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Osteopontin levels in human milk vary across countries and within lactation period: Data from a multicenter study

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ABSTRACT

Objectives

Osteopontin (OPN) is a multifunctional protein expressed in many cell types, tissues and body fluids with the highest concentrations found in milk; significantly higher in human than in bovine milk. Intervention studies have indicated beneficial effects of supplementing infant formula with bovine OPN. In this multicenter study, we determined the OPN content in human milk samples from 629 Chinese, Danish, Japanese and Korean mothers.

Methods

At each study site, milk samples were collected and analyzed for OPN and protein concentration using ELISA and infrared spectroscopy, respectively.

Results

A total of 829 milk samples from 629 women were included. When delivering the first sample, mean maternal age was 31.4 years (SD 4.0), and median infant age was 13.4 weeks (IQR 4.6-17.9). The median OPN concentration varied across sites; from 99.7 mg/L in Danish, 182.5 mg/L in Japanese, 216.2 mg/L in Korean to 266.2 mg/L in Chinese mothers ($p < 0.001$), corresponding to 1.3%, 2.4%, 1.8% and 2.7% of the total protein content (OPN/protein%) ($p < 0.05$), respectively. Based on 75 Chinese and 33 Japanese mothers delivering more than one sample, multilevel (mixed model) linear regression analysis showed a decrease in OPN concentration with infant age ($\beta = -11.3$, 95% confidence interval (CI) = (-13.9)-(-8.8) and $\beta = -2.1$, 95% CI = (-3.2)-(-0.9), respectively).

Conclusions

In this large multicenter study, we observed statistically significant differences in the OPN concentration and the OPN/protein% in human milk samples between countries. Based on mothers delivering more than one sample, a significant decrease within the lactation period was observed.

KEYWORDS

Breast milk, milk composition, bioactive milk protein, observational study

WHAT IS KNOWN

- Osteopontin (OPN) is a multifunctional protein present in most tissues and body fluids where it is involved in e.g. immune maturation and regulation
- The OPN concentration in human milk is much higher than in bovine milk and infant formulas
- Current knowledge on the OPN level in human milk is based on a small study comprising few Danish mothers

WHAT IS NEW

- Multicenter study comprising 629 mothers from four countries in Asia and Europe
- OPN concentration differed significantly between countries, also when adjusting for infant age
- OPN concentration in human milk decreased with infant age

INTRODUCTION

Human milk provides an unsurpassed nutritional support for the development of infants. It is an optimal source of energy, essential amino acids and lipids, as well as specific oligosaccharides.

There is increased awareness regarding the content of bioactive proteins in human milk, which may deliver important signals or actions in the growth and development of the infant. These include lactoferrin, α -lactalbumin and caseins, which upon digestion can release peptides that have antibacterial and opioid agonist effects as well as immune stimulating and mineral binding activities (1–3). Many of the bioactive proteins are present in significantly lower concentrations in bovine milk compared to human milk, and therefore also lower in infant formulas based on bovine milk (4). This could contribute to the differences observed between breastfed and formula fed infants in both development and well-being.

Osteopontin (OPN) is another bioactive protein present in higher concentration in human milk than in bovine milk (5,6). OPN is a multifunctional protein expressed in most cells and tissues (7). OPN is present in all physiological fluids, e.g. plasma (approx. 35 $\mu\text{g/L}$) (4), urine (approx. 4 mg/L) (8) and with the by far highest concentration in human milk (approx. 138 mg/L) (4). The function of OPN in milk remains unclear; however, the high concentration of OPN in human milk, cord blood (263 $\mu\text{g/L}$) and infant plasma (342 $\mu\text{g/L}$) (4) suggests a role in infant growth and development. OPN can activate cellular signaling cascades by interaction with cellular receptors via its integrin binding sequences and has been shown to be a key cytokine in the regulation of the Th1/Th2 balanced immune response (9). Likewise, OPN is shown to activate macrophages and induce their phagocytosis of bacteria (10), suggesting that OPN could also play a more direct role in the immune response by interacting directly with invading pathogens.

Bovine milk contains much less OPN than human milk (18 mg/L and 138 mg/L , respectively) (4). Bovine and human milk OPN are structurally very similar (11–13) and it has been suggested to use bovine OPN in infant formulas to close the concentration gap to human milk. *In vitro* experiments have shown that bovine OPN is partly resistant to proteolysis by neonatal gastric juice (14). Furthermore, studies have shown that large OPN

fragments can be found in the plasma of animals ingesting bovine milk OPN (15–17). This makes OPN a bioactive component of milk and infant formulas.

Studies have shown that bovine OPN have beneficial effects on formula fed animal and human infants. In a study with rhesus monkeys, addition of OPN (125 mg/L) to the formula reduced the number of genes expressed differently between the breastfed and formula fed infants from 1017 to 217 (18), suggesting that bovine milk OPN shifts intestinal gene expression in a way that reduces the difference between formula fed and breastfed infants. In a randomized double-blind controlled human trial, mothers either breast- or formula fed their infants from 1 to 6 months of age. Formula was standard or with 65 or 130 mg/L bovine OPN added. The addition of OPN changed the plasma levels of several amino acids and cytokines to be more similar to breastfed infants. Interestingly, addition of OPN lowered the levels of the pro-inflammatory cytokine TNF- α and increased the levels of interleukin-2 that plays a key role in oral tolerance (19). Furthermore, the OPN groups had fewer days with fever compared to the group receiving standard infant formula (19). Supplementing infant formula with OPN also shifted the gene expression of peripheral blood mononuclear cells to be more similar to breastfed infants and increased the proportion of circulating T-cells (20,21).

Overall, studies indicate that OPN in milk plays an important role in infant immune development. Information about the level of OPN in human milk is important both in understanding the physiological role of the protein in milk and for use as a guideline in the production of infant formulas containing OPN. Currently, knowledge about the level and variation of OPN in human milk is based on a single study including only 29 Danish mothers (4). In this explorative, multicenter study we therefore aimed to investigate the OPN content in a large number of human milk samples from four different countries; China, Denmark, Japan and Republic of Korea.

METHODS

Participants and sample deliveries

Participating mothers were recruited from well-developed areas with comparable high level environmental and sanitary conditions and economic wealth. In China, mothers were from the city of Changsha. In Korea, mothers were from postpartum care centers located in the center of the city in the provinces of Seoul, Gyeonggi-do, Gangwon-do, Sejong-si and Chungcheongbuk-do. In Denmark, mothers were from the municipality of Odense. In Japan, mothers were from the prefectures of Hokkaido, Aomori, Niigata, Ibaraki, Saitama, Tokyo, Chiba, Kanagawa, Aichi, Shiga, Osaka, Wakayama, Hyogo, Okayama, Tottori, Fukuoka, Oita, Saga, Nagasaki, and Kagoshima. At each study site, mothers were invited to deliver a milk sample with no requirements regarding the sample being fore- or hind milk. In China and Japan, mothers delivered a single sample at up to three different time points (China: day 30, 60 and 90 postpartum (pp); Japan: approx. 8, 16 and 24 weeks pp). Japanese samples were collected once a day for seven consecutive days and were individually pooled and mixed afterwards. In Republic of Korea and Denmark, mothers delivered a single sample (Korea: approx. 5 weeks pp; Denmark: approx. three months pp). After delivery, samples were mixed and stored at -80° C until analysis. Due to ethical and legal conditions, transport of milk samples between the four study sites was not possible. Therefore, OPN and protein analyses were performed at each of the four study sites, whereas data management and statistical analyses were conducted at one site. Information about the milk sample collection is included as *Supplementary material*.

OPN analysis

At all sites, OPN was measured using the Quantikine® Human Osteopontin ELISA (R&D Systems, Minneapolis, USA), which is validated for quantitative determination of OPN in human milk. Prior to analysis, samples were thawed and centrifuged to remove residual cream or precipitate as described (4). The samples were diluted 40,000-fold after the following scheme: 20 µl milk + 980 µl H₂O (50X) from which 20 µl was diluted in 780 µl H₂O (40X) from which 10 µl was diluted in 190 µl of the RD5-24 diluent buffer (20X) supplied with the ELISA kit. Milk samples and dilutions were kept on ice until analysis. All samples were analyzed in duplicates and doublets that deviated more than 10% (Japan: 20%) from each other were re-analyzed. Sample

concentrations were calculated using logistic curve fitting. To ensure comparability of the ELISA measurements, all standard curves from the four study sites were inspected and compared. Parameters used for the logistic fit standard curves from the four sites are included as *Supplementary material*.

Protein analysis

Before analysis, samples were thawed at 5° C overnight, placed in a 37-40° C water bath and homogenized. Protein analysis was performed using either a mid-infrared transmission spectroscopy device (Miris HMA, Sweden) or a Fourier transformation infrared spectroscopy device (MilkoScan FT2, Foss Analytical, Denmark), both developed for in-hospital analysis of human milk. In China, Denmark and Japan, the Miris HMA was used, and the samples were tested once (China) or twice (Denmark and Japan). In Republic of Korea, the MilkoScan FT2 was used, and each sample was tested once. Since the MilkoScan FT2 output was total/crude and not true protein, a total of 10 samples was re-analyzed using a Miris HMA. These results were used to calculate a conversion factor (0.802926), by which the MilkoScan FT2 output was converted for comparison of protein concentrations across sites.

Statistics

Study data were collected and managed using REDCap™ electronic data capture tool hosted at University of Southern Denmark, Odense, Denmark (22). Descriptive statistics were performed to describe the participating mothers and their infants. Due to non-normal distribution (tested graphically and by Shapiro-Wilk test), OPN and protein concentrations, OPN as percentage of true protein (OPN/protein %) and infant age are presented as medians and interquartile ranges (IQR), whereas maternal age is presented as mean $\pm$ standard deviation (SD). The median OPN concentration and OPN/protein% were calculated based on the first milk sample provided, i.e. mothers delivering more than one sample were included only once. Due to unequal variances, differences between sites were investigated using linear regression, both unadjusted and adjusted for infant and maternal age.

Based on samples from mothers delivering more than one sample, multilevel (mixed model) linear regression analysis was performed to investigate any correlation between the dependent variable OPN concentration and the independent variables infant and maternal age and sample age when OPN analysis was performed. The multilevel approach takes into account the longitudinal aspect of data, i.e. the correlation between OPN concentrations within each mother over time.

Statistics were performed using STATA 15.0/IC (Texas, USA) and *p*-values below 0.05 were considered statistically significant.

Ethics

The participation and sample collection at the individual study sites complied with the Declaration of Helsinki II including informed consent. The Chinese study complied with the China Food and Drug Administration's (CFDA) guideline for good clinical practice (GCP), The International Conference on Harmonisation's (ICH) guideline for GCP and were approved by the ethics committee of Changsha Hospital for Maternal and Child Health Care (ref. 20160303). The Danish study was approved by the Danish Data Protection Agency (ref. 12/26892) and the Regional Committees on Health Research Ethics for Southern Denmark (ref. S-20090130, sub protocols 12, 18 and 37). The Japanese study was approved by the internal review board in Fukuda Clinic (ref. IRB20140621-03) and registered in the Japanese Clinical Trials Registry (ref. UMIN000015494). The Korean study was approved by the Institutional Review Boards at the Maeil Asia Human Milk Research Center (ref. 0627-201508-HRBR-002-04).

RESULTS

Participants

Across sites, a total of 629 mothers delivered 829 milk samples (521 delivered one, 16 delivered two and 92 delivered three samples). An overview of the sample deliveries, including maternal and infant characteristics

is shown in Table 1. Infant age, when the sample was provided, ranged from 1 (Republic of Korea) to 30 weeks (Japan, 3rd visit).

OPN content in human milk

OPN analysis was performed on all 829 samples from the 629 mothers. Across sites, the median OPN concentration based on the first sample was 157.0 mg/L (IQR 95.4-229.5, min-max 2.2-474.8). Based on the first sample from 495 mothers with a corresponding protein concentration available, the median OPN/protein% was 1.8% (IQR 1.3-2.6, min-max 0.1-16.5). An overview of the OPN content and the OPN/protein% across sites is shown in Table 1 and Figure 1. Linear regression (with Denmark as baseline) established that the mean OPN concentration differed statistically significant across sites; $F(3,625) = 135.1$, $p < 0.001$, R^2 and adj. R^2 both = 0.39, with coefficients (β) ranging from 77.4 to 154.2 (all $p < 0.001$). The size of β and the significance remained when adjusting for maternal, infant and sample age; $F(6,622) = 67.7$, $p < 0.001$, R^2 and adj. R^2 both = 0.39, coefficients (β) ranging from 51.2 to 119.5 (all $p < 0.05$). Except from Japan and Republic of Korea, all sites were pairwise significantly different from each other after adjustment for maternal, infant and sample age.

Protein content in human milk

Protein analysis was performed on 695 of the 829 samples. One Chinese sample was not analyzed due to device breakdown, and 133 of the Danish samples did not have enough material for the protein analysis. The median protein concentration was 1.0 g/100 mL (IQR 0.8-1.1) in the Chinese samples, 0.9 g/100 mL (IQR 0.8-0.9) in the Danish, 0.8 g/100 mL (IQR 0.8-0.9) in the Japanese and 1.3 g/100 mL (IQR 1.2-1.4) in the Korean samples (after conversion).

OPN content in relation to infant age

An overview of the OPN concentration in relation to infant age across sites is shown in Figure 2. To avoid clustering for mothers delivering more than one sample, only first time visit samples are included in the figure.

Based on the 108 mothers delivering more than one sample (75 from China and 33 from Japan), the multilevel regression analysis showed a decrease in OPN concentration with infant age; in the Chinese milk with $\beta = (-11.3)$ (95% CI (-13.9) - (-8.8) , $p < 0.001$), and in the Japanese milk with $\beta = (-2.1)$ (95% CI (-3.2) - (-0.9) , $p = 0.001$). For both the Chinese and the Japanese milk, the association remained when adjusting for maternal age and sample age at the time of OPN analysis. An overview is shown in Figure 3.

DISCUSSION

In this explorative multicenter study we aimed to investigate the OPN content in a large number of human milk samples from four different countries; China, Denmark, Japan and Republic of Korea. Based on 829 milk samples from 629 mothers, we observed varying levels of OPN, both absolute (mg/L) and relative to the protein concentration (OPN/protein%) across sites and within lactation period.

The highest OPN concentration and OPN/protein% were found in milk from Chinese mothers, followed by slightly lower levels in the Korean and Japanese mothers (Fig 1.), whereas the OPN level in the Danish samples was significantly lower. To examine whether this lower level could be due to longer storage period of the Danish samples, fresh milk samples from 13 Danish mothers were analyzed. The OPN level and OPN/protein% in these 13 fresh milk samples (data not shown) did not significantly differ from the level obtained in the Danish samples in the present study or from the level in samples from 29 Danish mothers previously reported (4). In conclusion, three individual sets of Danish human milk samples and analyses all showed significantly lower OPN levels than those observed in milk from Asian mothers.

The reason for the variations in OPN content between the involved countries is not clear. It could be the result of environmental and dietary differences, but it is more likely that the variation is based in genetic differences between the populations. It is known that the composition of human milk varies geographically and between ethnicities. Recently, it was shown that human milk oligosaccharide concentrations and profiles varied

extensively in milk samples from 11 international cohorts from four different continents (23). Likewise, the concentration of lactoferrin was significantly higher in milk from Ethiopian than Swedish mothers (24) and the levels of both lactoferrin and lactadherin in milk from Indian and South African mothers were significantly higher than in milk from American mothers (25). Finally, levels of transforming growth factor beta (TGF- β), which is involved in immune regulation and mucosal defense, have been shown to be present at significantly higher levels in colostrum from Japanese women compared to Nepalese women (26).

The level and variation of OPN in human milk has only been analyzed in a couple of studies comprising few mothers and milk samples. Based on a total of 97 samples across lactation from 20 Japanese mothers, the median OPN concentration was reported to be 1,493.4 mg/L in early milk (72 hours to 7 days pp) declining to 896.3 mg/L (1 month pp), 550.8 mg/L (4-7 months pp) and 412.7 mg/L (11-14 months pp) (27). However, OPN concentrations were determined using an ELISA not validated for use in human milk, and later this assay was shown to overestimate the level of milk OPN up to 10-fold (4). In a study comprising 29 Danish mothers delivering a milk sample between day 6 and 58 pp, the OPN concentration (presented as mean $\pm$ SD (min-max)) was reported to be 138 ± 79 mg/L (18-322), corresponding to $2.1 \pm 1.4\%$ of the total milk protein concentration (4). In 2015, data were published on term (8 mothers, 32 samples) and preterm (14 mothers, 28 samples) human milk collected from American mothers, 2 to 58 days pp. The study found no significant difference in the OPN levels between the preterm and the term milk measured by peak ion counts in mass spectrometric analyses. The absolute levels of OPN in the milk samples were not reported (28).

In the present study, Chinese and Japanese mothers delivered more milk samples during the course of lactation. In both populations a significant decrease in OPN concentration with infant age was observed. Since OPN has been shown to be involved in immunological processes (9), it could be hypothesized that the presence of OPN is especially important in early milk. A similar scenario exists for other immunologically active milk proteins (29), like lactoferrin which is also known to decrease in concentration over the course of lactation (30).

Comparable protein concentrations were found in Chinese, Danish and Japanese milk, whereas the protein concentration was significantly lower in Korean milk. However, the observed Korean protein level is in line with a previous study comprising 2,632 Korean mothers (31).

By performing protein and OPN analyzes in four different places, a possible source of error is introduced in the comparison of data. The optimal study setup would have been to analyze all samples at one time point at the same laboratory or to perform ring analyses of a subset of samples. However, as described in the *Methods* section, this was not possible. Instead, actions were taken to minimize potential sources of error and ensure the comparability of the results. As for the OPN ELISA, milk samples had to be diluted 40,000-fold; this step represents the biggest source of experimental error in the process and in order to reduce this, laboratory staff at all sites were instructed to use the same dilution protocol and to follow the ELISA protocol very carefully. Likewise, all data used for generation of the ELISA standard curves obtained at the four study sites were inspected and compared to ensure comparability between sites.

In conclusion, this multicenter study investigated the OPN concentration in a large number of human milk samples from four different countries; China, Denmark, Japan and Republic of Korea. Based on the first visit, the median OPN concentration differed statistically significant across sites, from 99.7 mg/L in Danish, 185.0 mg/L in Japanese and 216.2 mg/L in Korean to 266.2 mg/L in Chinese mothers even after adjustment for infant, maternal and sample age. Based on the first sample from all mothers, a decreasing tendency in the OPN concentration with infant age was observed. This was confirmed when performing a multilevel regression analysis on data from mothers delivering more than one sample. Knowledge about the level of the bioactive protein OPN in human milk across different countries and within the lactation period provides a better understanding of human milk composition. This knowledge can aid in the development of improved infant formulas based on bovine milk proteins.

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FIGURE LEGENDS

Table 1: Maternal and infant characteristics, absolute (mg/L) and relative (%) OPN concentration at first sample delivery, $n = 629$. P -values based on linear regression with the study site with the lowest value as reference.

Figure 1: Median (IQR) OPN concentration in human milk samples, absolute (mg/L) and relative (OPN/protein%). Only milk samples delivered at first time visit, $n = 629$. Median (IQR) infant age was 4.3 (-), 17.4 (14.9-19.3), 8.0 (6.0-9.1) and 3.9 (3.0-4.9) weeks, respectively.

Figure 2: OPN concentration (mg/L) in relation to infant age (weeks). Samples delivered at first time visit, $n = 629$.

Figure 3: OPN concentration (mg/L) in relation to infant age. Only sites and mothers with repeated measurements are included, $n = 108$.

Table 1: Maternal and infant characteristics, absolute (mg/L) and relative (%) OPN concentration at first sample delivery, *n* = 629. *p*-values based on linear regression with the study site with the lowest value as reference.

	China	Denmark	Japan	Republic of Korea	<i>p</i> -value
<i>n</i>	76	318	118	117	-
Infant age (weeks), median (IQR)	4.3 ¹	17.4 (14.9-19.3)	9.1 (6.6-14.1)	3.9 (3.0-4.9)	< 0.001 ⁴
Maternal age (years), mean ± SD (min-max)	29.8 ± 3.6 (20.3-41.4)	31.4 ± 4.0 (21.7-43.4)	31.9 ± 4.2 (21.5-41.9)	32.2 ± 3.6 (22.3-42.2)	< 0.001 ⁵
OPN (mg/L), median (IQR)	266.2 (210.8-323.9)	99.7 (67.5-149.1)	185.0 (151.0-229.5)	216.2 (160.6-268.8)	< 0.001
OPN/protein%, median (IQR)	2.7 (2.2-3.6) ²	1.3 (0.9-1.7) ³	2.4 (1.8-2.9)	1.8 (1.3-2.1)	< 0.001 ⁶

1) 30 days postpartum (fixed)
2) *n* = 74
3) *n* = 185
4) *p*-value for China = 0.666
5) *p*-value for Denmark = 0.002
6) *p*-value for Republic of Korea = 0.035

Figure 1: Median (IQR) OPN concentration in human milk samples, absolute (mg/L) and relative (OPN/protein%). Only milk samples delivered at first time visit, $n = 629$. Median (IQR) infant age was 4.3 (-, 17.4 (14.9-19.3), 8.0 (6.0-9.1) and 3.9 (3.0-4.9) weeks, respectively.

Figure 2: OPN concentration (mg/L) in relation to infant age (weeks). Samples delivered at first time visit, $n = 629$.

Figure 3: OPN concentration (mg/L) in relation to infant age. Only sites and mothers with repeated measurements are included, $n = 108$.

SUPPLEMENTARY MATERIAL

Milk sample deliveries

In China, mothers were asked to deliver a breast milk sample of at least 20 mL during their visit to the hospital at day 30, 60 and 90 postpartum. In Denmark, mothers were given the opportunity to deliver a breast milk sample when their infant was seen for the first examination approx. three months postpartum as part of the Odense Child Cohort. The mothers were asked to deliver 30 mL if possible. In Japan, mothers were given the opportunity to deliver a breast milk sample when their infant was seen for the first examination approx. one month postpartum as part of the Japanese Human Milk Study in Japan. Healthy, breastfeeding mothers and infants aged less than 60 days were included in the study if the delivery of a breast milk sample would not disturb the breastfeeding. The use of a breast pump was mandatory, and the mothers were asked to deliver 20 mL (after breastfeeding their infant) for seven consecutive days if possible. In Korea, mothers from postpartum care centers (*joriwon*), baby fairs and breastfeeding seminars were given the opportunity to deliver a breast milk sample when their infant was seen for the first examination approx. five weeks postpartum as part of the activities of the Maeil Asia Human Milk Research Center (MAHRC). The mothers were asked to deliver 45 mL if possible.

Supplementary Table

The fitting parameters for four parameter logistic standard curves

Location	a	b	c	d	R ²
South Korea	0.04107	1.267	9.885	3.775	0.9933
	0.02008	1.127	19.18	2.214	0.9981
	0.1121	1.467	8.364	3.447	0.9967
Japan	0.01357	1.061	28.92	6.266	0.9999
	0.03101	1.164	21.61	5.321	0.9993
	-0.002271	1.06	28.17	6.091	0.9987
Denmark	0.021	1.276	15.19	3.81	0.9951
	0.005844	1.151	20.11	4.528	0.9992
	0.002293	1.294	16.42	4.122	0.9991
China	-0.03182	1.103	28.82	10.2	0.998
	0.0201	1.105	22.93	7.595	0.9964
	0.01737	1.125	20.65	8.269	0.9996

The curves were fitted to a four parameter logistic function $f(x) = d + ((a-d)/(1+(x/c)^b)$