Rocz Panstw Zakl Hig 2022;73(2):139-145

http://wydawnictwa.pzh.gov.pl/roczniki_pzh/ https://doi.org/10.32394/rpzh.2022.0214

REVIEW ARTICLE

HUMAN MILK METABOLOME: IMPACT OF GESTATIONAL AGE, LACTATION STAGE AND MATERNAL DIET

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ABSTRACT

Human breast milk due to its unique composition and the ability to adapt to the needs of the infant, is referred to as the "gold standard". Exclusive breastfeeding is recommended for the first 6 months of a inflant's life. The composition of breast milk and its metabolites is not constant and varies depending on the influence of various factors. Its analysis allows for rational management of infant nutrition. Intermediate and final metabolites of human milk are formed as a result of various metabolic processes in the mammary gland, and their role and the influence of various factors on them are not fully determined in the context of the proper development of infants. Metabolomic studies can be used to identify intermediate and terminal metabolites in breast milk. The aim of the study was to review the current literature on the variability of human milk metabolome depending on factors such as gestational age, lactation stage and mother's diet. A review of current research shows that the composition of human milk metabolome varies depending on various factors. Better understanding of metabolome of breast milk could be crucial in the future programming of metabolic processes in infants, which is crucial in preventing many diseases and maintaining health.

Key words: metabolome, metabolomics, human milk, gestational age, lactation stage, maternal diet

STRESZCZENIE

Mleko matki, ze względu na swój unikalny skład i możliwość dopasowania się do potrzeb niemowlęcia, jest określane jako „złoty standard". Wyłączne karmienie piersią zalecane jest przez pierwsze 6 miesięcy życia niemowlęcia. Skład mleka matki i jego metabolitów nie jest stały i zmienia się w zależności od wpływu różnych czynników. Jego analiza pozwala na racjonalne zarządzanie żywieniem niemowląt. Metabolity pośrednie i końcowe mleka ludzkiego powstają w wyniku różnych procesów metabolicznych w gruczole sutkowym, a ich rola i wpływ na nie różnych czynników nie są do końca określone w kontekście prawidłowego rozwoju niemowląt. Badania metabolomiczne można wykorzystać do identyfikacji pośrednich i końcowych metabolitów w mleku matki. Celem pracy był przegląd aktualnej literatury dotyczącej zmienności metabolomu mleka ludzkiego w zależności od czynników takich jak wiek ciążowy, okres laktacji i dieta matki. Przegląd aktualnych badań wskazuje na zróżnicowanie składu metabolomu mleka ludzkiego w zależności od różnych czynników. Lepsze poznanie metabolomu mleka matki może być kluczowe w przyszłym programowaniu procesów metabolicznych u niemowląt, co ma znaczenie w prewencji wielu chorób i utrzymaniu zdrowia.

Słowa kluczowe: *metabolom, metabolomika, mleko kobiece, wiek ciążowy, okres laktacji, dieta matki*

INTRODUCTION

Breastfeeding is defined as the "gold standard" in infant nutrition. Human milk is characterized by the complexity and uniqueness of its composition and provides nutrition and protection of the infant against diseases and microorganisms [36]. Human milk as a complex biological system consists of significant nutrients and bioactive components that interact with each other to form a unique bioliquid [8]. In addition, breastfeeding ensures optimal growth and development and health of infants, being not only food, but also a source of interacting bioactive ingredients [22]. Therefore, international organizations such as the World Health Organization (WHO), the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the American Academy of Pediatrics (AAP) recommend exclusive breastfeeding

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for the first 6 months of an infant's life, and then continuing breastfeeding as complementary foods are introduced, and breastfeeding continues for 2 years or more, as the mutual wishes of mother and infant [1, 13, 43]. The first 1000 days of life (from conception to 2 years of age) are extremely important for the proper development and prevention of diseases in the context of metabolic programming [32]. Metabolomics may be a tool that can help to understand the early health programming of infants from birth [37]. The metabolome of human milk is subject to changes depending on various factors. The identification of these factors will allow the determination and confirmation of the presence of many metabolites in breast milk that may play an important role in the prevention of metabolic diseases [6]. Due to the dynamically changing of breast milk, it is important to understand the influence of various factors on human milk metabolome. This knowledge can possibly improve and better manage infant nutrition [17].

DEVELOPMENT OF MODERN ANALYTICAL METHODS ENABLING THE DETERMINATION OF COMPOUNDS WITH SMALL MOLECULES

Metabolomics allows the analysis of small molecules and metabolites, making it a promising tool for the precise determination and understanding of intracellular metabolic pathways and biomarkers [35]. The most important groups of metabolites are amino acids, carbohydrates, nucleotides, lipids, coenzymes and cofactors, which are diverse in terms of structure and molecular structure, as well as properties and functions, posing an analytical challenge [47]. Metabolomics enables the study of the level of polar and lipid metabolites, which is the basis for understanding the pathophysiological mechanisms of metabolic diseases. Moreover, it enables the effective diagnosis of abnormal physiological processes and states, and can also be a tool for the prevention and treatment of diseases [21]. The development of modern analytical techniques for metabolomics and lipidomics has enabled the determination and identification of particles <1500 daltons [10]. The most frequently used analytical methods in metabolomics studies are nuclear magnetic resonance (NMR) and gas chromatography (GC), liquid chromatography (LC) and electrophoresis (CE) coupled with mass spectrometry (MS) [33]. The NMR technique is non-destructive and highly reproducible and automated, thanks to which it is possible to identify new compounds in particular in large-scale metabolomics studies [12]. However, the intensive development of MS techniques has made them less limited and more effective, selectable and sensitive. Moreover, the use of MS techniques allows the identification of a large number of different polar metabolites and/or lipids in samples even in very small amounts [15]. Currently, technological advances in the field of metabolomics reduce the difficulty of carrying out analyzes and reduce their costs [14]. Most of the current metabolomics and lipidomics studies are based on non-targeted analysis, which is free from hypotheses and allows to increase the knowledge about the unique composition of human milk and its metabolites [41]. Due to the complexity of the composition of human milk, the use of multiple analytical platforms allows the determination of as many metabolites as possible, which is often impossible using only one method [2]. It is worth emphasizing that the composition of breast milk is dynamic changes in both lactation progression and single feeding to cover the nutritional needs of the child, therefore the appropriate methods of its analysis should be selected [26]. In addition, metabolic processes taking place in the mammary gland result in the formation of intermediate and final metabolites of human milk, the role of which, as well as the influence of various factors on them, are not fully defined in the context of health and proper development of infants [29].

FACTORS INFLUENCING HUMAN MILK METABOLOME

Gestational age

According to a WHO report, it is estimated that approximately 15 million babies are born prematurely (before the 37th week of pregnancy) each year. This indicates that more than 1 in 10 infants are born preterm [42]. Nutrition of premature infants should be primarily aimed at achieving a postnatal growth rate similar to term infants [19]. Breastfeeding has invaluable benefits not only for healthy infants, but also for preterm infants. Brest milk feeding reduces the risk of necrotizing enterocolitis (NEC) and sepsis, and improves the neurological development of preterm infants [1]. Gestation age is one of the factors influencing the diversification of the human milk metabolome. Analysis of milk metabolome of preterm mothers may help to better understand changes in metabolite composition compared to the milk of full-term infants. Moreover, it may bring benefits from understanding the nutritional needs of preterm infants, which would help maintain an adequate nutritional status of this particularly vulnerable group [48]. *Sundekilde* et al. [40] investigated the effect of gestational age on metabolome of human milk. Milk samples were collected from 15 mothers of preterm infants, while 30 milk samples were collected from mothers who gave birth at term. Depending on the postpartum time, milk samples were divided into 3

categories: colostrum (<5 days postpartum; n = 5), transitional milk (6 days - 2 weeks postpartum; n = 4) and mature milk (> 2 weeks, n = 21). Milk metabolomes were compared by proton nuclear magnetic resonance (^{1H} NMR) spectroscopy. Comparing the metabolome of breast milk obtained from mothers of premature infants and mothers of full-term infants, it was found differences in the concentrations of carnitine, caprylate, caprate, pantothenate, urea, lactose, oligosaccharides, citrate, phosphocholine, choline and formate were noted. The gestational age can significantly affect the oligosaccharide content in human milk. In the study by *Perrone* et al [31], nuclear magnetic resonance (NMR) spectroscopy also was used to identify metabolites. In total, 18 mothers were recruited for the study: 6 mothers who gave birth to premature babies (from 29 to 31 weeks GA) and 12 mothers who gave birth at term. Higher concentrations of lactose and oligosaccharides, especially fucosylated, such as fucose, N-acetylneuraminic acid and N-acetylglucosamine were identified in the milk of premature infants compared to mothers of full-term infants. Changes in metabolomic profiles of milk of preterm infants may indicate different nutritional requirements of these babies. Another comparison between the metabolomic profile of milk of preterm mothers and milk of full-term mothers was performed by *Xu* et al. [46]. Lipidomic profiles were identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS). Colostrum samples were collected for the study from healthy mothers of preterm breastfeeding infants and mothers of full-term breastfeeding infants. Colostrum of premature mothers was characterized by a higher concentrations of phosphatidylethanolamine and phosphatidylcholine, as well as lower concentrations of diacylglycerol and ceramide compared to the milk of mothers who gave birth at term. *Spevacek* et al. [39] conducted a prospective observational study involving mothers of both term and premature babies. Human milk samples were collected 0-5 days postpartum and then 14-28 days later. ^{1H} NMR was used to identify and quantify human milk metabolome. The colostrum samples were rich in 3'-galactosyllactose (3'-GSL), 2-hydroxybutyrate, methionine and acetoine, but their amount decreased in term milk. Dimethylsulfone was also identified, it was characterized by a low content in the colostrum, while its concentration increased during the first month of lactation. Regardless of the age of pregnancy, the total concentration of oligosaccharides decreased during lactation. Interestingly, the concentrations of some amino acids and derivatives in breast milk also were changed. There was an increase in 2-aminobutyrate, alanine, carnitine, glutamate, glutamine, histidine, urea and valine, in contrast to the milk of mothers of premature infants. The concentration of fatty acids and their metabolites also changed, and an increase in lactose concentration was observed during the first month of lactation, both in preterm milk and in term milk. The study by *Longini* et al. [24] recruited 20 mothers who gave birth at 23-41 weeks of pregnancy, from whom 46 samples of human milk were collected. The metabolites of preterm and term milk were compared as well as formula milk. For metabolomic analysis, proton magnetic resonance spectroscopy (MRS) was used to determine water-soluble and lipid fractions extracted from human milk. Apart from noting changes between human milk and formula milk, the study found significant differences between the milk samples of mothers giving birth at 23-25 weeks of gestation compared to the milk of mothers giving birth at 29 weeks of gestation. A study by *Peila* et al. [30] recruited 36 breastfeeding mothers who gave birth prematurely between 23 and 33 weeks of gestation. In addition, three groups of mothers were distinguished according to different gestational age (GA): extreme (<28 GA week), very (29-31 GA week) or moderate (32-34 GA week). The milk samples were colostrum, transitional milk, and mature milk, metabolome of which was examined using nuclear magnetic resonance (NMR) spectroscopy. Depending on the gestational age, when comparing groups of extremely and moderately premature infants, the study showed significant but weak changes in the concentrations of some metabolites.

Lactation stage

Factors related to the time of feeding have a significant impact on metabolomic profile due to changes related to three main stages of lactation: colostrum, transitional milk and mature milk [4]. Colostrum, which is the first milk of the mother, is a rich source of active immunological substances and proteins [28]. Transitional milk is characterized by accelerated production in order to meet the infant's needs for its proper development and growth as much as possible. Mature milk is milk similar to transitional milk, but it is more stable [45].

Recent studies confirm the differentiation of metabolome of human milk depending on the stage of lactation. In the study by *Wu* et al. [44], nuclear magnetic resonance (NMR) spectroscopy was used to identify and quantify metabolites. Human milk samples were collected from a healthy 37-year-old woman (BMI = 24) in the morning and evening on days 9, 12, 24, 31, 60, 85, 86 and 87 (only the morning sample was taken on the last day). Human milk metabolomes in the early (9-24 days postpartum) and late (31-87 days postpartum) stages of lactation were statistically significantly different. The late stage of lactation was characterized by significantly increased concentrations of lactose, choline, alanine,

glutamate and glutamine, and decreased levels of citrate, phosphocholine, glycerophosphocholine and N-acetylglucosamine. The weakness of the study was that a sample was only tested from one person. Additionally, the effect of the storage time of the milk samples at the temperature of 20°C or 80°C was investigated, however, no significant differences in metabolome of human milk were found. The study by *Sundekilde* et al. [40] involved 45 women, including 15 mothers of preterm breastfeeding infants and 30 mothers of full-term breastfeeding infants. Milk metabolome was studied using proton nuclear magnetic resonance (^{1H}NMR) spectroscopy. In the colostrum of mothers of full-term infants, higher concentrations of valine, leucine, betaine and creatinine were found compared to mature milk. On the other hand, in mature milk a higher concentration of glutamate, caprylate and caprate was observed than in the colostrum of mothers of full-term infants. Mothers of premature infants had higher levels of oligosaccharides, citrate, and creatinine in the colostrum, while higher levels of caprylate, caprate, valine, leucine, glutamate and pantothenate were noted with time after delivery. Data on changes in metabolome of human milk was provided by a study by *Giuffrida* et al [18] among Chinese breastfeeding mothers. 540 healthy, breastfeeding women were recruited for the study and gave birth to one child at term. The milk was sampled over a period of eight months. Fatty acids were determined by gas chromatography coupled to a flame ionization detector (GC-FID), phospholipid classes were determined using liquid chromatography with evaporative light scattering detector (LC-ELSD), and for the determination of gangliosides, liquid chromatography with tandem mass spectrometry (LC-MS/MS). As a result, an increased content of polyunsaturated fatty acids (PUFA) and gangliosides (GD) was found in later stages of lactation. On the other hand, the concentration of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and phospholipids (PL) decreased during lactation. The study by *Azad* et al. [3] included the analysis of various factors influencing the variability in the human milk oligosaccharides (HMO) in 427 mother-child dyads, whose milk samples were delivered 3-4 months after delivery. The study focused on the content and type of HMO isolated by high-throughput solid phase extraction followed by HPLC analysis with fluorescence detection. HMO content was lower with longer lactation times in line with earlier literature, however, the study showed that disialyllacto-N-tetraose (DSLNT) and 3′-sialyllactose (3′-SL) concentrations were higher later in lactation. The metabolomic analysis performed using high-performance liquid chromatography-quadrupole-time of flight mass spectrometry (HPLC-QTOF-MS) in the study by *Li* et al. [23] was aimed at assessing changes in human milk metabolites during the lactation stages. Thirty healthy breastfeeding women were recruited for the study, from whom milk samples were collected at three stages of lactation (colostrum, transitional milk and mature milk) on days 1, 14 and 42 postpartum, respectively. In the study, a total of 84 metabolites were identified in the three stages of lactation. The longer the duration of lactation, the concentration of 9 dipeptides increased, such as: glycyl-valine, histidinyl-threonine, lysyl-threonine, threoninyl-tyrosine, tyrosyl-glutamine, tyrosyl-serine, valylvaline, valyl-alanine and histidinyl-histidine. There was also an increase in concentration of eicosanoid (prostaglandin E3), 1 fatty amide (octadecanamide) and 2 fatty esters (CE (15:1) and CE (14:0)), as well as free fatty acids: C10:0, C11:0, C14:0, C16:1n-7, C17:0, C18:1n-9 and C18:2n-6. Moreover, with longer lactation time, an increase in the concentration of 12 diacylglycerols in human milk was also noted DG (14:0/20:4n-6), DG (14:0/24:1n-9), DG (14:1n-5/18:3n-3), DG (15:0/20:4n-6), DG (18:0/18:1n-9), DG (18:4n-3/18:4n-3), DG (20:0/24:1n-9), DG 24:0/15:0), DG (18:3n-3/18:3n-3), DG (15:0/16:1n-7) and DG (14:0/14:1n-5). In addition, along with the longer lactation time, content of 11β-hydroxyprogesterone, cisaconitic acid and spermidine increased significantly, as well as nucleotides such as: 6-succinaminopurine, deoxyuridine, dUMP, purine and AMP 3'-phosphate. During the study, 7 vitamins were detected, of which the content of vitamin D3, 1,24,25- (OH) 3, vitamin D3, 25-hydroxyvitamin D2-25-glucuronide, vitamin D2 and biotin was positively associated with the lactation time, while the content of β-glucuronide retinoil and γ-tocopherol significantly decreased. The innovative use of a multiplatform approach combining HPLC-MS and ultra-performance LC-MS, GC-MS, CE-MS, and 1H NMR spectroscopy in the study by *Andreas* et al. [2] allowed for comprehensive identification and evaluation of metabolite changes in breast milk samples during lactation. Metabolomic profiling was performed on 70 samples of human milk collected between 2 and 80 postpartum, from 57 women who gave birth to healthy infants on time. The metabolites present in human milk changed dynamically during the first 3 months of lactation. At the beginning of lactation, an increase in the content of di- and tri-acylglycerols as well as lactose, some amino acids and short- and medium-chain fatty acids was noted. On the other hand, with increasing lactation time, many human milk oligosaccharides (HMOs), some phosphocholines as well as citrate and pyruvate decreased.

Factors related to maternal health and lifestyle

Maternal factors may play one of the key roles in shaping the metabolome of human milk. Factors such

as diet, nutritional status or maternal diseases and medical condition may have a significant effect on the increase or reduction of the concentration of certain metabolites in human milk.

Maternal diet

Maternal nutrition is one of the key factors influencing metabolome of human milk. Metabolomics can help to improve knowledge about the relationship between the nutritional status and metabolites found in body fluids, such as breast milk [27]. The study by *Smilowitz* et al [38] was aimed at examining the metabolome of breast milk depending on the phenotype and maternal diet. NMR technique was used to determine the metabolites. Metabolites such as mono-, di- and oligosaccharides, amino acids and derivatives, energy metabolites, fatty acids and related metabolites as well as vitamins, nucleotides and derivatives and others have been quantified. The following metabolites showed the greatest variability in breast milk: 2'-FL, fucose, LDFT, LNFP I, LNFP II, aspartate, lysine, proline, acetone, creatine phosphate, fumarate, acetate, azelate, butyrate, choline, niacinamide, hypoxanthine, formate and methanol. In addition, essential nutrients that come with the diet such as choline, niacinamide, ascorbate and pantothenate have also undergone various changes. Metabolomic analysis in the study by *Li* et al. [23] was carried out using HPLC-QTOF-MS technique. Eighty four metabolites were identified in the study, however only two were of dietary significance; the concentration of 1,24,25- (OH) 3 of vitamin D3 was positively associated with the consumption of meat and eggs, protein and fat in the diet, while the concentration of 11β-hydroxyprogesterone was negatively associated with the intake of fruit and carbohydrates in diet.

A study by *Marín* et al. [25] investigated the relationship between maternal nutrition and fatty acid composition of milk. 46 breastfeeding mothers who gave birth to healthy, full-term infants at 38-42 weeks of pregnancy were recruited for the study. Maternal nutritional status was assessed using body mass index (BMI), while maternal nutrition was assessed using the eating frequency questionnaires (FFQ). The questionnaire included questions about amount and frequency of consumption of high-fat foods per week (including whole milk and derivatives, fried meat, eggs, chocolate candies), as well as low-fat foods (including rice and other grains, vegetables and fruits, skim milk, low-fat fish, bread). The study did not show any effect of maternal nutritional status on human milk proteins. However, higher levels of lipids, linoleic acid and total n-6 acids and PUFA were observed in obese mothers.

Maternal overweight/obesity

Overweight and obesity are becoming a growing problem worldwide, posing a challenge to public health. Obesity may lead to the development of metabolic changes, causing e.g. diabetes, dyslipidemia, arterial hypertension, cancer or changes in the intestinal microbiota [5]. Although prevalence of obesity among children is lower than among adults, the rate of its spread is higher [16]. Maternal obesity before and during pregnancy may be associated with a higher prevalence of obesity in children [7]. Infants who are constantly exposed to factors related to maternal obesity may develop health problems later in life. Determining the milk metabolome of obese mothers can help prevent infant obesity later by ensuring optimal and sustainable growth and development. A study by *Saben* et al [34] aimed to assess the effect of maternal obesity on metabolome of human milk and to identify milk metabolites associated with infant obesity. Healthy women with BMI 18.5-24.9 were recruited for the study, divided into a group of obese and non-obese mothers. Human milk metabolome determination was performed using GC-time-of-flight-MS (GC-TOF-MS). The study showed that maternal obesity is related to metabolome of human milk, which is richer in monosaccharides and sugar alcohols compared to milk of non-obese mothers. The content of human milk monosaccharides, mannose and ribose as well as sugar alcohols, lixitol and ribitol were higher in mothers with higher BMI. Another study by *Isganaitis* et al. [20] analyzed the metabolome of breast milk in terms of its relationship to maternal obesity. For this purpose, non-directional liquid chromatography, gas chromatography and mass spectrometry were used. Six months after delivery, higher levels of acylcarnitines, monosaccharides and sugar alcohols in milk were noted in obese mothers, while the amount of amino acids and their metabolites decreased. This suggests association between obesity and changes in metabolome of human milk. The study by *De Luca* et al. [9] was aimed at determining changes in the concentration of free amino acids depending on obesity of a breastfeeding mother. Free amino acid (FAA) concentrations in breast milk were determined by ultra-performance liquid chromatography tandem mass spectrometry. The breast milk of the obese mothers contained more branched chain amino acids (BCAA) and more tyrosine than the milk of not obese mothers.

A study by *Ellsworth* et al. [11] compared changes in composition of milk from overweight and obese mothers to non-obese mothers. In milk of overweight and obese mothers the increase of long-chain polyunsaturated fatty acids (LC-PUFA) and the decrease of oleic acid (C18:1n-9) and conjugated linoleic acid (CLA) was found. Additionaly, increased level of insulin also was noted.

CONCLUSIONS

The composition of each mother's milk is personalized depending on the needs of the infant. Mother's milk is a unique bioliquid with unique properties, ensuring proper development and growth for infants. More research and application of various extraction and analysis techniques are needed to understand the influence of various factors on the composition and metabolic profile of human milk. It is especially important to study the influence of the diet of pregnant and breastfeeding women on the metabolome of breast milk, because this is a factor that can be relatively easily modified.

Conflict of interest

The authors declare no conflict of interest.

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Received: 17.05.2022
Accepted: 08.06.2022