 ± 0.03
Σ PUFA n-3	1.28 ± 0.10
18:3 n-3 (ALA)	0.83 ± 0.08
20:5 n-3 (EPA)	0.07 ± 0.02
22:6 n-3 (DHA)	0.24 ± 0.03
LA/ALA	13.64 ± 1.00
AA/DHA	1.65 ± 0.15
Total n-6/n-3	9.65 ± 0.58

[1] Values are means of means ± SD of data published from 8 French cities (n = 145 mature milk samples collected in 2007; calculated from ref 31). AA, arachidonic acid (C20:4 n-6); ALA, α-linolenic acid (C18:3 n-3); DHA, docosahexaenoic acid (C22:6 n-3); LA, linoleic acid (C18:2 n-6); PUFA, polyunsaturated fatty acids.

Glycerophospholipids and sphingolipids (polar lipids)

Milk phospholipids, mainly composed by glycerophospholipids and sphingomyelin (SM), and sphingolipids such as SM and gangliosides, are constituents of the milk fat globule membrane (MFGM) enveloping the triglyceride core of the globule. Phospholipids show a specific localization depending on their class: SM and phosphatidylcholine (PC) in the outer layer, and phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) in the inner layer (Figure 2A). The milk phospholipid fingerprint is specific to each mammalian species (88) and shows remarkable differences in human milk compared to cow milk mainly due to a higher level of SM and ethanolamine-plasmalogens (EPLAS), a class of glycerophospholipids that contains one vinyl-ether linked long chain alcohol group in place of one ester-linked fatty acid chain with a polar head group composed of ethanolamine (Figure 2A, NMR spectra). Interestingly, it was reported that the mature milk of Japanese mothers who delivered prematurely compared to mothers who delivered at term was richer in SM (43±8% total phospholipids versus 35±5%) and poorer in PC (23±4% versus 27±6%) (216). To note that formula dedicated to premature newborns will exhibit a different fat globule organization, and phospholipids composition and amount, depending on the lipid sources used by the industry (lecithins from soybean or egg, half-skimmed or skim cow milk concentrates) (Figure 2B). In addition, human milk phospholipids are peculiarly rich in LC-PUFA, AA (from 0.81 to 8.96% of total fatty acids) and DHA (from 0.93 to 4.34% of total fatty acids) (87). Whereas the concentration of phospholipids in human milk is very low (1-1.5% of total milk lipids) in comparison to triglycerides, the contribution of phospholipids to cover the requirements of DHA and AA for babies is around 20% and 10% respectively, and perhaps even more if one considers that the fatty acids of phospholipids are not known to be used in β-oxidation to produce energy (87).

Moreover, phospholipids provided by human milk may have a substantial role in human newborn intestinal health. Indeed, the addition of egg phospholipids (75% PC) to an experimental preterm formula (9g/100 g of total lipids) lowered the incidence of NEC in premature infants, in comparison to a classic formula (no LC-PUFA, 1% soy lecithins), by providing esterified choline at a much higher concentration than in mature human milk (i.e. 585 mg/L versus 158 mg/L), AA (0.4% of total fatty acids) and DHA (0.13% of total fatty acids) (41). The authors explained these beneficial effects in part by the fact that i) choline is essential to produce acetylcholine which is important for intestinal vasodilation and motility and that the esterified form of choline as PC has a higher bioavailability than free choline, and ii) prostaglandins E1 derived from AA is able to counteract the reduction in mesenteric blood flow and ameliorate the bowel injury induced by PAF. Orally administered PC in rats, at the dose of 100 mg/kg of body weight, seem to act directly on the mucosa to prevent gut permeability disturbances due to lipopolysaccharides (LPS, endotoxin of the outer membrane of gram-negative bacteria) (62).

SM, that is another main important polar lipid in milk, representing 20 to 29% of total phospholipids and ranging 18-113 µg/ml milk depending on milk specie (88), has a

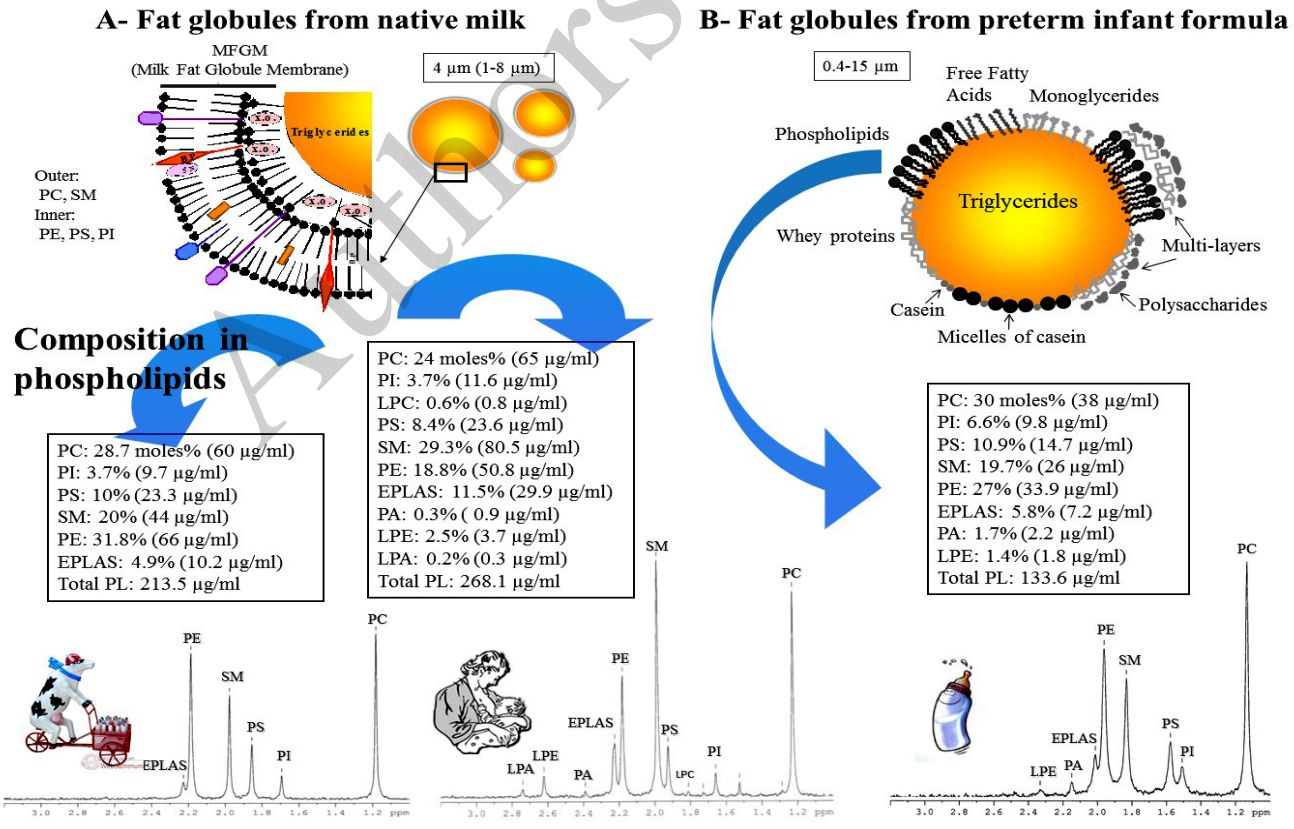


Figure 2. Cow and human milk fat globule organisation (A) compared to artificial fat globule from preterm infant formula (B), and fingerprint of phospholipids (^{31}P NMR spectra). Adapted from (12,88). EPLAS: ethanolamine-plasmalogens or plasmenylethanolamines; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PL: phospholipids; PS: phosphatidylserine; SM: sphingomyelin.

protective effect against bile salt-induced cytotoxicity and damages on intestinal cells *in vitro* (Moschetta et al. 2000). Furthermore, SM possibly accelerates intestinal development as it was reported in artificially reared rats that SM stimulates crypt cells differentiation and epithelial cells maturation at the top of the microvilli (173) at the daily-ingested doses of 50-150 mg SM. This amount is however not reached in very premature newborn during the first month of enteral feeding (3-27 mg/d) (86).

Gangliosides, that are molecules composed of a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acids (e.g. n-acetylneuraminic acid, NANA) linked on the sugar chain, may intervene in intestinal health by incorporation within the mucosa and thus restoring proper structure and function of this organ (166). Although the content and species of gangliosides vary with lactation, GD3 (N-acetylneuraminyl-2-galactosylglucosyl ceramide) is more predominant in colostrum (6.45±3.02 µg of sialic acid/ml), and GM3 (N-Acetyl-α-Neuraminidate-Gal-Glc-ceramide or monosialodihexosylganglioside) is the main form in mature milk (about 1.35 µg of sialic acid/ml) (231). GD3, at the concentration of 15 µg/ml formula, is able to decrease the incidence and histopathologic severity of NEC in newborn rat pups by augmenting mucosal regulatory immune responses (243). GM3 seems necessary for ensuring intestinal integrity and was shown to prevent the LPS-stimulated decrease in tight junctions between enterocytes in rats (166). In addition, the provision of dietary glycosphingolipids having antibacterial properties, especially GM1, GD3, GM3 and lactosylceramide, can prevent infection by direct binding to enterotoxigenic bacterias, by inhibiting toxin production and infectivity of several intestinal pathogens, thereby attenuating NFkB inflammatory signaling pathways (166, 179).

The role of plasmalogens in intestine development and protection is not documented, but these molecules could also play an indirect role by protecting milk LC-PUFA against peroxidation due to antioxidant properties (180, 244). A high correlation was found between the levels of plasmalogens and AA (rho = 0.63, $p = 0.019$) or DHA (rho = 0.70, $p = 0.009$) in human milk (87). Indeed, lipid hydroperoxides such as malondialdehyde, 4-hydroxyhexenal from DHA and 4-hydroxynonenal from AA, can be formed during the digestion steps of milk lipid containing LC-PUFA in the stomach and within the intestinal lumen. They will induce oxidative stress and inflammation in the upper intestine (15), in parallel to a decrease in the absorption rate of LC-PUFA (148) and thus of their bioavailability and further health benefits. Recently, a beneficial relationship between the amount of ingested ethanolamine-plasmalogens from human milk in very preterm neonates during the first month of enteral feeding and intestinal outcomes (less duration to reach complete enteral feedings, less gastrointestinal disorders, less infections) was reported in an abstract (89).

Cholesterol

Mammalians require cholesterol for normal physicochemical and functional properties of cell membranes, which will be covered both by endogenous biosynthesis and foods. Human milk provides substantial amount of cholesterol i.e. about 9-56 mg/100 ml (87) up to 150 mg/100 ml (178), in contrast to milk substitutes that usually contain less than 2 mg/100 ml. While the importance of dietary cholesterol in the intestinal development of human neonate is not elucidated yet, few studies conducted in animals highlight its role. Dietary cholesterol deprivation in neonatal piglets (2 mg versus 145 mg/100 ml of formula) was shown to alter the small intestinal epithelium biophysical properties with an increase in the fluidity of the microvillus membrane, a decrease in lactase activity, and a greater permeability possibly indicative of a delay in gut closure process (178). Interestingly, a synergistic effect of dietary LC-PUFA (AA: 0.33% of total fatty acids, DHA: 0.26% of total fatty acids), phospholipids (2.53% of formula) and cholesterol (0.1% of formula) was reported on the recovery of damaged proximal intestinal mucosa visible through a histological approach in neonatal piglets (143).

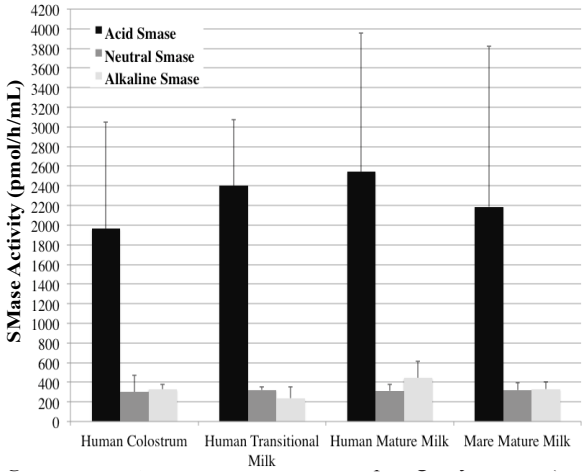
ROLE OF LIPOLYTIC ENZYMES FOUND IN HUMAN MILK

Sphingomyelinase

For an infant, milk is the only exogenous source of sphingomyelin (SM) that may have important roles in the development of the intestinal tract. Beneficial action of SM is linked to its hydrolysis into ceramide then in sphingosine (68). This is possible due to the presence of significant amounts early at birth of key enzymes in the intestinal tract of preterm newborns such as, respectively, endogenous alkaline sphingomyelinase (alk-SMase), whose activity is much higher than either acid or neutral SMase, and ceramidase (CDase) (64,66,67,140). The presence of SMase and CDase during digestion, especially in the neonatal period, is critical for the provision of SM-based lipids from food sources for cell membrane development and sphingosine-based cell signalling processes (68). Lacking of alk-SMase in mice was shown to result in sharp accumulation of SM and decreased formation of ceramide in the intestinal content and feces (246). In addition, human milk displays a SMase activity that is mainly acid rather than neutral or alkaline (Figure 3). This acid SMase activity found in human milk (ranging 304-5442 pmol/ml/h), but also in mare milk (ranging 302-2306 pmol/ml/h), varies highly between individuals (Figure 4), with no clear relationship with the amount of SM. To note that acid SMase level seems to decrease after pasteurization of human milk meaning that newborn receiving donor milk from lactarium will miss part of this milk enzyme (86). Milk SM digestion into ceramide plus phosphorylcholine might start in the stomach of premature babies, where pH ranges 5 to 5.5 over 1 hour after feeding (9), catalysed by the milk acid SMase, and will continue more importantly in the small intestine with the help of endogenous alkaline SMase (67). It was reported that about half of the SM amount ingested from one human milk feeding can theoretically be hydrolysed by the milk acid SMase in the newborn stomach within 1 hour (182). The final metabolite, sphingosine, has also been shown to display antibacterial effects mainly by attracting bacteria and by disrupting their membrane integrity (68,193).

Bile salt-stimulated lipase

The presence of a lipase stimulated by bile salts (BSSL) within breast milk, which is produced by lactating mammary glands, was originally discovered in the milk of humans and various other primates, and has since been found in the milk of many animals including dogs, cats, rats and



tivities in milk. Data are means ± SD from n = 5 (Human Colostrum and Human Transitional Milk), n = 8 (Human Mature Milk), or n = 6 (Mare Mature Milk) different samples. The activities of different types of SMase were analyzed as previously described (65) at pH 5.0, 7.5 and 9.0 for acid, neutral and alkaline SMase, respectively, after incubation at 37°C for 30 min with radioactive [^{14}C] sphingomyelin.

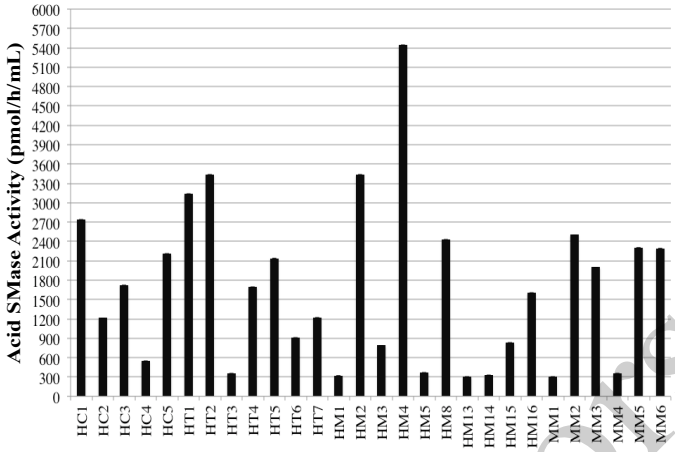


Figure 4. Acid sphingomyelinase (SMase) activity in milk. Data are means ± SD (n = 2 measurements) of individual values from n = 5 (Human Colostrum, HC), n = 7 (Human Transitional Milk, HTM), n = 10 (Human Mature Milk, HM), or n = 6 (Mare Mature Milk, MM) samples. The activity of acid SMase was analyzed as previously described (65) at pH 5.0 after incubation at 37°C for 30 min with radioactive [^{14}C] sphingomyelin.

rabbits (227), but not in cows, mares, ewes and goats. The BSSL provided by human milk, which exists in the form of different variants due to mother's genetic polymorphisms (225), plays different key roles in the newborn period.

Firstly, BSSL plays an important role in milk lipid digestion. Indeed, while the stomach digestive step of milk lipids is fully active in premature infants thanks to the gastric lipase that starts triglyceride hydrolysis (9), the intestinal digestive step is more critical due to a transient pancreatic insufficiency characterised by a low secretion of pancreatic lipases such as colipase-dependent triglyceride lipase currently called pancreatic lipase, carboxyl-esterlipase (CEL equivalent to BSSL), and phospholipase A2 (81). Intestinal milk lipid digestion in neonates is thus ensured by compensatory lipases to other lipolytic enzymes i.e. pancreatic lipase-related protein 2 (PLRP2) that seems mainly expressed during the neonatal period, and BSSL provided by human native milk at about 100 to 200 mg per liter of milk (7,9,21,120,225). This latter enzyme is naturally acid resistant (stays intact after passing through the stomach) and acts in the newborn intestine where the bile salts concentration is appropriate for its activity (2 to 3.5 mM). BSSL displays broad substrate specificity and is able to hydrolyze triglycerides, even containing LC-PUFA in addition to phospholipids, esterified cholesterol and esters of lipid-soluble vitamins (81,107). BSSL activity levels measured on triolein varie in milk samples from 12 to 52 U/mL (Figure 5) with no link to the length of pregnancy (9,83). By contributing to the triglyceride digestion into free fatty acids and *sn-2*-monoglycerides, and to phospholipid lipolysis into lysophospholipids, that are necessary for an efficient intestinal absorption rate of the end-lipolysis products (191), BSSL avoids intestinal epithelium disruption that can be caused by undigested lipids and/or accumulation of some non absorbed lipolysis products as shown in mice neonates (113). To note that BSSL is fully inactivated by pasteurization explaining why the activity of BSSL in milk from lactarium is very low-to-null (0-0.3U/mL) (9), and is not present in infant formula, leading to negative consequences on lipid absorption and growth in premature newborns (6). To solve these problems and improve lipid digestion in newborns, several options are available such as i) search for alternative treatment methods for donor human milk with, for example, the Ultraviolet-C Irradiation that was recently shown to protect BSSL (50); ii) production of human recombinant BSSL (175) that can be beneficially added at a dose physiologically found in human milk (0.15 g/L) (kiobrina from Biovitrum), in preterm formula (ClinicalTrials.gov NCT00658905) or in pasteurized breast milk (ClinicalTrials.gov NCT00659243); after positive safety and tolerability assessments and promising results on growth velocity and improvement of absorption rate of DHA and AA, a phase III clinical trials is under progress (149); iii) possible use of specific phospholipid types for constituting the surface of the lipid droplet (Figure 1) to highly enhance the BSSL activity for producing highly digestable infant formulas (11).

Secondly, BSSL displays a ceramidase activity and transformes, in a synergetic action with CDases from the pancreas and the intestinal brush border, one to two third of the ceramide, provided by the breakdown of milk SM by SMases, into sphingosine and free fatty acid (182).

Thirdly, BSSL displays an antiviral activity contributing to prevent diarrhea in newborns. The highest glycosylated form of BSSL (130 KDa) is able to inhibit the Norwalk virus ligand-binding to gut mucosa specific receptors by

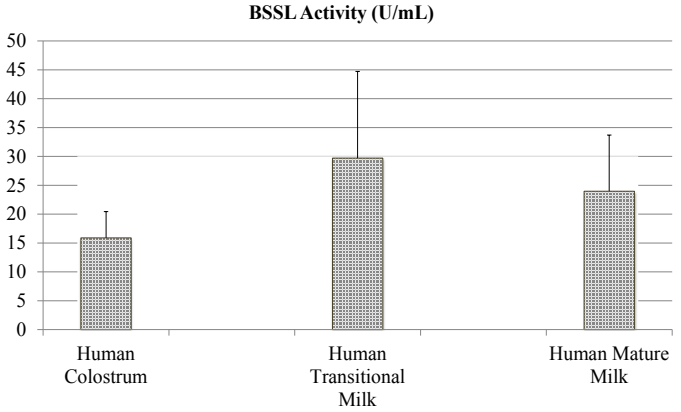


Figure 5. Bile Salt-Stimulated Lipase (BSSL) activity in human milk. Data are means ± SD from n = 5 (Colostrum and Transitional milk) or n = 9 (mature milk) samples. One unit is defined as one µmole of free fatty acid released per minute from triolein emulsion as previously published (9).

direct interaction of the enzyme glycosylated moities to virus, and thus appears as one of most effective decoy receptors found in milk (202).

IMPACT OF GROWTH FACTORS AND HORMONES

Human milk contains also a substantial number of growth factors and hormones that can highly impact on intestinal development (Table 4).

Growth factors and insulin

Human milk is a very important source of growth factors such as insulin-like growth factors (IGF-I and IGF-II, 7.5-7.65 kDa) (75,76,93,167,186,226), epidermal growth factor (EGF, 6 kDa) (44,71,93,127,181,185) and insulin (213,214,215). IGF-I and EGF are particularly high in colostrum (76,181,185,226) while insulin seems to be provided at constant levels in colostrum, transitional and mature milk (213,214). In addition, their receptors have been found at high levels in the gut of term and preterm newborns (60,72,163), suggesting a key role of such molecules during development. Indeed, exposure to IGF-I, or IGF-II in one study, leads to the proliferation of intestinal cells, and to increases in mucosal growth, enterocyte migration rates, villus height, brush border enzymes activity, and expression of glucose transporters in newborns piglets or rat pups (110,111,147,192,242). To note that the oral doses used were much higher than physiological amounts that could be ingested by human premature neonates (0.2-2 µg/ml of formula or 1 µg of recombinant human IGF-I daily for 3 days, or 200 µg/kg body weight/d, compared to 0.1 or 0.68 µg of IGF-I plausibly provided by 45 or 170 ml of human milk) in four of these studies (110,111,147,247), but was much plausible for one study (about 400-500 ng/d) (192). Interestingly, premature human neonates receiving during the first month of life a formula supplemented in IGF-I (extracted from cow milk whey fraction) at a concentration twice that found in colostrum (i.e. 100 µg/L) showed a lower permeability of the intestine compared to control at 14 days (54). Oral administration of EGF through formula at different concentrations (1.2 to 6 µg/ml) enhances intestinal growth (weight, length) and functional development (DNA content) in rat pups (19). Mice KO for EGF receptor have impaired gut development and enhanced risk of NEC occurrence (165), while EGF supplementation through formula (concentration 500 ng/ml, intake of 425 ng/d) decreases incidence and severity of inducted NEC in neonatal rats by acting at the site of damaged intestinal mucosa via an increase in EGF receptors (70) and a reduction of cells autophagy (160). Considering the fact that the rat pups intake of formula should be about 1 ml/d, the efficient doses of EGF through all these rat studies (425 ng to 6 µg/d) are in accordance with the amounts of EGF provided by mother's milk to the human premature neonates (about 1.82 to 6.89 µg/d for 45 to 170 ml of milk per day (86) at the average concentration of 40.53 ng/ml). This highly suggests that human milk EGF plausibly plays a key role in intestinal epithelium cell homeostasis through proliferation, differentiation and maturation of enterocytes, balance of apoptosis and autophagy processes, as well as it contributes to epithelial protection from injury and post-injury mucosal repair (72,160) in human neonates. Insulin provided by human milk influences also small intestinal development and maturation. Insulin used at the concentration of 3 mU/ml on cultured explants from human jejunum was shown to stimulate the proliferation of epithelial cells (163), but the concentration used was about 60 fold higher than in human colostrum (see Table 4). Oral feeding of milk insulin increases ileal mucosa mass and some disaccharidases activity (lactase and maltase) in newborn piglets at the high dose of 85 mU/ml (217), and reduces intestinal permeability and macromolecules penetration through the intestinal barrier in adrenalectomised suckling rats (102). More recently in an ongoing study, the addition of insulin to preterm infant formula results in a better growth and an accelerated intestinal maturation (212). Insulin may also protect from bacterial infections as shown in sucking mice treated during 7 days with E. Coli heat-stable endotoxin, and receiving orally 5 to 50 µg of insulin per day (5). Indeed, insulin administration increased the differentiation of intestinal brush border membrane and modified its structure/function in order to better respond to the infectious agent through upregulation of a specific endotoxin receptor (5).

Adipokines

Human milk contains several adipokines such as leptin and adiponectin (Table 4), and also ghrelin (ranges: 260-6000 pg/ml in whole milk) (16,69,122,203), obestatin (median (IQR): 846.6 (472.1) pg/ml, or 538.9 ± 46 pg/ml in colostrum and 528.5±39 pg/ml in mature milk) (17,203), and resistin (1710±68 pg/ml in colostrum or 670±18 pg/ml in 3-6 months mature milk, or median (IQR): 0.18 (0.44) ng/ml) (114,204). Among these adipokines, only leptin and adiponectin seem to demonstrate effects on gut maturation, and their receptors were found expressed in the small intestine of neonatal piglets (240) and mice (247), parallely to the presence of both hormones in the milk of these species (240). Human milk leptin concentrations exhibit a large dispersion from 0.16 to 105 ng/ml (Table 4) (17,22,35,43,63,112,153,162,168,199,204,220,238). Milk adiponectin concentrations highly vary from 1.25 to 90 ng/ml (Table 4) (35,69,153,162,205). Adiponectin is present as its different isoforms in human milk (trimeric low molecular weight, hexameric medium molecular weight and high molecular weight of large multimers of 12-18 subunits) but the most abundant form is the higher length one. Leptin and adiponectin concentrations in milk diminish through lactation, are positively correlated with the mother's BMI postpregnancy but are not linked to gender of the infants (35,112,153). More specifically, milk leptin concentrations are significantly higher in term milk compared to preterm milk (22,35,162), while milk adiponectin concentrations only tend to be higher in preterm milk compared to term milk (162) and exhibit variability due to ethnicity (lower in mexican women versus non-hispanic whites) (153). Neonatal piglets fed formula (leptin: 20.3 ± 2.5 ng/ml) showed a limited growth of their small intestine and a slower maturation of intestinal mucosa, regarding morphometry, cell proliferation and enterocytes brush border membrane activities, compared to piglets fed sow milk containing leptin (colostrum: about 63 ng/ml; transitional milk: 25.5 ± 2.5 ng/ml) (240). This was reversed, giving an intestinal development feature close to the one obtained with sow milk, by intragastric administration of exogenous leptin in formula fed animals but at the highest dose tested (about 50 µg leptin/d versus about 10 µg/d)

Table 4. Growth factors and hormones from human milk potentially involved in intestinal maturation and health[1]

Proteins	References	Time postpartum	Methods of measurement	n	Gestational age	Concentrations	Values ranges
IGF-I	(226)	13 ± 6 hrs	RIA on skim milk	15	Term	49 ± 17 µg/L	
		48 ± 20 hrs	RIA on skim milk	21	Term	29 ± 27 µg/L	
	(76)	1 d	RIA on skim milk	8	Term	52 ± 2.3 ng/ml	
		3 d	RIA on skim milk	8	Term	8.6 ± 3.0 ng/ml	
		4-8 d	RIA on skim milk	8	Term	3-6 ng/ml	
	(75)	7 d	RIA on skim milk	30	24-31 wks	2.8 ± 0.2 ng/ml	
		21 d	RIA on skim milk	19	24-31 wks	2.6 ± 0.2 ng/ml	
		7 d	RIA on skim milk	19	37-41 wks	2.3 ± 0.1 ng/ml	
		21 d	RIA on skim milk	16	37-41 wks	1.9 ± 0.1 ng/ml	
	(167)	3-18 d	RIA on skim milk	23	Term	3 ng/ml	
	(93)	5-46 d	RIA on skim milk	51	T and PT	2.16 ng/ml	0.26-14.14 ng/ml
	(186)	3 d	RIA on skim milk	29	Term	0.19 ng/ml	IQR: 0.09-0.35
		7 d	RIA on skim milk	29	Term	0.12 ng/ml	IQR: 0.06-0.26
		28 d	RIA on skim milk	29	Term	0.31 ng/ml	IQR: 0.16-0.49
		3 d	RIA on skim milk	29	Preterm	2.31 ng/ml	IQR: 1.49-3.07
		7 d	RIA on skim milk	29	Preterm	3.58 ng/ml	IQR: 2.47-4.70
		28 d	RIA on skim milk	29	Preterm	4.19 ng/ml	IQR: 3.12-5.70
IGF-II	(76)	1 d	RIA on skim milk	3	Term	10.5 ± 8.5 ng/ml	
		6 d	RIA on skim milk	3	Term	35 ± 21 ng/ml	
EGF	(71)	1-4 wks	RIA on skim milk	16	23-27 wks	7.9 ± 0.9 µg/ g protein	135-165 µg/ml
		1-4 wks	RIA on skim milk	16	32-36 wks	4.8 ± 0.5 µg/ g protein	62-110 µg/ml
		1-4 wks	RIA on skim milk	15	38-42 wks	4.9 ± 0.5 µg/ g protein	75-105 µg/ml
	(185)	1-5 d	ELISA on skim milk	20	22-31 wks	159.24 ± 51.05 ng/ml	
		1-5 d	ELISA on skim milk	28	32-36 wks	102.03 ± 55.43 ng/ml	
		1-5 d	ELISA on skim milk	33	Term	95.75 ± 48.58 ng/ml	
	(127)	> 3 wks	ELISA on skim milk	60	Term	129.01 ± 35.5 pg/dl	
	(93)	5-46 d	ELISA on skim milk	35	T and PT	40.53 ng/ml	13.5-78 ng/ml
	(44)	3 d	ELISA on skim milk	22	Term		70-90 µg/L
		10 d	ELISA on skim milk	22	Term		30 µg/L
		30 d	ELISA on skim milk	22	Term		25 µg/L
		3 d	ELISA on skim milk	10	Preterm		100-180 µg/L
		10 d	ELISA on skim milk	10	Preterm		50 µg/L
		30 d	ELISA on skim milk	10	Preterm		40 µg/L
		3 d	ELISA on skim milk	10	VPT		80-120 µg/L
		10 d	ELISA on skim milk	10	VPT		60 µg/L
		30 d	ELISA on skim milk	10	VPT		35 µg/L
	(181)	1-3 d	ELISA on skim milk	31	Term	131.6 ± 20.4 ng/ml	
		5 d	ELISA on skim milk	23	Term	35.9 ± 2.2 ng/ml	
		30 d	ELISA on skim milk	23	Term	24.7 ± 6.3 ng/ml	
Insulin	(213)	3 d-1 mo	RIA on skim milk	42	Term	60.23 ± 41.05 µU/ml	6.5-305.7 µU/ml
	(214)	3 d	RIA on skim milk	90	30-41 wks	50.1 ± 34.6 µU/ml	7-179 µU/ml
		10 d	RIA on skim milk	90	30-41 wks	41.1 ± 28.5 µU/ml	12-183 µU/ml
Leptin	(43)	1-5 d	WB on skim milk	34	-	1.35 ± 0.16 µg/L	0.35-4.6 µg/L
	(112)	-	RIA on skim milk	23	-	1.5 ± 0.87 ng/ml	0.5-5 ng/ml
		-	RIA on whole sonicated milk	23	-	10.1 ± 2.6 ng/ml	0.0-60 ng/ml
	(220)	-	RIA on skim milk	4	Term		1.1-1.45 ng/ml
		-	RIA on whole sonicated milk	4	Term	73.22 ± 39.03 ng/ml	45-105 ng/ml
		-	RIA on skim sonicated milk	4	Term		1.05-1.25 ng/ml
	(199)	14 d	RIA on lipolysed whole milk	11	23-34 wks	6.02 ± 8.97 ng/ml	
		28 d	RIA on lipolysed whole milk	11	23-34 wks	5.18 ± 4.96 ng/ml	
	(22)	2-3 d	RIA on whole sonicated milk	24	Term	1.34 ± 0.14 ng/ml	0.5-2.5 ng/ml
		4-5 d	RIA on whole sonicated milk	24	Term	0.92 ± 0.12 ng/ml	0.4-1.8 ng/ml
		4-6 wks	RIA on whole sonicated milk	24	Term		0.4-2 ng/ml
		2-3 d	RIA on whole sonicated milk	9	Preterm	0.63 ± 0.18 ng/ml	0.25-1.5 ng/ml
		4-5 d	RIA on whole sonicated milk	9	Preterm	0.46 ± 0.10 ng/ml	0.25-1 ng/ml
		4-6 wks	RIA on whole sonicated milk	9	Preterm		0.25-1.25 ng/ml
	(153)	1-7 mo	RIA on skim milk	30	Term	0.4 ng/ml	0.2-2.25 ng/ml
	(35)	2 d	ELISA on skim milk	56	Term	0.5 ± 0.05 µg/L	0-1.37 µg/L
		2 d	ELISA on skim milk	8	Preterm	0.3 ± 0.09 µg/L	
		2 d	ELISA on skim milk	48	Term	0.6 ± 0.05 µg/L	
	(238)	42 d	ELISA on skim milk	766	32-41 wks	0.28 ± 0.38 ng/ml	0-1.37 ng/ml
	(168)	1 mo	ELISA on whole milk	28	Term	0.156 ± 0.039 ng/ml	0-0.853 ng/ml
	(17)	2 d	EIA on skim milk	31	Term	2.01 ± 0.34 ng/ml	
		25 d	EIA on skim milk	15	Term	2.04 ± 0.67 ng/ml	
	(63)	1 d	RIA on skim milk	15	Term	5.69 ± 4.58 ng/ml	1.5-15.4 ng/ml
		21-30 d	RIA on skim milk	15	Term	23.84 ± 17.79 ng/ml	2.6-46.3 ng/ml
	(162)	6-8 d	ELISA on skim milk	15	24-31 wks		3-7.5 ng/ml
		6-8 d	ELISA on skim milk	5	Term		7-9 ng/ml
		13-15 d	ELISA on hind skim milk	15	24-31 wks		3-7 ng/ml
		13-15 d	ELISA on hind skim milk	5	Term		6-8 ng/ml
		20-22 d	ELISA on hind skim milk	15	24-31 wks		2-6 ng/ml
		20-22 d	ELISA on hind skim milk	5	Term		5-7 ng/ml
		27-29 d	ELISA on hind skim milk	15	24-31 wks		2-5 ng/ml
		27-29 d	ELISA on hind skim milk	5	Term		4-6 ng/ml
	(204)	< 6 mo	RIA on skim milk	23	Term	2.34 ng/ml	IQR: 5.73 ng/ml
Adiponectin	(153)	26-42 d	RIA on skim milk	19	Term	19.8 ng/ml (non-hispanic)	9-78.6 ng/ml
		31-40 d	RIA on skim milk	37	Term	11.7 ng/ml (hispanic)	6.2-39.8 ng/ml
		1wk-7 mo	RIA on skim milk	199	Term	17.7 ng/ml	4.2-87.9 ng/ml
	(35)	2 d	ELISA on whole milk	59	Term	13.7 ± 0.8 µg/L	3.9-30.4 µg/L
	(69)	1d	ELISA on whole milk	25	Term	29.5 ± 6.4 ng/ml	1.26-77.1 ng/ml
	(162)	6-8 d	ELISA on skim milk	15	24-31 wks		13-25 ng/ml
		6-8 d	ELISA on skim milk	5	Term		7.5-15 ng/ml
		13-15 d	ELISA on skim milk	15	24-31 wks		10-20 ng/ml
		13-15 d	ELISA on skim milk	5	Term		7.5-14.5 ng/ml
		20-22 d	ELISA on skim milk	15	24-31 wks		7.5-17.5 ng/ml
		20-22 d	ELISA on skim milk	5	Term		2.5-10 ng/ml
		27-29 d	ELISA on skim milk	15	24-31 wks		2.5-12.5 ng/ml
		27-29 d	ELISA on skim milk	5	Term		1.25-7.5ng/ml
	(205)	< 6 mo	ELISA on skim milk	46	Term	9.99 ng/ml	3.59-20.52 ng/ml

[1]Data are means ± SEM or SD, or median values or ranges; " - ": not indicated in the article cited; EGF: epidermal growth factor; EIA: enzyme immuno assay; ELISA: enzyme-linked immunosorbent assay; IGF: insulin-like growth factor; IQR: Interquartile range; RIA: radio-immuno assay; WB: western blot.
T: Term (37-42 weeks of gestation); PT: moderate or late Preterm (32-<37 weeks of gestation); VPT: Very preterm (28-<32 weeks of gestation).

that is also higher than the amount provided by sow milk (by calculation 12.5 µg to 25.5 µg leptin /d assuming a plausible ingestion of 500 ml to 1 L of sow milk) (240). This suggests that milk of the specie is more efficient due to a synergetic action of several bioactive compounds sharing trophic properties (leptin together with EGF for example). Moreover, the oral supplementation with 50 µg of leptin per day (10 µg/kg body weight each 8 hours) reduced cell apoptosis/autophagy and increased the mitosis-to-apoptosis ratio samely as sow milk in neonatal piglets (92). This is of peculiar interest because while in the adult apoptosis/autophagy represent processes necessary for a quick turnover of the gut cells, such phenomena have to be slowed down transiently during the suckling period for contributing to a rapid phase of intestinal growth. Fewer studies have been conducted with adiponectin, and showed that full-lenght adiponectin stimulates the growth of intestinal cells (HT29) *in vitro* (183).

WHEY PROTEINS AND PROTEINS FROM THE MFGM

Other proteins from human milk playing a key role in intestinal health of the newborn are located only in the aqueous fraction of human milk (such as lactoferrin) or only in the milk fat globule membrane (such as MUC-1, MUC-4, lactadherin), or partition between both fractions (sCD14, sIgA) (195, 209). Their respective levels in human milk are listed in Table 5.

Lactoferrin

Lactoferrin is a multifunctional whey iron-binding glycoprotein (80 kDa) which concentration is higher in human colostrum (ranging about 2.3-7.2 g/L) (94,135) then diminishes in mature milk over lactation (1-2 g/L) (94,171). Cow milk contains much less lactoferrin compared to human milk (1-2 g/L in cow colostrum and 0.01-0.1 g/L in mature cow milk). Preterm milk is about 1.6-fold richer in lactoferrin compared to term milk at least during the first 3 months of lactation (95,162). Considering the intake of 45 ml to 170 ml/d of own mother's fresh milk through the first month of postnatal life in very premature infants (86), the amount of lactoferrin ingested is comprised between 112 and 425 mg/d. In case of the use of donor milk, amounts will range 25-94 mg/d resulting from both a lower concentration in mature term milk and a loss of 63% of this protein during the pasteurization process (3). Lactoferrin resists in part to proteolysis through the infant's digestive tract (47,59,138) and is thus able to act in the gastrointestinal lumen. Lactoferrin plays a key role in the intestinal health of neonates through several mechanisms of action. Firstly, it can facilitate intestinal epithelium development, as suggested by *in vitro* studies on cell lines, by directly promoting enterocytes proliferation in a dose-dependant manner (from 0.1 µg/ml to 1 mg/ml), and also differentiation with maximum effects for a lower range of concentrations (1 or 100 ng/ml) (36,138). Secondly, lactoferrin exerts a broad-spectrum of primary defense activity by bacteriostatic and bactericidal action against bacteria, fungi, protozoa and viruses (232). Indeed, because lactoferrin is a strong and stable chelator of iron, it can compete efficiently with most of the bacterial siderophores and limitates the growth of a large broad of bacteria. Lactoferrin, or derived-peptides, can also directly bind to Gram- and Gram+ bacteria or to fungi that will destabilize/modify their cell wall, destroying them or preventing them to interact with the host cells. Lactoferrin helps also to fight against bacterial and viral infections by exerting a competition for binding to the different specific microbes receptors (GAGs and integrins as for examples) onto gut mucosa and thus preventing pathogens adherence and internalization into cells. Finally, lactoferrin is also able to recognize pathogen-associated molecular patterns (PAMPs), such as LPS, and in the same time to interact with immunologic system activating the innate immunity (most particularly macrophages, neutrophils, basophils, esosinophils, mastocytes and NK Cells) or adaptive immunity (B and T-cells, dendritic cells), either as a pro-inflammatory or an anti-inflammatory agent (74,136). Interestingly, a randomized clinical trial in very-low-birth-weight premature neonates is under progress to investigate the efficiency of an oral supplementation from birth until 30-45 days of life with cow milk lactoferrin (100 mg/d, GRAS status), that shares a high peptide homology with the human counterpart, alone or in association with probiotics (*Lactobacillus rhamnosus GG,* 6 x 10^9 CFU/g), to prevent late-onset sepsis (150). Preliminary results showed that both treatments compared to placebo were able to reduce the incidence rates of overall sepsis, the incidence and severity of NEC, and the rate of mortality (150). Furthermore, lactoferrin-derived peptides formed during partial digestion were shown to promote the growth of beneficial bacteria (like lactobacillus and bifidobacteria) *in vitro* (139). Data are more controversial in term infants studies showing no effect on faecal flora of formula containing bovine lactoferrin at the concentration of 10 mg/100 ml (200), or of 280 mg/100 ml administrated for 2 weeks (18), while a formula at a concentration of 100 mg/100 ml allowed the establishement of a « bifidus flora », comparable to the one of breastfed infants, in half of the infants studies at the age of 3 months (200).

Immunoglobulins

Among the immunoglobulins found in human milk (IgA, IgM, IgG), the secretory immunoglobulins A (sIgA, 75-95 kDa) are the most abundant antibodies and vary from 0.25 to 11.5 g/L (44,94,95,162,171). The sIgA concentration is generally higher in preterm milk through lactation compared to term milk (44,95,162), and is very high in colostrum (from 6.5 to 11.5 g/L in preterm and from 2 to 8 g/L in term) then diminishes in mature milk (0.4 to 2 g/L in preterm and 0.4 to 1 g/L in term) (44,94,95,162). Nevertheless, The sIgA concentration in mature milk is highly variable but not directly linked to the lactation stage as it can go up and down (from 1.0±0.3 mg/ml to 0.5±0.1 mg/ml) throughout lactation (94). This seems to be explained by immunologic pathways, linking the mother's gut and respiratory tract to the mammary gland, which might operate throughout lactation to provide antibodies protecting the infant against enteric and respiratory tract pathogens from the mother (94,101). The amount of sIgA provided by human milk to premature newborns is relatively high being about 22-90 mg to 85-340 mg/d considering a daily volume of administrated milk of 45 ml to 170 ml through the first month of enteral feeding (86), and could be of great interest to ensure local Ig-related gastrointestinal tract protection against viruses and bacteria due to its stability under digestion steps (47,101). Indeed, exogenous sIgA were shown to bind enteric pathogens and their tox-

Table 5. Bioactive whey and MFGM proteins from human milk potentially involved in intestinal maturation and health[1]

Proteins	References	Time postpartum	Methods of measurement	n	Gestational age	Concentrations	Values ranges
Lactoferrin	(94)	2-3 d	Electroimmunodiffusion	10	Term	5.3 ± 1.9 mg/ml	
		4-52 wks	Electroimmunodiffusion	11-4	Term	1.9 ± 0.3 – 0.8 ± 0.4 mg/ml	
	(95)	2-12 wks	Electroimmunodiffusion	8	Preterm (32 wks)	2-3 mg/ml	
		2-12 wks	Electroimmunodiffusion	13	Term	1-2 mg/ml	
	(171)	15-225 d	MEN-Immunoassay whole milk	497	-	2.2 g/L	IQR: 0.5-5.1 g/L
	(39)	8 mo	Immunobiosensor	2	-	98.5 ppm or 1.23 µM	
	(135)	5 d	Electrophoresis (WF)	14	Term	2.27 ± 0.19 mg/ml WF (Japan)	
		5 d	Electrophoresis (WF)	15	Term	2.64 ± 0.19 mg/ml WF (Thai)	
	(162)	6-8 d	EIA on skim milk	15	24-31 wks		400-600 µg/ml
		6-8 d	EIA on skim milk	5	Term		700-900 µg/ml
		13-15 d	EIA on hind skim milk	15	24-31 wks		225-400 µg/ml
		13-15 d	EIA on hind skim milk	5	Term		400-600 µg/ml
		20-22 d	EIA on hind skim milk	15	24-31 wks		225-400 µg/ml
		20-22 d	EIA on hind skim milk	5	Term		300-500 µg/ml
		27-29 d	EIA on hind skim milk	15	24-31 wks		80-225 µg/ml
		27-29 d	EIA on hind skim milk	5	Term		100-300 µg/ml
sIgAs	(94)	2-3 d	Fluo. immunoabsorbent assay	10	Term	2.0 ± 2.5 mg/ml	
		4-52 wks	Fluo. immunoabsorbent assay	11-4	Term	1.0 ± 0.3 – 0.5 ± 0.1 mg/ml	
	(95)	2-12 wks	Fluo. immunoabsorbent assay	8	Preterm (32 wks)	1-2 g/L	
		2-12 wks	Fluo. immunoabsorbent assay	13	Term	1 g/L	
	(171)	15-225 d	MEN-Immunoassay whole milk	495	-	1.2 g/L	IQR: 0.2-4.9 g/L
	(44)	3 d	ELISA on skim milk	22	Term		5-8 g/L
		10 d	ELISA on skim milk	22	Term		1 g/L
		30 d	ELISA on skim milk	22	Term		0.5 g/L
		3 d	ELISA on skim milk	10	Preterm		6.5-11.5 g/L
		10 d	ELISA on skim milk	10	Preterm		1 g/L
		30 d	ELISA on skim milk	10	Preterm		0.5 g/L
		3 d	ELISA on skim milk	10	VPT		1.5-3.5 g/L
		10 d	ELISA on skim milk	10	VPT		1 g/L
		30 d	ELISA on skim milk	10	VPT		0.5 g/L
	(162)	6-8 d	ELISA on skim milk	15	24-31 wks		600-1000 µg/ml
		6-8 d	ELISA on skim milk	5	Term		400-800 µg/ml
		13-15 d	ELISA on hind skim milk	15	24-31 wks		450-900 µg/ml
		13-15 d	ELISA on hind skim milk	5	Term		400-800 µg/ml
		20-22 d	ELISA on hind skim milk	15	24-31 wks		400-800 µg/ml
		20-22 d	ELISA on hind skim milk	5	Term		400-800 µg/ml
		27-29 d	ELISA on hind skim milk	15	24-31 wks		500-900 µg/ml
		27-29 d	ELISA on hind skim milk	5	Term		400-800 µg/ml
sCD14	(128)	all	Western Blot	22	-	52.9 ± 24 µg/ml	
		<6 d	Western Blot	10	-	67.09 ± 27.61 µg/ml	
		>7 d	Western Blot	12	-	41.12 ± 11.91 µg/ml	
		5 d	ELISA	10	-	20.1 ± 8.74 µg/ml	
		1 mo	ELISA	10	-	12.06 ± 4.77 µg/ml	
		3 mo	ELISA	10	-	12.16 ± 3.75 µg/ml	
		6 mo	ELISA	10	-	15.05 ± 4.08 µg/ml	
	(233)	all	ELISA	40	-	14.84 ± 6.39 µg/ml	
		<6 d	ELISA	10	-	20.1 ± 8.74 µg/ml	
		>8 d	ELISA	30	-	13.09 ± 4.31 µg/ml	
	(25)	6-9 d	ELISA	15	Term	29.5 ± 15.4 µg/ml	
		12-16 d	ELISA	15	Term	25.1 ± 11.9 µg/ml	
		26-32 d	ELISA	15	Term	22.3 ± 14 µg/ml	
	(129)	3 mo	ELISA	38	Term	9 µg/ml	IQR: 7.1-11.8
	(51)	1-2 d	ELISA	43	Term	26.23 µg/ml	95% CI: 22-31
		1 mo	ELISA	44	Term	5.08 µg/ml	95% CI: 4.2-6.1
MUC-1	(187)	-		-	Term	40 µg/ml	
	(190)	<15 d	RIA (Mc5) on whole milk	15	Preterm	913 ± 131 µg/ml	130-2500 µg/ml
		<15 d	RIA (BrE3) on whole milk	15	Preterm	932 ± 135 µg/ml	347-2940 µg/ml
		>15-90 d	RIA (Mc5) on whole milk	26	Preterm	554 ± 52 µg/ml	94-1240 µg/ml
		>15-90 d	RIA (BrE3) on whole milk	26	Preterm	643 ± 73 µg/ml	53-1530 µg/ml
Lactadherin	(190)	<15 d	RIA (Mc3) on whole milk	15	Preterm	139 ± 17 µg/ml	37-333 µg/ml
		>15-90 d	RIA (Mc3) on whole milk	26	Preterm	66 ± 5 µg/ml	28-126 µg/ml
	(179)	1 wk-mo	RIA on whole milk	31	Term	26.2-48.4 µg/ml	5.6-180 µg/ml

[1]Data are means ± SEM or SD, or median values or ranges; " - ": not indicated in the article cited; CI: confidence interval; EIA: enzyme immuno assay; ELISA: enzyme-linked immunosorbent assay; IQR: Interquartile range; MEN-Immunoassays: microparticle-enhanced nephelometric immunoassays; MUC-1: Mucin-1; RIA: radio-immuno assay; sCD14: soluble cluster of differentiation 14; WF: whey fraction.
T: Term (37-42 weeks of gestation); PT: moderate or late Preterm (32-<37 weeks of gestation); VPT: Very preterm (28-<32 weeks of gestation).

ins, thus preventing their adherence to intestinal cells, and to limit the bacterial translocation through the gut mucosa in baby rabbits (6.25 mg/kg/d of human sIgA) and mice models (103,159). This is of peculiar importance given the low levels of circulating antibodies in preterm newborns. Nevertheless, a limitation in sIgAs action can be due to the fact that immunoglobulins are heat-sensitive, and partially denaturated during pasteurization of own mother milk (necessary when pathogens have been detected) or of donor milk that will be delivered to very premature infants (3), depriving them of these protective molecules.

Interestingly, the total IgA concentrations in milk from mothers supplemented with *Bifidobacterium lactis* (9x 10^9 CFU/day), starting at 2-5 weeks before delivery and continuing for 6 months after, were higher compared to not supplemented women (about 67% more IgA in transitional milk at 1 week, and about 8-fold higher level at 3 months) (194).

sCD14 and sTLR2

Soluble forms of Toll-like receptors (sTLR2, 83-, 70-, 66-, 40-, and 38 kDa) and of the TLR's co-receptor CD14 (bacterial pattern recognition receptor sCD14, cluster of differentiation 14, 48 kDa) are both present in human milk. They may contribute to immune programming through the set-up of transient activation and subsequent downregulation of intestinal TLR2 and TLR4, involved in microbial recognition with the help of a membrane co-receptor (mCD14). Because the activation of such TLRs leads to the production of a variety of proinflammatory molecules, the set-up of this system has to be done in a tightly regulated way to prevent unremitting or excessive inflammation in the face of microbial exposure (133,134,233). The sCD14 is present in human milk at much higher concentrations (9-67 µg/ml) than in serum (3-4 µg/ml) that suggests a strong health impact in breast-fed newborns and infants (25,51,128,129,233). The levels of sTLR2 in human milk mirrore those of sCD14 suggesting that these molecules act synergetically (133). Milk sCD14 resists in part to proteolysis in the digestive tract by forming a complex with α-lactalbumin and it is thus able to display bioactive effect in the intestine (25,221). Nevertheless, it has to be noted that milk sCD14 is very sensitive to heat treatment and was reported to be not detectable in pasteurized human milk from lactarium (86). In the light of studies conducted on fetal intestinal epithelial cells and on animal models, it is suggested that premature newborns are highly sensitive to NEC by developing inappropriate strong inflammatory response to intestinal microorganisms (presenting a high density of antigens from a wide spectrum of species) because their intestinal cells, unlike adult intestine, expressed very high levels of TLR4 and TLR2 being thus hyperresponsive to LPS (1,119,161,177). The milk sCD14 might counteract the lack of enterocytes mCD14, by forming a complex with LPS that will activate the neonatal TLR2 and TLR4 for cytokines production (128,133,233). In order to avoid an excessive local inflammation, sTRL2 can intervene as a modulator by interacting directly with sCD14 or with cellular TLR2 (forming dimers) or by binding to the microbial components recognized by TLR2 acting as a milk decoy receptor, thus diminishing the activation of the TLRs (133).

MUC and lactadherin

Among the major proteins located in the MFGM (195), some have been shown to play a key role in intestinal health, especially the mucin MUC-1 and the lactadherin, by conferring protection against bacteria (such as Salmonella, E. Coli) (142,208) and viruses (poxvirus, rotavirus, Norwalk) (99,187,245). Both glycoproteins concentrations are very variable between mothers and are higher in early milk (< 15 days postpartum) than in later milk (15-90 days postpartum) (190) (concentrations in Table 5). They are proteins exhibiting resistance to degradation in the stomach of premature newborn (190), probably due to a high degree of glycosylation together with a pH preventing optimal pepsin activity (pH 5-6), and also to degradation in the intestine totally or in part (187).

MUC-1 is a large (400 kDa) transmembrane highly O-glycosylated (50%) protein (100,187). MUC-4 was also reported in human milk (142,202). Their large oligosaccharide part, exhibiting substantial sialic acid at their termini, accounts for binding to specific gut receptors recognized by pathogens (galectin 3, as example) or for binding directly certain pathogenic microorganisms, through different glycosylated motifs within the proteins (fucosylated glycans, or mannose or neuraminic acid motifs, as examples) (142,202,208). MUC-1 is a more effective competitive inhibitor of Salmonella binding to receptors on the epithelium than MUC-4 (IC 50 7-8 µg/L versus 33-39 µg/L) (142). This difference may be explained by carbohydrates composition but also by different peptide motifs that could account also for the biological activity (142). Human milk mucin fraction is agglutinated by S-fimbriated E. coli via direct interaction through neuraminic-acid of the glycoprotein (208). MUC-1 is able to aggregate poxviruses prior to their entry into host cells preventing their effects (99). MUC-1 and MUC-4 act as decoy receptors in milk and inhibit the attachement of viruses to their glycan receptors on gut epithelium, such as Norwalk virus that binds to a 2-linked fucosylated glycan as receptors (202). Deglycosylation of the mucin complex results in the loss of antiviral activity on rotavirus (245).

The lactadherin (homologue to PAS 6/7 or MFG-E8) is a N-glycosylated 46-53 KDa protein containing a RDG (ArgGlyAsp)-cell adhesion sequence that binds to integrin and a C-terminal sequence containing a phosphatidylserine-binding site (198). Lactadherin plays two different roles in intestinal health as it contributes to inhibit pathogens binding and infectivity in addition to repair intestinal mucosa. Lactadherin inhibits the ability of different rotavirus strains, responsible for much of the diarrhea in infants worldwilde, to infect intestinal cells *in vitro* via a dose-dependant manner; the highest inhibition rates are obtained for a concentration of 2.5 g/L (50-90%) and lower inhibition rates for a physiological concentration of 0.25 g/L (10-60%) (179). The removal of sialic acid from the protein conducts to the loss of the antiviral property suggesting that lactadherin inhibits the binding of rotavirus to host cell glycoprotein receptor that contains also a terminal sialic acid. As MUC, lactadherin acts as a decoy receptor in milk. The variation of the concentration of lactadherin in milk is of clinical relevance for rotavirus infection as it was reported that infants receiving milk samples containing about 48 µg lactadherin per ml (range 5.6-180 µg/ml) kept asymptomatic unlike to infants receiving milk with lower lactadherin concentrations (median: 29 µg/ml, range: 6.2-

103.4 µg/ml) (179). Exogenous lactadherin accelerates the rate of migration of stem cells from the intestinal crypts onto the villus surface possibly by binding to a patch of exposed phosphatidylserine at the posterior region of the migrating cells (198). This phenomenon contributes to the gut cells turnover. Furthermore, in mice model, the endogenous lactadherin expression is dramatically reduced following sepsis reducing the enterocyte migration from the crypts to the villi, and the administration of exogenous lactadherin restores crypt cell migration (198).

PROBIOTICS & PREBIOTICS

Human milk is a symbiotic food because it contains probiotics and prebiotics. Human milk probiotics are mainly composed of *Lactobacillus* (*fermentum*, *salivarius*, *plantarum*, *gasseri*), *Bifidobacterium* (*longum*, *bifidum*, *breve*, *adolescentis*, *dentium*, *animalis*, *catenulatum*), *Streptococcus* and *Staphylococcus* spp. (98,152,154,155,156,188). Their level, from 10^3 to 10^5 per ml of milk, their composition and diversity, are highly variable within and between mothers (151,155,188) due to the lactation stage and metabolic status (less bifidobacteria, more lactobacillus and reduced microbial gene richness in mature milk from obese mothers) (38). For more detailed information on this topic, we suggest to the readers to consult recent reviews (10,80). Probiotics are transferred from the mother's gut to the mammary gland through intestinal translocation and transport via leucocytes (188). Human milk probiotics will contribute to intestinal health and maturation of immunity in neonates by several different ways. The more general way is due to their major implication in the development of the microbiota in the early life through their high abilities to survive in the gastrointestinal environment (152). More specifically, some microorganisms interact with specific apical surface receptors on the enterocytes; this interaction triggers a response that prevents an overexpression of inflammatory cytokines, thus providing protection from pathogen-induced mucosal damage (176). Also, probiotics are able to produce antimicrobial compounds against pathogenic bacteria (bacteriocins such as defensins, hydrogen peroxide, lactic acid), and they will ferment carbohydrates (lactose, prebiotics) helping themselves to proliferate (increase of the mass of microbiota) and producing some of the antimicrobial molecules, and short chain fatty acids, especially butyric acid for a good development of the intestinal cells (10,104,152). One main problem in preterm neonates-feeding practice when milk from donors is used is that such milk is submitted to pasteurization to destroy potential pathogens (such as cytomegalovirus), which eliminates at the same time the favourable bacteria. In addition, intestinal microbiota development can be delayed by comparison to neonates born at term due to antibiotherapy in intensive care units (79,237). It was reported that premature neonates fed native own mother's milk have less risk of developing NEC by comparison to the neonates fed pasteurised milk from milk bank or formula (207). These data highly suggest that another trigger for the intestinal inflammation in preterm neonates could be the quality of their microbiota. It was thus suggested that the administration of certain probiotics might be beneficial for preventing or treating NEC. Randomized clinical trials conducted in premature newborns have shown that the administration of *Lactobacillus Rhamnosus GG* (ATCC 53103) for 7 days reduced the incidence of NEC (57), and that the administration of *Bifidobacterium breve* or mixtures of probiotics (*Bifidobacterium infantis* + *Lactobacillus acidophilus* or *Bifidobacterium infantis* + *Streptococcus thermophilus* + *Bifidobacterium bifidum*) since birth reduced incidence and severity and/or mortality by NEC (23,141,176). Furthermore, the administration of three species of bifidobacteria (*B. breve* M-16V, *Bifidobacterium longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536; 5×10^8 CFU of each strain) resulted in reduced rates of clostridium through the earlier formation of a bifidobacteria-predominant fecal microbiota and maintenance of this microbiota (118). Recently, it was shown *in vitro* that the probiotic *Lactobacillus acidophilus* counteracts PAF-induced inflammation cascade in intestinal epithelial cells models (30). Interestingly, as another example of synergetic action between components of human milk, it was shown that LC-PUFA enhance the adhesion of probiotics to mucosal surfaces augmenting their health promoting effects (58); thus, a combination of LC-PUFA and probiotics could offer an additional protection.

Concerning prebiotics, human milk contains mostly indigestible oligosaccharides (HMOs) that represent the third most abundant component reaching concentrations of 11-23 g/L in colostrum and in transitional milk, and 9-20 g/L in mature milk compared to about less than 1g/L in cow milk (53,85). The molecular structure of these prebiotics, synthesized by the mammary gland from the elongation of lactose, is highly variable (more than 100 different molecular structures) and more complex than other mammalian milk (61,179). The main molecules involved are glucose, galactose, N-acetylglucosamine, fucose and sialic acid. Almost all HMOs consist of a lactose core that can be conjugated, in more complex HMOs, with repeats of lacto-N-biose (β 1-3 linkage) or with N-acetyllactosamine (β 1-6 linkage) that can be further fucosylated, via the action of fucosyltransferases, or sialylated at the terminal positions (85,179). HMOs vary quantitatively and qualitatively depending especially on lactation period, mother's lewis blood group (related to four basic phenotypic HMOs groups through fucosyltransferases genes expression) and on gestational duration (preterm versus term milk) (61,85,179,219,228). Human milk oligosaccharides will protect the intestine in neonates by different mechanisms. Firstly, due to their prebiotic capability, HMOs are able to stimulate the growth of specific beneficial commensal bacteria species such as bifidobacteria preferentially (53), and thus promote the establishment of a beneficial bifidus-predominant microbiota well characteristic of breastfed infants in the early life (*Bifidobacterium breve* in 70% of cases, *B. longum* subsp. *infantis* in 41%, *B. longum* subsp. *longum in* 37%, *B. bifidum* in 22%) (158). Indeed, it was shown *in vitro* that some bifidobacteria are genetically adapted to substantially (*B. bifidum PRL2010, B. longum* subsp. *Infantis ATCC 15697*) or moderately (*B. breve*) grow using HMOs as substrates with some specific preference of the constituents to be fermented (glucosamine, fucose and sialic acid for *B. breve* strains and *B. infantis*) (201,229,235). These bifidobacteria grown on HMOs will enhance the intestinal barrier through the production of short chain fatty acids such as acetate having trophic and anti-inflammatory effects on the gut (84), and via the expression of tight junction proteins decreasing epithelial permeability as shown in animal model or cell lines

(49,77). Several clinical studies in healthy term infants have shown that formulas enriched in an oligosaccharides mixture composed of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (0.8 g/100 ml) affect the early microbiota composition and activity in a similar manner than human milk (184). Furthermore, fucosylated HMOs function as soluble receptor analogs competing for bacterial binding to the epithelial cell surface glycans of the intestinal mucosa, preventing the adhesion and translocation of pathogens; thus they can stop the action of bacteria-induced diarrhea, of stable toxin of *Escherichia coli* which stimulates the production of cyclic GMP by binding to the extracellular domain of cells guanylate cyclase producing futher diarrhea, or of enteric viruses (179). Milk specimens containing higher levels of α-2-linked fucosyloligosaccharides compared to α-3/4-linked ones seem more effective (179).

MICRO RNA

MicroRNAs (miRNAs), small noncoding RNAs of 19-24 nucleotides that play a key role in regulating the gene expression in various biological processes by targeting mRNAs, were recently identified in human milk (125,174,236,248) and also in cow milk (48). They are carried within breast milk exosomes or microvesicles of 30-300 nm in size (248) or even by milk fat globules (174). While the biological function is still unclear, it has been postulated that they represent a way of communication between the mother and the infant via the digestive tract route. Among the 429-602 miRNAs found in human milk (236,248), a considerable number are immune-related miRNAs and are highly expressed especially in the first 6 months of lactation (125, 248). Milk miRNAs are very stable during storage and multiple freeze-thaw of milk, to RNase digestion, and to very acidic conditions probably protected by their lipidic vehicles (125, 248). This suggests that they can reach intact the intestinal lumen, can be absorbed and act as innate immune-regulatory agents to protect the newborn from various infections by microbes through intestinal homeostasis and mucosal immunity. The miRNAs that could be especially involved are miR-125, the Let-7 family, miR-146 along with miR-132 and miR-155 which downregulate cellular LPS sensitivity and prevent excessive inflammation, miR-212 along with miR-146 and miR-132 that are implicated in the establishment and maintenance of postnatal TLR tolerance, and miR-21 which enhances the production of cytokine IL-10 decreasing the pro-inflammatory response (174,222,236). The let-7 family is also associated with cell differentiation and development (222). Recently, a potential role for miR-200b was reported in maintaining intact the intestinal epithelium through the inhibition of epithelial mesechymal transition and in promoting intestinal epithelial cells (48). Finally, miR-375 is a key regulator of intestinal epithelial properties, that are necessary for securing epithelium-immune system cross-talk, and of intestinal goblet cells differentiation (24). The miR-584, that is present in mature milk but not in colostrum (236), is involved in the post-transcriptional expression of lactoferrin receptor in the small intestinal cells during the perinatal period (137,138) and might contribute by this way to the beneficial effect of milk lactoferrin. To note that all the listed miRNAs above, except miR-584, were also reported in cow milk (48). The expression pattern of human milk miRNAs is very variable between individuals due to mother's living environment such as diet and probably genetics, metabolic status (obese versus lean), and during stages of lactation for the same mother (125,174).

CONCLUSION & FUTURE PROSPECTS

To summarize, it is now well established that human milk is a complex fluid with multifunctional roles within the developing gastrointestinal tract. It is the unique nature's first functional food for the neonate to establish and maintain intestinal cells homeostasis, allowing digestion/absorption, gut barrier function, and the development of immune tolerance, as well as to contribute highly to the set-up of the microbiota and to the protection against pathogens indispensable for extra-uterine survival. Indeed, among all the numerous biological molecules found in human milk, some exhibit powerful trophic properties, or are part of the intestinal cells membrane, others educate the innate immune system to recognize and respond appropriately to bacteria components, leading to the emerging concept that human milk influences the neonatal immune system's perception of « danger », while many of them display direct fighting against pathogens, or are dedicated to the establishment of the gut microbiota (Table 6). Interestingly, many of the bioactive compounds of human milk are multifunctional agents. Most of these biological actions are mediated via interaction with specific receptors present at the intestinal mucosa. For some of these bioactive molecules, the high potential interest for intestinal health early in life has been reported by clinical studies in newborns with enriched infant formulas. This is the case for the immune benefits of LC-PUFA (AA: 0.6%, DHA: 0.4%) on T-cell development and function (82), for the protection against NEC of LC-PUFA-phosphatidylcholines (41), for the benefit of recombinant BSSL on intestinal absorption rate of lipids (149), for the faster decrease in gut permeability by IGF-I (54), for the protective effects against sepsis and NEC of lactoferrin (150), for the protection of probiotics against clostridium invasion (118) and against NEC (23,57,141,176), and for the prebiotic ability of oligosaccharides (53,184). The intestinal health interest of other bioactive molecules in premature newborns has been pointed-out through human observational studies as for plasmalogens (89) and for lactadherin regarding its protection against rotavirus infection (179). For the rest of the molecules cited in this review, clinical trials still have to be conducted. Most importantly, clinical trials taking into account a mixture of most of all the bioactive molecules, at concentrations close to human milk, should be envisaged, as some synergy of action between compounds have been revealed or are higly suggested through the literature cited in this review.

In intensive neonatal care units, the use of own mother's milk is promoted to feed premature infants but this practice is not always possible due to the difficulty encountered by some mothers to produce milk in this extremely hard emotional time of having a premature baby. The use of donor's milk from milk-bank is the second option but the composition of such milk is not always well adapted to the premature neonate requirements in term of biological molecules. Firstly, because it can be mature term milk of several months, and the concentration of many bioactive

Table 6. Synthesis of the plausible intestinal effects of the main bioactive molecules from human milk.

Bioactive agents	Trophic effect	Cell membranes	Innate microbial recognition	Gut closure	T cells proliferation/ maturation/ activation	Fight against pathogens	Microbiota establishment
Lipids							
Fatty acids (C8, C12, C14, C18:1, C18:2 n-6)						X	
sn-2 monoglycerides (C8-C12)						X	
PUFA (LA)		X					
LC-PUFA (AA, DHA)	X	X		X	X		X
Branched chain fatty acids							X
Phosphatidylcholine		X		X			
Sphingomyelin	X	X				X	
Gangliosides		X		X		X	
Plasmalogens		X					
Cholesterol		X		X			
Enzymes							
SMase						X	
BSSL				X		X	
Growth factors & Hormones							
IGF	X			X			
EGF	X						
Insulin	X			X		X	
Leptin	X						
Adiponectin	X						
Whey & MFGM proteins							
Lactoferrin	X				X	X	X
sIgA						X	
sCD14			X				
sTLR2			X				
MUC-1						X	
Lactadherin	X					X	
Others							
Probiotics	X			X		X	X
Prebiotics	X			X		X	X
miRNA	X		X		X		

AA: arachidonic acid (C20:4 n-6); BSSL: Bile Salt-Stimulated Lipase; DHA: docosahexaenoic acid (C22:6 n-3); EGF: epidermal growth factor; IGF: Insulin-like growth factor; LA: linoleic acid (C18:2 n-6); LC-PUFA: long-chain polyunsaturated fatty acids; MFGM: Milk Fat Globule Membrane; MUC-1: Mucin-1; PUFA: polyunsaturated fatty acids; sCD14: soluble cluster of differentiation 14; sIgA: secretory immunoglobulins A; SMase: sphingomyelinase; sTLR2: soluble Toll-Like receptor 2.

compounds diminished during the lactation period or/and are different in preterm milk compared to term milk. Secondly, because donor's milk is most often heat-treated by pasteurization to eliminate possible pathogens that will destroy at the same time, partly or fully, some of the bioactive proteins. For instance, the regular pasteurization process applied in milk bank (i.e. 62.5-63°C for 30 min or 70°C for 20 min, cycle of 1.5 to 2.5 hours) destroys 100% BSSL, about 100% of sCD14, probiotics, 34% of IgA, 100% of IgM, 63% of lactoferrin, 64% of leptin, 30% of acid SMase, 39% of IGF-I and 10% of IGF-II (3,9,86,93,199). Alternative technological processes are currently investigated in order to preserve as far as possible the precious bioactive molecules from human milk, and some of them seem promising. The flash pasteurization, recommended in developing countries to prevent HIV transmission from milk (72.9°C during a short time then quick cooling), preserves 80% IgA and 70% IgG (46). A quick pasteurization (62.5°C during 5 seconds) preserves fully the amount of IGF-I and IGF-II (93). The high-pressure processing (no more than 400 MPa at 12°C) totally preserves IgA (189). Ultrasounds with then a quick heat treatment protect IgA, lactoferrin and BSSL (55). More recently, ultraviolet-C irradiation was shown to eliminate bacteria with no loss of BSSL and no change of fatty acid profile (50). To counteract the low levels of bioactive compounds, the use of specific new types of fortifiers could be envisaged. The fortifier currently used should be improved by containing indispensable lipid sources (DHA, AA, phospholipids) instead of mainly medium-chain and saturated triglycerides such as in liprocil (100% lipids, 82% saturated fatty acids, 78.6% MCT, 4.2% oleic acid and 14.5% linoleic acid) and Liquigen (50% lipids, 47.1% saturated fatty acids, 46.2% MCT), as well as more numerous specific bioactive proteins. The last possibility relies on the production of more functional preterm infant formulas, the most appropriate possible for programming good intestinal health since the beginning of birth, on the basis of clear new recommendations that should be urgently set-up by pediatricians and scientists expert in the field of infant nutrition.

Human milk is clearly vital through so-called early « programming effects » that lead to long-term biological and physiological outcomes important for, among others, intestinal health later in life. Our knowledge on the crucial components provided by human milk is now strong enough in this 21 century to help us to improve substantially the specific clinical care practices for premature newborns. For illustration of this remark, it may be noted that despite the fact that we know that DHA is very important, no action is actually proposed in neonatology to ensure a

sufficient intake, both due to the lack of consensual guidelines and of adapted industrial products (86,130). In order to give the premature newborns the same chances for an optimal development when own mother's fresh milk is not available, substantial efforts have to be made for proposing more appropriate donor milk (selection by date after postpartum, accordingly to DHA and AA levels that will have to be measured, as for some examples), adapted and well-thoughtful milk fortifiers, and functional better-defined preterm infant formulas.

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