Peer-reviewed version available at Nutrients 2017, 9, 1229; doi:10.3390/nu9111229

Article

Concentrations of carotenoids and tocopherols in breast milk from urban Chinese mothers and their associations with maternal characteristics: a cross-sectional study

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Abstract: This study aims to quantify carotenoids and tocopherols in human milk from healthy Chinese women, and to explore their associations with region, lactation stage, and maternal socio-economic and obstetric factors. Human milk was obtained from 509 healthy mothers and the compounds of carotenoids and tocopherols were analyzed by high-performance liquid chromatography after mild saponification and solvent extraction. Socio-economic and obstetric characteristics of the mothers and their dietary intakes through a single 24-hour dietary recall were evaluated. The median content of each component [μg/100mL, median (interquartile range)] in colostrum and mature milk was, respectively, β-carotene 8.0 (4.7-15.2) and 1.8 (1.4-2.7), β-cryptoxanthin 6.2 (2.4-12.9) and 1.8 (1.1-3.4), lutein 5.7 (2.9-10.2) and 3.4 (1.5-6.0), lycopene 6.3 (4.0-9.9) and 1.4 (1.1-2.0), zeaxanthin 1.0 (0.6-1.5) and 1.0 (0.6-1.4), α-tocopherol 645 (388-1176) and 211 (131-321), γ-tocopherol 68 (48-121) and 77 (45-120). The levels of all those vitamins presented regional differences, and decreased as lactation stage increased except for zeaxanthin and γ-tocopherol. Associations of carotenoid contents with maternal education, delivery mode, and present body mass index were found in multivariate analyses. These results suggest that some region, lactation stage, obstetric and socio-economic factors are associated with human milk concentrations of carotenoids and tocopherols in healthy Chinese mothers.

Keywords: Breast milk; Carotenoids; Tocopherols; Colostrum; Lactation stage; Cross-sectional study

1. Introduction

According to World Health Organization, exclusive breastfeeding is recommended for the first six months of life [1], period within which breast milk is the sole source of nutrition, providing all necessary nutrients to maintain health and permit normal growth. Thereafter, complementary feeding should be introduced while breastfeeding continues up to 2 years of age or beyond, so that breast milk is still a significant source of nutrients, at least in some parts of the world [2]. The recommended micronutrient intake for infants is currently based on the amounts provided by human milk from well-nourished women [3, 4]; although this has been questioned by some authors, given the high variability observed among individuals [5, 6]. Nevertheless, in absence of better studies to determine optimal intake, milk composition remains the best estimate to determine infant requirements.

Vitamin A designates a family of compounds having the biological activity of retinol. Vitamin A is present in breast milk in the forms of preformed retinol (as retinyl esters) but also present as provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthine). Carotenoids may represent a significant source of vitamin A for humans [7, 8] and, in particular, for the nursing infant, especially in developing countries [3, 9]. Vitamin A is required in many essential metabolic functions for the growing infant. Some of these processes, such as haematopoiesis, bone development, maintenance of epithelial cells, mucous membranes and immune functions can be supported by all forms of vitamin A (including provitamin A carotenoids); while vision and reproduction specifically require retinol [10] Since infants are born with very low reserves of vitamin A in the liver regardless on the mother's nutritional status [3], they rely entirely on breast milk to support growth and build up liver storage. In addition, non-provitamin A carotenoids (lutein, zeaxanthine and lycopene) have been associated with varied health benefits, from antioxidant to anti-inflamatory, immune or visual functions and cancer prevention [11-15].

Vitamin E is a family of eight naturally occurring compounds sharing a common structure, namely α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol. The most frequently present in nature and the most active form is by far α-tocopherol (showing 100 % vitamin E activity), followed by β-tocopherol, α-tocotrienol and γ-tocopherol. Breast milk contains primarily α-tocopherol, followed by smaller amounts of γ- and β-tocopherol. The main function of vitamin E in the human body is as an antioxidant, protecting fatty acids from oxidation in cell membranes and lipoproteins, but also improves immunity and prevents inflammatory conditions [16, 17].

Given the importance of all these compounds for the newborn and the specific risk of vitamin deficiency in lactating women and breastfed children [18, 19], the need to determine their concentrations in breast milk and how they depend on external or internal factors has been identified. A large number of reports in the composition of milk from Chinese mothers have focused on macronutrients [20-22], minerals [20, 23], and fatty acids [23-29], while only a few studies report fat soluble vitamin or carotenoid values [9, 30, 31]. Shi et al. [30] provide data on the vitamin content of milk from Chinese mothers from Inner Mongolia, which might be difficult to extrapolate to the whole country, partially due to the low number of data points and partially to the fact that it represents only a part of the country. The multinational study of Canfield et al. [9] showed differences in carotenoid patterns between countries, reflecting that each one the dietary carotenoid supply had no indication on the geographical origin of all samples, although this might be limited. Another multinational study [31] showed that, regardless of the difficulty to detect trends due to high individual variability, some carotenoids presented clear differences between countries, confirming previous results, but presenting the same drawbacks related to the limited geographical variability of the samples. Thus, considering the geographical extension and multivariate lifestyles and diet in different parts of China, it would be necessary to study the vitamin and carotenoid composition of milk from Chinese women from different regions through lactation. Vitamin composition data are available in mothers from other countries [9, 31-39] and can be used for data comparison.

The changes in milk composition depending on different factors such as stage of lactation or duration of the feeding [38-40], maternal diet, supplementation and nutritional status [36, 41-48] have been demonstrated. In recent years, association studies have found other potential factors influencing the concentrations of vitamins in breast milk, including maternal socio-economic [34, 49, 50], obstetric or physiological factors [34, 38, 48, 49, 51, 52]. Indeed, maternal socio-economic and obstetric factors are changing in China, such as the rate of cesarean delivery, which increased from 3.4 % in 1988 to 39.3 % in 2008 [53] and to 54.5 % in 2011 [54]; as well as the increase in inappropriate gestational weight gain (GWG), partly due to over nutrition and rising of different dietary habits [55], which may have an impact on the micronutrient status of lactating women and the composition

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of their milk. Therefore, it is deemed necessary to research the associations of carotenoids and tocopherols in breast milk with maternal characteristics, obstetric and nutritional factors in China.

The aims of this study were 1) to determine the composition of carotenoids and tocopherols in breast milk from healthy mothers from urban areas of China along lactation (zero to eight months) 2) to evaluate their interregional differences, and 3) to explore associations with nutrient intake. In addition, the associations with maternal socio-economic and obstetric characteristics, such as age, offspring gender, education, household income, delivery mode, body mass index (BMI), and GWG, were investigated. This study is part of the larger initiative Maternal Infant Nutrition Growth (MING) study.

2. Materials and Methods

2.1 Background of participants

The MING study was a cross-sectional study designed to investigate the dietary and nutritional status of pregnant women, lactating mothers and young children aged from birth up to three years living in urban areas of China. In addition, the human milk composition of Chinese lactating mothers was characterized. The study was conducted between 2011 and 2012. Three cities (Beijing, Suzhou and Guangzhou) were chosen for the characterization of human milk according to the geographical location and status of economic development. In each city, one grade A hospital and one maternal and child care hospital were randomly selected at each site, mothers at lactation period 0 to 240 days were randomly selected based on child registration information. Subjects period 0-5 days were recruited at the grade A hospital, and subjects period 6-11 days and 12-30 days were contacted by phone to join the study whereas other subjects were recruited at maternal and child care hospital; if participation was dismissed a replacement was made. Recruitment, and milk sampling as well as baseline data collection were done in separate days.

A stratified sampling of 540 lactating mothers in six lactation periods of 0-4, 5-11, 12-30, 31-60, 61-120 and 121-240 days was obtained in MING study. Eligibility criteria included women between 18-45 years of age giving birth to a single, healthy, full-term gestation and exclusive breastfeeding at least until 4 months. Exclusion criteria included gestational diabetes, hypertension, cardiac diseases, or acute communicable diseases. Lactating women who had nipple or lacteal gland diseases, been using of hormone in recent three months, postpartum depression, or insufficient skills to understand study questionnaires were also excluded.

In this cross-sectional study, carotenoids and tocopherols were quantified in 509 breast milk samples collected at different stages from early to late lactation in healthy Chinese women from three different cities (Beijing: n = 151; Guangzhou: n = 180; Suzhou: n = 178). Figure 1 displays the recruitment flowchart from eligibility to sample analysis.

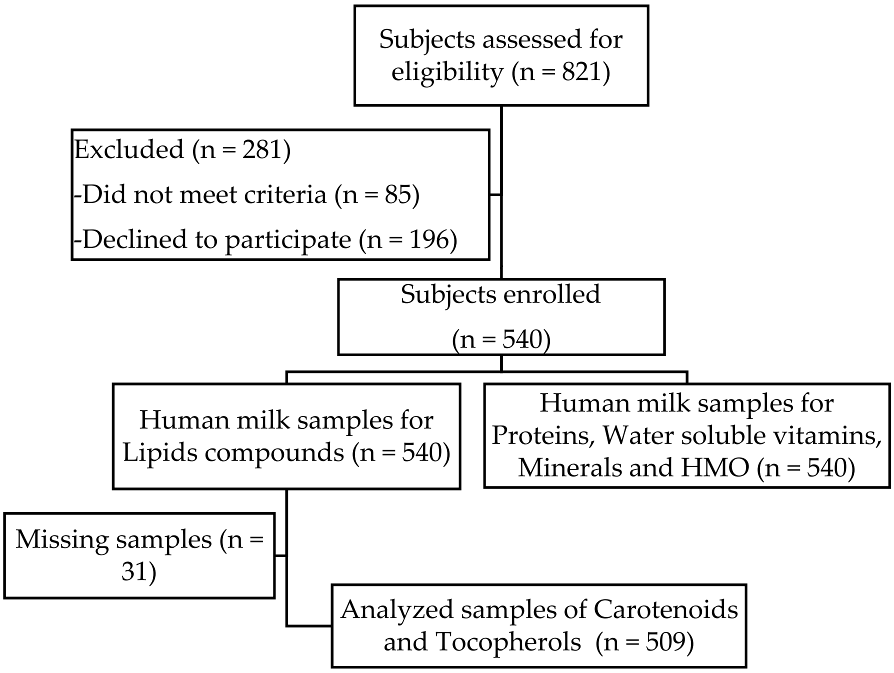

Figure 1. Study flow chart subjects enrolled

2.2 Data collection

All subjects completed a structured questionnaire including socio-economic and lifestyle aspects of the mother such as household income, maternal education and age. Self-reported weight at the beginning and at the end of pregnancy, number of gestational weeks at delivery, and delivery mode were also recorded. Additionally, a physical examination evaluated basic anthropometric parameters including height and weight which were explored to calculate the current BMI (kg/m2), and BMI < 18.5, 18.5-24.9, 25.0-29.9, and ≥ 30 kg/m^2 were defined as underweight, normal weight, overweight, and obese, respectively. These data was obtained to calculate the GWG. According to the guidelines from the Institute of Medicines (IOM) in the United States [56] suggesting that underweight women gain 12.5 kg to 18 kg, normal weight women gain 11.5 kg to 16 kg, overweight women gain 7 kg to 11.5 kg, and obese women gain 5 kg to 9 kg respectively, inadequate, adequate, and excessive weight gain were confirmed.

Dietary intake was assessed using one 24-hour dietary recall. Trained interviewers asked lactating mothers about all foods, beverages and supplements consumed on the previous day. A picture booklet of common foods consumed in China and measurement aids were used to estimate the amount of foods and beverages consumed. Details about food ingredients of homemade foods or meals eaten out were also asked and recorded. In addition, information on the use of dietary supplements was collected, including the name and brand of the supplement, age when supplement was first given and the amount used. A list of dietary supplements commonly used in China was used to identify the supplements reported during the interview.

Data collection was done through face-to-face interviews during the day of human milk sample collection. In addition, date of birth and gender information of the baby was collected after the data collection since the data was not included in the initial questionnaires. Subjects were contacted by phone and were asked to clarify these two aspects retrospectively.

2.3 Dietary assessment

After revision of questionnaires, food records were entered in a database and individual intakes of vitamin A, total carotenoids, retinol, vitamin E, and α-tocopherol were processed with a food composition database created for this study that included data from Chinese Food Composition

(CFC) tables 2004 & 2009 [57, 58], the Japanese Food Composition (JFC) tables 2005 [59] and branded products and supplements from China. In total, it contained information of 1773 foods with 36 nutrients. Finally we also compiled nutritional information from 75 dietary supplements sold in China.

2.4 Sample collection

Breast milk sampling was standardized for all subjects and an electric pump (Horigen HNR/X-2108ZB, Xinhe Electrical Apparatuses Co., Ltd) was used to sample the milk. Samples were collected at the second feeding in the morning (9-11 am) to avoid circadian influence on the outcomes. Single full breast was emptied and an aliquot of 15 mL for colostrum and 40 mL for the remaining time points was secured for characterization purposes. The rest of the milk was returned to the mother for feeding to the infant. Each sample was distributed in 1mL freezing tubes, labeled with subject number, stored at -80℃ and then transported to Lausanne (Switzerland) for analysis within 6 months of collection.

2.5 Sample preparation

Briefly, 5 μL of ethanol containing butylated hydroxytoluene (BHT) (79 g/L), 10 μL of an aqueous solution of deferoxamine mesylate (10 mg/mL), 4 mL methanol, and 1 mL aqueous solution of potassium hydroxide (KOH) (30 % w/w) were added successively to 1 mL of milk into a 15-mL tube. After mixing, the tube was placed for 30 minutes in a shaking water bath at 37 ℃ for saponification. The samples were then cooled down on ice, 5 mL of hexane containing 350 mg/L BHT added and mixed vigorously for 30 seconds. Then the tubes were centrifuged at 2500 rpm for 10 min at 4 ℃ and the upper organic phase transferred to a clean 15-mL tube by means of a glass Pasteur pipette. The liquid/liquid extraction process was repeated and the organic phases combined in the same tube. Once completely dried under nitrogen at room temperature, the residue was dissolved in 70 μL of dioxane/ethanol (1/1, v/v) and 70 μL acetonitrile were finally added. The samples were centrifuged at 2500 rpm for 10 minutes at room temperature and transferred into adapted low volume Ultra Performance Liquid Chromatography (HPLC) vials before analysis.

2.6 Sample analysis

All the compounds (α-tocopherol, γ-tocopherol, β-carotene, β-cryptoxanthin, lutein, lycopene and zeaxanthin were determined using a Waters Acquity Ultra Performance Liquid Chromatography UPLC® system (Waters, Milford, MA, USA) equipped with a 2.1 mm × 150 mm Waters Acquity UPLC® HSS T3 column, 100 Å (particle diameter, 1.8 μm) placed in a column oven set at 35 °C, while autosampler was set at 20 °C.

A 5 μL-aliquot of the final extract was injected into the analytical system. The binary gradient eluting system pumped the mobile phase at a flow rate of 0.4 mL/min. Solvent A was a solution of ammonium acetate 0.05 M in water, and solvent B was a mixture of acetonitrile/diethyl ether/methanol (589/71/119, w/w/w). The eluting gradient program was: 0-20 min, 75 % B; 20-22 min, 78 % B; 22-22.1 min, 80 % B; 22.1-30 min, 100 % B; 30-42 min, 100 % B; 42-42.1 min, 75 % B; 42.1-55 min, 75 % B. Quantification was performed by external calibration using pure standards. Concentration of standards was determined by spectrophotometry with corrections made for chromatographic purity. Carotenoids were detected and quantified using ultra violet (UV) at different wavelengths (lycopene, 472 nm; β-carotene, β-cryptoxanthin, lutein, and zeaxanthin, 450 nm), while α-tocopherol and γ-tocopherol were detected and quantified by fluorescence ($\lambda_{\text{excitation}}$: 298 nm, $\lambda_{\text{emission}}$: 328 nm). The standard calibration curve for each compound was constructed by plotting the response (Peak area) versus the concentration using a weighted linear regression model.

2.7 Statistical analysis

The database was established by using Epi Data version 3.0, and a double data entry was carried out. For the information of demographic characteristics, the data were presented as count

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with percentage for categorical data and median with interquartile range for continuous data with non-normal distribution. Before the progress of data analysis, Shapiro-Wilk test was used to determine whether carotenoids, and tocopherols in breast milk, and vitamins intake had a normal distribution or not. Because of non-normal distributions, median values (interquartile range) were performed to describe and ln transformations were applied when doing multivariate analysis and correlation analysis. Differences in breast milk vitamins were compared among stages of lactating period (0-4 days, 5-11 days, 12-30 days, 31-60 days, 61-120 days, and 121-240 days *postpartum*) and cities (Beijing, Suzhou, and Guangzhou cities) by using nonparametric Kruskal-Wallis test, then nonparametric Mann-Whitney U tests were employed to detect specific differences between the abovementioned groups further. According to demographic characteristics of lactating women and their offspring, comparisons in carotenoids and tocopherols concentration were carried out by using covariance analysis models adjusted with stages of lactating period and research cities. Furthermore, multivariate linear regression models were explored to research the demographic influencing factors of carotenoids and tocopherols concentrations in breast milk. To research the correlations between these contents in breast milk and dietary vitamins intake, Partial-correlation adjusted with stages of lactation and research cities were performed. All of the analyses were carried out using version SPSS 20.0 (SPSS Inc. Chicago, IL, USA), and all tests were two-tailed with statistical significance set at $p <$ 0.05.

3. Results

The socio-economic characteristics of the lactating women are summarized in Table 1. The mean age of the lactating women was 27.4 ± 4.0 years. The majority of lactating women were unemployed, had completed high school, and a monthly household income representative of urban China. Although the majority of women had normal BMI at present, 44 % of them had excessive GWG and up to 48% lactating women had a cesarean delivery. According to the stage of lactating period, there were no significant differences in the socio-economic characteristics of the lactating women such as age, offspring gender, family’s per capita income, current BMI, GWG, and pregnancy duration. However, it was found that less lactating women during 121-240 days *postpartum* had college education level or above when compared with the others ($p < 0.05$). Meanwhile, more lactating women during 0-4 days and 31-60 days *postpartum* underwent cesarean delivery when compared with those women during 5-11 days, 61-120 days, and 121-240 days *postpartum* ($p < 0.05$), and more lactating women during 31-60 days, 61-120 days, and 121-240 days *postpartum* received dietary supplement than those women during 0-4 days *postpartum* ($p < 0.05$).

Table 1. Demographic characteristics of lactating mothers with different stages of lactating period.

	0-4 days (n = 77)	**5-11 days (n = 89)**	**12-30 days (n = 73)**	**31-60 days (n = 90)**	**61-120 days (n = 90)**	**121-240 days (n = 90)**	***P*-value**
Age, years [1]							0.097
< 25	22 (28.6)	27 (30.3)	26 (35.6)	18 (20.0)	26 (28.9)	36 (40.0)	
25-30	35 (45.5)	41 (46.1)	29 (39.7)	44 (48.9)	50 (55.6)	39 (43.3)	
> 30	20 (26.0)	21 (23.6)	18 (24.7)	28 (31.1)	14 (15.6)	15 (16.7)	
Offspring gender [1]							0.158
Male	35 (45.5)	51 (57.3)	39 (53.4)	48 (53.3)	54 (60.0)	43 (47.8)	
Female	42 (54.5)	38 (42.7)	31 (42.5)	39 (43.3)	36 (40.0)	44 (48.9)	
Education [1]							0.003 *
Middle school or blow	17 (22.1)	12 (13.5)	16 (21.9)	26 (28.9)	22 (24.4)	39 (43.3)	
High school	23 (29.9)	31 (34.8)	27 (37.0)	22 (24.4)	25 (27.8)	23 (25.6)	
College or above	36 (46.8)	45 (50.6)	29 (39.7)	42 (46.7)	41 (45.6)	26 (28.9)	
Family's per capita income, Yuan/month [1]							0.140
< 2000	16 (20.8)	19 (21.3)	16 (21.9)	24 (26.7)	26 (28.9)	31 (34.4)	
2000-4000	30 (39.0)	37 (41.6)	34 (46.6)	41 (45.6)	40 (44.4)	41 (45.6)	
> 4000	27 (35.1)	30 (33.7)	17 (23.3)	23 (25.6)	22 (24.4)	18 (20.0)	
Unclear	4 (5.2)	3 (3.4)	6 (8.2)	2 (2.2)	2 (2.2)	0 (0.0)	
Delivery mode [1]							0.002 *
Vaginal delivery	29 (37.7)	50 (56.2)	35 (47.9)	37 (41.1)	55 (61.1)	55 (61.1)	
Cesarean delivery	48 (62.3)	37 (41.6)	38 (52.1)	53 (58.9)	35 (38.9)	34 (37.8)	
Present BMI [1]							0.075
Underweight	1 (1.3)	5 (5.6)	2 (2.7)	2 (2.2)	4 (4.4)	8 (8.9)	
Normal	48 (62.3)	54 (60.7)	47 (64.4)	57 (63.3)	69 (76.7)	65 (72.2)	
Overweight	24 (31.2)	26 (29.2)	23 (31.5)	26 (28.9)	16 (17.8)	16 (17.8)	
Obesity	4 (5.2)	3 (3.4)	1 (1.4)	5 (5.6)	1 (1.1)	1 (1.1)	
Gestational weight gain [1]							0.300
Inadequate	17 (22.1)	11 (12.4)	14 (19.2)	17 (18.9)	19 (21.1)	26 (28.9)	
Adequate	27 (35.1)	29 (32.6)	28 (38.4)	32 (35.6)	36 (40.0)	25 (27.8)	
Excessive	33 (42.9)	48 (53.9)	29 (39.7)	41 (45.6)	34 (37.8)	39 (43.3)	
Dietary supplements intake [1]							0.028 *
Yes	5 (6.5)	13 (14.6)	17 (23.3)	17 (18.9)	22 (24.4)	13 (14.4)	
No	72 (93.5)	76 (85.4)	56 (76.7)	73 (81.1)	68 (75.6)	77 (85.6)	
Pregnancy duration, weeks [2]	39 (38-40)	39 (39-40)	39 (38-40)	39 (38-40)	39.5 (39-40)	40 (39-40)	0.332

BMI, body mass index, was calculated as body weight by height squared (kg/m^2). Data are expressed as medians (interquartile ranges) for continuous variables and count (percentage) for categorical variables. * Indicates a significant difference among six stages of lactating period ($p < 0.05$). [1] Compared by Kruskal-Wallis test. [2] Compared by chi-square test.

The concentrations of the different compounds studied at different periods of lactation are shown in Figure 2 and Supplementary Table 1. As expected, significant differences ($p < 0.001$) according to the different periods of lactation were observed for many compounds. The concentrations of most of them (except for lutein and zeaxanthin) were significantly higher in colostrum (0-4 days *postpartum*) than in transitional (5-11 days and 12-30 days *postpartum*) and mature milk (31-60 days, 61-120 days, and 121-240 days *postpartum*) ($p < 0.01$); a decrease was observed with advancement of lactation until reaching stable levels from 12 days *postpartum*. Furthermore, lutein concentrations in milk from lactating women during 0-4 and 5-11 days *postpartum* were significantly higher compared with those during other periods ($p < 0.01$), while zeaxanthin and γ-tocopherol concentrations remained stable over time.

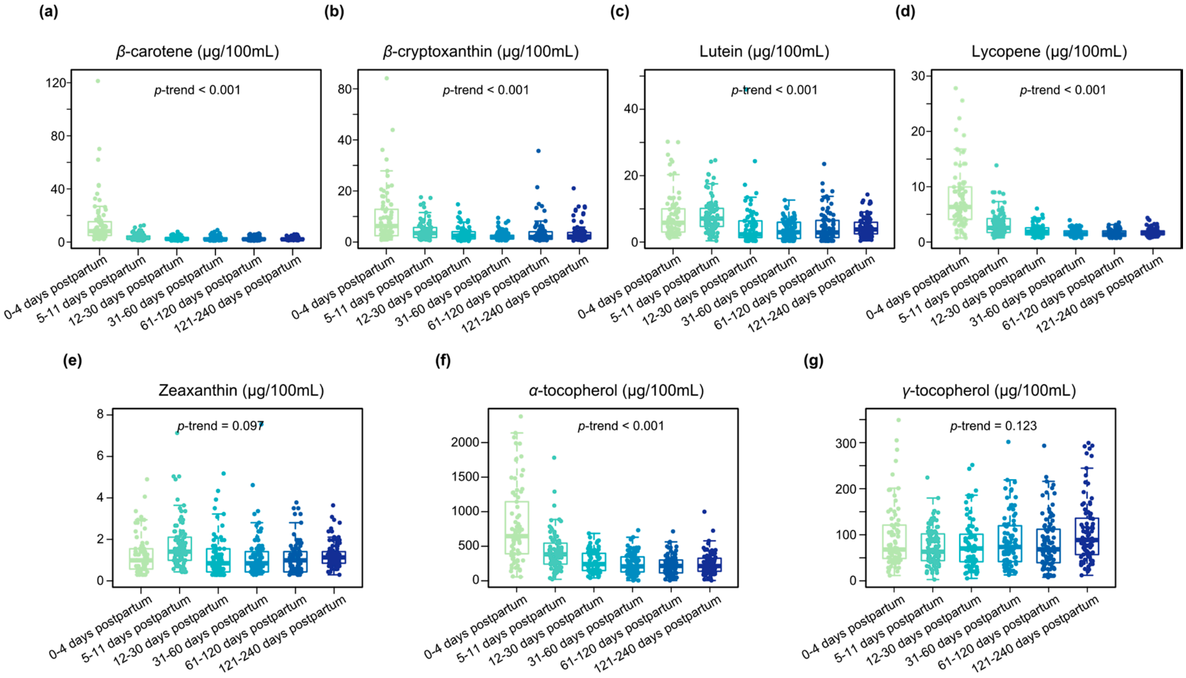

Figure 2. Carotenoids and tocopherols of human milk at different lactation stages (μg/100mL). Analysis of variance, ANOVA. Data are presented as the median (interquartile range). Linear trends of carotenoids and tocopherols levels along with lactation stages were tested by One-Way ANOVA after ln transformations.

Carotenoids and tocopherol concentrations in breast milk from lactating women in the three cities are shown in Figure 3 and Supplementary Table 2. The lycopene content in milk from Guangzhou city was significantly higher compared with those from Beijing and Suzhou cities ($p < 0.001$). Similarly, the majority of carotenoids (β-carotene, β-cryptoxanthin, lutein, and zeaxanthin) and α-tocopherol concentrations in milk from Beijing were significantly lower than those from Suzhou and Guangzhou ($p < 0.01$). While γ-tocopherol concentrations in mothers from Suzhou were the highest among the three cities ($p < 0.001$).

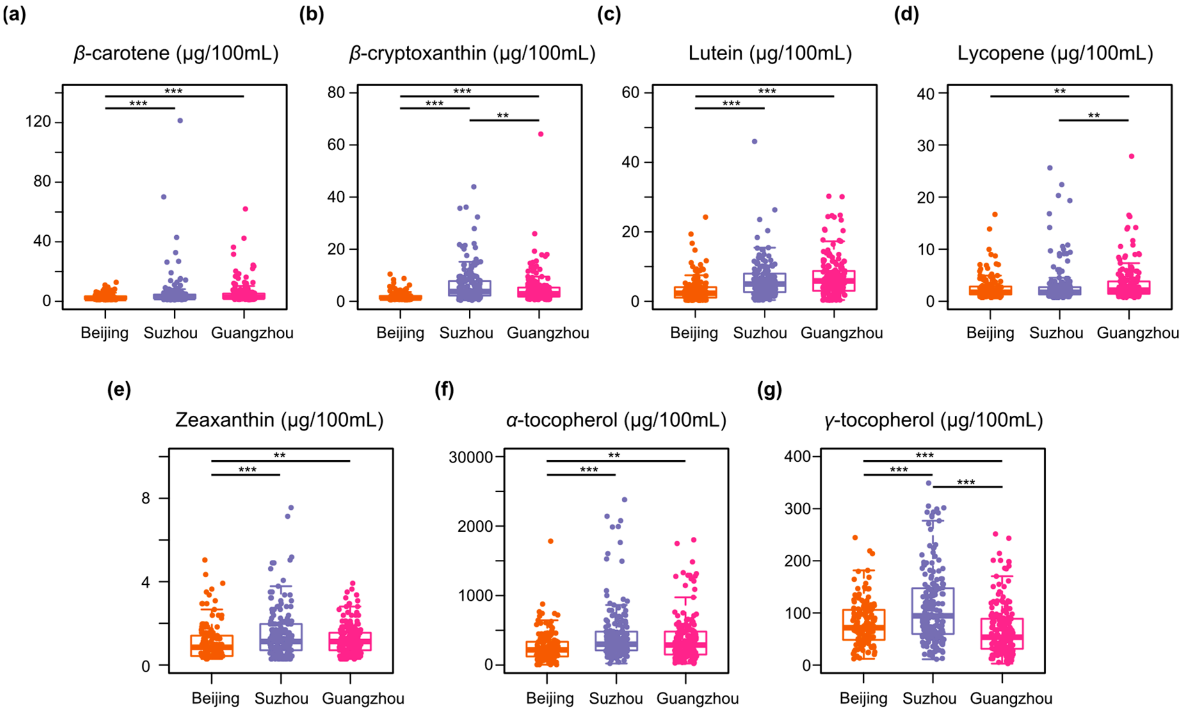

Figure 3. Carotenoids and tocopherols concentration of human milk from different cities (Beijing, Suzhou, and Guangzhou cities). Data are presented as the medians (interquartile ranges). Compared by Mann-Whitney U test with adjusted alpha value ($\alpha' = 0.01$). **, $p < 0.01$; ***, $p < 0.001$.

Comparisons of the concentrations by characteristics of lactating women and their offspring are provided in Table 2 and Supplemental Table 3. There were no significant associations detected between maternal age, offspring gender, household income, maternal GWG, dietary supplement intake, and tocopherols in breast milk ($p > 0.05$). However, zeaxanthin concentrations in lactating women with vaginal delivery were significant higher compared with those with cesarean delivery ($p < 0.05$). In addition, zeaxanthin concentrations in women with college education level or above was significant lower than in women with middle school education level or below ($p < 0.01$). Besides, β-carotene and zeaxanthin concentrations associations with maternal BMI were found, which indicated that β-carotene concentrations in lactating women with normal BMI were higher than those with overweight, while zeaxanthin in milk from underweight lactating women were higher than those from mothers with normal BMI.

Table 2. Comparisons of the carotenoids concentration in human milk by the characteristics of lactating women.

	β-carotene		β-cryptoxanthin		Lutein		Zeaxanthin	
	Adjusted [1] β (95%CI)	**SEM**	**Adjusted [1] β (95%CI)**	**SEM**	**Adjusted [1] β (95%CI)**	**SEM**	**Adjusted [1] β (95%CI)**	**SEM**
Age, years								
< 25	-0.05 (-0.18, 0.08)	0.07	0.11 (-0.06, 0.28)	0.09	-0.13 (-0.35, 0.08)	0.11	-0.01 (-0.14, 0.15)	0.07
25-30	Reference		Reference		Reference		Reference	
> 30	0.10 (-0.03, 0.23)	0.07	0.11 (-0.07, 0.29)	0.09	-0.03 (-0.15, 0.19)	0.11	0.12 (-0.03, 0.27)	0.08
Education								
Middle school or below	Reference		Reference		Reference		Reference	
High school	0.03 (-0.11, 0.17)	0.07	-0.18 (-0.36, 0.01)	0.09	0.12 (-0.11, 0.35)	0.12	-0.14 (-0.30, 0.02)	0.08
College or above	0.09 (-0.04, 0.23)	0.07	-0.12 (-0.30, 0.06)	0.09	0.08 (-0.14, 0.31)	0.11	-0.15 (-0.31, -0.00) *	0.08
Delivery mode					0			
Vaginal delivery	0.04 (-0.07, 0.15)	0.05	0.03 (-0.11, 0.17)	0.07	0.14 (-0.04, 0.31)	0.09	0.13 (0.02, 0.25) *	0.06
Cesarean delivery	Reference		Reference		Reference		Reference	
Current BMI					0			
Underweight	0.01 (-0.25, 0.26)	0.13	-0.02 (-0.35, 0.32)	0.17	0.32 (-0.09, 0.74)	0.21	0.29 (0.01, 0.57) *	0.14
Normal	Reference		Reference		Reference		Reference	
Overweight	-0.17 (-0.29, -0.05) *	0.06	-0.16 (-0.32, 0.00)	0.08	-0.11 (-0.31, 0.09)	0.10	-0.07 (-0.21, 0.07)	0.07
Obesity	-0.24 (-0.54, 0.07)	0.16	-0.16 (-0.57, 0.24)	0.21	0.16 (-0.34, 0.66)	0.26	-0.18 (-0.52, 0.16)	0.17

CI, confidence interval; SEM, standard error of mean. Multivariate linear regression model considering carotenoids in breast milk as the dependent variable and the other variables studies as independent variables. [1] Adjusted for periods of lactation (0-4 days, 5-11days, 12-30days, 31-60days, 61-120days, and 121-240days *postpartum*), cities (Beijing, Suzhou, and Guangzhou cities), and other independent influencing factors listed above. * Indicates a significant difference when compared with the reference ($p < 0.05$). β-carotene: $R^2 = 0.482$, $p < 0.001$; β-cryptoxanthin: $R^2 = 0.366$, $p < 0.001$; Lutein: $R^2 = 0.282$, $p < 0.001$; Zeaxanthin: $R^2 = 0.124$, $p < 0.001$.

The results from 24-hour food intake recall showed that dietary vitamin A and total carotenoids were not associated with all the carotenoids in breast milk when adjusted with different cities and lactation period ($p > 0.05$) (Table 3). Similarly, no significant associations were found between vitamin E and α-tocopherol intake and α- and γ-tocopherol in human milk ($p > 0.05$).

Table 3. The associations between vitamins intake and concentrations of carotenoids and tocopherols in breast milk.

		β-carotene	**β-cryptoxanthin**	**Lutein**	**Lycopene**	**Zeaxanthin**	**α-tocopherol**	**γ-tocopherol**
Dietary intake of vitamin A	*r*	0.022	0.026	0.027	-0.007	0.075	—	—
	p[1]	0.618	0.562	0.537	0.881	0.093	—	—
Dietary intake of total carotenoids	*r*	0.055	0.002	0.007	-0.038	0.003	—	—
	p[1]	0.220	0.963	0.880	0.398	0.948	—	—
Dietary intake of vitamin E	*r*	—	—	—	—	—	-0.083	0.006
	p[1]	—	—	—	—	—	0.063	0.885
Dietary intake of α-tocopherol	*r*	—	—	—	—	—	-0.033	-0.084
	p[1]	—	—	—	—	—	0.456	0.058

[1] Partial-correlation was performed to analyze the correlations adjusted with cities (Beijing, Suzhou, and Guangzhou cities) and periods of lactating (0-4 days, 5-11 days, 12-30 days, 31-60 days, 61-120 days, and 121-240 days *postpartum*).

4. Discussion

According to literature [40, 48, 60, 61], colostrum is the first milk secretion after delivery, persisting until the seventh or tenth day *postpartum*; it is followed by secretion of transitional milk from around the eight and up to fifteenth day *postpartum*; from then, mature milk is secreted, which shows a relative stable composition. In the present study, due to the nature of its design it was difficult to exactly classify the human milk collected within 4-11 days and 12-30 days *postpartum* as either colostrum or transitional milk. On the contrary, human milk collected within 0-4 days and 30-240 days *postpartum* was clearly classified as colostrum and mature milk respectively.

Highest concentrations of carotenoids and tocopherols were found in colostrum, after that the concentrations of the different compounds observed in milk at 12-30 days *postpartum* were close to those collected in 30-240 days *postpartum*, which may mean that the changes on carotenoids and tocopherols milk composition gradually slow-down until reaching a relatively stable level after 12 days *postpartum*. In accordance with previous studies [35, 40, 60, 61], the levels of most of the compounds (β-carotene, β-cryptoxanthin, lutein, lycopene, and α-tocopherol) except for zeaxanthin and γ-tocopherol decreased along with lactating stage. The evolution trend of γ-tocopherol concentrations in our study were generally comparable with those in Japan [35] (0.111 ± 0.048 mg/100mL in 6-10 days *postpartum*; 0.155 ± 0.126 mg/100mL in 11-20 days *postpartum*, 0.105 ± 0.059 mg/100mL in 21-89 days *postpartum*; 0.120 ± 0.046 mg/100mL in 90-180 days *postpartum*; 0.086 ± 0.043 mg/100mL in 6-10 days *postpartum*).

The carotenoid content in human milk has been studied in several multinational studies, but very few studies report data for a large lactation period (0-240 days *postpartum*). Canfield et al. [9] assessed the levels of carotenoids in human milk from nine countries (five in Asia or the Pacific Rim, three in Americas, and one in Europe) and found that the concentrations varied greatly among the countries, with only moderate disparities in β-carotene. Regional variability was also found in the longitudinal study of Lipkie et al [31]. In the present study, we found that the median β-carotene concentration in mature milk to be in the same range as that found in Australian, Canadian, Chilean, Japanese, Mexican, Filipinos, English, American and German mothers in several studies [9, 37, 39, 40]; while the median β-carotene concentrations in colostrum milk were lower than those reported in Germany [39] or Japan [37].Regarding the relative distribution of carotenoids in Chinese milk, lutein was found to be the major component in mature human milk, which is in agreement with data published by Lipkie et al. [31] and Canfield et al. [9]. On the contrary, β-carotene was the major carotenoid in colostrum, in agreement with a previous longitudinal study in the United States by Song et al. [62].

Differences in carotenoid content of the milk from the three different cities were also observed, milk from Beijing contained significantly lower amounts than milk from Guangzhou or Suzhou for most of carotenoids; while the concentration of lycopene in samples from Guangzhou was higher than the other cities. Taking into consideration that all the samples were collected with the same protocol and analyzed by the same laboratory, it seems reasonable to believe that the main reason for this difference are environmental factors such as dietary habits since Suzhou and Guangzhou had higher intake of fruits and vegetables compared to Beijing.

Median α-tocopherol concentrations (211 μg/100 mL) in Chinese mature milk were lower than those found by researchers in Germany [40], Greece [63], Turkey [34], Poland [61], Canada [64], and Japan [35]. In general, the α-tocopherol content in Chinese milk, not only in colostrum but also in mature milk was lower than those in industrialized countries, and similar with non-industrialized countries. The large inter-subject variation might primarily due to dietary habits, use of dietary supplements, food fortification or genetic differences among different ethnicities; or to methodological factors such as the postpartum date of collection, collection of foremilk or hindmilk, or the collection from a single breast of from both breasts.

To date, there is no much evidence of the tocopherols concentration in Chinese human milk. In our study, the median colostrum α-tocopherol concentrations (645 μg/100mL) were similar to those reported in different groups of Chinese lactating women [65, 66] and Polish women [60], but lower than those from German [40] and Brazilian [67], and higher than those of Tunisian [51], Japanese [35], and Inner Mongolia in China [30]. Our results in mature milk (30-240 days *postpartum*) align well to published data [30, 34, 35, 39, 40, 50, 60, 63, 64]. Intra-country variability was also found with the highest levels of tocopherols (26.8 mg α-tocopherol) found in mothers from Suzhou. Environmental factors such as dietary intake are likely accounting for this.

Some nutritional, obstetric, and socio-economic factors have been implicated as being associated with the vitamins A and E concentrations in milk [34, 51, 67]. In our study, there were no associations detected between the contents of β-cryptoxanthin, lutein, lycopene, α-tocopherol, and γ-tocopherol and socio-economic characteristics of women and their offspring such as maternal age, household income, maternal GWG or supplements intake. This data provides evidence to suggest that the concentrations of these compounds in breast milk are independent of socio-economic conditions and nutrition in pregnancy. On the contrary, we found associations between the human milk β-carotene and zeaxanthin concentrations with current BMI. These inverse correlations between some of the carotenoids and current BMI may be due to the underlying mechanism that excess of body fat increases the consumption of all antioxidant elements in the diet [68] so that lactating women with higher BMI and more body fat consumed more vitamins A and E than those with lower BMI, resulting in lower carotenoids in human milk. Our results were similar with the previous finding [49] that the percentage of body fat in the lactating women was negatively associated to the concentration of vitamin A in breast milk. In addition, maternal education presented an inverse relationship with lower median concentrations of zeaxanthin among women with high levels of education. Mothers undergoing cesarean delivery presented lower zeaxanthin levels in human milk. Previous studies have associated cesarean delivery with lower colostrum protein content [69] and decreased oxidative stress in colostrum [70], suggesting that cesarean delivery may be detrimental for human milk. Considering that carotenoids contribute to the total antioxidative effect of human milk, this relationship of carotenoids and delivery mode requires further investigation to elucidate the possible causal pathways of these mechanisms including organismal regulation, nutrition, and environment.

Previous studies [7, 34, 49] suggested vitamins A and E in human milk were associated to maternal stores, dietary supplements, fortified foods and dietary intake. However, our results indicated that neither vitamin A nor E intakes from one 24-hours dietary recall did not associate with

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vitamin A and E in human milk, which may be due to the inherent intake variability of one single 24-hour dietary recall questionnaire, which did not allow to estimate individual's usual diet, and therefore it is likely to under or overestimate some nutrient intakes. Moreover, Jiang et al. [71] found no significant correlation between dietary constituents and α-tocopherol, in line with our findings.

There were some limitations to the present study. Firstly, nutrient intake determined by one 24-hour recall may introduce some bias by under- or overestimating long-term dietary habits. This variability may result in difficulty to accurately estimating individual's intake when compared with three days dietary recall. Secondly, little is known about the levels of vitamins A and E in maternal plasma associated with the corresponding milk, which would be better than dietary intake to assess maternal nutrient status, due to impossibility to collect such samples in our research. Thirdly, the fact that our design was a cross-sectional study, we could not collect direct evidence about changes in vitamins A and E with lactation stages, these points should be addressed in future studies.

5. Conclusions

The total concentrations of carotenoids (β-carotene and β-cryptoxanthin, lutein, lycopene and zeaxanthin) and vitamin E (as α- and γ-tocopherol) were studied in human milk from healthy Chinese women.In summary, our results agree with previous studies and suggest that stage of lactation, regional differences, obstetric, and socio-economic factors may have an effect on human milk concentrations of carotenoids and tocopherol in healthy Chinese mothers. In view of the great importance of these compounds in human milk to ensure optimal growth and development of infants, research should continue to provide biological significance of such results and improve knowledge on the unique composition of human milk.

Supplementary Materials: Table S1: Carotenoids and tocopherols concentration of human milk at different lactation stages (µg/100mL)., Table S2: Carotenoids and tocopherols concentration of human milk from different cities (Beijing, Suzhou, and Guangzhou cities)., Table S3: Comparisons of carotenoids and tocopherols concentration according to characteristics of lactating women and their offspring (µg/100mL).

Acknowledgments: The authors would like to thank the participants who volunteered for this study, Lawrence Li for project support and guidance, Celia Ning for project management, Qiaoji Li for clinical project management, Yindong Zheng and Carlos Antonio de Castro for statistical analysis advice and Emilie Ba for data management. Special acknowledgment to Ai Zhao and the project staff at Peking University School of Public Health, to Dr. Jiaji Wang at Guangzhou University School of Public Health and Dr. Liqiang Qin at Soochow University School of Public Health for recruitment and data collection. Nestlé Research Center and Nestlé Nutrition Institute China sponsored the study. The views and opinions expressed in this manuscript are those of the authors and do not necessarily reflect the opinions and recommendations of Nestlé.

Author Contributions: Y.X. and E.C.G. interpreted the results, drafted, reviewed and revised the initial manuscript. K.M.R, A.L. and L.A. contributed to analysis of samples. G.V. contributed to the study design, drafted, and reviewed the initial manuscript. Y.X. contributed to statistical design. Y.Z. and P.W. contributed to study design and field collection. S.K.T contributed to the study design, breast milk sampling protocol, interpretation of the results. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Ethics approval and consent to participate: The study was conducted according to the guidelines in the Declaration of Helsinki. All of the procedures involving human subjects were approved by the Medical Ethics Research Board of Peking University (No.IRB00001052-11042). Written informed consent was obtained from all subjects participating in the study. The study was also registered in ClinicalTrials.gov with the number identifier NCT01971671.

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