Macronutrients, immunoglobulin A and total antioxidant capacity of human milk during prolonged lactation

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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 the milk after one year postpartum. The aim of the study was to determine the longitudinal changes in human milk macronutrients, immunoglobulin A (IgA) and total antioxidant capacity (TAC) during prolonged 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. IgA levels and TAC were determined by ELISA and a Trolox equivalent antioxidant capacity (TEAC) assay, respectively. Pearson Correlation Coefficient and Spearman's Rank Correlation Coefficient were used to determine the association of milk composition with month of lactation.

Results: The concentrations of fat, energy and IgA were positively correlated with the duration of lactation (r = 0.229, p < 0.01; r = 0.229, p < 0.01 and r = 0.304, p < 0.01, respectively). No significant correlations of protein, carbohydrate concentrations and TAC with the duration of lactation was observed (r = 0.106, p = 0.15; r = -0.032, p = 0.67; r = -0.056, p = 0.45, respectively).

Conclusions: We demonstrated that fat, energy and IgA contents increased during prolonged lactation lasting up to two years postpartum, while protein and carbohydrate concentration and TAC were not related to lactation duration. Based on the results, lactating mothers should be encouraged to continue breastfeeding for at least two years postpartum.

Background

Human milk is widely accepted as an 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, antioxidants and immunologic factors that help against infection and inflammation [1-3]. In addition to supporting normal growth and development, breastfeeding offers numerous advantages, including psychological, economic, and environmental benefits. Recent advances in molecular biological 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) reaffirms 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].

It is well established that human milk is a dynamic fluid whose composition continually changes throughout the lactation period. Approximately two weeks after birth, the colostrum and the transitional milk change rapidly, whereas mature milk is relatively similar in its composition with subtle changes up to weaning [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].

Our study aim was to examine the longitudinal changes in human milk macronutrient compositions, IgA, and total antioxidant capacity (TAC) between one and 24 months of lactation.

Methods

Study Design

This cross-sectional study included one hundred eighty-four breastfeeding mothers with full-term healthy children who had no underlying medical conditions and 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 lactation room 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. The mothers were eligible to participate in the study if they did not have an

underlining disease and had given birth to a term healthy infant who was between 1 and 24 months old. 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.

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 participants' weight was measured before their milk samples were collected. The pump was left on for approximately 15 minutes or until there was no further milk that 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 were stored at 0°C and were analyzed within 72 hours to preserve the antioxidant activity.

Biochemical Analyses of Human Milk

Carbohydrate Content

Total carbohydrate in human milk was estimated by 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 DI H_2O was added to reach a total volume of 100 mL; this solution is referred to as the working DNS solution. The milk samples were primarily 25X diluted with DI H_2O, and 500 µL of the diluted samples were mixed with 500 µL of working DNS solution. The mixture was then boiled for five minutes and cooled down in running tap water. Four milliliters 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 the carbohydrate in the milk was calculated from the D-lactose (Sigma, 61345) standard curve with a concentration range of 0-100 mg/mL.

Protein Content

Total protein in human milk was determined by Lowry's method using Folin-Ciocalteu solution (VWR Chemicals, 31360.264). The milk samples were primarily 100X diluted with DI H_2O, and 500 µL of the diluted samples were mixed with 2.5 mL of 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 the protein in the milk was calculated from the 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 (creamatocrit) in the human milk was examined by capillary centrifugation followed by calculating the lipid content and energy yield The milk samples were individually loaded into each capillary tube with a height of 4/5 of the tube capacity, and the filled tube was capped with clay. The tubes were placed on a microcentrifuge

(Hettich Haematokrit, Germany) and run for 15 minutes. The thickness of cream (A) and the total solution heights (B) were measured. The creamatocrit was calculated as 100 (A÷B), lipid content (g/L) was calculated as (creamatocrit x 5.57) - 3.08, and the energy (kcal/100) was calculated as (creamtocrit x 5.57) + 45.13.

Immunoglobulin A (IgA) Determination

IgA in human milk was determined by commercial ELISA kits (Aviva System Biology, OKEH00516) according to the manufacturer's protocol. Briefly, the human milk samples were diluted 200,000X 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 the ELISA plate. Incubation was performed at 37°C for two hours, and the solution in each well was replaced by 100 µL of biotinylated IgA detector antibody. Incubation was performed 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 incubation was performed 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 into each well, and the plate was read at 450 nm absorbance with the Synergy H4 Hybrid Reader (BioTek®, USA). The concentration of IgA in the milk was calculated from the IgA standard curve with a concentration range of 0-4,000 pg/mL.

Total Antioxidant Capacity (TAC)

TAC in human milk was determined by Trolox equivalence antioxidant capacity (TEAC) using ABTS solution, which was prepared by mixing two equal volumes of 0.768g% of ABTS® (AppliChem, A1088,0005) and 0.132g% of $K_2S_2O_8$ (VWR Chemical, 26915.291). The mix was incubated at RT for 12 hours, and the working ABTS was made by 50X dilution of the stock solution 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 the absorbance at 734 nm was read with Genesys™20 (Thermo Scientific, USA). The TAC in each human milk sample was calculated using a Trolox (Sigma, 238813) standard curve with a concentration range of 0-5 mM, and the TAC was reported as mM Trolox equivalence.

Statistical Analysis

This study was a cross-sectional study. Data are presented as descriptive statistics, including the mean, standard deviation (SD), frequency (n), and percentage (%). Kruskal-Wallis test was used to test the differences in macronutrients, and energy content in breast milk by month of lactation, whereas One-Way ANOVA test was used to test the differences in IgA and TAC in breast milk by month of lactation. Pearson Correlation Coefficient and Spearman's Rank Correlation Coefficient were used to determine the association of milk composition with month of lactation and to determine the association of maternal body mass index (BMI), maternal age and breastfeeding frequency with milk composition. Statistics 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
Maternal age [1] (mean ± SD, median, years)	32.6 ± 3.6, 32.0	30.9 ± 4.6, 31.0	32.2 ± 4.8 32.0	32.0 ± 4.5 32.0	0.279
Maternal BMI [2] (mean ± SD, median, kg/m^2)	23.2 ± 4.5, 22.5	22.6 ± 4.8, 21.3	22.3 ± 4.1, 21.6	22.3 ± 3.3, 21.7	0.777
Educational level [3] (n, %)					
Undergraduate degree	9 (20.9)	10 (21.3)	20 (40)	16 (36.4)	0.085
Graduate degree	34 (79.1)	37 (78.7)	30 (60)	28 (63.6)	
First ANC [2] (mean ± SD, median, months)	2.0 ± 1.3, 2.0	2.3 ± 1.6, 2.0	2.3 ± 1.3, 2.0	2.1 ± 1.2, 2.0	0.784
Gestational age [2] (mean ± SD, median, days)	270.6 ± 6.4, 272.0	271.3 ± 8.1, 272.0	271.5 ± 6.4, 273.0	272.3 ± 8.3, 272.5	0.840
Birth order [4] (n, %)					
First born	27 (62.8)	31 (66)	33 (66)	25 (56.8)	0.711
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)	
Parental status [4], couple (n, %) [4]	42 (97.7)	47 (100)	48 (96.0)	44 (100)	0.458
Breastfeeding frequency [2], mean ± SD, median, times per day	6.4 ± 5.6, 6.0	6.0 ± 4.8, 5.0	5.7 ± 3.8, 6.0	6.1 ± 3.9, 5.0	0.984

[1] One-Way ANOVA test, [2] Kruskal-Wallis test, [3] Chi-square test, [4] Fisher's exact test were used for statistical calculations, and a *p*-value lower than 0.05 was regarded as significant. BMI, body mass index; ANC, antenatal care; SD, standard deviation

All the participants were Thais.

Correlation of Human Milk Composition with Duration of Lactation

Macronutrients

The concentrations of fat, and energy in human milk expressed by mothers who had been lactating from 1-24 months showed a positive correlation with the duration of lactation ($r = 0.229$, $p < 0.01$ and $r = 0.229$, $p < 0.01$, respectively) (Figure 1b,1c.)

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 human milk collected from 6-12 months and 12-18 months postpartum (2.39 ± 0.52 g/dL and 2.40 ± 0.75 g/dL, $p < 0.01$, respectively). The fat concentration and energy content 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 those in the other groups (1-6 months and 12-18 months of lactation, fat concentration 3.67 ± 1.30 g/dL and 3.90 ± 1.32 g/dL, respectively, $p < 0.01$; energy content 84.86± 12.93 kcal/dL, and 87.91 ± 13.23 kcal/dL, respectively, $p < 0.01$).

There was no significant correlations of 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).

Table 2. Comparison of Macronutrients, IgA and Total Antioxidant Capacity of Human Milk by Month of Lactation.

:feeding ‘ation	1-6 months n=43[a]		6-12 months n=47[b]		12-18 months n=50[c]		18-24 months n=44[d]		P- value
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
dL) [1]	2.56 ± 0.62	2.53	2.39 ± 0.52	2.23	2.40 ± 0.75	2.36	2.84 ± 0.90	2.66	0.002** bd,cd
[1]	3.67 ± 1.30	3.79	3.96 ± 1.36	3.53	3.90 ± 1.32	3.85	4.64 ± 1.61	4.61	0.008**ad,cd
al/dL) [1]	84.86 ± 12.93	86.09	87.77 ± 13.61	83.54	87.91 ± 13.23	86.70	94.64 ± 16.13	94.28	0.008**ad,cd
ates (g/dL) [1]	9.62 ± 1.04	9.43	9.34 ± 0.59	9.27	9.31 ± 0.84	9.35	9.39 ± 0.81	9.42	0.650
.) [2]	110.82 ± 14.06	111.48	129.59 ± 16.67	130.33	124.29 ± 10.80	125.07	127.16 ± 14.59	126.07	< 0.001**ab, ac, ad
	1.61 ± 0.94	1.62	1.61 ± 0.67	1.64	1.84 ± 0.85	1.84	1.60 ± 0.97	1.27	0.117

[1] Kruskal-Wallis test, [2] One-Way ANOVA test were used for statistical calculations, and a *p*-value lower than 0.05 was regarded as significant. * p < 0.05, ** p < 0.001

Total Antioxidant Capacity (TAC) unit is mM, Trolox equivalent.

Immunoglobulin A (IgA)

The concentration of IgA in human milk showed a positive correlation with 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 of longer duration groups (6-12 months, 12-18 months and 18-24 months of lactation, 129.59 ± 16.67 g/dL, 124.29 ± 10.80 g/dL and 127.16 ± 14.59 g/dL, respectively, p < 0.001).

Total Antioxidant Capacity (TAC)

Similar to carbohydrate content, there was no significant correlation between the antioxidant capacity of human milk and the length of lactation(r = -0.056, p > 0.05) (Figure 1f).

Factors Affecting Human Milk Composition

Correlations among maternal BMI, maternal age, breastfeeding frequency, and milk composition were tested using Spearman's Rank correlation coefficient and Pearson's correlation coefficient (Table 3). Maternal BMI was positively correlated with fat concentration and energy content in human milk (r = 0.233, p < 0.001 and r = 0.233, p < 0.001, respectively) and negatively correlated with carbohydrate content (r = -0.193, p < 0.05). Maternal age was positively associated with changes in the carbohydrate concentration (r = 0.148, p < 0.05), while breastfeeding frequency was negative associated with carbohydrate content in human milk (r = -0.182, p < 0.05).

Table 3. The Correlation Coefficient of Maternal BMI, Maternal Age and Breastfeeding Frequency with Human Milk Composition.

	Protein (g/dL)[a]	Fat (g/dL)[a]	Energy (kcal/dL)[a]	Carbohydrate (g/dL)[a]	TAC (mM)[b]	IgA (mg/dL)[b]
Maternal BMI	0.081	0.233**	0.233**	-0.193*	-0.033	-0.116
Maternal age	0.02	-0.112	-0.112	0.148*	0.094	-0.082
Breastfeeding frequency (times per day)	-0.116	0.002	0.003	-0.182*	0.068	-0.048

[a] Spearman's Rank correlation coefficient was used for statistical calculations, [b] Pearson correlation coefficient was used for statistical calculations, *p*-value lower than 0.05 was regarded as significant, *p < 0.05, **p < 0.01

Discussion

There were no significant differences between the groups with respect to demographics or baseline characteristics. However, the participants have an education level higher than the average level in Thailand. This is because the recruitment flyers were distributed in

hospital's lactation room in an urban area, and those who were interested in participating would need to travel to our lactation room by themselves. Breastfeeding frequency was not found to be statistically different across two years of breastfeeding. This could be because in order to maintain milk supply, frequent nursing throughout the lactation phases is necessary.

Macronutrients

In our study, we found that protein concentration was not related to lactation duration.

Conversely, two recent studies [12, 13] demonstrated that the protein concentration significantly increased during the second year postpartum. In 2018, Czosnykowska-Łukacka et al. [12] reported a positive correlation between the concentration of protein and true protein with duration in milk expressed by mothers who had lactated from 1 to 48 months postpartum ($N = 137$, $r = 0.44$; $p < 0.05$ 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 (N=131), and reported that the total protein concentration of human milk increased longitudinally in the second year postpartum. They also compared the protein content in human milk in the second year postpartum (N=79) with milk bank samples (N=31) that were pooled from 51 donors, with an average time of lactation of 4.8 $\pm$ 3.3 months. They found that the samples from prolonged lactation contained a significantly higher concentration of protein than the milk bank samples [13].

Mandel et al. [14] demonstrated that human milk expressed by mothers who had been lactating over one year had a significant increase in fat content compared to that expressed by mothers who had been lactating for shorter periods. This finding was consistent with our study and with Czosnykowska-Łukacka et al. [12] who showed that the fat content significantly increased in human milk expressed by mothers lactating beyond 18 months postpartum. However, Shehadem et al. [15] and Perrin et al. [13] concluded that fat concentration was not related to the duration of lactation.

Limited studies examining carbohydrate concentration in human milk beyond the first year of lactation have shown equivocal results. Czosnykowska-Łukacka et al. [12] showed that carbohydrate content decreased significantly in the group of 12 to 18 months of lactation compared to that in those lactating between 1 and 12 months, while no change was observed in our study and others [13, 15].

Immunoglobulin A (IgA)

Prior research on the concentration of IgA in the context of prolonged lactation has reported inconclusive results. Our study and Perrin et al. [13] demonstrated that the IgA concentration of human milk increased in the second year postpartum. Conversely, other studies [16, 17] showed that the IgA concentration was stable during extended lactation. A semilongitudinal study of rural Gambian mothers (N=152) found that the concentration of IgA decreased in the first year of lactation and then remained stable up to 26 months [16]. As reported by Hennart et al. [17], a cross-sectional study in Zairean mothers (N=127) found that the concentration of IgA was stable throughout the 18 months of lactation.

Total Antioxidant Capacity (TAC)

Antioxidant components in human milk might offer infants protection against the development of complications induced by oxidative stress. Our study found that TAC were 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. Their final samples measured colostrum at 2$\pm$1 days after birth (n=115), transitional milk at 7$\pm$3 days (n=97) and 30$\pm$3 days (n=102), mature milk at 90$\pm$7 days (n=100) and 180$\pm$10 days after birth (n=91). They reported the TAC in milk was significantly higher in colostrum than in transitional and mature milk [18]. The same pattern of TAC 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 antioxidant components and TAC were at their highest levels in colostrum and declined during early lactation [18-20]. To our knowledge, this was the first study to describe a longitudinal change in TAC in breast milk that had no observed change in antioxidant capacity in the second year postpartum.

Factors Affecting Milk Composition

There are many factors affecting the composition of human milk. Some factors are well examined with repeated positive associations, such as lactational stage, diurnal variation, and time point in breastfeeding session (foremilk and hindmilk) [1]. However, the association between many factors (e.g. smoking, maternal age and parity number) and milk composition has not demonstrated a well-defined effect. Body composition and maternal diet potentially influence human milk composition. Ours and other studies have shown

a positive relationship between maternal BMI and fat content [21-23]. Others have reported a correlation between BMI and protein content [22, 24], while the majority of studies have reported that the maternal diet had little or no effect on many nutrients in human milk [22, 25, 26].

Limited studies examining association between carbohydrate concentration and breastfeeding frequency. Negative association was found between carbohydrates concentrations and breastfeeding frequency in our study. In contrast, Gridneva et al. demonstrated that no significant associations were found between carbohydrates concentrations and 24-h breastfeeding frequency [27].

Decreasing volume and mammary gland involution during the weaning process have been correlated with human milk composition. These factors may affect human milk components, especially in longitudinal studies. Garze et al. [27] documented that protein and fat concentrations increased during weaning . A significant increase in protein, but a decrease in the lactose concentration, was observed during gradual weaning only when milk volume was below 400 mL/day, as reported by Neville et al. [28]. The concentration of IgA was also affected by maternal nutritional status and the stage of lactogenesis (weaning and nonweaning) [29, 30]. A limitation of this study was that it was not possible to infer causality, because of the study design. Furthermore, both genetic variation [8, 31] and environmental factors [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 the potential effects.

Conclusions

We demonstrated that the concentration of fat, energy and IgA significantly increased ($p < 0.05$), while no change was observed in carbohydrate or antioxidant capacity over prolonged lactation of up to two years postpartum. Based on these results, lactating mothers should be encouraged and supported to continue breastfeeding for at least two years postpartum.

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 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.

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Authors' 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

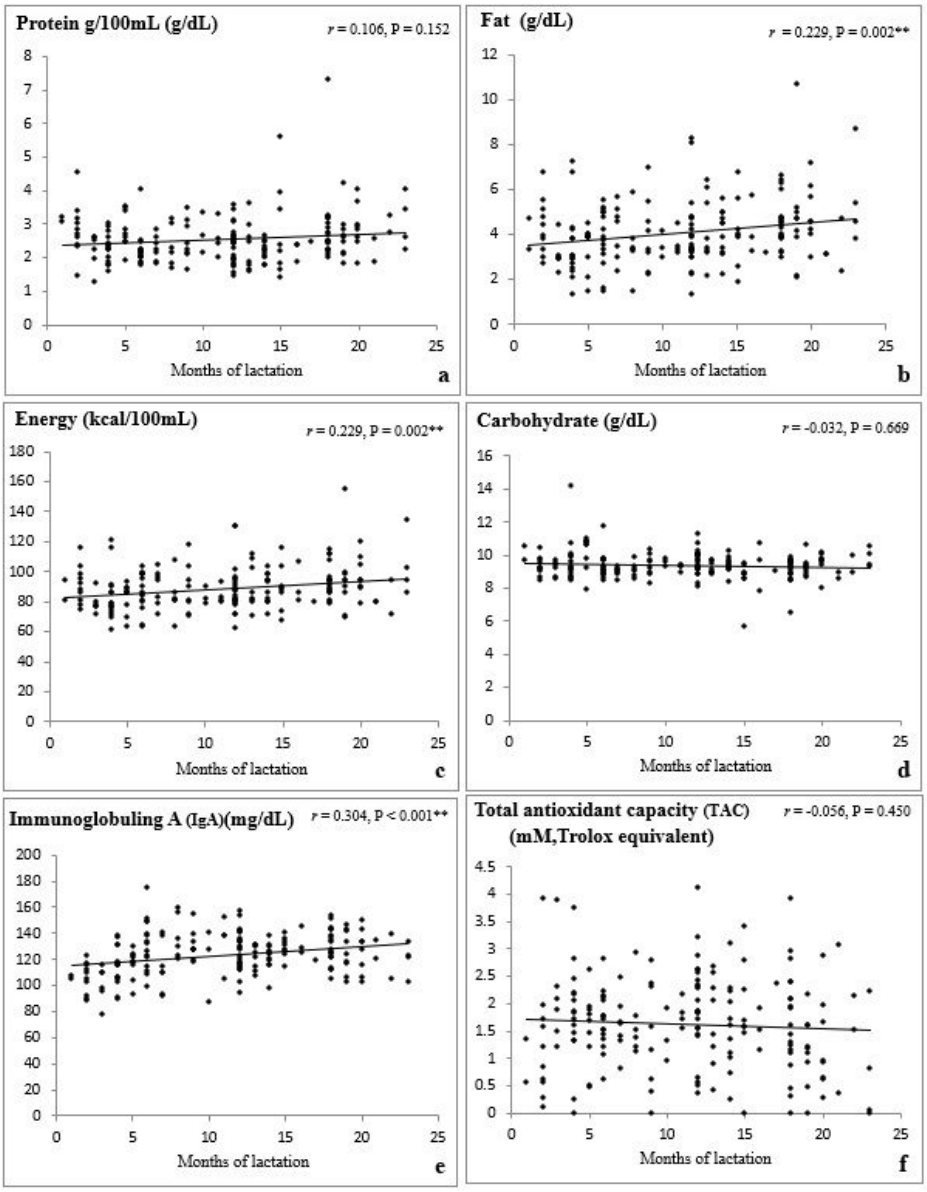

Figure 1

(a-f) The Correlation of Macronutrients, IgA and TAC of Human Milk with MontData of protein, fat, energy, and carbohydrate were analyzed by using Spearman's Rank Correlation Coefficient, Data of IgA and TAC were analyzed by using Pearson Correlation Coefficient, * $p < 0.05$, ** $p < 0.01$ of Lactation.