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ORIGINAL CONTRIBUTION

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Effect of the stage of lactation in humans on carotenoid levels in milk, blood plasma and plasma lipoprotein fractions

Summary In mammals the composition of milk changes during early lactation, with a rapid decline of fat-soluble vitamins and a continuous increase in total lipids. The mechanisms underlying this phenomenon are not well understood, but might involve selective mechanisms related to mammary uptake or secretion into the milk. Since carotenoids are specifically distributed among the lipoprotein fractions in plasma, the simultaneous determination of carotenoids in plasma, lipoprotein fractions and milk might offer an opportunity to gain insight into this phenomenon. In 21 healthy mothers carotenoids in plasma and lipoprotein fractions were investigated at day 2 and 19 and milk on day 4 and 19 after delivery. Plasma levels of α-tocopherol and cholesterol as well as lutein, zeaxanthin and cryptoxanthin were significantly lower later in lactation (day 19) than shortly after birth ($P < 0.01$). The stage of lactation had no effect on the distribution of carotenoids and α-tocopherol among the plasma lipoprotein fractions. In milk, triacylglycerol increased ($P < 0.01$). In contrast, levels of carotenoids, α-tocopherol and vitamin A were highest in colostrum and declined ($P < 0.01$). Because the magnitude of decrease was not the same in all carotenoids, the carotenoid pattern changed substantially. In colostrum the carotenoid pattern resembled those of plasma and the low-density lipoprotein fraction. In mature milk it was similar to the pattern found in the high density lipoprotein fraction. Based on these observations a selective mechanism might be responsible for the transfer of these components in milk involving different lipoprotein fractions at specific times of lactation.

Key words carotenoids – fat-soluble vitamins – colostrum – lipoproteins – human

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Introduction

The colostrum initially secreted by the mammary gland after birth is distinct in composition. Besides hormones and growth factors [1], it is a rich source of fat-soluble vitamins A and E as well as carotenoids. In cattle and horses the characteristic yellow coloration is mainly due to β-carotene [2–5], while in humans a wide variety of carotenoids are observed [6–8]. Because the transfer of fat-soluble vitamins via the placenta is limited and plasma levels are usually low in newborn babies [9], colostrum rich in fat-soluble vitamins and carotenoids is an important source of vitamins and pro-vitamins for the newborn. In adults and infants carotenoids, especially β-carotene, α-carotene and β-cryptoxanthin are important precursors of vitamin A [10, 11]. Carotenoids and α-tocopherol are also reported to be important either by acting as antioxidants [12], or by enhancing the immune response [13, 14]. Therefore, it is of importance that the newborn is supplied with fat-soluble vitamins and carotenoids with the colostrum. Problems with regard to a sufficient supply are reported for premature babies, who are prone to develop hemolytic anemia, characterized by an increased susceptibility of red cells to oxygen due to an insufficient supply of α-tocopherol. Furthermore, broncho pulmonary dysplasia and intraventricular hemorrhagia can be reduced by administra-

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tion of vitamin A and/or E, indicating the importance of these two milk constituents for lung development [15].

With the progress of lactation, a rapid decline of carotenoids, vitamins A and α-tocopherol as well as hormones and growth factors can be observed, while the amount of total lipids (basically triacylglycerol) increases substantially in many species [16–18]. Despite the observation that most fat-soluble micronutrients are enriched only in colostrum, very little is known concerning the mechanism involved in the transfer of carotenoids and fat-soluble vitamins from blood plasma to the secretion of the mammary gland. Based on the observation that β-carotene, α-tocopherol and vitamin A decrease with the progress of lactation in most mammals investigated so far while total lipids increase at the same time, it can be assumed that the transfer of these lipophilic components is not a mere reflection of the transfer of lipids into colostrum and milk, and a specific mechanism of transfer is assumed [2, 5, 8].

Cholesterol, phospholipids, carotenoids and α-tocopherol are presented to the mammary gland bound to the lipoprotein fractions of different density such as lipoproteins of very low, low and high density [19]. Because these components occur in all three lipoprotein fractions, it is difficult even with the use of labeled components to determine which individual lipoprotein fraction contributes to the transfer of a given component into milk. In contrast to cholesterol, phospholipids or α-tocopherol, the distribution of carotenoids among the major lipoprotein fractions is specific. Thus, the individual lipoprotein fractions are characterized by a specific carotenoid pattern [20].

The study was designed to investigate possible differences in the carotenoid pattern between plasma, lipoproteins and milk at two stages in lactation (colostrum and mature milk) which differ significantly in milk carotenoid composition that might give an idea about the mechanism involved in the transfer of carotenoids and lipids other than triacylglycerol from plasma to milk at different stages of lactation.

Subjects and methods

Subjects

Twenty-one healthy women on a regular diet without supplements containing carotenoids or vitamin A were recruited from mothers attending the Department of Obstetrics, Charité, Campus Virchow Klinikum, Berlin. All mothers were sampled within a period of eight weeks. Mothers giving birth before the 37th week of pregnancy were excluded. The local ethics committee approved the study protocol and informed consent was obtained from each participant. The average age was 30 ± 6 (20–39) years. All women had delivered at term 40 ± 1 (37–42) weeks, underwent uncomplicated pregnancies, and gave birth to healthy new born.

Blood and milk samples

First maternal blood and milk samples were obtained as close as possible to parturition (blood 2 ± 2 days post partum (pp); milk 4 ± 2 days pp) and second maternal blood and milk samples were obtained at 19 ± 2 day pp. Blood samples (5 ml) were collected by antecubital puncture and centrifuged (1500 x g; 10 min) to obtain plasma. The total milk volume of one breast was collected. After collection, the milk and plasma samples were transported on ice in the dark directly into the laboratory, where milk and plasma samples were aliquoted and stored at –80 °C and analyzed within one month after collection, a period and temperature at which analytes have been reported to be stable [21].

Selective precipitation of lipoproteins in plasma

High-density lipoproteins (HDL) were isolated from plasma using dextran sulfate and magnesium chloride to precipitate the very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions. Plasma (400 µl) was transferred into a microtube and 40 µl reagent added. The tube was immediately vortexed. After 10 min incubation at room temperature in the dark, the sample was centrifuged for 30 min at 1500 x g. The supernate containing the HDL particles was then removed and kept at 4 °C in the dark until further analysis [22].

Analysis of carotenoids and α-tocopherol in plasma, lipoprotein fractions and milk

A modified gradient reversed-phase HPLC-system was used for separation and quantification of carotenoids (lutein, zeaxanthin, canthaxanthin, α-carotene, β-carotene, β-cryptoxanthin, lycopene), α-tocopherol and vitamin A (retinol and retinyl esters) in plasma, plasma high density lipoprotein fraction and milk [8, 23]. Briefly, 200 µl of ethanol was added to 100 µl plasma or lipoprotein fraction diluted with 100 µl H_2O. After vortexing for 30 s, plasma or lipoprotein fractions were extracted twice with n-hexane (1 ml each time stabilized with 0.05 % butylated hydroxytoluene (BHT)) and vortexed for 3 min. The supernatants were removed, pooled, and evaporated under nitrogen and reconstituted in 200 µl isopropanol and injected into the HPLC system (Waters, Eschborn, Germany). For separation of compounds we used a C30 carotenoid column (5 µm, 250 × 4.6 mm; YMC, Wilmington, USA) in line with a C18

pre-column (Luna, Phenomenex, Germany) with a solvent system consisting of solvent A with methanol (Roth Chemicals Germany):water (90:10; v:v, with 0.4 g ammonium acetate in 1l H_2O) and solvent B with methanol:methyl-tert-butyl-ether (Sigma Deisenhofen, Germany):water (8:90:2; v:v:v, with 0.1 g ammonium acetate in 1l H_2O).

Carotenoids, α-tocopherol and vitamin A were extracted from milk samples according to a modified saponification method as described in detail elsewhere [24]. Briefly, milk lipids and total carotenoids, vitamin A and α-tocopherol were first extracted together with total lipids using n-hexane and methanol. To remove the triacylglycerol, the organic extract had to be saponified. Prior to this, however, polar carotenoids, susceptible to saponification, were extracted into ethanol. After saponification samples were combined and were further treated like plasma samples.

Accuracy and precision of the analyses were verified using a standard reference material (SMR 968a fat-soluble vitamins in human serum; National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA). Coefficient of variability over time using control plasma was less than 4% for carotenoids, vitamin A (retinol, retinyl esters) and α-tocopherol. The recovery rate was above 95% for all carotenoids, retinol and α-tocopherol. Total plasma carotenoids were computed by summing the individual carotenoids. Carotenoid standards of lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopene were provided by Hoffmann-La Roche (Basel, Switzerland), α-carotene, β-carotene, α-tocopherol, retinol and retinyl palmitate were from Sigma (Deisenhofen, Germany).

Concentrations of cholesterol, phospholipids and triacylglycerol in plasma, lipoprotein fractions and milk were determined enzymatically using commercial kits (Boehringer Mannheim, Germany).

Statistical procedures

Values are expressed as means and standard deviation (SD). Statistical analysis was carried out by Student's t-test. Values of $p < 0.05$ were considered significant.

Results

In all milk and plasma samples, carotenoids (lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene), α-tocopherol and retinol were observed. With regard to retinol in milk it has to be remembered that milk samples were saponified prior to analysis so that retinyl esters present in milk were hydrolyzed to retinol. Although differences in the concentration can be observed, similar carotenoids were observed in plasma, colostrum and mature milk. Table 1 summarizes the results for the analytes in plasma at the two time points during lactation. With the progression of lactation a significant ($P < 0.01$) decrease was observed in plasma for triacylglycerol, phospholipids α-tocopherol, lutein, zeaxanthin and β-cryptoxanthin, but not for α-carotene, β-carotene, lycopene or cholesterol.

With regard to the distribution of carotenoids between the lipoprotein fractions, Table 1 shows that irrespective of when the plasma sample was obtained, lutein and zeaxanthin were distributed more or less equally be-

Table 1 Levels of triacylglycerol, cholesterol phospholipids, retinol, α-tocopherol, and carotenoids in human plasma 2 and 19 days after delivery (mean ± SD)

	Plasma		% of total recovered in HDL	
	Day 2 pp	Day 19 pp	Day 2 pp	Day 19 pp
Triacylglycerol[1] (mmol/L)	1.66±0.50***	0.87±0.30	Nd[2]	Nd[2]
Cholesterol[1] (mmol/L)	5.75±0.89	6.47±1.51	Nd[2]	Nd[2]
Phospholipids[1] (mg/ml)	2649.6±341.7**	2413.2±389.5	Nd	Nd
Retinol[1] (μmol/L)	1.67±0.30	1.76±0.31	Nd	Nd
α-Tocopherol (μmol/L)	42.3±5.8***	36.4±7.2	28.2±5.2	29.7±6.0
Carotenoids (nmol/L):				
Lutein	349.5±86.1***	265.1±65.7	47.0±5.0	48.8±8.9
Zeaxanthin	51.7±16.0***	36.7±14.9	45.0±4.8	46.9±11.6
Canthaxanthin	46.4±22.6	41.0±37.7	19.5±4.3	21.7±9.8
β-Cryptoxanthin	413.4±217.4**	310.1±177.5	27.7±4.2	28.1±12.1
α-Carotene	187.5±121.0	195.5±134.4	13.3±3.8	11.1±4.1
β-Carotene	652.2±363.1	725.8±424.2	9.9±2.9	7.5±2.7
9-*cis*-β-Carotene	21.0±9.1*	17.5±7.6	15.0±4.8	13.0±4.6
Lycopene	519.7±230.3	490.1±317.6	7.9±2.4	6.0±3.6

Differences between days 2 and 19 (plasma) are significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)
[1] No analysis was done for the lipoprotein fractions (Nd)

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tween HDL and the VLDL/LDL fractions, while all other less polar carotenoids as well as α-tocopherol were predominantly associated with the VLDL/LDL fractions, but the carotenoid pattern varied greatly between the different lipoprotein fractions (Fig. 1).

Significant differences were observed for most milk components with the progression of lactation (Table 2). For total and individual carotenoids, α-tocopherol and cholesterol, high values were found in the colostrum samples and low in mature milk ($P < 0.001$). The change was not the same for all carotenoids. The decrease was most pronounced in lycopene, followed by α-carotene, β-cryptoxanthin and β-carotene, and lowest for lutein and zeaxanthin. In contrast to the observed decrease of total carotenoids, α-tocopherol and cholesterol, the concentration of triacylglycerol increased significantly ($P < 0.01$). In any case, carotenoid concentrations in milk were always lower than in plasma as indicated by a plasma to milk ratio of greater than 1. The only exception to this was canthaxanthin, which had a concentration that was 4 times higher in colostrum and equal to plasma values in mature milk.

Table 2 Levels of triacylglycerol, cholesterol, vitamin A, α-tocopherol, and carotenoids in colostrum and mature human milk at days 4 and 19 after delivery (mean ± SD) and percentage of these components in mature milk compared to colostrum

	Colostrum	Mature milk	Relative changes[2]
Triacylglycerol (mmol/L)	32.1±11.2**	42.7±14.7	147.5±65.0
Cholesterol (μmol/L)	0.539±0.156***	0.361±0.084	72.5±24.3
α-Tocopherol (μmol/L)	51.1±31.1***	13.2±5.1	34.3±19.6
Vitamin A[1] (μmol/L)	5.35±2.53**	2.90±1.12	61.6±36.7
Carotenoids (nmol/L):			
Lutein	164.0±84.9**	88.1±37.8	57.5±24.3
Zeaxanthin	33.2±17.2**	19.5±10.2	64.8±33.8
Canthaxanthin	190.3±172.1***	34.8±23.1	27.7±21.5
β-Cryptoxanthin	238.8±156.1***	60.6±36.7	28.1±15.2
α-Carotene	170.4±147.5***	21.2±14.3	17.6±12.7
β-Carotene	423.4±326.6***	78.2±46.2	22.6±14.2
9-*cis*-β-Carotene	18.4±11.9***	4.3±2.4	25.1±13.2
Lycopene	508.9±421.7***	59.8±38.9	17.3±11.7

Differences between days 4 and 19 are significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)

[1] Milk was saponified prior to analysis, thus, retinyl esters present in milk were hydrolyzed to retinol

[2] Percentage of the concentration of analytes found in mature milk compared to that in colostrum

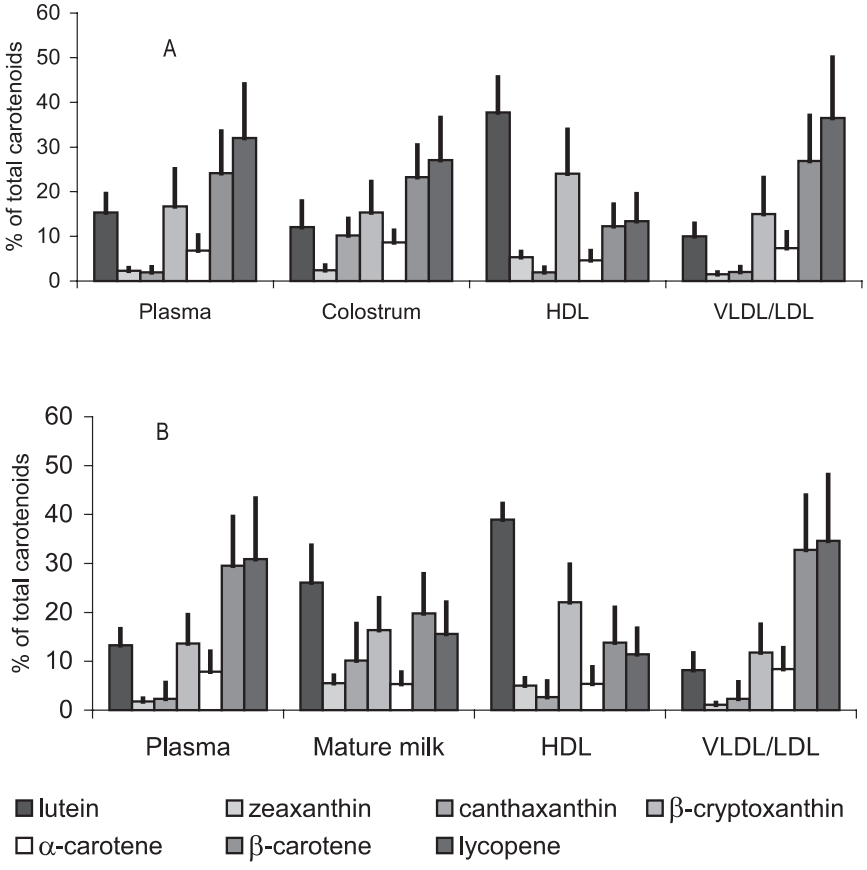

Fig. 1 Differences in carotenoid pattern (% of total carotenoids in the respective sample; mean ± SD) of plasma, milk and lipoprotein fractions at days 2 (plasma, lipoprotein fractions) and 4 (milk), respectively after delivery (**A**) and at day 19 after delivery (**B**)

Discussion

Both absolute plasma concentrations of lipids (triacylglycerol, phospholipids, cholesterol), α-tocopherol, retinol and carotenoids as well as the ratio of individual carotenoids to each other corresponded well to published data [9, 25–27]. The observed decrease between plasma samples obtained shortly after delivery and 3 weeks post partum has been reported previously for triacylglycerol, phospholipids, cholesterol and α-tocopherol and has been attributed to the decrease in plasma lipoproteins, the carriers of these plasma components [19].

Our results for the absolute levels of the individual carotenoids and α-tocopherol in milk correspond to published data [5, 8, 16, 26, 31–35]. Both the total amount of triacylglycerol in milk as well as the increase with the progress of lactation are in close agreement with previous reports [31, 36, 37]. Carotenoids, α-tocopherol and cholesterol in milk decreased over time with highest levels in colostrum and lowest levels in mature milk. This has not only been reported in humans [5, 16, 38, 33] but also in other mammalian species such as cows, horses and grey seals [2, 4, 39]. Similar to our results in humans, α-tocopherol, vitamin A and β-carotene levels in mature cow milk six weeks after parturition represent only 32, 20 and 6 % of the respective concentrations in colostrum [2].

These obvious differences between carotenoids, α-tocopherol and cholesterol, on the one hand, and triacylglycerol, on the other hand, shows that the high concentration of fat-soluble vitamins, carotenoids and cholesterol in colostrum is not merely secondary to lipid secretion into milk. It indicates that different mechanisms are involved in the transfer of lipids from blood plasma into milk as has been proposed for α-tocopherol in humans and seals [5, 39] and additionally for β-carotene in cattle [2]. Because of the similar trends observed in this study, their mechanism of transfer from blood plasma into the secretion of the mammary gland might be very similar for all investigated components in milk expect for triacylglycerol.

It has been reported that carotenoids are not equally distributed among the lipoprotein fractions in plasma. The determining factor for the distribution of carotenoids among the lipoprotein fractions may be the nature of the carotenoid. The less polar hydrocarbon carotenoids such as β-carotene, α-carotene, lycopene and also β-cryptoxanthin are primarily associated with the LDL fraction and represent 60–80 % of the total amount in plasma. The more polar carotenoids, including zeaxanthin and lutein, are distributed more or less equally between LDL and HDL [28, 29]. These observations are independent of the method used to isolate the lipoprotein fractions such as ultracentrifugation or selective precipitation [22]. They correspond to our results for the distribution of carotenoids between HDL and VLDL/LDL obtained by the selective precipitation of the VLDL/LDL fraction. Since carotenoid levels are strongly affected by dietary intake the percentage distribution as shown in Table 1 is a more appropriate measure than absolute values found in the individual lipoprotein fractions.

In contrast to plasma constituents carried by lipoprotein, concentrations of retinol, which is bound to its specific carrier, the retinol binding protein (RBP), remain more or less constant over the investigated period. This can be explained by the homeostatic control of plasma retinol levels [9, 30].

When comparing carotenoid patterns between plasma, lipoprotein fractions and milk, a few factors have to be considered. First, the carotenoid patterns differ significantly between colostrum and mature milk. Second, the distribution of carotenoids among the lipoprotein fractions and thus the carotenoid patterns do not change with the stage of lactation. Third, all three lipoprotein fractions have a distinct carotenoid pattern. As discussed previously, not only the carotenoid concentration but also the carotenoid pattern differ significantly between colostrum and mature milk, showing a change from a higher proportion of the less polar carotenoids β-carotene and lycopene in colostrum to the polar lutein and zeaxanthin in mature milk. At the same time the proportion of carotenoids in plasma and the lipoprotein fractions (HDL vs. VLDL/LDL) showed no obvious change between samples taken early in lactation and later on (Fig. 1).

Based on this first direct comparison between milk, plasma and lipoprotein fractions (Fig. 1), the differences in the distribution of the individual carotenoids among the lipoprotein fractions in plasma and the differences in the decrease of individual carotenoids in milk during the progress of lactation support the hypothesis that many lipophilic components other than triglycerides are transferred in most mammalian species from plasma into milk in a similar fashion and that this mechanism might be different during colostrogenesis and the transfer in lactation. The exact mechanism remains to be elucidated. It might involve a selection on the level of lipid uptake via specific lipoprotein fractions as indicated by similarities in carotenoid pattern, intracellular transfer and/or excretion into the colostrum and milk, respectively.

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