

Antiviral oxysterols are present in human milk at diverse stages of lactation

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ABSTRACT

Oxysterols are cholesterol oxidation derivatives. Those containing an additional hydroxyl group on the side chain of the cholesterol molecule result from a physiological enzymatic synthesis and include the majority of oxysterols present in the circulation. Among these, 25-hydroxycholesterol (25OHC) and 27-hydroxycholesterol (27OHC) are characterized by a broad antiviral activity and are now considered involved in the innate immune response against viruses. Despite the emerging role of these sterols in the innate antiviral defences, no data are available on their presence in human breast milk (BM) to date. In this study, we investigated the content of oxysterols of enzymatic synthesis in BM of twelve donor mothers at different stages of lactation (i.e. in colostrum, transitional milk, and mature milk) by gas chromatography-mass spectrometry analysis. The side-chain oxysterols 25OHC, 27OHC, and 24S-hydroxycholesterol (24SOHC) were actually present in BM in all stages of lactation, but the concentration of 27OHC showed a remarkable peak in colostrum. Antiviral assays revealed that all the colostrum samples contained 27OHC concentrations that were active in vitro against two relevant pediatric viral pathogens: the human rotavirus and the human rhinovirus. Overall, this study discloses new antiviral components of BM and suggests a passive transfer of these protective factors to the infant via breastfeeding, especially in the first few days of lactation.

1. Introduction

The composition of human breast milk (BM) has been studied for decades to unravel the molecular basis of its unique nutritional, trophic, and immunological functions [1,2]. The immunological and antimicrobial factors of BM contribute to stimulate the development of the immature immune system of the newborn and to provide the infant with a first line of defense against pathogens through a passive transfer of acquired and innate immunological factors [1,3]. These include cells

(macrophages, lymphocytes and stem cells), cytokines and chemokines, immunoglobulins (mostly secretory IgAs), antimicrobial proteins (e.g. lactoferrin, lactoperoxidase and lactadherin), mucins and oligosaccharides [3]. A large number of immunological and antimicrobial factors have been already identified but new ones are still being discovered and more research is necessary in to investigate their content and biological role in BM.

In BM, lipids are dispersed as globules formed by a core (rich in triglycerides) surrounded by a specialized plasma membrane. These

Abbreviations: 25HC, 25-hydroxycholesterol; 27HC, 27-hydroxycholesterol; BM, breast milk; 24SHC, 24S-hydroxycholesterol; 7KC, 7-ketocholesterol; 7 β OHC, 7 β -hydroxycholesterol; MFGM, milk fat globules; COP, cholesterol oxidation products; GC-MS, gas chromatography-mass spectrometry; DMEM, Dulbecco's Modified Eagle Medium; HRoV, Human rotavirus; HRhV, Human rhinovirus; FFU, focus-forming units; MOI, multiplicity of infection; SEM, standard error of the mean; EC₅₀, antiviral effective concentration; MAP, medically assisted procreation; C, colostrum; TM, transitional milk; MM, mature milk; CS, cesarean section; S, spontaneous delivery

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structures are known as milk fat globules (MFGM), and provide a source of cholesterol, essential for the synthesis of bile acids, hormones, vitamin D, and lipoproteins [4]. Metabolites of cholesterol biosynthesis, including lathosterol, lanosterol and desmosterol, are also present in BM [5,6]. The cholesterol in BM is prone to auto-oxidation, resulting into the formation of several cholesterol oxidation products, also known as oxysterols, containing an additional hydroxyl, ketone or epoxide group on the sterol nucleus [7]. These compounds have received much attention due to their potential toxicity, and hence 7-ketocholesterol (7KC), is currently used as a biomarker of cholesterol oxidation in milk subjected to industrial processes or storage procedures [8]. However, cholesterol auto-oxidation and the formation of oxysterols in fresh BM is very low due to the low oxygen concentration [7]. A distinct class of oxysterols containing an additional hydroxyl group on the side chain of the cholesterol molecule, results from a physiological synthesis catalyzed by specific enzymes named cholesterol hydroxylases, and includes the majority of oxysterols present in the circulation.

Oxysterols participate in different aspects of lipid metabolism, play an important role in the regulation and maintenance of whole-body cholesterol homeostasis and have been ascribed a number of roles in connection with atherosclerosis, inflammation, apoptosis and immunosuppression [9,10]. Over the past few years, an increasing number of studies showed that some defined side-chain oxysterols of enzymatic origin, in particular 25-hydroxycholesterol (25OHC) and 27-hydroxycholesterol (27OHC) have a broad spectrum antiviral activity [11–15] and thus are now considered as effectors of the innate immune response against viruses [16,17]. 25OHC is by far the most studied member of the family, since its production is regulated by an interferon-inducible enzyme, the cholesterol 25-hydroxylase [11,12]. The production of 27OHC by the ubiquitous mitochondrial hydroxylase CYP27A1 [18] is constitutive and 27OHC is the most abundant oxysterol in human plasma [19,20]. Despite the emerging role of these oxysterols in the innate antiviral defences, to date no data are available on their presence in BM.

Here we report on the presence of the three side-chain oxysterols 25OHC, 27OHC, and 24SOHC in BM at different stages of lactation, i.e. in colostrum (C), transitional milk (TM) and mature milk (MM). Notably, of the three oxysterols, only 27OHC showed a remarkably high concentration in the colostrum. A sort of bolus administration of this oxysterol by the mother to the baby. Only for antiviral purposes? A fascinating open question.

2. Materials and methods

2.1. Ethics statement

An ethical review process was not required for this study since it was not a clinical trial. Each milk donor involved in this research signed a written consent form where mother's and infant's data protection were assured. Besides, donors were informed that milk samples stored in excess would be used only for research purposes strictly related to the present study.

2.2. Milk samples

Twelve healthy mothers, admitted to Sant'Anna Hospital (Città della Salute e della Scienza di Torino) for term delivery, were enrolled in the study between November 2017 and March 2018. Colostrum (days 1–5 postpartum), transitional milk (days 6–14 postpartum) and mature milk (beyond day 15 postpartum) samples were longitudinally obtained. Five milliliters samples were collected by an electric breast pump in disposable sterile polypropylene BPA-free bottles, and immediately aliquoted and stored at -80°C until use.

2.3. Blood samples

Immediately after delivery mothers' blood samples were collected, during routine blood exams, in EDTA tubes. As soon as possible, by centrifugation process, plasma was separated and collected in Eppendorf tubes and stored at -80°C in order to minimize every degeneration process.

2.4. Sterols quantification by gas chromatography-mass spectrometry (GC-MS)

To a screw-capped vial sealed with a teflon septum, 0.25 ml of plasma or 0.5 ml of milk or colostrum were added together with 1000 ng of lathosterol-25, 26, 26, 26, 27, 27, 27-d₇ (Avanti Polar Lipids Inc. USA, SKU: 700056 P, used also as internal standard for desmosterol), 50 ng of lanosterol-26, 26, 26, 27, 27, 27-d₆ (Avanti Polar Lipids Inc. USA, SKU: 700090 P), 50 ng of 7 β -hydroxycholesterol-25, 26, 26, 26, 27, 27, 27-d₇ (C/D/N Isotopes Inc. Canada, SKU: D-4123), 50 ng of 7-ketocholesterol-25, 26, 26, 26, 27, 27, 27-d₇ (C/D/N Isotopes Inc. Canada, SKU: D-6045), 50 ng of 24(R/S)-hydroxycholesterol-25, 26, 26, 26, 27, 27, 27-d₇ (Avanti Polar Lipids Inc. USA, SKU: 700018 P), 50 ng of 25-hydroxycholesterol -26, 26, 26, 27, 27, 27-d₆ (C/D/N Isotopes Inc. Canada, SKU: D-6774), 50 ng of 27-hydroxycholesterol -25, 26, 26, 26, 27, 27-d₆ (Avanti Polar Lipids Inc. USA, SKU: 700059 P) as internal standards, 50 μl of butylated hydroxytoluene (5 g/L) and 50 μl of K3-EDTA (10 g/L) to prevent auto-oxidation.

Each vial was flushed with argon for 10 min to remove air. Alkaline hydrolysis was allowed to proceed at room temperature (22°C) with magnetic stirring for 60 min in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml of cyclohexane. 3 ml of the cyclohexane extract were used for sterols analysis. The oxysterols were separated from cholesterol and sterols by elution of the remaining 7 ml on SPE cartridge (SI 100 mg columns, Isolute) with isopropanol:hexane 30:70 v/v. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA (60°C for 60 min).

Analysis was performed by gas chromatography - mass spectrometry (GC-MS) on a Clarus 600 gas chromatograph (Perkin Elmer, USA) equipped with Elite-5MS capillary column (30 m, 0.32 mm, 0.25 μm . Perkin Elmer, USA) connected to Clarus 600C mass spectrometer (Perkin Elmer, USA). The oven temperature program was as follows: initial temperature of 180°C was held for 1 min, followed by a linear ramp of $20^{\circ}\text{C}/\text{min}$ to 270°C , and then a linear ramp of $5^{\circ}\text{C}/\text{min}$ to 290°C , which was held for 10 min. Helium was used as carrier gas at a flow rate of 1 ml/min and 1 μl of sample was injected in splitless mode.

Mass spectrometric data were acquired in selected ion monitoring mode (OTMSi-ethers) at $m/z = 465$ for lathosterol-d₇, $m/z = 458$ for lathosterol (Sigma-Aldrich Inc. USA, SKU: C3652), $m/z = 343$ for desmosterol (Sigma-Aldrich Inc. USA, SKU: D6513), $m/z = 463$ for 7 β -hydroxycholesterol-d₇, $m/z = 456$ for 7 β -hydroxycholesterol (Sigma-Aldrich Inc. USA, SKU: H6891), $m/z = 399$ for lanosterol-d₆, $m/z = 393$ for lanosterol (Sigma-Aldrich Inc. USA, SKU: L5768), $m/z = 413$ for 24(R/S)-hydroxycholesterol-d₇ and 24(S)-hydroxycholesterol (Avanti Polar Lipids Inc. USA, SKU: 700,061 P), $m/z = 462$ for 25-hydroxycholesterol- d₆, $m/z = 456$ for 25-hydroxycholesterol (Sigma-Aldrich Inc. USA, SKU: H1015), $m/z = 479$ for 7-ketocholesterol- d₇, $m/z = 472$ for 7-ketocholesterol (Sigma-Aldrich Inc. USA, SKU: C2394), $m/z = 462$ for 27-hydroxycholesterol- d₆ and $m/z = 456$ for 27-hydroxycholesterol (Avanti Polar Lipids Inc. USA, SKU: 700,021 P).

Inter-assay CV ranged between 2.3% for lathosterol up to 5.3% for 25-hydroxycholesterol. Tested recovery was 95% for desmosterol, 101% for lathosterol, 99% for lanosterol, 98% for 7 β OHC, 102% for 24SOHC, 101% for 27OHC and 104% for 25OHC [21–26].

For cholesterol quantification, to a screw-capped vial sealed with a teflon septum, 50 μl of plasma or 0.2 ml of milk or colostrum were

added together with 100 µg of epicoprostanol (Sigma-Aldrich Inc. USA, SKU:C2882) as internal standard, 50 µl of butylated hydroxytoluene (5 g/L) and 50 µl of K3-EDTA (10 g/L) to prevent auto-oxidation. Each vial was flushed with argon for 10 min to remove air. Alkaline hydrolysis was allowed to proceed at room temperature (22 °C) with magnetic stirring for 60 min in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml of cyclohexane. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA (60 °C for 60 min).

Analysis was performed by gas chromatography - mass spectrometry (GC-MS) on a Clarus 600 gas chromatograph (Perkin Elmer, USA) equipped with Elite-5MS capillary column (30 m, 0.32 mm, 0.25 µm. Perkin Elmer, USA) connected to Clarus 600C mass spectrometer (Perkin Elmer, USA). The oven temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20 °C/min to 270 °C, and then a linear ramp of 5 °C/min to 290 °C, which was held for 10 min. Helium was used as carrier gas at a flow rate of 1 ml/min and 1 µL of sample was injected in split (1:50) mode. Mass spectrometric data were acquired in selected ion monitoring mode (OTMSi-ethers) at $m/z = 370$ for epicoprostanol and $m/z = 368$ for cholesterol (Sigma-Aldrich Inc. USA, SKU: C8667). Inter-assay CV was 1.8%. Recovery was 98% [27].

2.5. Cell line and virus

African green monkey kidney epithelial MA104 cells (ATCC® CRL-2378.1) and human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2™) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 1%(v/v) penicillin/streptomycin solution (Euroclone, Milan, Italy) and heat inactivated, 10% (v/v) fetal bovine serum (Sigma). Human rotavirus (HRoV) strain Wa (ATCC® VR-2018) and Human rhinovirus (HRhV) 1A (ATCC® VR-1559™) were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). HRoV and HRhV were propagated in MA104 and HeLa cells, respectively, as previously described [13]. Viral titers are expressed as focus-forming unit (FFU) per ml.

2.6. Antibodies and reagents

25OHC, 27OHC, and 24SOHC (Sigma-Aldrich, Milan, Italy; SKU: 700019P, SML2042, and SML1648, respectively) were dissolved in sterile ethanol at 3 mM. Mouse monoclonal antibody (MAb) directed to HRoV VP6 (2B4) was purchased from Covalab (Villeurbanne, France). MAb directed to HRhV (R16-7) was purchased from Antibodies-online.com (Aachen, Germany). The secondary antibody peroxidase-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

2.7. Antiviral assays

The antiviral efficacy of the oxysterols was determined by focus reduction assay on confluent MA104 or HeLa monolayers plated in 96-well trays, as described elsewhere [13]. Cells were treated for 20 h at 37 °C with 25OHC, 27OHC or 24SOHC at concentrations ranging from 0.07 to 16.7 µM. Control samples (100% of infectivity) were prepared by treating cells with culture medium supplemented with equal volumes of ethanol, corresponding to 0.6% (v/v) to 0.0014% (v/v) in cell media. Alternatively, cells were treated with a mix of the three oxysterols, at the respective concentrations assessed in colostrum. Cells were then washed with medium and infected with HRoV or HRhV at a multiplicity of infection (MOI) of 0.02 FFU/cell for 1 h. Cells were incubated for 16 h, then fixed with cold acetone-methanol (50:50), and viral titers were determined by indirect immunostaining.

2.8. Statistical analysis

Continuous data were inspected and tested to determine whether distributions were normal by Kolmogorov-Smirnov normality test and compared using ANOVA with the Holm-Sidak method for Multiple Comparison in repeated samples. Values for statistical significance were set at $P < 0.05$. Correlations were computed using Pearson's coefficient. All analyses were performed with Sigmapstat 3.01 (Sigma-Aldrich, St Louis, MO, USA).

Blockade of viral infectivity were expressed as mean % \pm standard error of the mean (SEM). One-way ANOVA, followed by Bonferroni test, was used to assess the statistical significance of the differences between treated and untreated samples, where appropriate (significance was set at $P < 0.05$). Where possible, half-maximal antiviral effective concentration (EC_{50}) values were calculated by regression analysis using the dose-response curves generated from the experimental data using GraphPad PRISM 7 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Characteristics of the study population

The study group included 12 mothers of which 9 spontaneous pregnancy and 3 medically assisted procreation (MAP), 7 cesarean section and 5 spontaneous delivery. Gestational ages ranged from 37 + 2–41 + 2 (week + day). The main clinical characteristics of the study population are reported in Table 1.

3.2. Sterols quantification in milk and blood samples by GC-MS

We assessed the variation of sterols and oxysterols content in BM during lactation.

To this aim, BM samples were collected from different mothers ($n = 12$) corresponding to different lactation periods: colostrum (C, 1–5 days), transitional milk (TM, 6–14 days), and mature milk (MM, 15–45 days). The analysis included cholesterol, three metabolites of cholesterol biosynthesis which are known components of BM (i.e. lanosterol, desmosterol and lathosterol), three oxysterols of enzymatic origin (i.e. 24SOHC, 25OHC, 27OHC), two markers of cholesterol auto-oxidation (7KC, and 7 β -hydroxycholesterol, 7 β HC). The same sterols and oxysterols were also quantified in the serum of each BM donor to evaluate the correlations between these two biological fluids.

The amount of sterols and oxysterols are presented in Table 2.

According to the GC-MS measurements, the mean contents of cholesterol, lathosterol, desmosterol, and lanosterol were consistent with the data shown in literature [6]. Cholesterol in MM and LM was lower

Table 1
Main clinical characteristics of study population.

N. of mothers	12
Maternal age	32 (28-39)
Previous pregnancies (N. of mothers)	
0	7
1	3
2	2
N. of infants	
M	6
F	6
Gestational age (weeks + days - range)	37 + 2 (41 + 2)
Delivery (N. of mothers)	
S	5
CS	7
Type of pregnancy (N. of mothers)	
S	9
MAP	3

M: male; F: female; S: spontaneous delivery; CS: cesarean section; MAP: medically assisted pregnancies.

Table 2
Cholesterol, precursor sterols and oxysterols in colostrum (C), transitional milk (TM), mature milk (MM) and serum from 12 healthy mothers.

	cholesterol (mg/L)	lanosterol (µg/L)	desmosterol (µg/L)	lathosterol (µg/L)	24SOHC (µg/L)	25OHC (µg/L)	27OHC (µg/L)	7βOHC (µg/L)	7KC (µg/L)
C	Mean ± Std Dev Median 25%-75% Min-Max	179.35 ± 94.38 172.09 94.24 - 266.48 63.51 - 333.02	3131.72 ± 1548.72 2957.63 1688.76 - 4427.10 1212.22 - 5684.25	119.49 ± 26.07 118.70 96.45 - 136.65 86.65 - 167.71	10.99 ± 2.42 10.95 8.95 - 12.89 7.68 - 15.49	8.76 ± 1.68 8.62 7.41 - 10.16 6.23 - 11.88	126.20 ± 60.87 116.83 77.78 - 163.64 51.98 - 249.08	7.91 ± 2.46 8.67 5.63 - 9.83 3.83 - 11.09	17.62 ± 7.60 19.89 10.04 - 23.86 7.06 - 27.38
TM	Mean ± Std Dev Median 25%-75% Min-Max	132.28 ± 54.75 131.00 73.70 - 275.05 41.16 ± 192.05	4393.89 ± 1760.21 4157.64 3131.14 - 5663.18 1933.66 - 7575.27	82.19 ± 22.10 78.88 64.75 - 100.13 46.8 - 120.12	9.37 ± 3.07 9.40 7.04 - 12.11 4.68 - 13.72	8.49 ± 1.83 8.22 6.93 - 9.75 5.92 - 11.76	5.58 ± 2.67 5.42 3.83 - 6.85 1.59 - 10.73	7.32 ± 0.98 7.19 6.51 - 8.04 6.04 - 9.25	12.51 ± 5.00 11.82 8.40 - 15.51 4.91 - 22.32
MM	Mean ± Std Dev Median 25%-75% Min-Max	157.36 ± 53.19 149.90 131.44 - 203.89 70.80 - 233.35	6213.88 ± 1978.43 6667.58 5255.70 - 7330.09 2890.72 - 9903.26	143.83 ± 55.65 162.27 98.44 - 181.01 52.63 - 219.52	14.75 ± 3.28 14.25 13.54 - 17.09 9.46 - 20.22	11.07 ± 3.90 11.12 7.78 - 13.69 5.19 - 17.79	6.84 ± 3.17 6.85 4.69 - 8.72 1.89 - 11.81	7.70 ± 1.52 7.63 6.66 - 8.80 5.52 - 10.34	10.38 ± 3.61 10.62 6.78 - 13.02 5.13 - 15.43
ANOVA	All	< 0.001	0.04	0.06	0.125	0.796	< 0.001	ns	0.472
Holm-Sidak	C vs TM	0.01	0.004	0.01	0.001	0.0328	< 0.001	ns	< 0.001
Comparison between groups	C vs MM	0.08	0.004	0.01	< 0.001	0.191	0.919	ns	0.326
Serum	Mean ± Std Dev Median 25%-75% Min-Max	2475.817 ± 465.658 2622.30 2064.20 - 2778.00 1696.20 - 3249.80	107.829 ± 30.795 111.44 85.26 - 138.50 54.33 - 146.88	1282.36 ± 324.081 1301.27 971.81 - 1492.29 783.11 - 1788.14	56.25 ± 10.64 57.59 49.37 - 61.73 37.81 - 76.08	14.28 ± 1.75 14.23 13.05 - 15.21 11.23 - 17.72	63.82 ± 11.97 62.10 53.81 - 71.01 49.52 - 84.24	6.58 ± 1.04 6.57 5.90 - 7.28 4.93 - 8.23	15.25 ± 4.27 15.17 11.61 - 19.11 8.62 - 21.16

24OHC: 24S-hydroxycholesterol; 25OHC: 25-hydroxycholesterol; 27OHC: 27-hydroxycholesterol; 7βOHC: 7β-hydroxycholesterol; 7KC: 7KC.

compared to C ($P < 0.001$), while we observed a progressive increase of sterol precursors from C to MM (lanosterol $p < 0.001$, desmosterol $P < 0.001$, lathosterol $P < 0.001$, see Table 2 for multiple comparisons statistic significances and Fig. 1). Desmosterol was the most abundant precursor sterol measured in C, TM and MM. Its concentration was about 10–20 times higher compared to lathosterol and lanosterol while lathosterol was the most abundant precursor sterol found in serum, as previously observed [6].

It clearly appears that cholesterol synthesis in mammary glands prefers the desmosterol pathway, but the reasons why this biochemical switch occurs very early after parturition definitely need further investigation to be disclosed. Right now we deem important to quote that desmosterol has been proven to be a very good ligand of liver X receptors [28], whose pivotal actions in cell metabolism, cell signaling, membrane homeostasis and function are fully recognized [29].

The concentrations of 7KC (18 µg/L in C; 13 µg/L in TM; 10 µg/L in MM) were relatively low and consistent with previous findings [8]. We could observe a progressive reduction of 7KC from C to MM ($P = 0.018$). Neither difference nor significant trend were observed for 7βOHC (see Table 2 and Fig. 1). The low levels of 7βOHC and 7KC are indeed indicative of a low degree of cholesterol autooxidation at least along the process of sample collection, storage and analysis.

The oxysterols with an enzymatic origin, 24SOHC, 25OHC, 27OHC, are present in human BM (Table 2, Fig. 1). The 27HC in colostrum was found as significantly ($P < 0.001$) higher (mean: 126 µg/L) when compared to the other oxysterols (11 µg/L for 24HC; 9 µg/L for 25HC; 8 µg/L for 7βHC). A positive trend from C to MM was observed in the case of 24SOHC ($P < 0.001$) as well as in the case of 25OHC ($P = 0.038$ for the absolute value and $P < 0.001$ for the 25OHC/cholesterol ratio). 27HC was significantly higher in C compared to TM ($P < 0.001$) and MM ($P < 0.001$) with no significant differences observed between TM and MM.

This result is consistent with the fact that 27OHC is the most represented enzymatic oxysterol in human blood [20]. While 24OHC is present in maternal blood at a concentration comparable to the one of 27OHC, it is significantly less represented than 27OHC in the colostrum. A possible explanation is that the higher concentration of 27OHC results from a specific production of this oxysterol in the mammary gland, rather than from a passive release of 27OHC from serum to C, as supported by the absence of any statistical significance in the correlation analysis between serum and C, TM or MM. Further, 27OHC was significantly higher in C compared to TM and MM ($P < 0.001$), a finding that suggests a most early overexpression of the CYP27A1 enzyme in BM, rapidly decreasing with its maturation.

Indeed, it has been shown in different cell types that CYP27A1 enzyme is markedly upregulated by estrogens [30,31]. It appears that the mother has its highest estrogen level at point of delivery and thus the expression of CYP27A1 in the mammary gland is at its top, decreasing with the lowering of the mother's estrogen level briefly after delivery.

In C, TM and MM we observed a high degree of correlation between cholesterol, sterols and the enzymatic oxysterols (usually $r > 0.7$ and $P < 0.001$). These observations are suggestive of the presence of metabolic relations between cholesterol synthesis, cholesterol amount and cholesterol conversion into oxysterols. 7βHC and 7KC were correlated each other but not with the other sterols and oxysterols, a fact that could be suggestive of an auto-oxidative generation which does not seem to be related to the cholesterol synthesis and enzymatic conversion.

Serum cholesterol was relative high (mean 2476 mg/L): actually, 10/12 women had total cholesterol above the recommended concentration (< 200 mg/dL = 2000 mg/L). Unexpectedly, weak or not significant were the correlations between cholesterol and the studied sterols and oxysterols which, instead showed significant degree of correlations with homogeneous compounds (sterols vs sterols, oxysterols vs oxysterols). It is likely that the high degree of dyslipidemia observed in some of the women might have affected the results in an

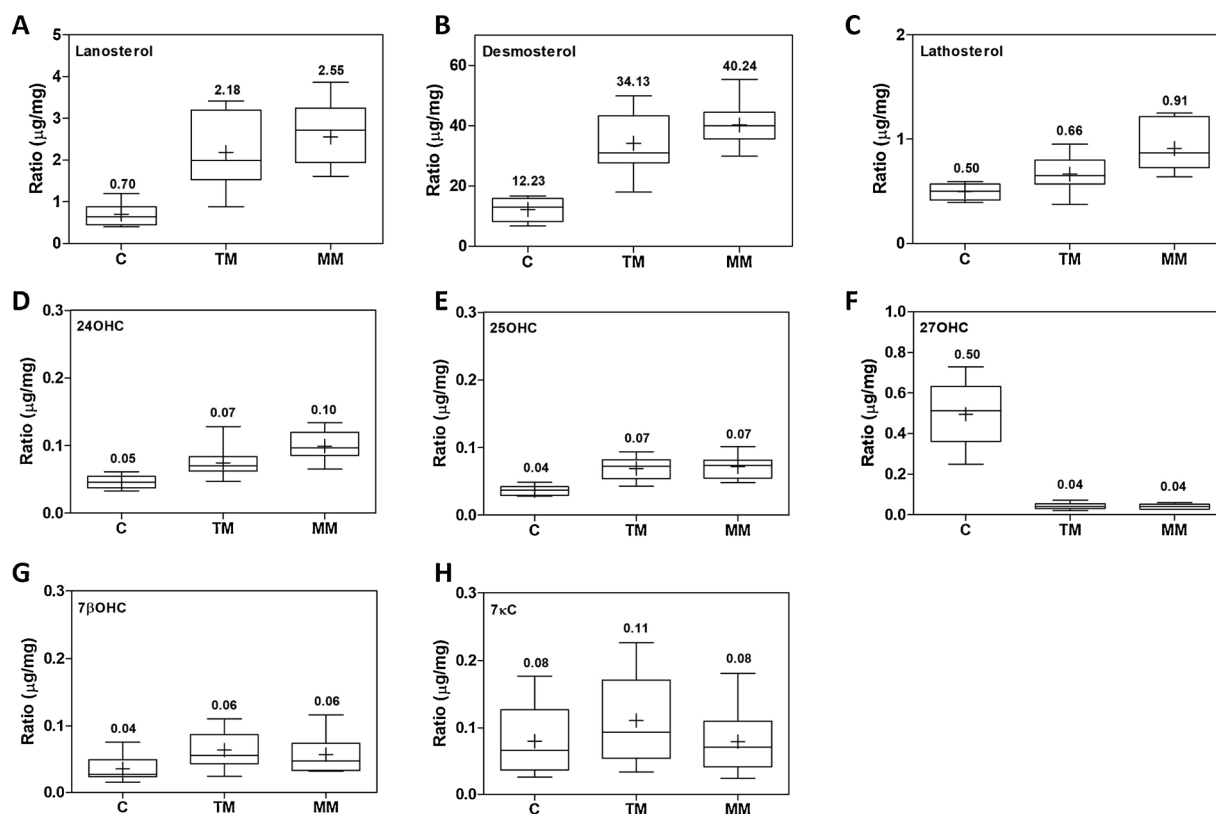


Fig. 1. Sterols/cholesterol ratios and enzymatic oxysterols/cholesterol ratios in maternal colostrum (C), transitional milk (TM), mature milk (MM). The mean amount ($n = 12$) is depicted above the respective bar. Results of One Way Repeated Measures ANOVA with all Pairwise Multiple Comparison Procedures (Holm-Sidak method) are reported. Significance level was set for $P < 0.05$.

unexpected way. No correlations were found between sterols and oxysterols measured in the blood serum with those detected in BM, thus supporting the hypothesis that sterols and oxysterols measured in BM are essentially produced by the mammary gland.

Further, an important information with respect to the increased amount of 27OHC in colostrum versus intermediate and mature milk comes from the observed double amount of cholesterol in the milk of the first few days after parturition (Table 2), when CYP27A1 is most likely up-regulated, as to that of the later days. The observed difference in cholesterol level between colostrum and later milks actually matches the data already present in the relevant literature [32] and contributes to explain the pick of 27OHC occurring in colostrum.

3.3. Antiviral activity assessment of side-chain oxysterols detected in BM

The finding of side-chain oxysterols in BM raised the question of their role in this biological fluid. 25OHC and 27OHC are well-documented antiviral molecules [16,17] whereas the antiviral activity of 24SOHC has never been investigated so far. Therefore, we first explored whether 24OHC was endowed with antiviral activity and then assessed whether the concentrations of the three side-chain oxysterols in colostrum and BM laid within the range of their *in vitro* antiviral activity. The antiviral assays were performed against human rotavirus (HRoV), a major cause of gastroenteritis in infants, and human rhinovirus (HRhV) a major cause of common cold. The two viruses were selected because they are relevant pediatric pathogens previously shown to be sensitive to 25OHC and 27OHC [13,15]. The dose-response curves in Fig. 2 clearly show that 24OHC is also endowed with antiviral activity, with an EC₅₀ corresponding to 155.6 µg/L against HRoV, and to 315.2 µg/L against HRhV, a potency that is intermediate between 27OHC and 25OHC.

Then we compared the EC₅₀ values of the three side-chain

oxysterols to their contents in BM samples to assess whether they are present in potentially antiviral concentrations. Fig. 3 shows that all the colostrum contained 27OHC concentrations that are potentially active *in vitro* against HRoV and HRhV, although to a different extent. Of note, nine out of twelve colostrum contained 27OHC concentrations higher than the EC₅₀ calculated *in vitro* against HRoV. By contrast, 24SOHC and 25OHC concentrations do not lay within the range of their *in vitro* antiviral activity in any BM sample examined.

Nevertheless, we put forward the hypothesis that the mixture of the three side-chain oxysterols present in colostrum, could elicit a more potent antiviral activity than the one exerted by each single molecule. To verify this hypothesis, we performed antiviral assays by treating cells with different combinations of 24SOHC, 25OHC, and 27OHC, at the respective concentrations found in colostrum. As expected, treatment with 24SOHC and 25OHC, or the combination of both, does not inhibit HRoV or HRhV infectivity, while 27OHC significantly ($p_{ANOVA} < 0.001$) blocks viral replication to a maximum of 63% (Fig. 4). Consistently, the combined treatment with 24SOHC and 27OHC, or with 25OHC and 27OHC does not improve the antiviral activity of 27OHC. Interestingly, the simultaneous treatment with 24SOHC, 25OHC, and 27OHC shows a significantly ($p_{ANOVA} < 0.001$) higher anti-HRoV and anti-HRhV activity (37% of infectivity), if compared to the previous oxysterol combinations or to 27OHC alone.

4. Conclusions

We reported here that 24SOHC, 25OHC and 27OHC are present in BM at any stage of lactation, therefore these molecules should be added to the still growing list of the components of human milk. This finding raises a number of questions including possible variations of their content in different study groups and whether they exert any effect on the breastfed infant. Clearly, these issues warrant further investigations.

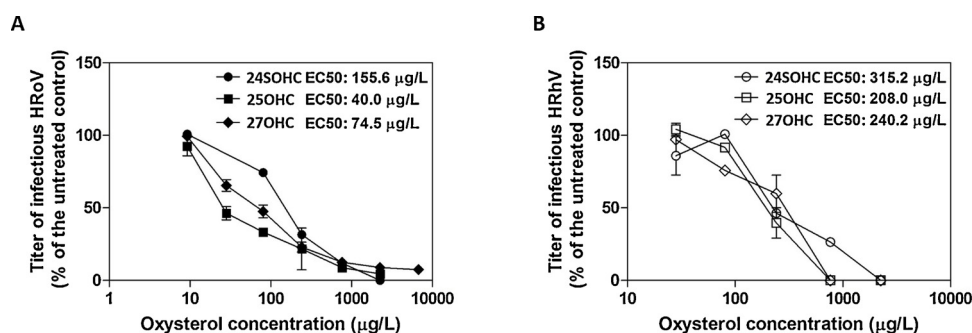


Fig. 2. Antiviral activity of 24OHC, 25OHC, and 27OHC against HRoV (panel A) and HRhV (panel B), assessed on MA104 and HeLa cells, respectively. Cells were treated for 20 h with increasing concentrations of oxysterols and then infected. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for triplicates. Half-maximal inhibitory concentrations (EC50) were calculated using GraphPad PRISM 7 (GraphPad Software, San Diego, CA, USA).

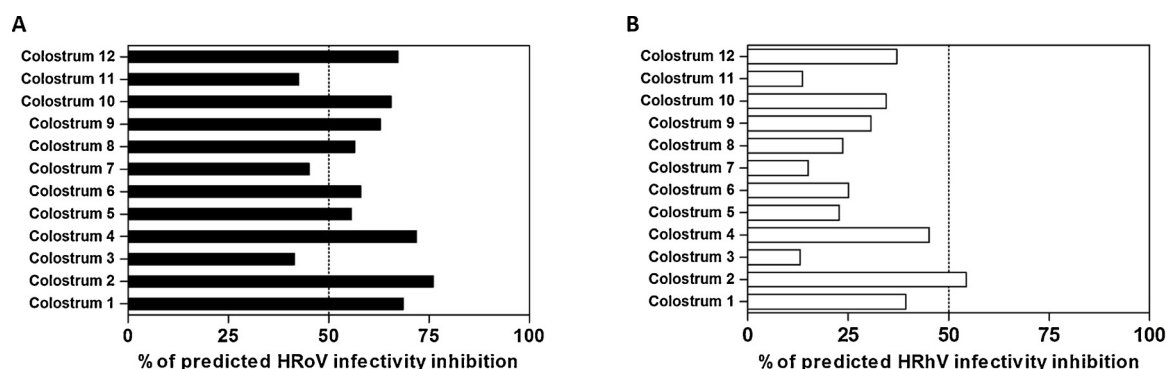


Fig. 3. Percentage of predicted HRoV or HRhV inhibition (panel A and B, respectively). The predicted % of inhibition were calculated using a non-linear interpolation method by exploiting the dose-response curves generated from the experimental data as standards, using GraphPad PRISM 7 (GraphPad Software, San Diego, CA, USA).

Cholesterol resulted to be the most preeminent sterol in human colostrum and milk: its levels were about 10 times lower compared to corresponding blood serum [33]. While lathosterol was the most abundant sterol found in blood serum, desmosterol resulted to be the most preeminent precursor sterol measured in colostrum, TM and MM. Its concentration was about 10–20 times higher compared to lathosterol and lanosterol. The sterols showed concentrations consistent with previous findings and we found a significant reduction of cholesterol and a progressive significant increase of precursor sterols from C to TM and MM as reported in previous studies [6,34–36]. It seems to be likely that along the process of BM maturation, the rate of cholesterol turnover markedly increases.

Previous studies reported that the delivery mode may influence the content of some components of BM. For instance, mothers undergoing cesarean delivery presented lower zeaxanthin levels in BM [37] and a decreased protein content and oxidative stress in colostrum [38,39].

Moreover, the mode of delivery was found to have large impact on the microbiota composition of colostrum [40]. In this study, no significant differences were observed in the 24SOHC, 25OHC and 27OHC contents between milk samples from mothers who underwent Cesarean section ($n = 7$) and those who had vaginal delivery ($n = 5$). Further studies are needed to confirm this finding in a large number of BM donors.

The finding of side-chain oxysterols in BM stimulates the search for a biological role. Since these molecules are not produced by spontaneous cholesterol autooxidation but, instead, they are synthesized by specific enzymes, it is reasonable to hypothesize that they could provide the breastfed infant with some sort of nutritional, trophic or immunological benefit. Of particular interest is the finding that the 27OHC content in colostrum is much higher than that of the 24SOHC and 25OHC, and declines over time to lower levels in TM and MM, probably due to a rapid reduction of the CYP27A1 activity.

These findings suggest a specific biological role during the first days

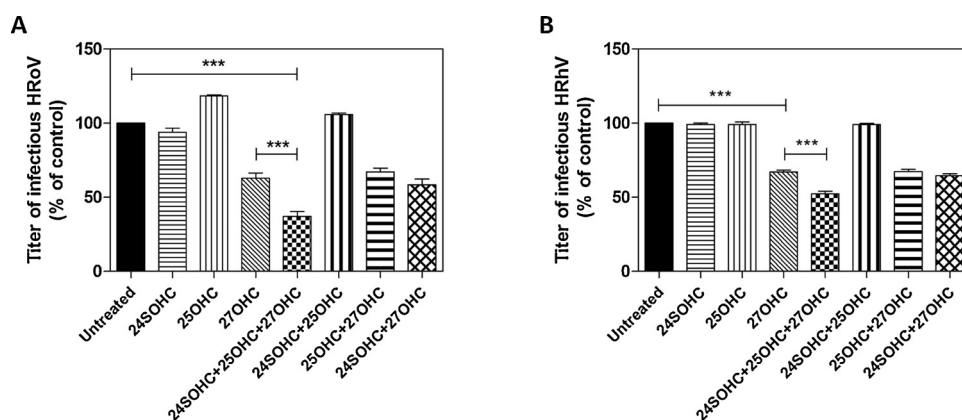


Fig. 4. Effect of combined treatment with side-chain oxysterols on HRoV and HRhV infectivity (panel A and panel B, respectively). Cells were treated for 20 h at 37 °C with 24SOHC, 25OHC, 27OHC, or a combination of them (i.e. 24SOHC + 25OHC + 27OHC, 24SOHC + 25OHC, 25OHC + 27OHC, or 24SOHC + 27OHC), at the respective concentrations as assessed in maternal colostrum. Cells were treated for 20 h with increasing concentrations of oxysterols and then infected. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for triplicates. ANOVA test followed by Bonferroni comparison post-hoc test was conducted for comparison of measured concentrations. Values of $p < 0.05$ were considered as significant. *** $p < 0.001$.

of breastfeeding and opens new lines of research. Among the several biological activities exerted by side-chain oxysterols, here we focused on their antiviral properties. Indeed, we demonstrated that the 27OHC concentration in colostrum lies within the range of its *in vitro* antiviral activity. Of note, most of the colostrum samples examined contained 27OHC concentrations higher than the EC50 calculated *in vitro* against HRoV. Such a finding suggests that this oxysterol might contribute to the known protective activity of human milk against gastroenteritis caused by HRoV [41,42]. Moreover, we found that the *in vitro* antiviral potency of 27OHC is increased by the concomitant presence of 24SOHC and 25OHC at the same concentrations found in colostrum. While this improvement is apparently not remarkable, it must be taken into account that the approximate usual intake of milk for a healthy term neonate is about 800 ml overall during the first 5 days of life, accounting for a total intake of about 0.010 mg of 24SOHC and 25OHC, and about 0.10 mg of 27OHC. This specific combination of oxysterols is therefore administered to the newborn at each breastfeeding (about 40 times for the first 5 days of life), so it is likely to hypothesize that the overall intake of side chain oxysterols is a biologically active dosage representing a passive transfer of innate immunity factors exerting a protective role during the first days of the newborn.

At least two main questions are generated by the claim here made of a primary antiviral effect of the 27OHC bolus dose received by the breastfed infant just after birth: would it be possible that the here demonstrated colostrum higher amounts of 27OHC are absorbed in the newborn's intestine? Where the suggested protection against viral infection could take place? With regard to the intestinal absorption of oxysterols, this has been demonstrated in a number of studies in rodents and rabbits, as quite recently reviewed by Otaegui-Arazola and colleagues [43]. Apparently only two reports are available that confirm the intestinal absorption of oxysterols also in humans. In one study the plasma recovery of non enzymatic oxysterols only was demonstrated following consumption of a powdered egg meal in volunteers [44]. In the second study, healthy young men were offered a salami- and parmesan-containing meal naturally rich in oxysterols and plasma and lipoprotein oxysterol concentrations were measured over the following 9 h. In this case, also a side-chain oxysterol was present in the test meal, namely 25OHC, and, interestingly, its recovery in plasma lipoproteins was extremely high [45]. Hence, even if not yet demonstrated directly, an intestinal absorption of 27OHC as well as that of 25OHC and 24SOHC in the intestine of breastfed infants appears very likely, and consequently, their protection against HRoV infections should mainly take place at the gut level. Of, interest, once incorporated in chylomicrons and other lipoproteins, oxysterols may be taken up by different tissues and cells, so it cannot be excluded that a consistent but moderate supply of oxysterols with BM could in the long run exert physiological effects also in cells other than enterocytes, for example on respiratory epithelial cells where HRhV replication takes place.

These and other issues must be addressed by further studies to understand the biological roles of side-chain oxysterols in BM, a totally unexplored field of research to date.

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