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Research Article

Oxidation products of polyunsaturated fatty acids in infant formulas compared to human milk – A preliminary study

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Information about lipid oxidation in fresh and stored human milk compared with infant formulas is scarce. We aimed to assess *n*-6 and *n*-3 PUFA oxidation in these milks by measuring the 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) content. Human milk samples ($n = 4$), obtained from volunteer mothers, were analyzed fresh and after 1 wk at 4°C or 24 h at 18°C. Vitamin E and malondialdehyde (MDA) were measured by HPLC and fatty acid profile by GC. The 4-HHE and 4-HNE contents were measured by GC-MS. Infant formulas ($n = 10$) were tested; their fat droplet size was measured by laser light scattering and observed by confocal laser scanning microscopy. Human milk samples contained 31.0 ± 6.3 g/L of lipids and 1.14 ± 0.26 mg/L of vitamin E. Fat droplets were smaller in infant formulas than reported in human milk. The (4-HHE/*n*-3 PUFA) ratio was 0.19 ± 0.01 μg/g in fresh human milk (unchanged after storage) *versus* 3.6 ± 3.1 μg/g in dissolved powder formulas and 4.3 ± 3.8 μg/g in liquid formula. (4-HNE/*n*-6 PUFA) was 0.004 ± 0.000 μg/g in fresh milk (0.03 ± 0.01 μg/g after storage) *versus* 1.1 ± 1.0 μg/g in dissolved powder formulas and 0.2 ± 0.3 μg/g in liquid formula. Infant formulas also contained more MDA than human milk. *n*-3 PUFA were more prone to oxidation than *n*-6 PUFA. Whether threshold levels of 4-HHE and 4-HNE would be of health concern should be elucidated.

Keywords: Lipid / Milk / Nutrition / Oxidation / Pediatrics

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1 Introduction

An important nutritional quality of breast milk lays in its *n*-6 and *n*-3 long-chain PUFA content [1]. Therefore, infant formulas are also enriched in PUFA to fulfill the infant nutritional needs. Though, these fatty acids (FAs) are prone to oxidation. Because breast milk is recommended for exclusive infant feeding up to 6 months of age and for further complementary feeding [2], some breastfeeding mothers working out of home express their milk. It is reported that expressed human milk can be stored up to 4–8 h at 25°C or 2–5 days at 4°C [3–5]. The bactericidal activity of human milk has been reported to decrease only beyond 72 h of refrigerated storage [6] and bacterial growth was not found significantly different after 4 days at 4°C [7], presumably due to the high lysozyme, lactoferrin, and other bacteriostatic agents in human milk [8]. Some authors report that for short-term storage, breast milk may also be stored safely at 15°C for 24 h [9–11]. However, maternal practice may vary from recommendations, which may affect lipid oxidation.

PUFA oxidation, assessed by hydroperoxides, headspeace aldehydes, and malondialdehyde (MDA), was found significant in infant formula (up to 1.2 μg of MDA *per* gram of formula) [12, 13] and other enteral formula after storage [14]. Breast milk has been found to contain less peroxidized linoleic acid than formula [15] and is reported to

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Abbreviations: BODIPY®FL C_5-lactosylceramide, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosyl 1-β-D-lactoside; **FA,** fatty acid; **4-HHE,** 4-hydroxyhexenal; **4-HNE,** 4-hydroxynonenal; **MDA,** malondialdehyde

provide better antioxidant power than do formulas [16, 17]. However, the impact of breast milk storage on PUFA oxidation has seldom been assessed; only MDA and peroxidized linoleic acid were reported to increase after storage at 4°C for 48 h and 4 days, respectively [15, 18]. Moreover, liquid infant formula are now available on the market, with a shelf life of 48 h in the refrigerator once the bottle is opened, but no result is available to date about the impact of liquid formula storage on lipid oxidation.

Specific end-markers of *n*-3 *versus* *n*-6 PUFA oxidation are, respectively, 4-hydroxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE) [19, 20]. 4-HNE, at concentrations higher than physiological level, exhibits *in vitro* some cytotoxic and genotoxic effects. Though, it is not known which threshold content in food products would present harmful health effects [20]. Moreover, oxidative stress is claimed to have a deleterious impact in the metabolic syndrome, and infants should be protected therefrom [17]. To our knowledge, 4-HHE and 4-HNE have never been measured to date in infant formulas and breast milk, neither fresh nor stored.

The aim of the present study was thus to perform a preliminary comparison of the 4-HHE and 4-HNE content in different infant formulas and in human milk, with regard to their FA and vitamin E composition and compared to overall lipid oxidation assessed by MDA, and to explore the impact of milk storage.

2 Materials and methods

2.1 Milk samples

Four lactating mothers of infants of less than 1 year of age were recruited, who were healthy, similar regarding age, BMI, and lactation frequency (Table 1), and familiar with expressing their milk. Informed consent of all volunteer mothers was obtained according to the rules of the local ethics committee. Mature human milk samples were collected in the laboratory by the volunteers using their own breast-milk pump, while their baby was nursing on the other breast and until complete emptying of the breast (three mothers expressed their milk within 10 min, one mother during 50 min; Table 1). This technique provided the most significant milk regarding quality and quantity, because these parameters vary during one feeding and whether milk is expressed with a pump or by the baby [21, 22]. Samples of fresh milks were kept under argon at −80°C prior to analysis. Other aliquots were stored either at 18°C for 24 h or at 4°C for 1 wk (according to a survey reported in Table 2, performed among 52 mothers from different French breastfeeding support organizations to assess the most common and the maximum storage times used by mothers). The (head space/milk surface) ratio in storage tubes was like in a bottle. After storage, these samples were kept at −80°C under argon and analyzed for 4-hydroxyalkenals after thawing at 37°C for 5 min.

Table 1. Characteristics of the volunteer mothers providing expressed human milk, of their baby and of milk expression (mean ± SD)

Maternal age (year)	32.5 ± 2.0
Maternal weight (kg)	64.9 ± 3.0
Maternal BMI (kg/m^2)	24.9 ± 2.2
Time postpartum (month)	6.1 ± 4.0
Number of children in family	2.3 ± 1.0
Total mother breastfeeding duration[a)] (month)	28.0 ± 17.4
Present breastfeeding duration (wk)	24.8 ± 14.4
Exclusive breastfeeding duration (wk)	19.3 ± 4.0
Lactation frequency (feeds/24 h)	8.9 ± 1.6
Number of feeds the day before experiment	7.5 ± 1.2
Number of feeds the night before experiment	2.8 ± 1.8
Time of last feed before experiment	06:45 ± 00:44
Usual milk expression duration[b)] (min)	16.3 ± 9.4
Expression duration during the experiment[c)] (min)	18.8 ± 21.0
Usual milk volume expressed[b)] (mL)	76.7 ± 24.0
Volume expressed during the experiment[c)] (mL)	100.0 ± 31.6

$n = 4$
a) Past breastfeedings + present breastfeeding.
b) According to the mothers.
c) Measured during the experiment, starting at 09:00.

Table 2. Storage practices used for human milk by mothers who breastfed their infants: usual storage time and common maximum storage time at room temperature and in the refrigerator

Room temperature storage			Refrigerator storage		
Time	Usual (%)	Max (%)	Time	Usual (%)	Max (%)
2 h	44.2	32.7	6 h	3.8	1.9
6 h	9.6	25.0	12 h	5.8	–
12 h	1.9	5.8	1 day	21.2	15.4
1 day	–	3.8	2 days	38.5	11.5
N.A.[a)]	44.2	32.7	3 days	11.5	17.3
–	–	–	4 days	7.7	3.8
–	–	–	5 days	1.9	17.3
–	–	–	6 days	1.9	9.6
–	–	–	1 wk	–	19.2
–	–	–	>1 wk	–	3.8
–	–	–	N.A.	7.7	–

Survey among breastfeeding mothers ($n = 52$) used to express their milk on a regular basis.
a) Not applicable or no answer.

Different types of infant formulas, used in local neonatology sections or commercially available on the market, were tested regarding 4-hydroxyalkenals: five powder formulas (samples coded PD) and five liquid formulas (samples coded L). The formulas enriched with long chain-PUFA will be coded with an asterix (DP3*, DP5*, L3*, and L5*). Powder formulas (boxes opened 3 days earlier) were dissolved in water (14%) at 37°C prior to analysis. Liquid formula bottles were opened extemporaneously and samples were kept under argon at −80°C prior to analysis. For three liquid formulas (L1, L2, and L3*), the bottle was kept

at 4°C after opening. Aliquots were then collected after 24 and 48 h of storage (L1 and L2) or after 1 and 24 h of storage (L3*), according to the manufacturers' instructions, and kept under argon at −80°C prior to analysis.

2.2 Protein

The protein content in human milks was measured by the method of Bradford [23] (BioRad protein assay, reference 500 – 0006, BioRad Laboratory).

2.3 Fatty acid

The FA composition of human milks was measured in triplicate using the methods of Lepage and Roy [24] and López-López *et al*. [25] with slight modifications. Human milk (100 μL) was mixed with 500 μg of glyceryl tritridecanoate (Sigma, internal standard) and 1 mL of toluene/methanol (2:1). After vortexing, 1 mL of 0.5 M sodium methoxide (Sigma) was added and allowed to react at 50°C for 10 min. Care has to be taken that water does not exceed 10% in this reaction medium [24]. The mixture was cooled on ice prior to add 1 mL of BF_3-methanol (14%, Sigma) in order to finish the transmethylation at 90°C for 10 min. After cooling on ice, reaction was stopped with 1 mL of K_2CO_3 (10%). Isooctane (2 mL) was added and after centrifuging 10 min at 1500 rpm, the upper phase was collected. The methyl ester extract was diluted (∼1/6) with isooctane prior to injection into the Hewlett Packard gas chromatograph (HP6890, Agilent Technologies, Les Ullis, France). The gas chromatograph was equipped with a flame ionization detector, a programmed temperature injector, and a fused-silica capillary column coated with stabilized poly-90% bis-cyanopropyl/10% cyanopropylphenyl siloxane (60 m × 0.25 mm; film thickness 0.25 μm, Supelco 24111-SP 2380, Supelco, Bellefonte, USA). The initial temperature of the 1 μL-splitless injection was 230°C. The oven temperature was 57°C for 2 min, increased from 57 to 130°C at 20°C/min and remained for 5 min, increased to 210°C at 1.5°C/min, and finally to 250°C at 10°C/min. The detector temperature was 270°C, under hydrogen flux (30 mL/min); the carrier gas was helium (160 kPa).

2.4 Vitamin E (α- and γ-tocopherol)

Human milk (200 μL) was vortexed with 300 pmol of tocol (internal standard), 300 μL water, and 500 μL ethanol. Then, 1.5 mL hexane was added. After vortexing and centrifuging at 3000 rpm, 10°C for 10 min, the upper hexane phases were collected and dried under nitrogen. Lipid extracts were diluted in 200 μL of methanol/water (85:15) prior to injection of 100 μL in the HPLC. A standard was analyzed, containing 200 pmol of α-tocopherol, γ-tocopherol, and tocol. HPLC mobile phase was methanol/water (98:2) at a flow rate of 1 mL/min. The column was Nucleosil C18 (5 μm, 10 cm). The characteristics of the fluorimetric detection were: excitation at 295 nm, emission at 340 nm.

2.5 4-Hydroxyalkenals

For 4-HHE and 4-HNE measurements, 500 μL of milk (human milks, dissolved powders PD1 to PD5*, liquid formulas L1 to L5*) or 200 mg of powders (PD1, PD2, and PD3*) was spiked with 15 – 20 ng of both 4-HHE-CD_3 and 4-HNE-CD_3 (internal deuterated standards). Citric acid (50 μL) 0.15 M and 1 mL of *O*-pentafluorobenzyl hydroxylamine (*O*-PFB) (50% in PIPES buffer, pH 6.5) were added. After vortexing, the reaction took place under nitrogen for 30 min at room temperature. The following reagents were then successively added, with vortexing for 1 min between each additions: methanol (2.5 mL), hexane pestipur (5 mL), and 98% H_2SO_4 (60 μL). After centrifugation at 1800 rpm for 5 min (Jouan, Saint-Herblain, France), upper phases were transferred into 4 mL conical glass tubes (Interchim, Montluçon, France). After evaporation under nitrogen, 100 μL of *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) (Sigma, St. Louis, MO, USA) was added. The reaction took place at 60°C for 30 min, after which samples were transferred into vials for injection into the GC-MS. GC-MS was carried out on a Hewlett-Packard quadrupole mass spectrometer interfaced with a Hewlett-Packard gas chromatograph (Les Ullis, France). The gas chromatograph was equipped with an HP-1MS fused-silica capillary column (30 m × 0.25 mm id, 0.25 μm film thickness) (Hewlett-Packard), which was held at 57°C. The following oven temperature program was used: 2 min at 57°C, then increased to 180°C at 20°C/min, followed by an increase to 280°C at 4°C/min. Samples were injected with a splitless injector with a head pressure of 7.9 psi. The interface, injector, and ion source were kept at 280, 260, and 130°C, respectively. Electron energy was set at 70 eV. Helium and methane were used as carrier and reagent gases, respectively. Mass spectra were acquired from 50 to 800 Da using both the negative ion chemical ionization (NICI) modes. The electron multiplier voltage was usually set at 1400 V.

2.6 Malondialdehyde

In additional experiments using three human milk samples, three powder formulas dissolved in water (PD1, PD2, and PD3*) and three liquid formulas (L1, L2, and L3*), overall lipid peroxidation was evaluated by quantitation of MDA by RP-HPLC according to the method of Therasse and Lemonnier [26]. Milk samples were mixed with thiobarbituric acid (TBA) (10 mM) and acetic acid in the presence of BHT (5 mM) and incubated at 95°C for 1 h. TBA – MDA adduct was extracted with ethyl acetate, separated on a Nucleosil C18 column 5 μm (4.6 mm × 250 mm), and

detected fluorimetrically (excitation 515 nm, emission 553 nm).

2.7 Fat droplet size distribution

Fat droplet size distribution of infant formulas, in liquid form or after powder dissolution in water, was measured by laser light scattering (Mastersizer2000, Malvern, UK). The Sauter diameter (volume-surface mean diameter) (d_{32}) and specific surface area were calculated therefrom as described previously [27].

2.8 Confocal laser scanning microscopy (CLSM)

CLSM was used to observe fat globules in human milk and fat droplets in infant formula. Nile red and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-sphingosyl 1-β-D-lactoside (BODIPY® FL C_5-lactosylceramide) were purchased from Molecular Probes (Invitrogen). Nile red was used to label total lipids and BODIPY FL C5-LacCer was used to label the milk fat globule membrane. Samples were incubated with 0.5 μg/mL Nile red or 5 μM BODIPY FL C5-LacCer for 5 min at room temperature. Specimens were then examined under a Zeiss LSM 510 META confocal microscope equipped with 100× Plan Neofluar objective. Images were analyzed using LSM 510 META 3.2 software. In micrographs, total lipids are coded in red and membrane in green.

2.9 Statistical analysis

A paired *t*-test was used to test whether concentration was significantly higher in stored than in fresh milk. Due to variance heterogeneity among groups, a nonparametric Mann–Whitney *U*-test was mandatory to test whether concentration was significantly different in infant formulas than in human milk or between different types of infant formulas. Results are expressed as mean ± SD.

3 Results and discussion

3.1 Human milk storage time

According to the survey results presented in Table 2, storage practices used by mothers who are used to express their milk on a nearly daily basis may vary significantly from official recommendations. In our survey, 61.5% of the mothers used to store their milk longer than 48 h in the refrigerator and 50% did not hesitate to store it occasionally between 5 days and 1 wk prior to baby feeding. Some mothers sometimes used their milk even if it had stand at room temperature between 12 and 24 h (Table 2). Some mothers argued that they did not observe any alarming alteration of the milk aspect or odor after such storage. On a biological point of view, such storage conditions (*e.g.*, 4–5 days at 4°C) have been reported to result in no deleterious bacterial development [9, 10]. However, we postulated that they might be critical on a lipid peroxidation point of view, this is why we compared lipid peroxidation after storage of human milk at positive temperature to that observed in different infant formulas.

3.2 Milk composition

Table 3 shows the composition of human milk samples and infant formulas, which was consistent with the literature [28, 29]. Vitamin E content was slightly lower than reported in the literature. However, we should stress that in the present study, milk samples resulted from full expression from one breast and after milk ejection reflex. They were thus a combination of foremilk, containing less fat, and hind milk, that is more fat. In the literature, hindmilk is often studied. Because hindmilk contains more fat [22], vitamin E content values may be sometimes overestimated compared to the real content in one entire feeding. The FA profile was consistent with the literature. However, we could detect more FAs with chain length up to 14 carbon atoms, certainly thanks to the use of the direct transmethylation method that avoids losses occurring during total lipid extraction. FA composition in breast milks was borderline regarding recommendations of 9–22% for linoleic acid and 1–3% for linolenic acid, while DHA content was adequate (0.4–1%) [30]. On an average, the *n*-6/*n*-3 PUFA ratio in human milks met the requirement of being lower than 5, except for one mother whose ratio *n*-6/*n*-3 was 8. The infant formulas contained up to twice more proteins than human milk and up to seven-fold more vitamin E (used to protect PUFA from oxidation). Only L3* and L5* contained few adjunction of vitamin E. The average *n*-6/*n*-3 PUFA ratio was higher in infant formulas than in human milk (Table 3).

3.3 Oxidation products from PUFA

Figure 1A shows that the mass ratios (4-HNE/*n*-6 PUFA) and (4-HHE/*n*-3 PUFA) were higher in infant formulas than in human milk ($p < 0.05$). Therefore, greater specific oxidation of these FAs occurred in infant formulas, either in dissolved powder or liquid form, than in human milk. Consistently, Fig. 1B shows that (MDA/PUFA) ratio was also higher in infant formulas than in human milk. These results are consistent with the better antioxidant power reported for human milk compared with formula [15–17], even if one study reported higher conjugated dienes, lipid peroxides, and TBARS in human foremilk than in infant formulas [31]. However, discrepancies among formulas were observed: the (4-HNE/*n*-6 PUFA) mass ratio was in the range of 0.02–0.03 ppm for L1 and L2, in the range of 0.62–0.77 ppm for L5* and PD1, and up to 2.7 ppm for PD4. The (4-HHE/*n*-3 PUFA) mass ratio was in the range of 0.55–0.84 ppm for L1 and L2, in the range of 1.7–2.6 ppm

Table 3. Composition of expressed human milks and infant formulas

	Milk	Min	Max	Mean ± SD
Protein (g/L)	Human	7.8	10.7	9.2[a)] ± 1.4
	Formula	12.0	23.9	16.2[b)] ± 4.4
Lipid (g/L)	Human	17.7	43.5	31.0[a)] ± 12.6
	Formula	31.0	44.0	35.9[a)] ± 4.6
Vitamin E[c)] (mg/L)	Human	0.54	1.60	1.14[a)] ± 0.52
	Formula	0.3	8.00	5.58[b)] ± 2.98
FA (g/100 g total FA)	Human			
8:0[d)]		0.14	0.19	0.17 ± 0.02
10:0		1.32	1.52	1.40 ± 0.08
12:0		5.33	6.48	5.97 ± 0.54
14:0		7.62	9.38	8.56 ± 0.74
14:1		0.26	0.39	0.30 ± 0.06
15:0		0.39	0.49	0.43 ± 0.04
15:1		–	0.09	0.06 ± 0.04
16:0		24.02	27.41	25.41 ± 1.44
16:1 *n*-9		0.28	0.32	0.31 ± 0.02
16:1 *n*-7		1.41	2.58	1.98 ± 0.50
17:0		0.28	0.38	0.33 ± 0.04
17:1		0.04	0.17	0.12 ± 0.06
18:0		5.60	8.89	7.26 ± 1.24
18:1 *n*-9 *trans*		0.18	0.34	0.27 ± 0.08
18:1 *n*-9 *cis*		29.89	36.50	33.05 ± 2.82
18:1 *n*-7		1.18	1.55	1.39 ± 0.16
Total CLA[e)]		0.30	0.48	0.38 ± 0.08
18:2 *n*-6 *cis*		7.51	10.87	8.97 ± 1.54
18:3 *n*-6		0.06	0.20	0.12 ± 0.06
18:3 *n*-3		0.52	1.18	0.86 ± 0.28
20:0		0.12	0.15	0.13 ± 0.02
20:1 *n*-9		0.21	0.55	0.42 ± 0.14
20:2 *n*-6		0.10	0.18	0.12 ± 0.04
20:3 *n*-9		–	0.05	0.02 ± 0.02
20:3 *n*-6		0.19	0.31	0.25 ± 0.06
20:4 *n*-6		0.26	0.32	0.29 ± 0.02
22:1 *n*-9		0.07	0.31	0.23 ± 0.12
20:5 *n*-3		0.10	0.37	0.20 ± 0.12
22:4 *n*-6		0.04	0.06	0.05 ± 0.00
22:5 *n*-3		0.14	0.30	0.20 ± 0.08
22:6 *n*-3 (DHA)		0.37	1.07	0.73 ± 0.32
Saturated		46.4	52.9	49.7 ± 2.6
Monounsaturated		35.8	40.9	38.1 ± 2.4
Polyunsaturated		11.3	13.4	12.2 ± 1.0
Total *n*-6 (g/100 g FA)	Human	8.3	11.5	9.8[a)] ± 1.4
	Formula	12.9	18.0	16.3[b)] ± 1.9
Total *n*-3 (g/100 g FA)	Human	1.4	2.7	2.0[a)] ± 0.6
	Formula	1.7	2.3	1.9[a)] ± 0.2
n-6/*n*-3 ratio	Human	3.1	8.0	5.3[a)] ± 2.0
	Formula	5.6	10.6	8.9[b)] ± 1.9

a, b) For a given parameter, two means in a column with different superscripts are significantly different ($p < 0.05$) between human milk and infant formula.
c) α- + γ-tocopherol.
d) Carbons: unsaturations.
e) Conjugated linoleic acids.

for PD2 and PD3*, and up to 5.6 ppm for L5*. Moreover, powder formulas dissolved in water presented a higher (MDA/PUFA) ratio than liquid formulas. Liquid formulas seem thus to result in lower *n*-6 FA oxidation than dissolved powders, maybe because the former are more readily used once the container is opened.

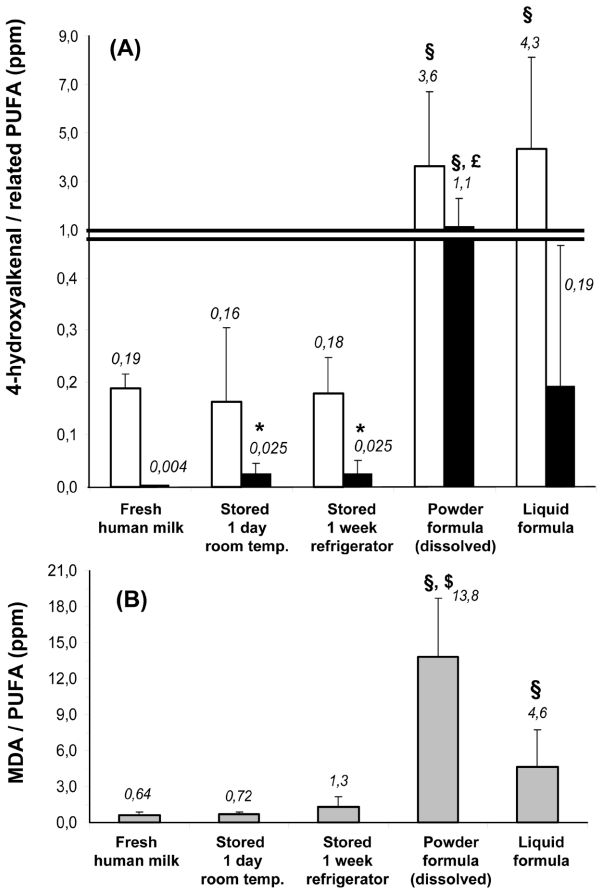

Figure 1. (A) (4-HHE/*n*-3 FA) mass ratio (□) and (4-HNE/*n*-6 FA) mass ratio (■) in expressed human milk (fresh or after storage 1 day at 18°C or 1 wk at 4°C, $n = 4$) and infant formula (powder dissolved in water, $n = 5$, samples PD1 – PD5*; or liquid, $n = 5$, samples L1 – L5*). Note scale break in the *y*-axis. (B) (MDA/PUFA) mass ratio (■) in expressed human milk (fresh or after storage 1 day at 18°C or 1 wk at 4°C, $n = 3$) and infant formula (powder dissolved in water, $n = 3$, samples PD1 – PD3*; or liquid, $n = 3$, samples L1 – L3*). *Significant increase in stored human milk compared with fresh human milk ($p < 0.05$); §significant difference between infant formula and fresh human milk ($p < 0.05$); $significant difference between powder formula (once dissolved) and liquid formula ($p < 0.05$); £trend between powder formula (once dissolved) and liquid formula ($p < 0.1$). Results are mean ± SD.

Complementary analyses were performed in powder formulas before and after dissolution in water in order to elucidate whether 4-hydroxyalkenals were present in dry formula or appeared during preparation. A significant increase of oxidation products was observed after powder dissolution in water ($p < 0.05$): (4-HHE/*n*-3 PUFA) ratio was 3.8 ± 2.8 ppm after dissolution in water *versus* 0.20 ± 0.04 ppm in powder; (4-HNE/*n*-6 PUFA) ratio was 0.35 ± 0.36 ppm after dissolution in water *versus* 0.010 ± 0.003 ppm in powder. This can be explained because in products such as powders, with a low water

activity close to the water monolayer, lipid oxidation is low due to a decreased catalytic effect of transition metals, quenching of free radicals and singlet oxygen and/or retardation of hydroperoxide decomposition. Once powder is in contact with water, lipids are more accessible to oxygen and catalysts [32, 33].

The greater (4-HHE/*n*-3 PUFA) ratio compared to (4-HNE/*n*-6 PUFA) ratio observed both in human milk and infant formulas can be explained by the greater number of unsaturations of *n*-3 PUFA, that are more prone to oxidation on a given mass basis. The same phenomenon has been observed in human plasma, that contained more 4-HHE than 4-HNE even though the concentration in *n*-6 PUFA was higher [19].

3.4 Oxidation products from PUFA during milk storage

In human milk, the ratios (4-HHE/*n*-3 PUFA) (Fig. 1A) and (MDA/PUFA) (Fig. 1B) did not vary during storage. The ratio (4-HNE/*n*-6 PUFA) was higher after storage than in fresh milk ($p = 0.04$), although still lower than in formulas. Infants would have ingested 7.0 ng of 4-HNE (0.4 pmol) if fed with these samples of breast milk stored in extreme conditions *versus* 1.1 ng of 4-HNE (0.07 pmol) ingested during one feed on the breast. The physiological impact of this difference should be investigated. The few lipid oxidation products detected in fresh milk may arise from mothers' circulating plasma lipids, or to primary oxidation occurring during milk expression and prior to analysis.

The (MDA/PUFA) ratio increased during the storage of liquid infant formula at 4°C after bottle opening (Fig. 2), especially regarding formula supplemented in long chain-PUFA. However, the storage of liquid formula (L1 and L2) up to 48 h at 4°C did not affect (4-HHE/*n*-3 PUFA) ratio (stable around 0.8 ± 0.2 ppm) and (4-HNE/*n*-6 PUFA) ratio (stable around 0.02 ± 0.01 ppm). This difference between MDA and 4-hydroxyalkenal during liquid formula storage may be explained because 4-hydroxyalkenals are more reactive than MDA: *e.g.*, 4-HNE can form Michael adducts with lysine-, cysteine-, and hystidine-containing proteins [34].

3.5 Milk concentration of oxidation products

Freshly prepared infant formulas contained more 4-hydroxyalkenals (4-HHE + 4-HNE) than in human milk (even after storage of the latter): 9.1 ± 7.8 µg/L in dissolved powder formulas and 4.5 ± 3.7 µg/L in liquid formulas *versus* 0.13 ± 0.09 µg/L in human milk ($p = 0.01$). The 4-hydroxyalkenal concentration varied greatly among infant formula samples, from 1.9 µg/L for PD3* up to 20.8 µg/L for PD4 *versus* 0.07 – 0.12 µg/L in stored human milk. MDA concentration was also higher in infant formulas than in human milk: 84.7 ± 32.2 µg/L in dissolved powder formulas (from 51.7 µg/L in PD1 to 116 µg/L in PD3*) and 29.6 ± 22.3 µg/L in liquid formulas (from 18.1 µg/L in L1 to 55.4 µg/L in L3*) *versus* 3.0 ± 1.2 µg/L in human milk ($p < 0.05$). The impact of such differences in oxidation product concentration on infant oxidative stress remains to be characterized.

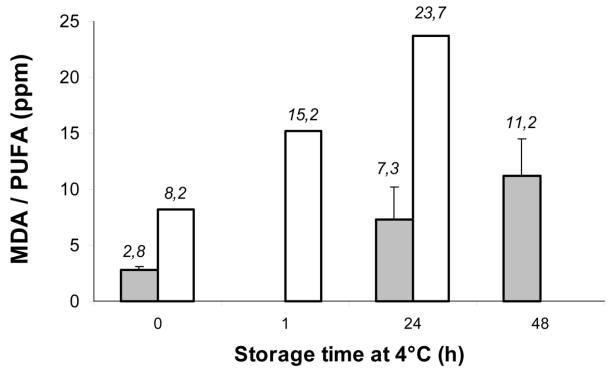

Figure 2. (MDA/PUFA) mass ratio during storage at 4°C in liquid infant formula: (■) not supplemented (L1 and L2) or (□) supplemented in DHA and arachidonic acid (L3*).

3.6 Fat droplet structure

The lower protection against PUFA oxidation of infant formulas might arise from their fat droplet structure (size distributions and interface coatings: phospholipids, proteins such as casein micelles or sodium caseinate). Figure 3 shows that the size of fat droplet is dramatically different between human milk and infant formula. The specific structure of the human milk fat globules is already known to provide unique nutritional properties compared to fat droplets from infant formulas [35, 36]. Moreover, when oxygen concentration is not limiting, conjugated diene formation was found to increase in emulsions when the fat droplet size decreased [37]. The mean fat droplet size in infant formulas was $d_{32} = 0.26 \pm 0.1$ µm, which is consistent with the literature [38]. Conversely, human milk contains native fat globules covered by a biological membrane (Fig. 3, coded in green), sizing $d_{32} \sim 3.5$ µm [27, 38]. Therefore, fat surface area is 27.2 ± 11.4 m^2/g of fat in infant formulas, much greater than in human milk (≤ 1.9 m^2/g [27]). This larger fat interface can enhance lipolysis and lipid oxidation [37]. Therefore, the higher amounts of 4-HHE, 4-HNE, and MDA measured in infant formulas in this study could be partly explained by differences in fat droplet structure.

4 Concluding remarks

For the first time to our knowledge we have shown that infant formulas contained more 4-HHE and 4-HNE than human milk, even after storage of the latter at positive tem-

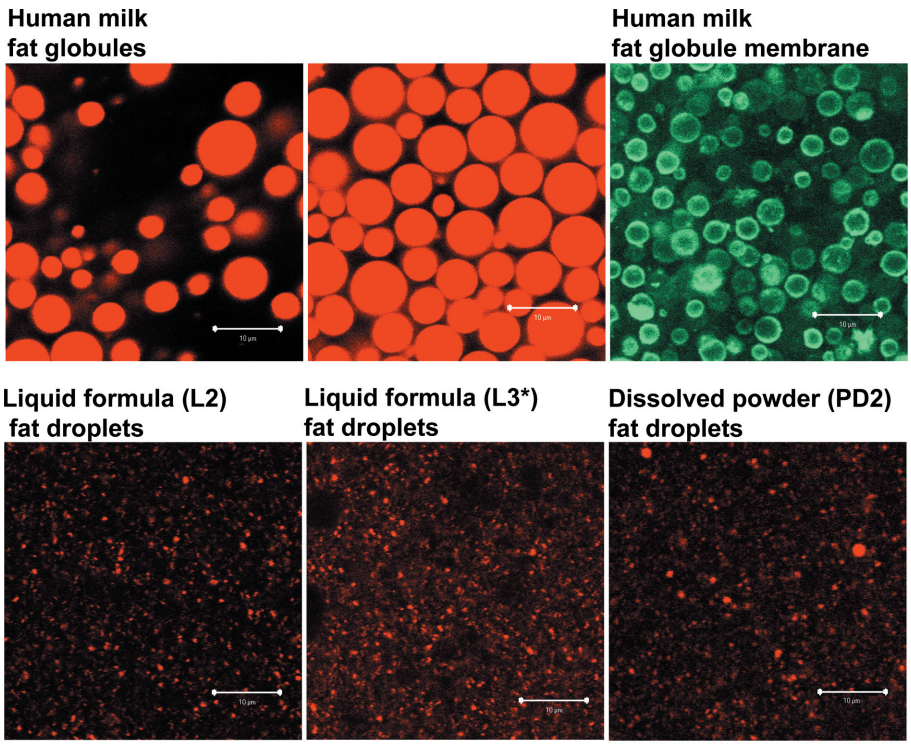

Figure 3. Confocal laser scanning micrographs of human milk (top) and infant formula (bottom). Total lipids are coded in red; biological milk fat globule membrane surrounding human milk fat globules is coded in green. Scale bar is 10 μm.

peratures. Such storage did not increase 4-HHE content in human milk. Low levels (<1 μM) of 4-HNE have recently been reported to have deleterious effects on hepatocyte function [39]. No significant cytotoxicity of 4-HNE was observed from 0.01 to 100 μM. However, from 0.1 μM, a concentration-dependent decreased phosphorylation of ERK1/2 occurred in hepatocytes. A loss of normal transcriptional activity was also observed in such conditions [39]. Therefore, we can wonder whether low but chronic 4-HNE exposure *via* oxidized dietary lipids would have deleterious effects on infant metabolism. Further research should thus determine threshold concentrations of 4-HHE and 4-HNE of possible health concern regarding infant oxidative stress and metabolism.

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