

# 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

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## Abstract

**Background:** A longer duration of breastfeeding of up to two years is encouraged by many health authorities, but information regarding the composition of milk after one year postpartum is limited. The goal of this study was to determine the associations of macronutrient contents, immunoglobulin A (IgA) levels, and the total antioxidant capacity (TAC) in human milk (HM) with the month of lactation from 1 to 24 months postpartum.

**Methods:** Milk samples were collected from mothers with healthy full-term children who had been lactating from 1 to 24 months from January 2019 to April 2019. HM was biochemically analyzed for protein and carbohydrate contents 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 the 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 and the month of lactation, and multiple regression analysis was used to assess the association between covariates and milk composition. Differences were considered significant at  $p < 0.05$ .

**Results:** One hundred eighty-four milk samples were analyzed. The month of lactation was positively associated with the fat concentration ( $B = 0.31$ ,  $SE = 0.09$ ,  $p = 0.001$ ), energy content ( $B = 3.11$ ,  $SE = 0.92$ ,  $p = 0.001$ ), and IgA ( $B = 4.17$ ,  $SE = 1.08$ ,  $p < 0.001$ ) but negatively associated with the carbohydrate concentration ( $B = -0.22$ ,  $SE = 0.01$ ,  $p = 0.04$ ). No association was observed between the month of lactation and the protein concentration and TAC after adjustment for maternal age, maternal BMI, birth order, and breastfeeding frequency.

**Conclusions:** Based on our results, fat, energy, and IgA contents in HM were positively associated with the month of lactation, and a slight but significant negative association was detected for the carbohydrate concentration up to two years postpartum.

## Background

Human milk (HM) is widely accepted as optimal food and provides essential components for the growth and development of infants. Apart from macronutrients and micronutrients, HM 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 HM 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].

HM 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 study designs. Moreover, immunoglobulin A (IgA), which is the predominant immunoglobulin in HM, and the 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 determine the associations of macronutrient and immunoglobulin A (IgA) contents and the total antioxidant capacity (TAC) of HM with the months of lactation from 1 to 24 months.

## 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 telephone, they were asked a set of questions corresponding to the inclusion and exclusion criteria. Lactating mothers who had given birth to a full-term infant were recruited for this study. The exclusion criteria were as follows: (a) any underlying disease in the mother or her offspring, (b) a maternal age under 18 years or above 40 years, (c) illiteracy in the Thai language, and (d) an inability of the mother to travel to our lactation room on her own. All eligible participants were then asked to set an appointment for milk collection. The participants completed a self-report questionnaire regarding baseline information including maternal age, education level, first antenatal care (ANC) visit, gestational age, birth order, parental status, and breastfeeding frequency. Paper-based questionnaires in the Thai language were used for data collection. The weight and height of each participant were measured before milk samples were collected. Before providing information and breast milk samples, all participants signed informed consent forms. The participants received no payments.

### *Sample Collection*

Participants were required to provide 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. Samples 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 HM 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), and then DI H<sub>2</sub>O 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<sub>2</sub>O, 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<sub>2</sub>O 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 HM was determined by Lowry's method using Folin-Ciocalteu solution (VWR Chemicals, 31360.264). The milk samples were diluted 100× with DI H<sub>2</sub>O, 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 of 0-100 mg/mL.

#### *Creamatocrit, Lipid Content, and Energy Conversion*

The percentage of cream (crematocrit) in the HM 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 creatocrit was calculated as  $100 (A \div B)$ , lipid content (g/L) as  $(\text{crematocrit} \times 5.57) - 3.08$ , and energy (kcal/100) as  $(\text{crematocrit} \times 5.57) + 45.13$ .

#### *Immunoglobulin A (IgA)*

IgA levels in HM were determined using a commercial ELISA kit (Aviva System Biology, OKEH00516) according to the manufacturer's protocol. Briefly, the HM 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, and then 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. An 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 of 0-4000 pg/mL.

#### *Total Antioxidant Capacity*

The TAC of HM was determined as the Trolox equivalent 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<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (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<sub>2</sub>O. Twenty microliters of HM 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™ 20 instrument (Thermo Scientific, USA). The TAC in each HM sample was calculated using a Trolox (Sigma, 238813) standard curve with a concentration range of 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 differences in macronutrient and energy contents in breast milk by months of lactation, whereas one-way ANOVA post hoc and independent-sample T tests were used to test differences in IgA levels and the TAC in breast milk by months of lactation. Pearson's correlation coefficient and Spearman's rank correlation coefficient were used to determine associations between milk composition and the months of lactation. Multiple linear regression analysis was used to assess the association between the months of lactation and milk composition with maternal age, maternal BMI, birth order, and breastfeeding frequency as covariates. 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). No significant differences between the groups were found with respect to demographic or baseline characteristics (Table 1).

**Table 1.** Characteristics of the Study Population.

	Breastfeeding 1-6 months (n = 43)	Breastfeeding months (n = 47)	6-12	Breastfeeding months (n = 50)	12-18	Breastfeeding months (n = 44)	18-24	p- value
Maternal age, y (mean ± SD, median, P25 <sup>th</sup> -75 <sup>th</sup> ) <sup>1</sup>	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
Maternal BMI, kg/m <sup>2</sup> (mean ± SD, median; P25 <sup>th</sup> -75 <sup>th</sup> ) <sup>2</sup>	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
Maternal educational level (n, %) <sup>3</sup>								0.13
Primary school	9 (20.9)	7 (14.9)		16 (32)		12 (27.3)		
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)		
Maternal ANC visit, months (mean ± SD, median; P25 <sup>th</sup> -75 <sup>th</sup> ) <sup>2</sup>	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
Maternal gestational age, days (mean ± SD, median; P25 <sup>th</sup> -75 <sup>th</sup> ) <sup>2</sup>	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
Birth order <sup>3</sup> (n, %)								0.71
First born	27 (62.8)	31 (66)		33 (66)		25 (56.8)		
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)		
Maternal marital status, couple (n, %) <sup>3</sup>	42 (97.7)	47 (100)		48 (96.0)		44 (100)		0.46
Maternal breastfeeding frequency, times per day (mean ± SD; median, P25 <sup>th</sup> -75 <sup>th</sup> ) <sup>2</sup>	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

<sup>1</sup> One-way ANOVA, <sup>2</sup> the Kruskal-Wallis test, and <sup>3</sup> 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, percentile

All participants were Thai.

### Correlation Between Human Milk Composition and the Duration of Lactation

#### Macronutrients

The fat and energy contents in HM expressed by mothers who had been lactating from 1-24 months showed a positive correlation with the duration of lactation ( $r = 0.23$ ,  $p = 0.002$  and  $r = 0.23$ ,  $p = 0.002$ , respectively) (Figure 1b, 1c.). No significant correlations were found between protein and carbohydrate concentrations and the length of lactation ( $r = 0.11$ ,  $p = 0.15$ ;  $r = -0.03$ ,  $p = 0.67$ , respectively) (Figure 1a, 1d). In the subsequent lactation period (Table 2), the protein concentration in HM after 18 months postpartum ( $2.84 \pm 0.90$  g/dL) increased significantly compared with that observed in HM collected from 6-12 and 12-18 months postpartum ( $2.39 \pm 0.52$  g/dL,  $p = 0.001$  and  $2.40 \pm 0.75$  g/dL,  $p < 0.001$ , respectively). The fat and energy contents were significantly higher in HM collected after 18 months ( $4.64 \pm 1.61$  g/dL and  $94.64 \pm 16.13$  kcal/dL, respectively) than in the other groups (1-6 and 12-18 months of lactation, fat concentration  $3.67 \pm 1.30$  g/dL,  $p \leq 0.001$  and  $3.90 \pm 1.32$  g/dL,  $p = 0.03$ , respectively; energy content  $84.86 \pm 12.93$  kcal/dL,  $p = 0.001$  and  $87.91 \pm 13.23$  kcal/dL,  $p = 0.03$ , respectively).

**Table 2.** Comparison of Macronutrients, IgA, and the TAC in Human Milk by the Months of Lactation.

Breastfeeding Duration	1-6 months (n = 43) <sup>a</sup>		6-12 months (n = 47) <sup>b</sup>		12-18 months (n = 50) <sup>c</sup>		18-24 months (n = 44) <sup>d</sup>		Mean rank or Mean diff. (SE)	p-value
	Mean ± SD	Median, P25 <sup>th</sup> -75 <sup>th</sup>	Mean ± SD	Median, P25 <sup>th</sup> -75 <sup>th</sup>	Mean ± SD	Median, P25 <sup>th</sup> -75 <sup>th</sup>	Mean ± SD	Median, P25 <sup>th</sup> -75 <sup>th</sup>		
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 <sup>ab</sup> 52.22, 42.51 <sup>ac</sup> 40.23, 47.68 <sup>ad</sup> 50.09, 47.98 <sup>bc</sup> 37.34, 55.25 <sup>bd</sup> 38.87, 57.31 <sup>cd</sup>	<0.001 <sup>1,**</sup> 0.08 <sup>2</sup> 0.08 <sup>2</sup> 0.17 <sup>2</sup> 0.71 <sup>2</sup> 0.001 <sup>2,**</sup> <0.001 <sup>2,**</sup>
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 <sup>ab</sup> 42.78, 50.63 <sup>ac</sup> 34.69, 53.10 <sup>ad</sup> 49.20, 48.81 <sup>bc</sup> 40.88, 51.47 <sup>bd</sup> 41.67, 54.13 <sup>cd</sup>	<0.001 <sup>1,**</sup> 0.14 <sup>2</sup> 0.16 <sup>2</sup> <0.001 <sup>2,**</sup> 0.94 <sup>2</sup> 0.06 <sup>2</sup> 0.03 <sup>2,*</sup>
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 <sup>ab</sup> 42.77, 50.64 <sup>ac</sup> 34.67, 53.11 <sup>ad</sup> 49.20, 48.81 <sup>bc</sup> 40.88, 51.47 <sup>bd</sup> 41.67, 54.13 <sup>cd</sup>	<0.001 <sup>1,**</sup> 0.14 <sup>2</sup> 0.16 <sup>2</sup> 0.001 <sup>2,**</sup> 0.94 <sup>2</sup> 0.06 <sup>2</sup> 0.03 <sup>2,*</sup>

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 <sup>ab</sup> 49.73, 44.65 <sup>ac</sup> 46.53, 41.52 <sup>ad</sup> 48.12, 49.83 <sup>bc</sup> 44.83, 47.25 <sup>bd</sup> 47.48, 47.52 <sup>cd</sup>	0.65 <sup>1</sup> 0.24 <sup>2</sup> 0.36 <sup>2</sup> 0.35 <sup>2</sup> 0.76 <sup>2</sup> 0.66 <sup>2</sup> 0.99 <sup>2</sup>
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) <sup>ab</sup> -13.47 (3.14) <sup>ac</sup> -16.34 (3.23) <sup>ad</sup> 5.31 (3.06) <sup>bc</sup> 2.43 (3.16) <sup>bd</sup> -2.87 (3.12) <sup>cd</sup>	< 0.001 <sup>3,**</sup> <0.001 <sup>4,**</sup> <0.001 <sup>4,**</sup> 0.08 <sup>4</sup> 0.44 <sup>4</sup> 0.36 <sup>4</sup>
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) <sup>ab</sup> -0.23 (0.18) <sup>ac</sup> 0.21 (0.18) <sup>ad</sup> -0.23 (0.18) <sup>bc</sup> 0.18 (0.18) <sup>bd</sup> 0.44 (0.18) <sup>cd</sup>	0.09 <sup>3</sup> 0.85 <sup>4</sup> 0.19 <sup>4</sup> 0.25 <sup>4</sup> 0.19 <sup>4</sup> 0.33 <sup>4</sup> 0.01 <sup>4,*</sup>

<sup>1</sup> The Kruskal-Wallis test, <sup>2</sup> the Mann-Whitney U test, <sup>3</sup> a one-way ANOVA post hoc test, and <sup>4</sup> independent-sample T test were used for statistical calculations.

\*p < 0.05, \*\*p < 0.001

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

#### Immunoglobulin A

The concentration of IgA in HM showed a positive correlation with the duration of lactation ( $r = 0.30$ ,  $p < 0.001$ ) (Figure 1e). The mean IgA concentrations varied between the four group based on the breastfeeding period, with the lowest concentration observed in the 1-6 month group ( $110.82 \pm 14.06$  g/dL) compared with the longer-duration groups (6-12, 12-18, and 18-24 months of lactation;  $129.59 \pm 16.67$ ,  $124.29 \pm 10.80$ , and  $127.16 \pm 14.59$  g/dL, respectively,  $p < 0.001$ , Table 2).

#### Total Antioxidant Capacity

The antioxidant capacity of HM showed no significant correlation with the lactation duration lactation ( $r = -0.06$ ,  $p = 0.45$ ) (Figure 1f).

#### Factors Affecting Human Milk Composition

Multiple linear regression analysis was used to assess the association between the months of lactation and milk composition with maternal age, maternal BMI, birth order, and breastfeeding frequency as covariates (Table 3). After adjusting for covariates, the number of months of lactation was positively associated with the fat concentration ( $B = 0.31$ ,  $SE = 0.09$ ,  $p = 0.001$ ), energy content ( $B = 3.11$ ,  $SE = 0.92$ ,  $p = 0.001$ ), and IgA ( $B = 4.17$ ,  $SE = 1.08$ ,  $p < 0.001$ ) but negatively associated with the carbohydrate concentration ( $B = -0.22$ ,  $SE = 0.01$ ,  $p = 0.04$ ). In addition, maternal BMI was positively associated with the fat concentration ( $B = 0.09$ ,  $SE = 0.02$ ,  $p < 0.001$ ) and energy content ( $B = 0.88$ ,  $SE = 0.24$ ,  $p < 0.001$ ) but negatively associated with the carbohydrate concentration ( $B = -0.04$ ,  $SE = 0.01$ ,  $p = 0.02$ ).

**Table 3.** Associations between Months of Lactation, Maternal Age, Maternal BMI, 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)	95% CI
Months of lactation	0.09 (0.05)	-0.01, 0.19 (p=0.06)	0.31 (0.09)	0.13, 0.49 (p=0.001)	3.11 (0.92)	1.30, 4.92 (p=0.001)	-0.22 (0.01)	-0.22, -0.01 (p=0.04)	4.17 (1.08)	2.03, 6.31 (p<0.001)	-0.04 (0.06)	-0.15, 0.05 (p=0.54)
Maternal age	-0.01 (0.01)	-0.03, 0.02 (p=0.62)	-0.03 (0.02)	-0.08, 0.01 (p=0.18)	-0.32 (0.23)	-0.78, 0.14 (p=0.17)	0.02 (0.01)	-0.004, 0.05 (p=0.09)	-0.40 (0.28)	-0.95, 0.14 (p=0.15)	0.03 (0.02)	-0.01, -0.05 (p=0.10)
Maternal BMI	0.02 (0.01)	-0.003, 0.048 (p=0.09)	0.09 (0.02)	0.04, 0.14 (p<0.001)	0.88 (0.24)	0.40, 1.36 (p<0.001)	-0.04 (0.01)	-0.06, -0.01 (p=0.02)	-0.40 (0.29)	-0.96, 0.14 (p=0.17)	-0.02 (0.02)	-0.05, 0.05 (p=0.35)
Birth order	-0.02 (0.09)	-0.19, 0.16 (p=0.85)	0.19 (0.16)	-0.13, 0.52 (p=0.25)	1.92 (1.64)	-1.33, 5.16 (p=0.25)	-0.22 (0.16)	-0.22, 0.27 (p=0.77)	-0.15 (1.94)	-3.98, 3.69 (p=0.94)	-0.003 (0.11)	-0.21, 0.19 (p=0.98)
Breastfeeding frequency	-0.01 (0.01)	-0.04, 0.01 (p=0.27)	-0.001 (0.023)	-0.05, 0.04 (p=0.97)	-0.01 (0.23)	-0.46, 0.44 (p=0.97)	-0.04 (0.02)	-0.04, 0.01 (p=0.39)	-0.20 (0.27)	-0.73, 0.33 (p=0.45)	0.02 (0.02)	-0.004, 0.05 (p=0.10)

A p-value less than 0.05 was regarded as significant. CI, confidence interval; B, unstandardized beta; SE, standard error. Maternal age, maternal BMI, birth order, and breastfeeding frequency were analyzed as covariates.

## Discussion

We reported that the number of months of lactation was positively correlated with fat, energy, and IgA contents but negatively correlated with carbohydrate concentrations in HM, while no association was found for the protein concentration and TAC. The factors exhibiting an association with HM composition in our study were the months of lactation and maternal BMI.

The demographics or baseline characteristics of our participants did not show significant differences between the groups. However, the participants' education level was higher than that in the general population in Thailand because the recruitment process was limited to populations in urban areas and populations with internet access. In Thailand, socioeconomic differences are prominent. Therefore, those who were informed about the project were more likely to have received more education than individuals in the general population. Breastfeeding frequency was not significantly different across two years of breastfeeding. Our results are consistent with those of previous studies [12,13] reporting no significant differences in the mean number of breastfeeding episodes per day. Mandel et al. [12] reported that the feeding frequencies of a short-duration group (6-12 months) and a long-duration group (12-39 months) were 7.1 and 5.9 feedings/day, respectively. Shehadeh et al. [13] reported mean lactation frequencies of 7.1 and 6.9 feedings/day for participants with breastfeeding durations less than one year and longer than one year, respectively. These results can be explained by the need to maintain frequent nursing throughout the lactation phases to preserve the milk supply.

### Macronutrients

We observed that the protein concentration was not related to the lactation duration. In contrast, two recent studies demonstrated that the protein concentration significantly increased during the second year postpartum [14,15]. In 2018, Czosnykowska-Lukacka et al. [14] reported a positive correlation between the concentration of protein and true protein and the lactation 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. [15] described longitudinal changes in HM composition in the second year postpartum. They recruited 19 lactating women who were 9–11 months postpartum at the time of enrollment and provided monthly milk samples for at least 18 months postpartum. This study showed that the total protein concentration was increased longitudinally in the second year postpartum and contained higher protein concentrations than pooled milk samples from 51 approved donors whose breastfeeding duration was less than one year. We observed that fat and energy contents were positively correlated with the duration of lactation, which is consistent with previous results reported by Mandel et al. [12] who demonstrated that HM expressed by mothers who had been lactating for more than one year (12-39 months) showed a significant increase in fat content compared with milk expressed by mothers who had been lactating for shorter periods (6-12 months). Czosnykowska-Lukacka et al. [14] showed that the fat content significantly increased in HM expressed by mothers lactating beyond 18 months postpartum, whereas Shehadem et al. [13] and Perrin et al. [15] concluded that the fat concentration was not related to the lactation duration. A negative correlation between carbohydrate concentrations and the duration of lactation were observed in this study. Few prior studies have examined the carbohydrate concentration in HM beyond the first year, providing inconclusive results. Czosnykowska-Lukacka et al. [14] showed that the carbohydrate content decreased significantly in a group of women lactating from 12 to 18 months compared with women lactating between 1 and 12 months, while no change was observed in our study or in others [13,15].

We assessed the macronutrient composition of HM and changes in the concentrations of the components among four periods. We observed that the protein concentration in HM after 18 months postpartum significantly increased compared with that in HM collected from 6-12 and 12-18 months postpartum. The fat and energy contents were higher in HM 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 during 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 the lactose concentration was observed during gradual weaning when the milk volume was below 400 mL/day [29].

### ***Immunoglobulin A***

Significant increases in IgA contents were observed during extended lactation lasting up to two years postpartum. Similar to our results, Perrin et al. [15] reported that the IgA concentration gradually increased ( $p < 0.05$ ) over a study period of 11-17 months postpartum. According to a limited previous study regarding the correlation between the duration of lactation and the IgA concentration, the results remain inconclusive. Prentice et al., 1984 [16] measured the concentration of IgA in the HM of 153 rural Gambian mothers who lactated from 14 days to 26 months postpartum and described that IgA concentrations decreased significantly ( $p < 0.001$ ) during the first year of lactation. On the other hand, Hennart et al., 1991 [17] observed that the concentration of IgA remained stable throughout 18 months of lactation. They also compared IgA concentrations in 54 milk samples from urban mothers with those in 73 milk samples from rural mothers. The IgA concentrations were significantly higher in the samples from the rural mothers than from the urban mothers ( $p < 0.05$ ). These studies highlighted that urban mothers had substantially higher milk yields ( $612 \pm 27$  mL/day) than rural mothers ( $307 \pm 16$  mL/day), and that the mean breastfeeding frequency was significantly higher among urban mothers (10.1 times/day) than among rural mothers (6.8 times/day,  $p < 0.05$ ). With respect to the lactation period, we observed that the mean IgA concentrations varied between the four groups based on the breastfeeding period, with the lowest concentration observed in the 1-6 month group compared with the longer-duration groups. This variation may have been observed 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 the stage of lactogenesis (weaning and non-weaning) [16-17,29-30].

### ***Total Antioxidant Capacity***

The results of our study showed that the TAC was not related to the lactation duration. A few studies have focused on the relationship between the TAC in breast milk and postnatal age. In 2009, Zarban et al. [18] measured the TAC at five different times in 115 healthy mothers of full-term infants for colostrum at  $2 \pm 1$  days after birth ( $n = 115$ ), transitional milk at  $7 \pm 3$  days ( $n = 97$ ) and  $30 \pm 3$  days ( $n = 102$ ), and mature milk at  $90 \pm 7$  days ( $n = 100$ ) and  $180 \pm 10$  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 changes in the TAC in HM during the first month of lactation. Based on limited research, the highest levels of antioxidant components and the TAC can be concluded to be present in colostrum, which decreased during early lactation [18-20]. To the best of our knowledge, this is the first study to describe longitudinal changes in the TAC in breast milk, with no change in the antioxidant capacity observed in the second year postpartum.

### ***Factors Affecting the Milk Composition***

We investigated potential predictors of the HM composition, including **the** months of lactation, maternal age, maternal body mass index (BMI), birth order, and breastfeeding frequency. Apart from the months of lactation as discussed above, the factor showing an association with HM composition in our study was maternal BMI, which was significantly positively associated with the fat concentration and energy content in HM. A positive correlation between fat content and maternal BMI has been repeatedly reported [21-24], while information on the association between maternal BMI and carbohydrate contents remains limited and inconclusive. The same results have been shown by Chang et al. [24]. The authors measured the concentrations of macronutrients in 2632 mature breastmilk samples (1-8 months postpartum) and found that maternal BMI was negatively associated with the lactose content. HM is a dynamic fluid that can vary in composition according to maternal diet. Elucidating the reason for the association between maternal BMI and HM macronutrient contents is difficult because diet type and eating behavior may differ for each group (normal or abnormal BMI). However, most studies have reported that maternal diet has a slight effect on the contents of many nutrients in HM [22, 25-26].

Some limitations should be noted. First, the average education level of our participants was higher than that of the general population. Applying the findings of this study to the general population would require further investigation. Second, the underlying health conditions of the mothers and their offspring were evaluated by self-report questions without medical documentation. Third, milk volume [17], genetic variation [8, 31], and environmental factors such as dietary intake, the time since the last feeding, and ethnicity [32,33] have been shown to influence HM composition, and these factors were uncontrollable and beyond the scope of this study. Future research should include a prospective cohort study to reduce individual bias at each time point with careful adjustments for the potential effects of such factors.

## **Conclusions**

The number of months of lactation was positively associated with fat, energy, and IgA contents in HM, but no association was observed for the protein concentration and TAC in HM for up to two years postpartum, while a slight but significant negative association was detected for the carbohydrate concentration.

## **List Of Abbreviations**

Immunoglobulin A (IgA)

Total antioxidant capacity (TAC)

Body mass index (BMI)

## 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 data sets 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.

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### 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.

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## Figures

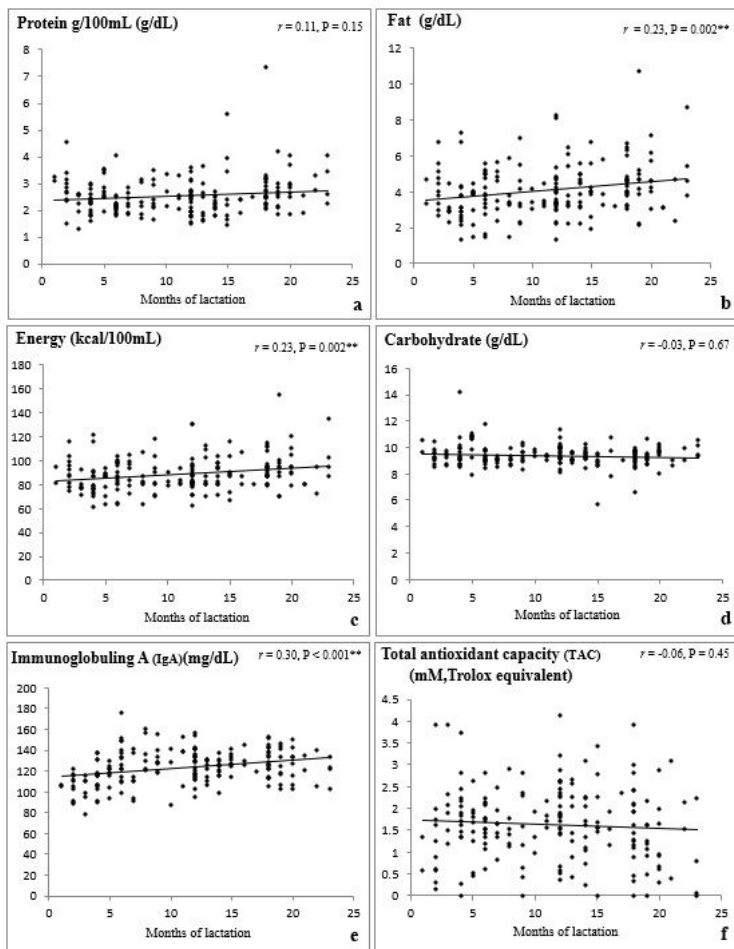


Figure 1

(a-f) Correlations of Macronutrients, IgA, and the TAC of Human Milk with the Months of Lactation

## Supplementary Files

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