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Human breast milk concentration of neopterin at various stages of lactation and during a single feeding

Abstract: The aim of this work was to determine the physiological level of neopterin in human breast milk, and to study its variability depending on the duration of a single feeding and the lactation stage. Breast milk samples from 74 women were collected between 2 and 4 days after delivery, and at 15, 30, and 90 days after delivery. Additionally, breast milk samples from eight women were collected before and after 7 and 15 min of breastfeeding. The concentration of neopterin in breast milk was determined by an immunoenzymatic assay. The range of breast milk neopterin concentration at various stages of lactation amounted to 15.4–19.2 nmol/L at 2–4 days after delivery, 20.2–23.0 nmol/L at day 15, 20.8–24.5 nmol/L at day 30, and 16.9–20.4 nmol/L at day 90. The level of neopterin 2–4 days after delivery was significantly lower than that at days 15 and 30; moreover, the concentration of neopterin at day 30 was significantly higher than that at day 90. No significant differences were documented between neopterin concentrations at various phases of a single feeding. While the breast milk concentration of neopterin changes depending on the stage of lactation, it remains stable throughout a single feeding.

Keywords: breastfeeding; immunoenzymatic assay; inflammatory markers; neonatology; reference level.

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Introduction

Neopterin, 2 amino-4-hydroxy-6-(D-erythro-1,2,3-trihydroxypropyl)pterin, is a low-molecular-weight compound belonging to the group of pteridines. It was isolated from human urine by Sakurai and Goto for the first time in 1967 [1]. Twelve years later, Wachter et al. [2] documented an elevated level of neopterin in patients with neoplasms and viral infections. Currently, neopterin is known to be the activity marker of cellular immune response [3], and its increased concentration is observed in the course of various infections, autoimmune disorders, and graft-versus-host reactions. Neopterin was demonstrated to modulate the cytotoxicity of free radicals, including reactive oxygen species (ROS) [4], inducing or inhibiting antioxidant reactions [5]. Owing to the positive correlation between neopterin concentration and the level of ROS, this compound is considered to be an indirect marker of the severity of immune-mediated oxidative stress [6].

Owing to the involvement of neopterin in the immune response and antioxidative processes, determining the concentration of this compound may be useful in three clinical functions: establishing a differential diagnosis, monitoring the course of the disease, and formulating a prognosis. Neopterin was detected in an array of body fluids, including blood, urine, cerebrospinal fluid, synovial fluid, pancreatic juice, saliva, ascitic fluid, and breast milk [7–13]. To date, the reference ranges of neopterin have been determined for blood [14], urine [15], cerebrospinal fluid, and saliva [16, 17]. Aside from pathological processes, the level of neopterin in biological material can also be modulated by other patient characteristics such as race, sex, age, blood group, and physiological status [15, 18–22].

Aside from nutrients and the components of passive immunity, human breast milk may also constitute a vector of numerous factors negatively affecting neonatal health status. Because of the relatively non-selective permeability of the blood-milk barrier, breast milk may contain harmful chemical, physical, and biological factors to which the lactating woman was exposed. Moreover, the

pathological processes of the maternal body can have a detrimental effect on the nutritional profile of the breast milk. Consequently, there is a need for a universal marker of breast milk safety. Owing to its involvement in immune and antioxidative processes, neopterin seems a natural candidate to constitute this type of marker. However, little is known on the physiological level of this compound in human breast milk and its potential modulators. Perhaps this results from difficulties in obtaining experimental material or a variety of factors that can potentially modulate the composition of human milk. Many previous studies showed that aside from environmental and maternal factors, the composition of breast milk is modulated by lactation characteristics that include the stage of lactation and the phase of a single feeding.

Taking into account the above-mentioned considerations and having access to a relatively large and homogeneous group of lactating women, we decided to determine the physiological level of neopterin in human breast milk, and to study its variability depending on the duration of a single feeding and on the lactation stage.

Materials and methods

Participants

The study was conducted in 2009–2010 at the Obstetrical Ward of the Pomeranian Traumatology Center in Gdansk (northern Poland). The study group (n=74) included postpartum Caucasian women who met the following inclusion criteria: uncomplicated pregnancy, normal spontaneous full-term delivery, neonates in good general status, and with normal birth weight. The exclusion criteria comprised acute and chronic disorders (including gestational diabetes, hypertension, and thyroid disorders), active smoking during pregnancy and lactation, and pharmacotherapy other than vitamin supplementation.

Ethics

All procedures were approved by the local Ethics Committee of the Medical University in Gdansk. The subjects gave their informed consent before the start of any procedure.

Samples

Breast milk samples were collected four times: (i) between the 2nd and 4th days after delivery (n=57), (ii) 15 days after delivery (n=48), (iii) 30 days after delivery (n=47), and (iv) 90 days after delivery (n=38). The mammary gland was evacuated completely with an aid of an electric lactator 2 h after the first morning feeding. The milk was collected into sterile glass containers. After careful mixing, 10 mL samples were taken and placed into another sterile container and immediately frozen at –80°C. The remaining milk was fed to the infants. Additionally, breast milk samples from eight women were collected manually before and after 7 and 15 min of breastfeeding, and treated as above.

Determination of neopterin concentration

The concentration of neopterin in breast milk samples was determined with an ELISA ELItest Neopterin (BRAHMS, Hennigsdorf, Germany) for quantitative determination of neopterin in serum, plasma, and urine. Before the determination, the samples of breast milk were thawed at room temperature and mixed. During thawing, the samples were protected against sun exposure by using aluminum foil. The immunoenzymatic kit used for neopterin determination included plates coated with polyclonal sheep anti-neopterin antibodies. The samples of milk (50 μL), control sera, and standards were incubated with an enzymatic conjugate (neopterin/alkaline phosphatase conjugate) competing with breast milk neopterin for binding to antibodies coating the wells. The solution prepared in this manner was applied onto the plates to obtain solid-phase bound immune complexes of antibody-enzymatic conjugate or antibody-neopterin. Unbound elements were removed by washing. Subsequently, 4-nitrophenyl phosphate was added to initiate enzymatic reaction catalyzed by alkaline phosphatase contained in the conjugate. As a result of this reaction, the 4-nitrophenyl phosphate was degraded to yellow 4-nitrophenol. The enzymatic reaction was stopped by alkalization with sodium hydroxide. The concentration of neopterin was determined on the basis of the optical density of bound enzymatic conjugate read at 405-nm wavelength (Ultrospec III spectrophotometer; Pharmacia LKB, Woerden, Netherlands).

Dietary assessment

To exclude the potential modulatory effect of diet on the neopterin level, each participating woman was assessed by four dietary surveys regarding their diet within a month before the delivery, and the detailed dietary history from 3 days before each breast milk sampling was obtained. Data on the dietary intake of protein, fat, and carbohydrates were analyzed with use of Dieta 4.0 package (National Institute of Food and Nutrition, Poland).

Other confounders

To exclude the potential confounding effect of other maternal variables on neopterin level, we analyzed the associations between this parameter and the participant's age, blood group, parity, and use of mineral-vitamin preparations during pregnancy and lactation. All data were obtained from medical documentation and/or standardized interview.

Statistical analysis

The normality of continuous variable distribution was tested with the Kolmogorov-Smirnov test. Depending on the type of distribution, statistical characteristics of continuous variables were presented as

arithmetic means and their standard deviations (SDs), or medians and interquartile ranges. The range of neopterin concentration in human breast milk at various stages of lactation was defined as a 95% confidence interval of the mean (95% CI). The significance of changes in neopterin concentration during a single feeding or throughout lactation was analyzed with Friedman's ANOVA. The direction and power of the effect of continuous variables on neopterin level were assessed on the basis of the Spearman's rank coefficients of correlation (R), while the effects of discrete variables were analyzed with the Mann-Whitney U-test or the Kruskal-Wallis test. All calculations were conducted with Statistica 10 (StatSoft, Tulsa OK, USA) package, with the level of statistical significance set at $p \leq 0.05$.

Results

The characteristics of study participants are presented in Table 1.

Breast milk level of neopterin

The range of breast milk neopterin concentration at various stages of lactation, defined as 95% CI, amounted to 15.4–19.2 nmol/L at 2–4 days after delivery, 20.2–23.0 nmol/L at day 15, 20.8–24.5 nmol/L at day 30, and 16.9–20.4 nmol/L at day 90.

We observed significant differences between the neopterin concentration at various stages of lactation ($p<0.001$): the level of this compound 2–4 days after delivery was significantly lower than that at day 15 ($p=0.003$) and day 30 ($p<0.001$); moreover, the concentration of neopterin at day 30 was significantly higher than that at day 90 ($p=0.02$).

In contrast, no significant differences were documented between neopterin concentrations at various phases of a single feeding ($p=0.69$; Table 2).

Potential confounders

We did not find a significant influence of the participant's age ($R=-0.15$, $p=0.26$) and parity ($R=-0.08$, $p=0.53$) on the breast milk level of neopterin. Also, maternal blood group and the use of mineral and vitamin preparations during pregnancy and lactation did not influence the level of the studied parameter at various stages of lactation (Table 3).

Table 1: Characteristics of the studied group.

Parameter	Range	Mean±SD or median (IQR)
Maternal age, years	20–40	28.8±4.8
Parity, n	1–6	2 (1–2)
Gestational age at birth, weeks	38–42	40 (39–41)
Birth weight, kg	2.75–4.55	3.543±0.4156
Apgar score at 1 min, points	7–10	10 (10–10)

Table 2: Breast milk concentration of neopterin, nmol/L, at various phases of a single feeding.

Minute	n	Mean	SD	Median
0	8	23.13	14.84	20.95
7	8	22.94	14.44	23.50
15	8	23.29	14.95	20.70

Discussion

Using the confidence interval method enabled us to determine the levels of neopterin concentration in human breast milk obtained at various times after delivery. In the first of a small number of reports on the breast milk content of neopterin, Dhondt et al. [23] revealed that 1 week after delivery, the concentration of this compound was equal to 15.8±7.4 nmol/L, thus being similar to the level observed in our study during the initial stage of lactation.

Our study showed that the breast milk concentration of neopterin changes depending on the stage of lactation: it is the lowest at 2–4 days after delivery, subsequently increases, and reaches its peak value at about day 30. Next, it decreases; as a result, on the 90th day of lactation, the breast milk concentration of neopterin is similar to that observed immediately after delivery. Iizuka et al. [24] reported slightly different dynamics of neopterin concentration in human milk. These authors analyzed changes in the breast milk concentration of neopterin during the first 30 days after delivery. In contrast to our study, the concentration of neopterin was the highest at 1 day after delivery and then gradually decreased, reaching its minimal level on day 8. Although this parameter increased in subsequent days, it did not return to its baseline level until the end of the studied period [24]. We were unable to confirm if the level of neopterin really decreases within 1 week of delivery because the second measurement was taken no earlier than on lactation day 15.

Many previous studies demonstrated that the composition of human breast milk changes not only depending on the lactation stage but also during a single feeding. However, our study did not confirm this phenomenon with regard to neopterin, as its baseline concentration did not change significantly at 7 and 15 min of feeding. The previously unreported relationship can prove useful

Table 3: Breast milk concentration of neopterin, nmol/L, depending on maternal blood group and the use of various micronutrient supplements.

Variable	Day 2–4		Day 15		Day 30		Day 90	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Blood group								
0	18.32	7.45	22.53	3.66	23.66	5.85	21.90	11.24
A	19.35	6.18	20.58	5.45	20.91	6.45	18.78	3.57
B	17.42	8.37	22.16	4.37	22.59	4.23	19.06	4.95
AB	24.21	6.65	23.51	4.48	25.47	3.91	20.92	3.42
p-Value		0.35		NS		NS		NS
Type of micronutrient supplement[a]								
Feminatal	17.64	7.92	19.71	4.24	22.52	7.43	22.14	12.19
Elevit	18.35	5.95	20.65	2.48	21.00	3.98	16.99	4.62
Prenatal	17.78	8.87	20.97	4.55	22.28	5.46	19.07	3.37
Falvit	19.56	7.26	25.86	2.09	22.97	6.17	20.09	3.35
Materna	17.32	4.91	21.14	4.71	17.95	4.35	19.71	5.28
p-Value		0.96		0.41		0.49		0.84

NS, non-significant; exact p-value was not determined owing to the small sample size. [a]Contents of antioxidative vitamins and minerals: Feminatal – 0 mg vitamin A, 12 mg vitamin E, 180 mg vitamin C, 0 μg selenium, 15 mg zinc, 1 mg copper, 70 mg magnesium, and 3 mg β-carotene; Elevit – 1500 μg vitamin A, 10.5 mg vitamin E, 100 mg vitamin C, 0 μg selenium, 7.5 mg zinc, 1 mg copper, 100 mg magnesium, and 0 mg β-carotene; Prenatal – 0 mg vitamin A, 10 mg vitamin E, 100 mg vitamin C, 20 μg selenium, 15 mg zinc, 1 mg copper, 50 mg magnesium, and 3 mg β-carotene; Falvit – 800 μg vitamin A, 10 mg vitamin E, 60 mg vitamin C, 25 μg selenium, 15 mg zinc, 0 mg copper, 30 mg magnesium, and 0 mg β-carotene; Materna – 0 mg vitamin A, 12 mg vitamin E, 110 mg vitamin C, 25 μg selenium, 7 mg zinc, 0 mg copper, 100 mg magnesium, and 2 mg β-carotene.

in clinical practice, as our findings suggest that milk samples for neopterin determination can be obtained at any time during the feeding. Thus, neopterin can be used as a safety marker of human breast milk.

Our study also documented the lack of significant influence of pregnancy duration and maternal age on the level of neopterin, which substantiates using the level of this compound as the independent safety marker. Many previous studies showed that pregnancy is associated with a significant increase in the blood concentration of neopterin. Immediately after delivery, the blood level of this compound is similar to that observed in the third trimester; then, it gradually decreases, reaching its prepregnancy level at about week 6 [22, 25, 26]. Therefore, the dynamics of blood neopterin concentration after delivery are not consistent with that documented in breast milk from various periods of lactation. Consequently, it cannot be excluded that neopterin contained in human milk does not originate in the blood of breastfeeding women but is synthesized *in situ* in the mammary gland. Also, Ganglberger et al. [27] proved that the breast milk concentration of neopterin is not associated with its blood or urinary concentration. Similar conclusion can be drawn from the results of previous studies dealing with the breast milk concentration of other two pteridines, biopterin and tetrahydrobiopterin, which, similarly to neopterin, derive from dihydroneopterin triphosphate [24, 28].

Aside from the duration of pregnancy, the patient's age also exerts a significant effect on the blood concentration of neopterin. Frick et al. [29] analyzed the blood concentration of neopterin in individuals between 34 and 93 years of age, and observed a significant positive correlation between the level of this compound and age. Also, Schennach et al. [20, 21] observed that the blood concentration of neopterin in individuals older than 41.5 years is significantly higher than in the younger subjects. Age-progressing increase in the blood concentration of neopterin is interpreted as a potential response of this parameter to ongoing subclinical pathological processes, such as atherosclerosis and dementia [20, 21, 29]. The lack of a significant relationship between the breast milk level of neopterin and the age of participating mothers, documented in our study, confirms our previously mentioned hypothesis on the independence of blood and milk concentrations of this compound and its local synthesis within the mammary gland. This finding would constitute another argument for using neopterin as a specific safety marker of human milk. However, it cannot be excluded that the lack of age-related changes in the breast milk concentration of neopterin resulted from the relatively wide range of participant ages (20–40 years). The oldest individual in our group did not reach the age that, according to the literature, is associated with elevated blood concentrations of neopterin [20, 21].

The potential limitations of this study should be examined in view of interpreting our findings. Undoubtedly, the small size of our sample, particularly with respect to later pregnancy stages, represents the main flaw of our experiment. This is a frequent and widely emphasized problem of studies addressing questions related to human breast milk composition. We tried to overcome this limitation by providing the highest possible homogeneity of the examined group and excluding the potential confounding effects of maternal variables and environmental factors. The lack of a constant monitoring of neopterin concentration throughout consecutive stages of lactation was another potential limitation of this study. Previously mentioned discrepancies between our results and the findings of Iizuka et al. [24] suggest that even 2-week-long intervals between consecutive measurements can be too long to reveal considerable changes

in the breast milk concentration of neopterin. Finally, it should be emphasized that we measured the concentration of neopterin only in human milk. The lack of information about the level of this compound in other body fluids, including blood and urine, precluded the direct verification of the hypothesis on the local synthesis of neopterin in the mammary gland and its independent character as a potential safety marker of human breast milk.

Despite these limitations, further studies on the role of breast milk concentration of neopterin as a potential diagnostic marker seem justified. The next stage of such research should unambiguously verify the independence of blood and milk concentrations of neopterin, and study the potential changes of the latter parameter induced by maternal pathologies and/or harmful factors present in breast milk.

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