Macronutrient, immunoglobulin A and total antioxidant capacity profiles of human milk: cross-sectional surveys at ages 6, 12, 18 and 24 months.

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Research

Keywords: Breastfeeding, Human milk, Immunoglobulin, Macronutrient composition, Prolonged lactation, Total antioxidant capacity

Posted Date: July 27th, 2020

DOI: https://doi.org/10.21203/rs.2.19567/v4

Version of Record: A version of this preprint was published on October 30th, 2020. See the published version at https://doi.org/10.1186/s13006-020-00333-5.

Abstract

Background: A longer duration of breastfeeding of up to two years is encouraged by many health authorities, but there is limited information regarding the composition of milk after one year postpartum. The goal of this study was to determine the association between human milk macronutrient, immunoglobulin A (IgA), and total antioxidant capacity (TAC) profiles during extended lactation.

Methods: One hundred eighty-four breastfeeding mothers with full-term healthy children who had been lactating from 1 to 24 months were recruited from January 2019 to April 2019. Human milk was biochemically analyzed for protein and carbohydrate content by colorimetric assays. The fat content was determined by capillary centrifugation, and the energy content was calculated from the results of centrifugation assays. IgA levels and TAC were determined by ELISA and a Trolox equivalent antioxidant capacity (TEAC) assay, respectively. Pearson's correlation coefficient and Spearman's rank correlation coefficient were used to determine associations between milk composition with month of lactation, and multiple regression analysis was used to assess the association between covariate variables and milk composition. Differences were considered significant at $p < 0.05$.

Results: The fat, energy and IgA contents were positively correlated with the duration of lactation ($r = 0.229$, $p = 0.002$; $r = 0.229$, $p = 0.002$ and $r = 0.304$, $p < 0.001$, respectively). No significant correlations between protein, carbohydrate concentrations and TAC with the duration of lactation were observed ($r = 0.106$, $p = 0.15$; $r = -0.032$, $p = 0.67$; $r = -0.056$, $p = 0.45$, respectively). After adjusting for the covariates, the month of lactation was negatively associated with the carbohydrate concentration ($p = 0.04$), while similar results were observed for other components.

Conclusions: We demonstrated that fat, energy, and IgA contents increased during extended lactation lasting up to two years postpartum. A slightly but significant decrease was detected in carbohydrate concentration. No association was observed in protein concentration and TAC with the duration of lactation. Based on these results, lactating mothers should be encouraged and supported to continue breastfeeding for at least two years postpartum.

Background

Human milk is widely accepted as optimal food and provides essential components for the growth and development of infants. Apart from macronutrients and micronutrients, human milk contains various nonnutritive bioactive compounds, including prebiotics, growth factors, hormones, and antioxidants, as well as components that protect against infection such as lysozyme, lactoferrin, oligosaccharide, and IgA [1-3]. In addition to supporting normal growth and development, breastfeeding offers numerous advantages, including psychological, economic, and environmental benefits. Recent advances in molecular biology techniques have shown that human milk plays an essential role as an epigenetic modulator of gene expression in milk recipients and may positively impact life-long metabolic programming [4, 5].

A longer duration of breastfeeding is encouraged by the World Health Organization (WHO) [6], which recommends exclusive breastfeeding for the first six months, along with continued breastfeeding for at least two years. The American Academy of Pediatrics (AAP) has reaffirmed the recommendation of exclusive breastfeeding for approximately the first six months followed by continued breastfeeding as complementary foods are introduced with the continuation of breastfeeding for at least one year of life [7].

Human milk has been well established to be a dynamic fluid with a composition that continually changes throughout the lactation period. During the colostrum and transitional stage of lactation (within the first 10-14 days postpartum), the composition of breast milk undergoes remarkable changes. Mature milk gradually replaces transitional milk after approximately two weeks postpartum and remains relatively similar in its composition, with subtle changes occurring during the weaning period [8]. Although the composition of the milk produced during the first six months postpartum has been widely reported, information on milk composition during the second year postpartum is limited and inconclusive due to small sample sizes, nonstandardized sample collection protocols, and limitations associated with the study designs. Moreover, immunoglobulin A (IgA), which is the predominant immunoglobulin in human milk, and antioxidant capacity, which supports the immature immune system by neutralizing pathogens and removing free radicals, are rarely reported [9-11].

The goal of this study was to examine the association between the human milk macronutrient, immunoglobulin A (IgA), and total antioxidant capacity (TAC) profiles during extended lactation.

Methods

Study Design

This cross-sectional study included 184 breastfeeding mothers who had been lactating for 1 to 24 months. Participants were recruited from January 2019 to April 2019 through study posters posted in the well-baby clinic and the lactation rooms of 4 hospitals in Chiang Mai City. Participants were also recruited from a Facebook parenting group. After interested mothers contacted the study staff via a phone call, they were asked a specific set of inclusion and exclusion criteria questions. Lactating mothers who had given birth to a full-term infant were recruited for this study The exclusion criteria were as follows: (a) the mother or their offspring with any underlying disease, (b) the maternal age of the mother was under 18 years old or above 40 years old, (c) the mother was illiterate in Thai, and (d) the mother could not travel to our lactation room on her own. Paper-based questionnaires were used for data collection using the Thai language. All participants completed a self-report questionnaire on baseline information that included maternal age, education level, first antenatal care (ANC), gestational age, birth order, parental status, and breastfeeding frequency. We corrected the frequency of breast milk feeding by a latch on to the breast (do not bottle-feed breast milk). The weight and height of each participant was measured before their milk samples were collected. All eligible participants were then asked to make an appointment for milk collection. Before providing information and breast milk samples, all participants signed informed consent forms. The participants received no payments.

Sample Collection

Participants were required to collect milk samples in the lactation room of Maharaj Nakorn Chiang Mai Hospital, Nakornping Hospital, Health Promotion Hospital Region One and Lampang Hospital. To minimize possible circadian influences [9] and to ensure uniformity of the samples, all breast milk samples were expressed between 8:00 AM and 12:00 PM using a Lactina Electric Selection pump (Medela®, Switzerland). The pump was left on for approximately 15 minutes or until no further milk could be expressed for at least five minutes. For storage, the samples were aliquoted into 1.5-mL microcentrifuge tubes and frozen at -80°C until further analysis. Sample collected for antioxidant activity measurements

were stored at 0°C and analyzed within 72 hours to preserve the antioxidant activity.

Biochemical Analyses of Human Milk

Carbohydrate Content

The total carbohydrate content in human milk was estimated using a 3,5-dinitrosalicylic acid (DNS) solution prepared by solubilizing one gram of DNS (Sigma, 128848) in a 2 M NaOH (VWR Chemicals, 28244.295) solution containing 30 g Na-K tartrate (VWR Chemicals, 27068.233), after which DI H_2O was added to reach a total volume of 100 mL; this solution was referred to as the working DNS solution. The milk samples were diluted 25× with DI H_2O, and 500 µL of each diluted sample was mixed with 500 µL of working DNS solution. The mixture was then boiled for five minutes and cooled down in running tap water. Then, 4 mL of DI H_2O was added to each reaction, and the absorbance was read at 540 nm with a Synergy H4 Hybrid Reader (BioTek®, USA). The concentration of carbohydrates in the milk was calculated from a D-lactose (Sigma, 61345) standard curve with a concentration range from 0-100 mg/mL.

Protein Content

The total protein content in human milk was determined by Lowry's method using Folin-Ciocalteu solution (VWR Chemicals, 31360.264). The milk samples were diluted 100× with DI H_2O, and 500 µL of each diluted sample was mixed with 2.5 mL of an alkaline solution and 250 µL of the Folin-Ciocalteu solution. The mixture was incubated at room temperature (RT) for ten minutes, and the absorbance was read at 650 nm with a Synergy H4 Hybrid Reader (BioTek®, USA). The concentration of protein in the milk was calculated from a bovine serum albumin (GE Healthcare, K41-001) standard curve with a concentration range from 0-100 mg/mL.

Creamatocrit, Lipid Content, and Energy Conversion

The percentage of cream (creamatocrit) in the human milk was examined by capillary centrifugation followed by calculation of the lipid content and energy yield. The milk samples were individually loaded into each capillary tube to 4/5 of the tube capacity, and the filled tube was capped with clay. Then, the tubes were microcentrifuged (Hettich Haematokrit, Germany) for 15 minutes. The thickness of the cream (A) and the total solution heights (B) were measured. The creamatocrit was calculated as 100 (A÷B), lipid content (g/L) as (creamatocrit × 5.57) - 3.08, and energy (kcal/100) as (creamatocrit × 5.57) + 45.13.

Immunoglobulin A (IgA)

IgA levels in human milk were determined using a commercial ELISA kit (Aviva System Biology, OKEH00516) according to the manufacturer's protocol. Briefly, the human milk samples were diluted 200,000× in water as well as assay diluent buffer. Then, 100 µL of the diluted samples and the IgA standard were loaded into each well of an ELISA plate. The samples were incubated at 37°C for two hours, after which the solution in each well was replaced with 100 µL of biotinylated IgA detector antibody. The samples were incubated at 37°C for an hour, and the solution in each well was discarded and washed. The avidin-HRP conjugate mixture was added at 100 µL into each well and incubated at 37°C for another hour. Next, the solution in the well was discarded, and the plate was washed. Then, 90 µL of TMB substrate was added to each well, and the plate was incubated in the dark at 37°C for 15 minutes. Finally, 50 µL of the stop solution was added to each well, and the plate was read at an absorbance of 450 nm with a Synergy H4 Hybrid Reader (BioTek®, USA). The concentration of IgA in the milk was calculated from an IgA standard curve with a concentration range from 0-4000 pg/mL.

Total Antioxidant Capacity

The TAC of human milk was determined as the Trolox equivalence antioxidant capacity (TEAC) using ABTS solution, which was prepared by mixing two equal volumes of 0.768 g% of ABTS® (AppliChem, A1088,0005) and 0.132 g% of $K_2S_2O_8$ (VWR Chemical, 26915.291). The mixture was incubated at RT for 12 hours, and the working ABTS was made by diluting the stock solution 50× in DI H_2O. Twenty microliters of human milk sample was mixed with 2 mL of the diluted ABTS solution. The reaction was allowed to run for six minutes, and then the absorbance at 734 nm was read with a Genesys^{TM}20 instrument (Thermo Scientific, USA). The TAC in each human milk sample was calculated using a Trolox (Sigma, 238813) standard curve with a concentration range from 0-5 mM, and the TAC was reported as the millimolar Trolox equivalence.

Statistical Analysis

This was a cross-sectional study. The data are presented as descriptive statistics, including the mean, standard deviation (SD), frequency (n), percentage (%), median, interquartile range, and range. Kruskal-Wallis and Mann-Whitney tests were used to test the differences in macronutrient and energy contents in breast milk by month of lactation, whereas one-way ANOVA post hoc tests were used to test the differences in IgA levels and TAC in breast milk by month of lactation. Pearson's correlation coefficient and Spearman's rank correlation coefficient were used to determine associations between milk composition with month of lactation, and multiple linear regression analysis was used to assess the association between month of lactation, maternal age, maternal body mass index (BMI), birth order, and breastfeeding frequency and milk composition. Differences were considered significant at $p < 0.05$.

Results

The participants were divided into four groups based on breastfeeding periods: 1-6 months (n = 43), 6-12 months (n = 47), 12-18 months (n = 50), and 18-24 months (n = 44). There were no significant differences between the groups with respect to demographics or baseline characteristics (Table 1).

Table 1. Characteristics of the Study Population.

	Breastfeeding 1-6 months (n = 43)	Breastfeeding 6-12 months (n = 47)	Breastfeeding 12-18 months (n = 50)	Breastfeeding 18-24 months (n = 44)	p-value
ternal age, y (mean ± SD, median, P25th-75th) [1]	32.6 ± 3.6, 32, 29-35	30.9 ± 4.6, 31, 28-35	32.2 ± 4.8, 32, 33-36	32.0 ± 4.5, 32, 29-36.7	0.28
ternal BMI, kg/m2 (mean ± SD, median; P25th-75th) [2]	23.2 ± 4.5, 22.5, 19.7-24.8	22.6 ± 4.8, 21.3, 19-25.1	22.3 ± 4.1, 21.6, 20.2-23.8	22.3 ± 3.3, 21.7, 19.8-25.1	0.78
ıcational level (n, %) [3]					
Primary school	9 (20.9)	7 (14.9)	16 (32)	12 (27.3)	0.13
Secondary school/ Certificate	0 (0)	3 (6.4)	4 (8)	4 (9.1)	
Graduate degree	34 (79.1)	37 (78.7)	30 (60)	28 (63.6)	
st ANC, months (mean ± SD, median; P25th-75th) [2]	2.0 ± 1.3, 2, 1-2	2.3 ± 1.6, 2, 1-3	2.3 ± 1.3, 2, 1-3	2.1 ± 1.2, 2, 1-3	0.78
stational age, days (mean ± SD, median; P25th-75th) [2]	270.6 ± 6.4, 272, 266-273	271.3 ± 8.1, 272, 266-278	271.5 ± 6.4, 273, 266-275	272.3 ± 8.3, 272.5, 266-280	0.84
th order [3] (n, %)					
First born	27 (62.8)	31 (66)	33 (66)	25 (56.8)	0.71
Second born	15 (34.9)	15 (31.9)	13 (26)	16 (36.4)	
Third-fifth born	1 (2.3)	1 (2.1)	4 (8)	3 (6.8)	
ental status, couple (n, %) [3]	42 (97.7)	47 (100)	48 (96.0)	44 (100)	0.46
eastfeeding frequency, times per day (mean ± SD; median, 25th-75th) [2]	6.4 ± 5.6, 6, 1-10	6.0 ± 4.8, 5, 2-9	5.7 ± 3.8, 6, 3-8	6.1 ± 3.9, 5, 3.2-8	0.98

[1] One-way ANOVA, [2] Kruskal-Wallis test, [3] Fisher's exact test were used for statistical calculations, and a p-value less than 0.05 was regarded as significant.

BMI, body mass index; ANC, antenatal care; SD, standard deviation; P, percentiles

All participants were Thais.

Correlation Between Human Milk Composition and Duration of Lactation

Macronutrients

The fat and energy contents in human milk expressed by mothers who had been lactating from 1-24 months showed a positive correlation with the duration of lactation (r = 0.229, p = 0.002 and r = 0.229, p = 0.002, respectively) (Figure 1b,1c.). There were no significant correlations between protein and carbohydrate concentrations with the length of lactation (r = 0.106, p = 0.15; r = -0.032, p = 0.67, respectively) (Figure 1a,1d).

In the subsequent lactation period (Table 2), the protein concentration in human milk after 18 months postpartum (2.84 ± 0.90 g/dL) increased significantly compared with that observed in human milk collected from 6-12 and 12-18 months postpartum (2.39 ± 0.52 g/dL, p = 0.001 and 2.40 ± 0.75 g/dL, p < 0.001, respectively). The fat and energy contents were significantly higher in human milk collected after 18 months (4.64 ± 1.61 g/dL and 94.64 ± 16.13 kcal/dL, respectively) than in the other groups (1-6 and 12-18 months of lactation, fat concentration 3.67 ± 1.30 g/dL, p ≤ 0.001 and 3.90 ± 1.32 g/dL, p = 0.03, respectively; energy content 84.86± 12.93 kcal/dL, p = 0.001 and 87.91 ± 13.23 kcal/dL, p = 0.03, respectively)

Table 2. Comparison of Macronutrients, IgA, and TAC of Human Milk by Month of Lactation.

Breastfeeding Duration	1-6 months (n = 43) [a]		6-12 months (n = 47) [b]		12-18 months (n = 50) [c]		18-24 months (n = 44) [d]		Mean rank or Mean diff. (SE)	p-value
	Mean ± SD	Median, $P25^{th}$-75^{th}	Mean ± SD	Median, $P25^{th}$-75^{th}	Mean ± SD	Median, $P25^{th}$-75^{th}	Mean ± SD	Median, $P25^{th}$-75^{th}		
Protein (g/dL)	2.56 ± 0.62	2.53, 2.21-2.92	2.39 ± 0.52	2.23, 2.05-2.69	2.40 ± 0.75	2.36, 1.87-2.61	2.84 ± 0.90	2.66, 2.29-3.13	50.49, 40.94 [ab] 52.22, 42.51 [ac] 40.23, 47.68 [ad] 50.09, 47.98 [bc] 37.34, 55.25 [bd] 38.87, 57.31 [cd]	<0.001 [1,**] 0.08 [2] 0.08 [2] 0.17 [2] 0.71 [2] 0.001 [2,**] <0.001 [2,**]
Fat (g/dL)	3.67 ± 1.30	3.79, 2.92-4.33	3.96 ± 1.36	3.53, 3.17-4.83	3.90 ± 1.32	3.85, 3.17-4.68	4.64 ± 1.61	4.61, 3.8-5.13	41.26, 49.38 [ab] 42.78, 50.63 [ac] 34.69, 53.10 [ad] 49.20, 48.81 [bc] 40.88, 51.47 [bd] 41.67, 54.13 [cd]	<0.001 [1,**] 0.14 [2] 0.16 [2] <0.001 [2,**] 0.94 [2] 0.06 [2] 0.03 [2,*]
Energy (kcal/dL)	84.86 ± 12.93	86.09, 77.42-91.55	87.77 ± 13.61	83.54, 79.94-96.55	87.91 ± 13.23	86.70, 79.94-95.01	94.64 ± 16.13	94.28, 86.24-99.48	41.26, 49.38 [ab] 42.77, 50.64 [ac] 34.67, 53.11 [ad] 49.20, 48.81 [bc] 40.88, 51.47 [bd] 41.67, 54.13 [cd]	<0.001 [1,**] 0.14 [2] 0.16 [2] 0.001 [2,**] 0.94 [2] 0.06 [2] 0.03 [2,*]

Breastfeeding Duration	1-6 months (n = 43) [a]	6-12 months (n = 47) [b]	12-18 months (n = 50) [c]	18-24 months (n = 44) [d]	Mean rank or Mean diff. (SE)	p-value	Breastfeeding Duration	1-6 months (n = 43) [a]	6-12 months (n = 47) [b]
Carbohydrates (g/dL)	9.62 ± 1.04	9.43, 8.97-10.12	9.34 ± 0.59	9.27, 8.97-9.71	9.31 ± 0.84	9.35, 8.95-9.74	9.39 ± 0.81	9.41, 8.96-9.73	48.90, 42.39 [ab] 49.73, 44.65 [ac] 46.53, 41.52 [ad] 48.12, 49.83 [bc] 44.83, 47.25 [bd] 47.48, 47.52 [cd]
IgA (mg/dL)	110.82 ± 14.06	111.48, 103-117.63	129.59 ± 16.67	130.33, 115.03-140.5	124.29 ± 10.80	125.07, 116.35-131.37	127.16 ± 14.59	126.06, 115.73-139.57	-18.77 (3.18) [ab] -13.47 (3.14) [ac] -16.34 (3.23) [ad] 5.31 (3.06) [bc] 2.43 (3.16) [bd] -2.87 (3.12) [cd]
TAC	1.61 ± 0.94	1.62, 1.2-2.04	1.61 ± 0.67	1.64, 1.89-2.05	1.84 ± 0.85	1.84, 1.38-2.38	1.60 ± 0.97	1.27, 0.69-2.09	0.03 (0.18) [ab] -0.23 (0.18) [ac] 0.21 (0.18) [ad] -0.23 (0.18) [bc] 0.18 (0.18) [bd] 0.44 (0.18) [cd]

[1] Kruskal-Wallis, [2] one-way ANOVA post hoc, and [3] Mann-Whitney U tests were used for statistical calculations.

*p < 0.05, **p < 0.001

The total antioxidant capacity (TAC) unit is in mM, Trolox equivalent; SD, standard deviation; P, percentile; Mean diff., mean difference; SE, standard error.

Immunoglobulin A

The concentration of IgA in human milk showed a positive correlation with the lactation duration (r = 0.304, p < 0.001) (Figure 1e). The mean IgA concentration was lowest from 1-6 months (110.82 ± 14.06 g/dL) compared with that observed in the longer duration groups (6-12, 12-18, and 18-24 months of lactation; 129.59 ± 16.67, 124.29 ± 10.80, and 127.16 ± 14.59 g/dL, respectively, p < 0.001).

Total Antioxidant Capacity

The antioxidant capacity of human milk showed no significant correlation with the lactation duration lactation (r = -0.056, p = 0.45) (Figure 1f).

Factors Affecting Human Milk Composition

The association between month of lactation, maternal age, maternal body mass index (BMI), birth order, and breastfeeding frequency with milk composition was tested using multiple linear regression analysis (Table 3.) After adjusting for covariates, the month of lactation was positively associated with the fat

concentration (B = 0.311, SE = 0.092, p = 0.001), energy content (B = 3.111, SE = 0.916, p = 0.001), and IgA (B = 4.169, SE = 1.083, p < 0.001) but negatively associated with the carbohydrate concentration (B = -0.216, SE = 0.005, p = 0.04). In addition, the maternal BMI was positively associated with the fat concentration (B = 0.088, SE = 0.024, p < 0.001) and energy content (B = 0.881, SE = 0.243, p < 0.001) but negatively associated with the carbohydrate concentration (B = -0.035, SE = 0.014, p = 0.015).

Table 3. Associations between Maternal Age, Maternal BMI, Infant Age, Birth Order, Breastfeeding Frequency, and Human Milk Composition Using Multiple Linear Regression.

	Protein (g/dL)		Fat (g/dL)		Energy (kcal/dL)		Carbohydrate (g/dL)		IgA (mg/dL)		TAC (mM)
	B (SE)	95% CI	B (SE)	95% CI	B (SE)	95% CI	B (SE)	95% CI	B (SE)	95% CI	B (SE)
nth of tation	0.092 (0.049)	-0.005, 0.189 (p=0.064)	0.311 (0.092)	0.130, 0.492 (p=0.001)	3.111 (0.916)	1.302, 4.919 (p=0.001)	-0.216 (0.005)	-0.218, -0.008 (p=0.04)	4.169 (1.083)	2.031, 6.307 (p <0.001)	-0.036
ternal age	-0.006 (0.013)	-0.031, 0.019 (p=0.623)	-0.032 (0.023)	-0.078, 0.014 (p=0.175)	-0318 (0.233)	-0.778, 0.142 (p=0.174)	0.023 (0.014)	-0.004, 0.05 (p=0.091)	-0.404 (0.276)	-0.948, 0.14 (p=0.145)	0.025 (
ternal BMI	0.023 (0.013)	-0.003, 0.048 (p=0.085)	0.088 (0.024)	0.040, 0.136 (p <0.001)	0.881 (0.243)	0.401, 1.361 (p <0.001)	-0.035 (0.014)	-0.063, -0.007 (p=0.015)	-0.395 (0.287)	-0.962, 0.14 (p=0.171)	-0.015
th order	-0.017 (0.088)	-0.191, 0.157 (p=0.846)	0.192 (0.164)	-0.133, 0.516 (p=0.245)	1.915 (1.643)	-1.326, 5.157 (p=0.245)	-0.218 (0.161)	-0.216, 0.274 (p=0.768)	-0.145 (1.942)	-3.977, 3.687 (p=0.941)	-0.003
astfeeding quency	-0.014 (0.012)	-0.038, 0.011 (p=0.268)	-0.001 (0.023)	-0.046, 0.044 (p=0.970)	-0.008 (0.228)	-0.458, 0.442 (p=0.972)	-0.038 (0.015)	-0.039, 0.014 (p=0.391)	-0.20 (0.269)	-0.731, 0.332 (p=0.459)	0.024 (

A p-value less than 0.05 was regarded as significant. CI, confidence interval; B, unstandardized beta; SE, standard error.

Discussion

The aim of this report is to evaluate the association between human milk macronutrient, IgA, and TAC profiles during extended lactation. The duration of lactation was positively correlated to fat, energy, and IgA contents, but it was negatively correlated to carbohydrate concentrations. In contrast, protein and TAC were not correlated to month of lactation. Comparisons of milk components with the duration of lactation were made using four groups (1-6, 6-12, 12-18, and 18-24 months). In the subsequent lactation period, the IgA concentration was significantly lower from 1-6 months than in the other groups (6-12, 12-18, and 18-24 months). The factors exhibiting an association with human milk composition in our study were month of lactation and maternal BMI. The demographics or baseline characteristics of our participants did not show significant differences between the groups. However, a higher than average education level was observed among individuals in Thailand, which may affect the population diversity. Because the recruitment flyers were distributed in the hospital's lactation room in an urban area, those who were interested in participating would need to travel to our lactation room by themselves. Breastfeeding frequency was not significantly different across two years of breastfeeding. Our results are consistent with previous studies [12,13] reporting no significant differences in mean of numbers of breastfeeding per day. Mandel et al. [12] reported that the feeding frequency of a short-duration group (6-12 months) and a long-duration group (12-39 months) was 7.1 and 5.9 feedings/day, respectively. Shehadeh et al. [13] reported mean lactation frequencies for participants whose breastfeeding duration was under one year (approximately 3 months postpartum) and longer (approximately 14 months postpartum) of 7.1 and 6.9 feedings/day, respectively. These results could be explained by the need to maintain frequent nursing throughout the lactation phases to preserve the milk supply. Macronutrients In our study, we observed that protein concentration was not related to lactation duration. In contrast, two recent studies [14,15] demonstrated that the protein concentration significantly increased during the second year postpartum. In 2018, Czosnykowska-Lukacka et al. reported a positive correlation between the concentration of protein and true protein with duration in milk expressed by 136 mothers who had lactated from 1 to 48 months postpartum (r = 0.44; p < 0.05 and r = 0.45; p < 0.05, respectively). In 2016, Perrin et al. recruited 19 lactating women who provided monthly milk samples from 11 to 17 months postpartum (131 milk samples) and reported that the total protein concentration in human milk increased longitudinally in the second year postpartum. They also compared the protein content in human milk in the second year postpartum with unpasteurized milk samples from 51 approved donors less than one year postpartum from milk banks. The mean protein content for participants whose breastfeeding duration was 11-17 months postpartum was significantly higher than the protein content of milk from a milk bank (average time of lactation was 4.8 + 3.3 months) [15]. We reported that the fat and energy contents positively correlated with the duration of lactation. Similarly, Mandel et al. [12] demonstrated that human milk expressed by mothers who had been lactating over one year (12-39 months) showed a significant increase in fat content compared with that expressed by mothers who had been lactating for shorter periods (6-12 months). Czosnykowska-Łukacka et al. [14] showed that the fat content significantly increased in human milk expressed by mothers lactating beyond 18 months postpartum. However, Shehadem et al. [13] and Perrin et al. [15] concluded that the fat concentration was not related to lactation duration. Negative correlations between carbohydrate concentrations and the duration of lactation were observed in this study. The few studies that have examined the carbohydrate concentration in human milk beyond the first year of lactation have produced conflicting results. Similarly,

Czosnykowska-Łukacka et al. [14] showed that the carbohydrate content decreased significantly in a group of women from 12 to 18 months of lactation compared with women lactating between 1 and 12 months, while no change was observed in our study or others [13,15]. We assessed the macronutrient composition of human milk and changes in concentrations of the components among four periods of time assayed. We observed that the protein concentration in human milk after 18 months postpartum significantly increased compared with human milk collected from 6-12 and 12-18 months postpartum. The fat and energy contents were higher in human milk after 18 months than in the other groups (1-6 and 12-18 months of lactation). This variation may be due to decreases in volume and mammary gland involution during the weaning process, which regularly occur in longitudinal breastfeeding. Garze et al. [28] reported that protein and fat concentrations increased during weaning. Neville et al. reported a significant increase in protein, but a decrease in lactose concentration was only observed during gradual weaning when the milk volume was below 400 mL/day [29]. A decreasing volume and mammary gland involution during the weaning process have been correlated with human milk composition. Immunoglobulin A A significant increase in IgA contents was observed during extended lactation lasting up to two years postpartum. Similar to our results, Perrin et al. [15] observed that the IgA concentration gradually increased ($p < 0.05$) over a study period from 11-17 months postpartum. Prentice et al., 1984 [16], measured the concentration of IgA in the mature breast milk of 153 rural Gambian mothers who lactated from 14 days to 26 months postpartum. In contrast to our study, they observed that IgA concentrations decreased significantly ($p < 0.001$) during the first year of lactation. The breast milk volume of the women in their study peaked at 2-3 months postpartum and decreased in the first 12 months of lactation. In contrast to our results, Hennart et al., 1991 [17], studied 127 Zairean mothers (54 urban and 73 rural mothers) who lactated between the first week to 18 months postpartum. They observed that the concentration of IgA remained stable throughout the 18 months of lactation and reported that the IgA concentration in milk was significantly higher in rural than in urban mother ($p < 0.05$). The urban mothers had much higher milk yields (612 +27 ml/day) than the rural mothers (307 + 16 ml/day), and the mean breastfeeding frequency was significantly higher ($p < 0.05$) in the urban mothers (10.1 times/day) than the rural mothers (6.8 times/day). With respect to lactation period, we observed that the mean IgA concentration was significantly lower from 1-6 months than that observed in the longer duration groups. We also observed a non-significant decrease in the mean IgA concentration from 12-18 months. This variation may be because our samples were collected from different women, and we did not control for factors potentially influencing the IgA level, such as breastfeeding frequency, milk output per day, geographical region (rural or urban area), maternal nutritional status, and stage of lactogenesis (weaning and non-weaning) [16-17,29-30]. Total Antioxidant Capacity The results of our study showed that TAC was not related to lactation duration. A few studies have focused on the relationship between TAC in breast milk and postnatal age. In 2009, Zarban et al. [18] measured TAC at five different times from 115 healthy mothers of full-term infants for colostrum at 2+1 days after birth (n = 115), transitional milk at 7+3 days (n = 97), and 30+3 days (n = 102), mature milk at 90+7 days (n = 100), and 180+10 days after birth (n = 91). They reported that the TAC in milk was significantly higher in colostrum than in transitional and mature milk [18], The same TAC pattern was reported by Quiles et al. [19], who evaluated the changes in TAC in human milk during the first month of lactation. Based on limited research, it can be concluded that the highest levels of antioxidant components and TAC were observed in colostrum and decreased during early lactation [18-20]. To the best of our knowledge, this is the first study to describe a longitudinal change in TAC in breast milk where there was no observed change in antioxidant capacity in the second year postpartum. Factors Affecting the Milk Composition We investigated the potential predictors of the human milk composition, including month of lactation, maternal age, maternal body mass index (BMI), birth order, and breastfeeding frequency. Apart from the month of lactation as discussed above, the factor showing an association with human milk composition in our study was maternal BMI, which was significantly positively associated with the fat concentration and energy content in human milk and negatively associated with carbohydrate concentration in the multiple regression analysis. This finding is consistent with previous studies [21-24]. Bzikowska et al. [21] measured the body composition and analyzed the data at three-time points: during the first (n = 40), third (n = 22), and sixth (n = 15) month of lactation, a positive correlation was observed between human milk fat content and maternal BMI in the first month postpartum ($r = 0.33$; $p = 0.032$). Hahn et al. [23] evaluated the association between the fat concentration in human milk and interactions between maternal age and BMI and energy in a study where the participants were subgrouped by age and BMI. They observed that both maternal age and BMI significantly affected the fat, energy, and carbohydrate contents, while the protein content in human milk was only affected by maternal BMI. A positive correlation between fat content and maternal BMI has been repeatedly reported, while there has been limited and inconclusive information on the association between maternal BMI with protein and carbohydrate contents. In contrast to our results, Chang et al. [24] measured the concentrations of macronutrients from 2632 mature breastmilk samples (1-8 months postpartum) and observed that maternal BMI was negatively associated with lactose content. Human milk is a dynamic fluid that can vary in composition according to the maternal diet. Elucidating the reason for the association between maternal BMI and human milk macronutrient contents is difficult because diet type and eating behavior may differ for each group (normal or abnormal BMI). However, the majority of studies have reported that maternal diet has a slight effect on the contents of many nutrients in human milk [22, 25-26]. A limitation of this study was that it was not possible to infer causality because of the study design. The average education level of our participants was higher than that of the general population, and implementing the findings of this study in the general population would require further investigation. Furthermore, milk volume [17], genetic variation [8, 31], and environmental factors such as dietary intake, time since last feeding, and ethnicity [32,33] have been shown to influence human milk composition, and these factors were uncontrollable and beyond the limitations of this study. Future research should include a prospective cohort study to reduce individual bias at each time point with careful adjustments for their potential effects.

Conclusions

In this study, we demonstrated that with an overextended lactation of up to two years postpartum, the fat, energy, and IgA contents of human milk significantly increased. A slight but significant decrease was detected in the carbohydrate concentration, whereas no association was observed in protein concentration or TAC. Based on these results, lactating mothers should be encouraged and supported to continue breastfeeding for at least two years postpartum.

Declarations

Ethics Approval and Consent to Participate

The protocol for this study was approved by the Research Ethics Committee 4, Faculty of Medicine, Chiang Mai University (No.158/2018). This study complied with the principles set forth in the Declaration of Helsinki (1964) and all of its subsequent amendments. Written informed consent was obtained from all

participants.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

Funding

The study was financially supported by the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (grant number 017/2562). The funding bodies had no role in the design of the study; the collection, analysis, and interpretation of data; or the writing of the manuscript.

Author Contributions

OK designed the study, managed the study approval, drafted the initial manuscript, and revised the manuscript. RJ supervised the sample collection and the sample analysis and revised the manuscript. OK, SP (1), and MR participated in fieldwork management, sample collection, and analysis. SR and AP analyzed the data. KK and SP (2) critically reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank the mothers who provided milk samples for the project. We would also like to thank Chotiros Phanpong, Darunnee Limtrakul, Pattana Lerkdumnernki, Nitthinan Yousaibua, Autcharaporn Nakrit, and Jaruwan Ruangjit for helping to collect samples from the study participants.

List Of Abbreviations

Immunoglobulin A (IgA)

Total antioxidant capacity (TAC)

Body mass index (BMI)

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Figures

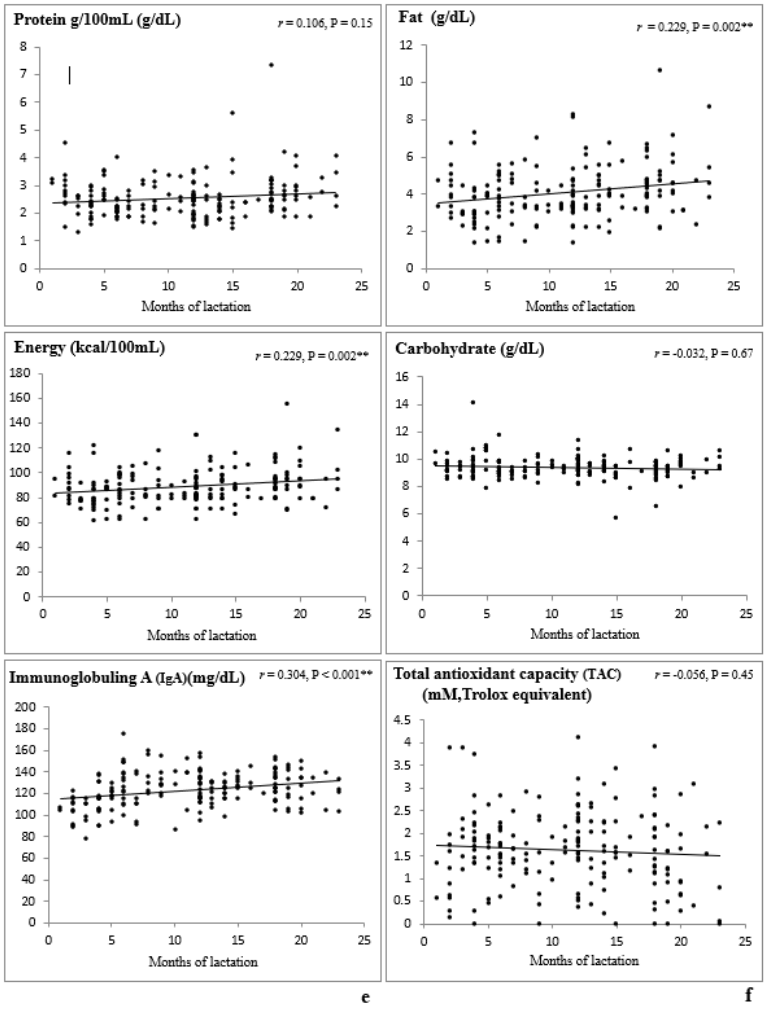

Figure 1

(a-f) The Correlations Between Macronutrients, IgA, and TAC of Human Milk with Month of Lactation.

Supplementary Files

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- reference3.pdf
- reference11.pdf