VILNIUS UNIVERSITY

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Changes in Human Milk Macronutrient Content of Non-Breastfeeding Mothers after Delivery and Factors or Procedures Affecting its Composition

SUMMARY OF DOCTORAL DISSERTATION

Medicine and health sciences,
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VILNIAUS UNIVERSITETAS

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Nežindančių motinų pieno maistinių medžiagų koncentracijos pokyčiai po gimdymo ir veiksniai bei procedūros, turintys įtakos jo sudėčiai

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ABBREVIATIONS

AAP – American Academy of Pediatrics

BMI – body mass index

ELISA – enzyme-linked immunosorbent assay

EMBA – European Milk Bank Association

ESPGHAN – European Society for Paediatric Gastroenterology, Hepatology and Nutrition

ELBW – extremely low birth weight (< 1 000 g)

GA – gestational age (weeks)

HIV – human immunodeficiency virus

HMO – human milk oligosaccharides

NMR – nuclear magnetic resonance

VLBW – very low birth weight (< 1 500 g)

VUH SK – Vilnius University Hospital Santaros Klinikos

WHO – World Health Organization

INTRODUCTION

Background

Human milk is a unique source of food for newborns and infants. With human milk, the infant receives not only nutrients (proteins, fats, carbohydrates, vitamins and microelements), but also biologically active components (hormones, growth factors, cytokines, chemokines, immunoglobulins, oligosaccharides), specific microbiome (lactobacteria and bifidobacteria), as well as specific cells contained in milk (epithelium, immune, multipotent stem cells, etc.) [1–5]. Breastfeeding ensures a close bond between the mother and her child, and contributes to the activation of various metabolic processes determining the infant's growth, development of organs systems, migration and differentiation of nervous cells, colonisation of the intestines with microorganisms, and maturation of the immune system [2]. The composition of each mother's human milk is unique and ensures specific needs of her child in the best manner. Fresh human milk is the best food both to healthy full-term newborns and to ill or premature newborns. It has been proved that children who were breastfed from birth instead of giving them infant formula, fall ill more seldom and develop better [6, 7].

Human milk is special not only because of its composition, but also because of its continual changes. Changes affect not only the amount of the macronutrient, but also the "live" part of human milk, namely, bioactive components. Differences of and changes in human milk composition are determined by various factors: duration of pregnancy and lactation [8, 9], circadian rhythm [10, 11], mode of delivery [12], a newborn's gender [12–14], a woman's age and ethnicity [15], nutrition and lifestyle [16–18], a woman's diseases [19], as well as all procedures performed with human milk (manner of expression, storage, processing, etc.) [20–23].

It might seem that human milk composition has long been explored in detail. However, improvement in laboratory equipment

and emergence of new possibilities of analysis make it possible for scientists to keep discovering many interesting facts about human milk – a "live" source of food. More thorough examination of human milk has been enabled by emergence of OMICS technologies. For instance, human milk's metabolome analysis enables the characterisation of the full composition of the milk's metabolites and assessment of changes in milk composition determined by various factors, as well as disclosure of how it affects a breastfed newborn's adaptation, microbiome, and development of organs systems [24]. Established specific metabolome changes provide knowledge about the body's pathophysiological processes, and can contribute to diagnostics of diseases and monitoring of treatment. Still, the functions of many composite human milk components are not fully known yet [2, 3].

Currently, there is no doubt that fresh human milk is the best food for newborns and infants. Breastfeeding is promoted by international organisations – WHO, AAP, ESPGHAN and others [6, 25, 26]. Even though human milk is extremely important for premature newborns with very low (VLBW) and extremely low birth weight (ELBW), it is not sufficient on its own to ensure adequate growth of these newborns and their bone mineralisation; therefore, special supplements are recommended for them in addition to human milk – the so-called human milk fortifiers [27]. In the preparation of a nutrition plan for premature ELBW and VLBW newborns, knowing the composition of human milk is crucial in order to avoid unreasonable (insufficient or surplus) prescription of human milk fortifiers.

Donor human milk should be the second choice of nutrition for premature newborns when own mother‘s milk is lacking [28, 29]. Such milk must be specially prepared in the donor milk bank in compliance with appropriate safety requirements [30]. All procedures performed with human milk can influence changes in its composition. Therefore, it is crucial to look for optimum methods of human milk collection, storage and preparation for safe use, which

would enable preservation of fresh human milk composition with minimum changes.

The Scientific Novelty of the Study and Implementation in Clinical Practice

This is the first study of the kind conducted in Lithuania, which was focused on the assessment of changes in human milk macronutrient content during the first weeks after delivery, as well as various factors and procedures having an impact on human milk composition. We were the first in Lithuania to use the mid-infrared transmission spectroscopy method to establish human milk macronutrient and energy value. The findings we provide can be directly applied in the clinical practice of health specialists taking care of newborns and infants, in particular, to address issues with regard to insufficient growth of weight of a newborn (or an infant), preparation of a nutrition plan, and there is no possibility to analyse human milk composition.

In cooperation with Italian researchers, innovative human milk metabolome analysis was conducted in the NMR laboratory of the Department of Chemical and Geological Sciences, University of Cagliari (Italy); the analysis enabled assessment of pasteurisation impact on human milk metabolome composition and provided knowledge about oligosaccharide composition of human milk of women residing in Lithuania.

Clinical practice, especially nutrition of premature newborns, witnesses increasingly wider use of pasteurised donor human milk. Since scientific literature contains a lot of contradictory information on the impact of pasteurisation on human milk composition, it was critical to establish how this process influences and/or changes the composition of human milk macronutrient, biologically active proteins and metabolome.

The Aims of the Study

To investigate the composition of milk of non-breastfeeding mothers living in Lithuania, to establish physiological factors depending on the mother and the newborn, which have an impact on human milk content as well as the impact of freezing and Holder pasteurisation on human milk composition.

The Objectives of the Study

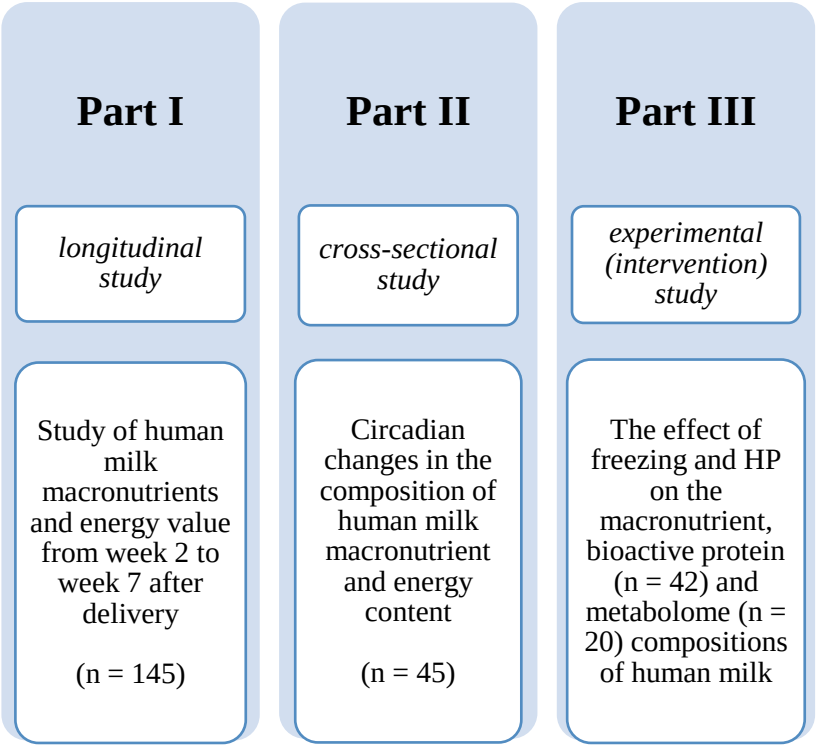
1. To assess changes in the composition of macronutrients and energy value of human milk of non-breastfeeding mothers living in Lithuania during the first seven weeks after delivery.
2. To establish whether the mother's age, her body mass index (BMI), pregnancy duration, mode of delivery and a newborn's gender have an impact on human milk macronutrient composition.
3. To assess circadian changes in human milk macronutrient composition and energy value.
4. To assess the impact of freezing and Holder pasteurisation on human milk macronutrient composition, energy value and the composition of biologically active proteins (lactoferrin, lysozyme) and metabolome.

Defended Statements

1. Macronutrient content and energy value of human milk of mothers living in Lithuania change depending on the duration of lactation.
2. Various physiological factors as well as factors related to the mother and newborn are important for the human milk composition of macronutrients and metabolome.
3. Pasteurised and frozen human milk retains a large share of unchanged important components of fresh human milk.

MATERIALS AND METHODS

For the most part, the study was conducted in 2017–2020 at the Neonatology Centre of Vilnius University Hospital Santaros Klinikos (VUH SK). Human milk metabolome analysis was performed at the NMR laboratory of the Department of Chemical and Geological Sciences, University of Cagliari (Italy). The present study was approved by the Vilnius Regional Research Ethics Committee. The study was divided into three parts (Figure 1).



HP – Holder pasteurisation

Figure 1. Parts of the biomedical study

Calculation of the Sample of the Study

To establish the samples of the study, in all three parts G*Power (version 3.1.9.4) programme was used. The level of significance of the criterion was chosen as follows: $\alpha = 0.05$. Account was taken of the fact that the criterion would be no less powerful than 0.8 ($0.8 < \beta < 0.95$). The chosen amount of samples was in conformity with the requirements.

Study of human milk macronutrients and energy value from week 2 to week 7 after delivery

The first part aimed at establishing the changes in human milk composition of macronutrients and energy value during the first seven weeks after delivery, as well as establishing whether such factors as pregnancy duration, a newborn's gender, the mother's age and BMI, mode of delivery have an impact on human milk composition.

The study was conducted at the Neonatology Centre of VUH SK from October 2017 to April 2020.

By way of convenience sampling, the following women hospitalised at the Neonatology Centre of VUH SK were included into the study: healthy women not using medication who did not breastfeed their newborns due to premature birth, a newborn's disease or other reasons, but fed them with their own expressed milk. If need be, the women participating in the study were supplied with human milk electric pumps and taught how to use them appropriately.

Table 1 below shows the criteria of inclusion into the study and rejection from the study.

Table 1. Study inclusion and rejection criteria

Inclusion criteria	**Rejection criteria**
Healthy mothers not using medication;	Mother's diseases: infectious (tuberculosis, hepatitis B and C, HIV, mastitis), oncological, endocrinal (diabetes); Regular use of medication;
Mothers not following specific diets;	Vegetarians, vegans;
Single foetus pregnancy;	Multiple foetus pregnancy;
Human milk expressed using a mechanical or electric pump;	Breastfeeding mothers; Human milk expressed by hand;
Amount of expressed human milk exceeds a newborn's daily nutrition needs;	All expressed human milk is used for a newborn's feeding;
Regular human milk expression (at least four times in 24 hours);	Human milk expression more seldom than four times in 24 hours;
Signing the informed person's form of consent	Refusal to participate in the study

Milk composition of each study participant was analysed once a week, starting with the second week and ending with the seventh week after delivery: week 2 (on 8–10th days after delivery), week 3 (15–17th days after delivery), week 4 (22–24th days after delivery), week 5 (29–31st days after delivery), week 6 (36–38th days after

delivery), week 7 (43–45th days after delivery). Human milk was fully expressed from one or both breasts with the help of an electric or mechanical breast pump and collected for 24 hours.

The milk's macronutrient and energy content was evaluated by mid-infrared spectroscopy using the Miris Human Milk Analyser (Miris AB, Sweden), which was operated using the calibration mode for processed (homogenised) milk. According to the manufacturer's instructions, prior to analysis, the milk samples were warmed to 40 °C and homogenised for 1.5 s/ml using the Miris Ultrasonic Processor. A daily calibration check was performed using the calibration solution (Miris check), which was provided by the supplier.

The study involved 145 participating women. 74 women completed the study fully (that is, expressed milk for analysis for seven weeks after delivery). The remaining women finished their participation in the study earlier for various reasons (lack of human milk, breastfeeding, travelling to another city, etc.).

Circadian changes in the composition of human milk macronutrient and energy content

The purpose of part II of the study was to assess circadian changes in the composition of human milk macronutrient content.

The cross-sectional study was conducted from October 2017 to May 2018 at the Neonatal Center of VUH SK.

Criteria of inclusion and non-inclusion into the study were the same as in part I of the study.

Milk samples from 45 mothers (the mothers of 27 preterm and 18 full-term newborns) were collected on a single day chosen between the 14th to 16th days after delivery. The samples were taken four times during the chosen day at the collection points of 12 PM, 6 PM 12 AM and 6 AM (±1 hour).

After one or both breasts were fully emptied with an electrical or manual breast pump and the milk was carefully mixed in the container, samples of 10 ml milk were immediately collected in separate sterile plastic containers. After being labelled (ID number, data and correct time of collection), each sample was stored in a refrigerator at +4 °C. Further analysis of the milk's macronutrient composition was performed within 22 to 25 hours after the beginning of the collection (at 12 PM).

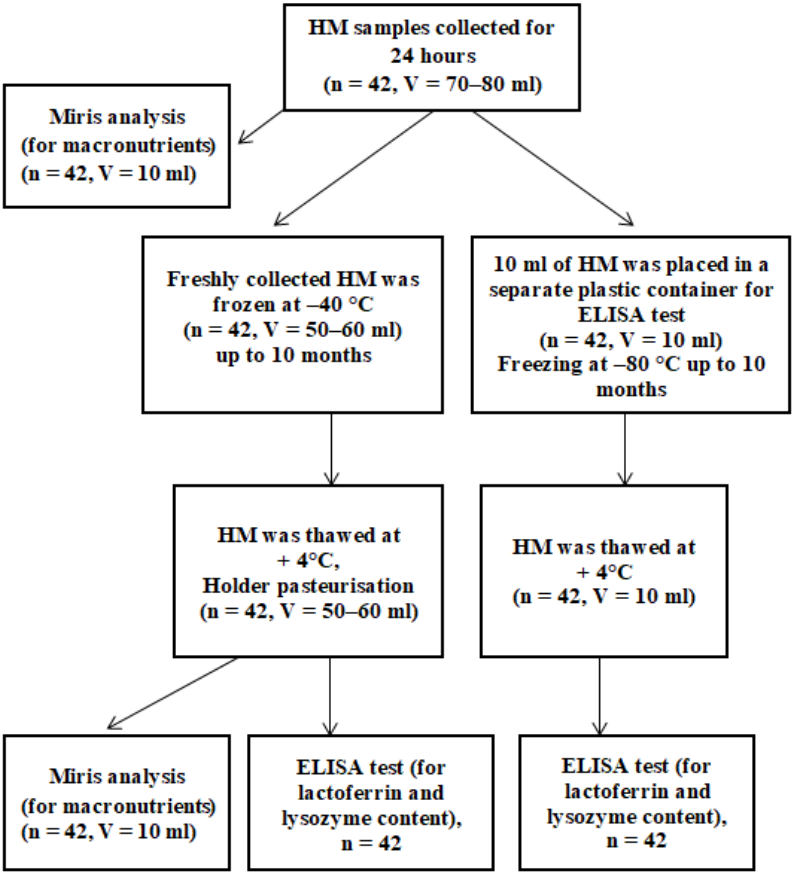
The milk's macronutrient and energy content was evaluated by mid-infrared spectroscopy using the Miris Human Milk Analyser (Miris AB, Sweden), which was operated using the calibration mode for processed (homogenised) milk. According to the manufacturer's instructions, prior to analysis, the milk samples were warmed to 40 °C and homogenised for 1.5 s/ml using the Miris Ultrasonic Processor.

The effect of freezing and Holder pasteurisation on the macronutrient, bioactive protein and metabolome compositions of Human Milk

The purpose of part III of the study was to analyse the impact of Holder pasteurisation on the macronutrient, bioactive protein (lysozyme, lactoferrin) and metabolome compositions of human milk.

This study was conducted at the Neonatology Centre of VUH SK from October 2017 to July 2018. Criteria of inclusion and non-inclusion into the study were the same as in parts I and II of the study.

A flow diagram for this study is shown in Fig. 2.



HM – human milk; V – volume per sample (ml); n – number of samples.

Fig. 2. Procedure diagram.

Human milk samples were collected for 24 hours on days 14 to 16 after delivery. Milk was expressed from one or both breasts with an electric or mechanical breast pump until the breast(s) were fully emptied. Women admitted to the neonatal unit to nurse their babies normally express milk every three hours, i.e., 7–8 times a day. Women were asked to place a 10-ml milk sample from each portion of expressed milk (until the milk is warm and unsettled) into a sterile

plastic container for testing. The remaining expressed milk was used for infant feeding. After each expression, the milk samples were drawn into syringes and stored in a refrigerator for about one hour before being placed into a test container to ensure a uniform temperature of the milk collected at different times. Milk samples with a volume of 70–80 ml were collected daily and stored in the refrigerator at +4 °C (for approximately 24 hours from the start of collection) until macronutrient analysis. A total of 42 human milk samples were collected from the mothers of 22 preterm infants (gestational age < 37 weeks) and 20 term infants (gestational age ≥ 37 weeks).

Macronutrient composition was tested twice, once in freshly collected human milk and again in thawed pasteurised milk. A volume of 10 ml was taken from the daily sample of freshly collected milk (well mixed by rotation), and the macronutrients were analysed using the Miris Human Milk Analyser (Miris AB, Sweden). Before analysis, the milk samples were heated to +40 °C in a water bath and were then homogenised using an ultrasound homogeniser (Miris Sonicator).

The concentrations of bioactive proteins (lactoferrin and lysozyme) were also measured twice, once in unpasteurised thawed and again in pasteurised human milk. Volumes of 10 ml from the daily sample of freshly collected human milk were placed in separate plastic containers, and the samples were transported to the VUH SK laboratory on ice and centrifuged at 500 x g, at +4 °C, for 15 min. The supernatant was stored at –80 °C until testing by enzyme-linked immunosorbent assay (ELISA).

The remaining part of the daily milk sample (50–60 ml) was kept at –40 ºC in a freezer until pasteurisation and then repeatedly assayed for macronutrient content and by ELISA to measure lactoferrin and lysozyme concentrations (Fig. 2). The pasteuriser PAS 10000/1 (HSC, France) was used for milk pasteurisation. The night before pasteurisation, all the frozen human milk samples were thawed at +4 °C overnight in a refrigerator. Another 10 ml of milk was

collected from each sample after Holder pasteurisation for repeated measurements of macronutrient composition using the Miris analyser. Remaining pasteurised milk was delivered to the VUH SK laboratory on ice to be immediately analysed to determine the lactoferrin and lysozyme concentrations. Enzyme-linked immunosorbent assays were simultaneously performed for pasteurised milk samples and thawed unpasteurised samples (using one ELISA kit each for the analysis of lactoferrin and lysozyme).

Samples were collected for 10 months until pasteurisation and analysis by ELISA. Lactoferrin concentration was measured with ELISA using the commercial Human Lactoferrin ELISA kit (BIOVendor, Czech Republic). The lysozyme concentration was measured by means of ELISA using the commercial CircuLex Human Lysozyme ELISA Kit (MBL, Japan).

After completion of all the tests, the content of macronutrients, energy and bioactive proteins (lactoferrin and lysozyme) in human milk was compared before and after Holder pasteurisation.

In cooperation with scientists from Cagliari University (Italy), effect of Holder pasteurisation on human milk metabolome content was assessed. For this purpose, additional 40 paired samples of human milk were collected from 20 mothers in November 2018 – March 2019. The study involved healthy mothers not using medication, having delivered full-term newborns (GA > 37 weeks) and expressing milk with a breast pump. Morning human milk samples (between 9 AM and 12 PM) were collected between 15–17 days after delivery. The women were asked to express 50 ml of human milk using a breast pump. After collection, human milk samples were separated into two parts (10 ml and 40 ml), immediately frozen and stored at the temperature of –80 °C for up to five months to the utmost. On the day of pasteurisation, one part of frozen samples (40 ml) was thawed, pasteurised using Holder method and again immediately frozen at –80 °C. All frozen human milk samples (fresh and pasteurised human milk, n = 40) were sent to Italy, the NMR laboratory of the Department of Chemical and

Geological Sciences, University of Cagliari, on dry ice and using depersonalised data (human milk collection date, identification number). Milk aliquots for NMR analysis were defrosted and prepared for metabolomics analysis. In order to remove residual lipids and proteins, milk samples were centrifuged at 10 000g for 30 min at 4 °C using Amicon Ultra 0.5 ml 10 kDa spin filters (Millipore, Billerica, MA, USA). Each filtered sample (350 μl) was mixed with 350 μl of 0.1 M phosphate buffer solution (pH 7.4) containing sodium trimethylsilyl-(2,2,3,3-2H4)-1-propionate (final concentration 2 mM) and then transferred into a 5 mm wide NMR tube. 1 H NMR experiments were performed at 300 K on a Varian UNITY INOVA 500 spectrometer (Agilent Technologies, Inc., Santa Clara, CA), operating at a frequency of 499.83 MHz. One-dimensional (1D) 1 H NMR spectra were obtained using a standard pulse sequence (1D NOESY) with presaturation during relaxation and mixing time for water suppression. The NMR spectra were processed using MestReNova, version 12 (Mestrelab Research SL, Santiago de Compostela, Spain) and analysed by multivariate statistica analysis using SIMCA 14 (Umetrics, Umeå, Sweden).

Following the receipt of the findings, human milk metabolome data were compared before and after Holder pasteurisation.

Statistical analysis of data

Statistical analysis of the data was conducted using IBM SPSS Statistics 23 programme package. When assessing quantitative indicators, arithmetic mean, standard deviation and median were calculated. To verify the independence of qualitative variables, the criterion of chi square (χ^2) was applied. To verify the data normality condition, the Shapiro-Wilk test was used. To compare two dependant samples, the paired Student t or Wilcoxon criteria were applied, while independent samples were compared using the Student *t* test or the Mann-Whitney-Wilcoxon rank sum criterion,

taking into account the data normality condition. To compare three and four independent samples, the one-factor dispersive analysis ANOVA, the Bonferroni test and the Kruskal-Wallis test were applied, while to compare dependent samples – the Friedman criterion was used. Differences were considered statistically significant, when the p value was < 0.05.

RESULTS

Study of human milk macronutrients and energy value from week 2 to week 7 after delivery

80 % of study participants came from Vilnius county (80 % out of which were residents of Vilnius city). The remaining mothers came from different regions of Lithuania (Utena, Panevėžys, Šiauliai, Klaipėda, Marijampolė and Alytus counties).

Following the analysis of human milk samples, changes in human milk macronutrient and energy content were assessed depending on lactation duration in groups of mothers who gave birth to newborns of different gestational age: Group I – mothers of extremely preterm newborns, GA of 22^{+0}–27^{+6} weeks; Group II – mothers of very preterm newborns, GA of 28^{+0}–31^{+6} weeks; Group III – mothers of moderately to late preterm newborns, GA of 32^{+0}–36^{+6} weeks; Group IV – mothers of term newborns, GA of 37^{+0}–41^{+6} weeks. A comparison was also made of macronutrient amount and energy value in human milk in the said four groups of study participants after the same number of weeks post-delivery. No statistically significant differences between groups have been established taking into account mothers‘ age, ethnicity, BMI, number of births, delivery mode and a newborn‘s gender (Table 2).

Table 2. Comparative characteristics of studied groups of mothers

Groups of mothers according to their newborns GA / Characteristics	Group I 22^{+0}–27^{+6} wks (n = 35)	Group II 28^{+0}–31^{+6} wks (n = 40)	Group III 32^{+0}–36^{+6} wks (n = 37)	Group IV 37^{+0}–41^{+6} wks (n = 33)	*p* value
Maternal age (years), $\bar{x} \pm s$	31 ± 4	32 ± 5	34 ± 6	32 ± 5	0.09
Ethnicity (Lithuanian / other*), n (%)	29 / 6 (83 / 17)	31 / 9 (77.5 / 22.5)	30 / 7 (81 / 19)	28 / 5 (85 / 15)	0.47
BMI :					
underweight (< 18.5), n (%)	3 (8.5)	4 (10)	4 (11)	1 (3)	
normal (≥ 18.5–≤ 25), n (%)	22 (63)	24 (60)	23 (62)	24 (73)	0.96
overweight / obese (> 25), n (%)	10 (28.5)	12 (30)	10 (27)	8 (24)	
Parity, $\bar{x} \pm s$	2 ± 1	2 ± 1	2 ± 1	2 ± 1	0.75
Delivery mode (VD/CS), n (%)	17 / 18 (49 / 51)	27 / 13 (67.5 / 32.5)	20 / 17 (54 / 46)	25 / 8 (76 / 24)	0.08
Newborn's birth weight (g), $\bar{x} \pm s$	871 ± 182	1496 ± 278	2129 ± 531	3301 ± 445	< 0.01
Newborn's gender (male / female), n (%)	22 / 13 (63 / 37)	21 / 19 (52.5 / 47.5)	23 / 14 (62 / 38)	23 / 10 (70 / 30)	0.50

$\bar{x} \pm s$ – mean value ± standard deviation; *other ethnicities: Russians, Poles, Ukrainians; BMI – body mass index; VD – vaginal delivery; CS – caesarean section; GA – gestational age

Data on human milk macronutrient content and energy value in the seven weeks after delivery are provided in Table 3.

Table 3. Changes in human milk macronutrient content and energy value depending on the time after delivery

	Week after delivery	$\bar{x} \pm SN^*$				*p* value
		Group I	Group II	Group III	Group IV	
Protein, g/100 ml	2	1.6 ± 0.4	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	0.97
	3	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	0.47
	4	1.4 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	**0.04**
	5	1.3 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	**0.03**
	6	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	0.16
	7	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	0.43
Fat, g/100 ml	2	4.0 ± 1.1	4.0 ± 0.9	3.7 ± 0.9	4.0 ± 0.7	0.68
	3	4.2 ± 0.8	4.0 ± 0.8	4.1 ± 0.7	4.1 ± 0.7	0.78
	4	4.3 ± 0.7	4.0 ± 0.8	4.0 ± 0.8	4.0 ± 0.9	0.40
	5	4.1 ± 0.7	4.1 ± 0.8	4.0 ± 0.8	3.8 ± 0.9	0.46
	6	4.0 ± 0.7	4.0 ± 0.9	3.9 ± 0.6	4.1 ± 0.9	0.53
	7	4.1 ± 0.6	3.9 ± 1.0	4.0 ± 0.9	3.9 ± 0.8	0.10
Carbo - hydrate, g/100 ml	2	7.0 ± 1.5	7.4 ± 0.5	7.5 ± 0.6	7.4 ± 0.7	0.25
	3	7.5 ± 0.6	7.7 ± 0.5	7.7 ± 0.5	7.6 ± 0.7	0.17
	4	7.6 ± 0.5	7.7 ± 0.4	7.8 ± 0.5	7.7 ± 0.5	0.90
	5	7.7 ± 0.5	7.6 ± 0.5	7.7 ± 0.5	7.7 ± 0.5	0.56
	6	7.7 ± 0.5	7.6 ± 0.5	7.7 ± 0.6	7.5 ± 0.5	0.97
	7	7.6 ± 0.4	7.6 ± 0.4	7.5 ± 0.6	7.7 ± 0.4	0.56
Energy, kcal/ 100 ml	2	73.3 ± 16.7	74.9 ± 8.2	72.9 ± 7.2	75.3 ± 5.9	0.96
	3	76.7 ± 8.0	75.4 ± 7.3	76.5 ± 6.7	76.1 ± 6.5	0.83
	4	77.43 ± 6.6	74.6 ± 7.6	75.0 ± 7.8	74.4 ± 8.0	0.24

Continued table.

	Week after delivery	$\bar{x} \pm SN^*$				p value
	5	75.15 ± 6.9	75.0 ± 7.3	74.7 ± 7.5	72.8 ± 8.6	0.63
	6	74.68 ± 7.1	73.9 ± 9.0	73.1 ± 6.4	74.6 ± 8.3	0.48
	7	74.78 ± 6.0	73.2 ± 7.9	72.8 ± 9.1	73.1 ± 7.7	0.07

* mean value ± standard deviation; statistically significant differences are presented in bold font; Group I – mothers of extremely preterm newborns, GA of 22^{+0}–27^{+6} weeks; Group II – mothers of very preterm newborns, GA of 28^{+0}–31^{+6} weeks; Group III–mothers of moderately to late preterm newborns, GA of 32^{+0}–36^{+6} weeks; Group IV – mothers of term newborns, GA of 37^{+0}–41^{+6} weeks.

Protein concentration in human milk in all the four groups of GA gradually decreased from week two to week seven post-delivery. Significant changes were observed in protein concentration between groups during week four and week five post-delivery. Analysis of human milk protein concentration in week four resulted in statistically significant differences between Groups I and II (p = 0.04), Groups I and III (p = 0.03), and Groups I and IV (p < 0.001). Analysis of human milk protein concentration in week five resulted in statistically significant differences between Groups I and III (p = 0.01), Groups I and IV (p = 0.01). The Wilcoxon dependent samples test was applied.

Fat and carbohydrate concentration showed marked individual differences, but no significant differences in different GA groups were observed. Depending on the number of weeks post-delivery, fat concentration did not show pronounced changes, while carbohydrate concentration was lower during week two (in transitional human milk) than during weeks 3–7 after delivery.

Changes in human milk energy value showed little difference with fat concentration fluctuations.

Figure 3 shows graphical changes in human milk macronutrient content and energy value, while Table 4 shows differences in transitional and mature human milk.

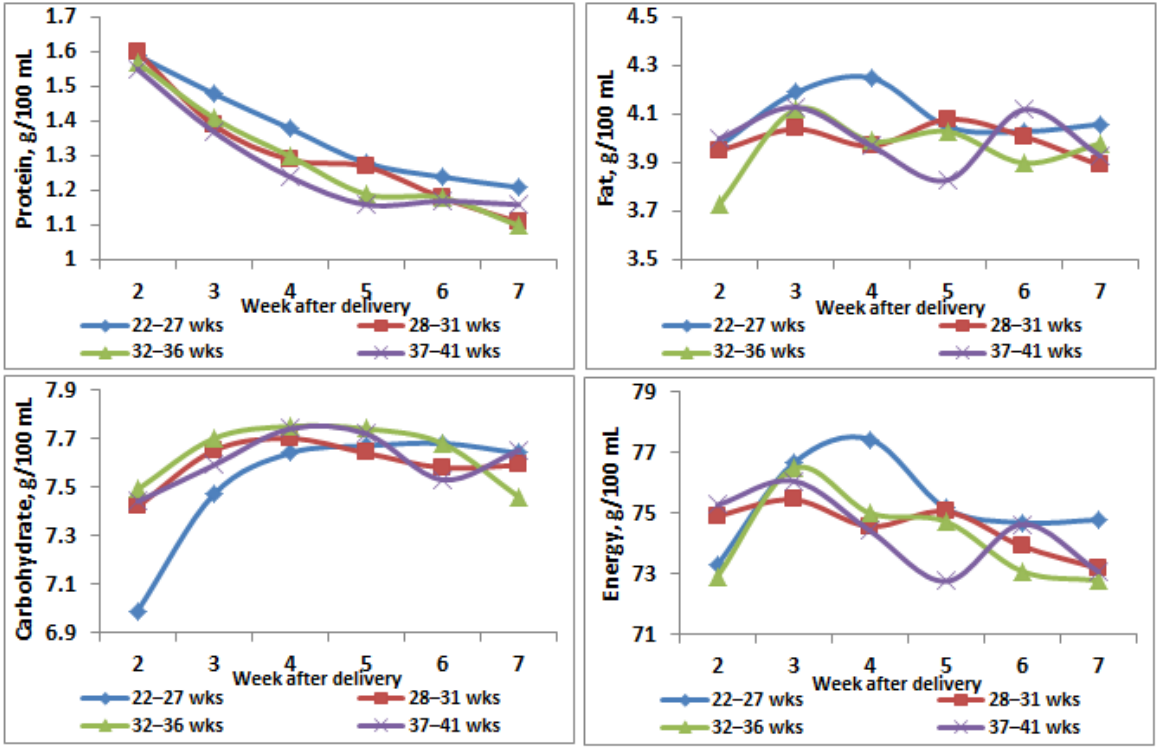


Figure 3. Changes in human milk macronutrient content and energy value depending on the duration of lactation

Table 4. Changes in macronutrient content and energy value in transitional (2 weeks post-delivery) and mature (6 weeks post-delivery) human milk

	Protein, g/100 ml		Fat, g/100 ml		Carbohydrate, g/100 ml		Energy, kcal/100 ml	
	Preterm milk	Term milk	Preterm milk	Term milk	Preterm milk	Term milk	Preterm milk	Term milk
Week 2	1.6	1.6	3.9	4.0	7.3	7.4	73.8	75.3
Week 6	1.2	1.2	4.0	4.1	7.6	7.5	74.0	74.6
Difference	–25 %	–25 %	3 %	3 %	4 %	1 %	0.3 %	–1 %
p value	**< 0.001***	**0.001***	0.53	0.23	**< 0.001***	0.05	0.47	0.78

Preterm milk: groups I – III (22^{+0}–36^{+6} wks), term mik – group IV (37^{+0}–41^{+6} wks); *significant differences

Evaluation of the impact of other factors on human milk content resulted in a finding that during week two post-delivery, breast milk of mothers who bore boys had a higher fat concentration than breast milk of mothers who bore girls (respectively, 4.1 ± 0.8 and 3.7 ± 1.0 g/100 ml, p = 0.02). For this reason, transitional milk of mothers feeding boys had a higher calorific value than that of mothers of girls (respectively, 75.6 ± 7.4 and 71.7 ± 2.8, p = 0.03). These differences were not statistically significant in mature human milk (that is, during week five post-delivery). Concentration of fats and carbohydrates did not show significant differences in the milk of mothers of newborns of different genders.

A higher fat concentration and calorific value were observed during week two after delivery in the milk of mothers who bore their children naturally as compared with those who underwent the caesarean section (respectively, 4.0 ± 1.0 and 3.7 ± 0.7 g/100 ml, p = 0.02 and 75.1 ± 11.5 and 72.5 ± 6.5 kcal/100 ml, p = 0.01). These differences also disappeared in the mature human milk (during week five post-delivery).

Mother's age and BMI did not have a significant impact on human milk macronutrient content and calorific value.

Circadian changes in the composition of human milk macronutrient and energy content

A total of 180 samples from 45 lactating mothers were collected and analysed for macronutrient and energy content. All participating women were permanent residents of Lithuania. The main characteristics of the women enrolled in the study are presented in Table 5. The mothers of term and preterm newborns were similar regarding newborn gender, delivery mode, age, ethnicity and number of deliveries.

Table 5. Characteristics of the mothers of preterm and term newborns

Characteristics	Term (GA ≥ 37^{+0} wks), n = 18	Preterm (GA < 37^{+0} wks), n = 27	*t*	*df*	*p* value
Gestational age (weeks), $\bar{x} \pm s$ Range (weeks)	38.7 ± 1.0 37–40	30.2 ± 2.5 24–36	–15.61	37.04	< 0.001
Birth weight (g), $\bar{x} \pm s$ Range (g)	3265 ± 589 1590–3960	1477 ± 405 845–2380	–12.09	43	< 0.001
Gender (male / female), n (%)	11 / 7 (61.1 / 38.9)	18 / 9 (66.7 / 33.3)	0.15	1	0.70
Delivery mode (VD/CS), n (%)	15 / 3 (83.3 / 16.7)	16 / 11 (59.3 / 40.7)	2.92	1	0.09
Number of deliveries, $\bar{x} \pm s$ Range, n	1.6 ± 0.7 1–3	1.7 ± 0.9 1–5	0.22	43	0.83
Maternal age (y), $\bar{x} \pm s$ Range (y)	30.9 ± 6.2 19–40	32.9 ± 4.3 24–43	1.28	43	0.21
Ethnicity (Lithuanian, other*), n (%)	14 / 4 (77.8 / 22.2)	23 / 4 (85.2 / 14.8)	0.25	1	0.69

$\bar{x} \pm s$ – mean value ± standard deviation; VD – vaginal delivery; CS – caesarean section; GA – gestational age; *t* – test value; *df* – degrees of freedom; *other ethnicities: Russians, Poles, Ukrainians

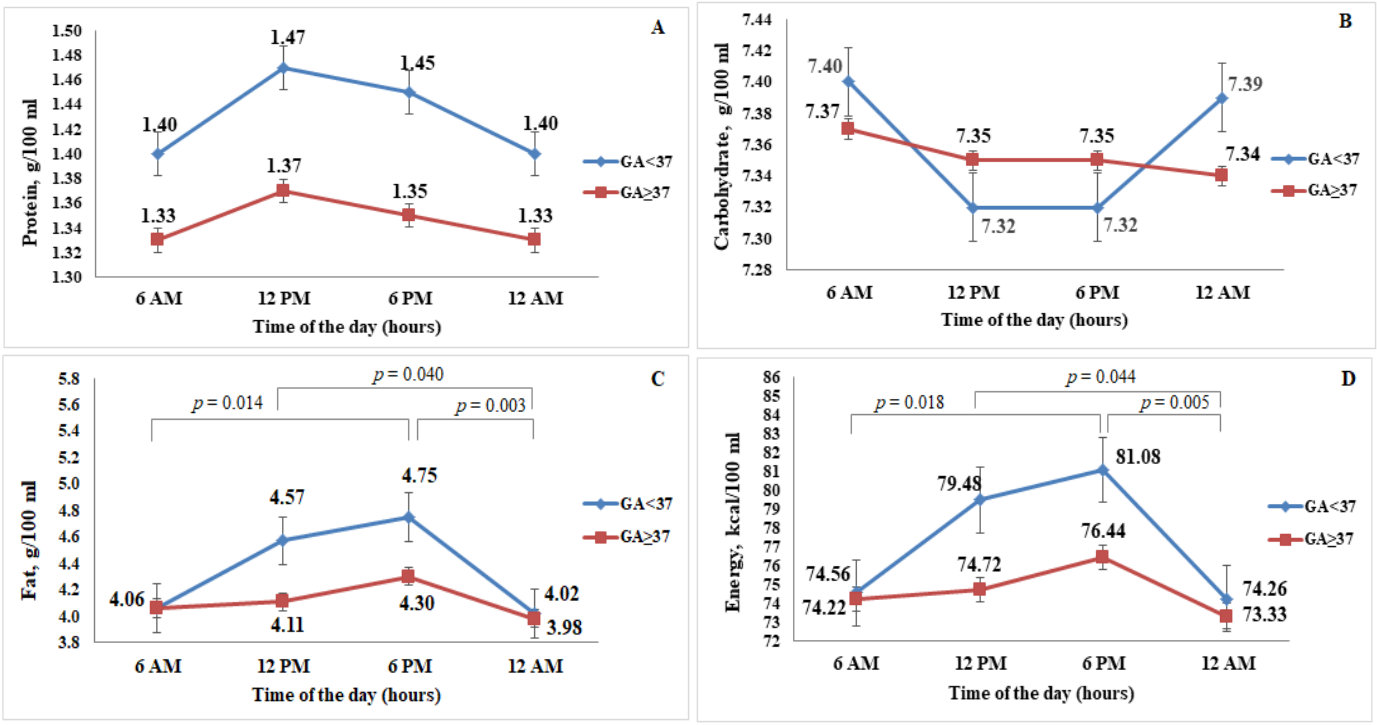
Analysis of all 180 samples from the 45 women enrolled in the study showed significant diurnal variation of protein, fat, and energy content, with the highest levels of these macronutrients and energy content during day expressions (at 12 PM and 6 PM) and with the lowest during night expressions (at 12 AM and 6 AM). The carbohydrate content in the breast milk did not reveal significant diurnal fluctuations (Table 6).

Table 6. Macronutrient and energy content in preterm and term human milk (n = 45) during the daytime

		Time of day (hours)					
		6 AM – 12 PM	**6 AM – 6 PM**	**6 AM – 12 AM**	**12 PM – 6 PM**	**12 PM – 12 AM**	**6 PM – 12 AM**
Protein, g/100 ml,	$\bar{x} \pm s$	1.37 ± 0.2 1.43 ± 0.2	1.37 ± 0.2 1.41 ± 0.2	1.37 ± 0.2 1.37 ± 0.2	1.43 ± 0.2 1.41 ± 0.2	1.43 ± 0.2 1.37 ± 0.2	1.41 ± 0.2 1.37 ± 0.2
	p value	**< 0.001****	**0.02***	0.90	0.24	**0.001***	**0.02***
Fat, g/100 ml	$\bar{x} \pm s$	4.06 ± 0.9 4.39 ± 1.1	4.06 ± 0.9 4.57 ± 1	4.06 ± 0.9 4.00 ± 0.7	4.39 ± 1.1 4.57 ± 1.0	4.39 ± 1.1 4.00 ± 0.7	4.57 ± 1.0 4.00 ± 0.7
	p value	**0.04***	**0.001***	0.65	0.28	**0.02***	**< 0.001****
Carbohydrate, g/100 ml	$\bar{x} \pm s$	7.39 ± 0.2 7.33 ± 0.3	7.39 ± 0.2 7.33 ± 0.2	7.39 ± 0.2 7.37 ± 0.2	7.33 ± 0.3 7.33 ± 0.2	7.33 ± 0.3 7.37 ± 0.2	7.33 ± 0.2 7.37 ± 0.2
	p value	0.12	0.14	0.40	0.88	0.27	0.22
Energy, kcal/ 100 ml	$\bar{x} \pm s$	74.42 ± 9.1 77.58 ± 10.0	74.42 ± 9.1 79.18 ± 9.7	74.42 ± 9.1 73.89 ± 6.8	77.58 ± 10.0 79.18 ± 9.7	77.58 ± 10.0 73.89 ± 6.8	79.18 ± 9.7 73.89 ± 6.8
	p value	**0.03***	**< 0.001****	0.59	0.29	**0.01***	**< 0.001****

*$p < 0.05$; **$p < 0.001$

A comparison of macronutrient content changes in preterm and term human milk separately during the time of the day is presented in Figure 4. Macronutrients and energy content did not differ significantly when comparing the preterm and term human milk samples, but the diurnal variations were more pronounced in the preterm milk samples.



GA – gestational age (weeks). Significant differences in fat and energy content were found only in the preterm group (GA < 37 wks).

Fig. 4. Comparison of macronutrient content in preterm (n = 18) and term (n = 27) human milk by time of day

Although protein content revealed similar diurnal fluctuations in both the preterm and full-term milk samples, with the highest levels during day expressions (at 12 PM and 6 PM), these differences were not significant. The preterm milk samples also contained more protein than the full-term samples, but the differences were not significant (Figure 4A).

Carbohydrate content did not show apparent diurnal fluctuations in the full-term samples but showed more apparent diurnal fluctuations in the preterm milk samples, although these differences did not reach significance. In contrast to the case for proteins, the highest concentration of carbohydrates in preterm milk was observed during night expression, and the lowest was observed during the day (Figure 4B).

We found significant differences in the diurnal fluctuations of fat and energy, with peak concentrations at 12 PM and 6 PM in the preterm milk samples, but these differences did not reach significance in the full-term milk samples (Figures 4C and 4D).

The effect of freezing and Holder pasteurisation on the macronutrient, bioactive protein and metabolome compositions of Human Milk

A total of 84 paired human milk samples were analysed.

Holder pasteurisation did not significantly affect the mean concentration of macronutrients or energy content in human milk (Table 7). However, Holder pasteurisation did significantly reduce the concentrations of lactoferrin and lysozyme (difference of > 99 % and 35 %, respectively) (Fig. 5).

Table 7. Difference in the mean macronutrient concentrations in human milk before and after Holder pasteurisation (n = 42)

	Before pasteurisation	After pasteurisation	*p* value
Protein, g/100 ml, $\bar{x} \pm s$	1.4 ± 0.2	1.4 ± 0.2	0.87
Fat, g/100 ml, $\bar{x} \pm s$	4.3 ± 0.7	4.2 ± 0.7	0.54
Carbohydrate, g/100 ml, $\bar{x} \pm s$	7.3 ± 0.2	7.3 ± 0.2	0.14
Energy, kcal/100 ml, $\bar{x} \pm s$	77 ± 7	76 ± 7	0.48

$\bar{\boldsymbol{x}} \pm \boldsymbol{s}$ – mean; ± standard deviation

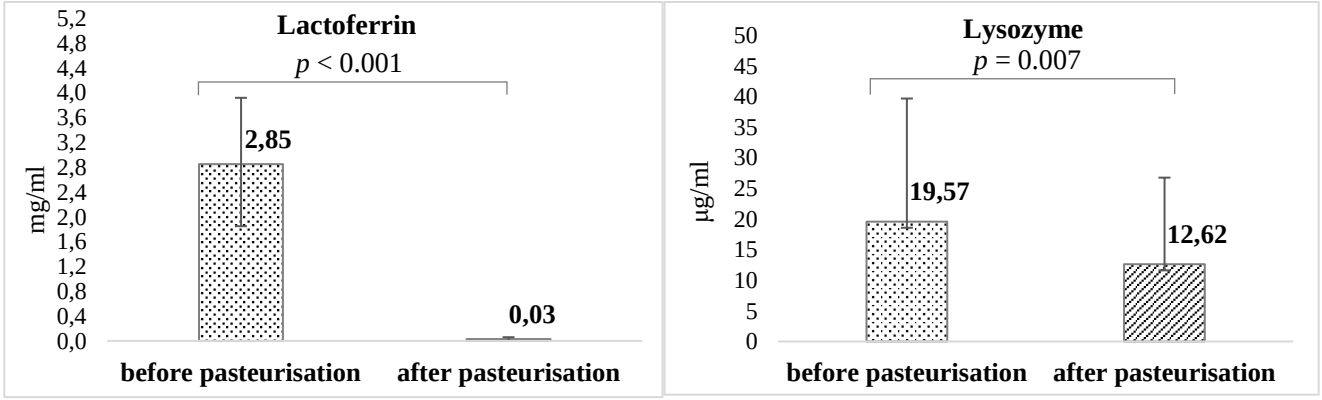
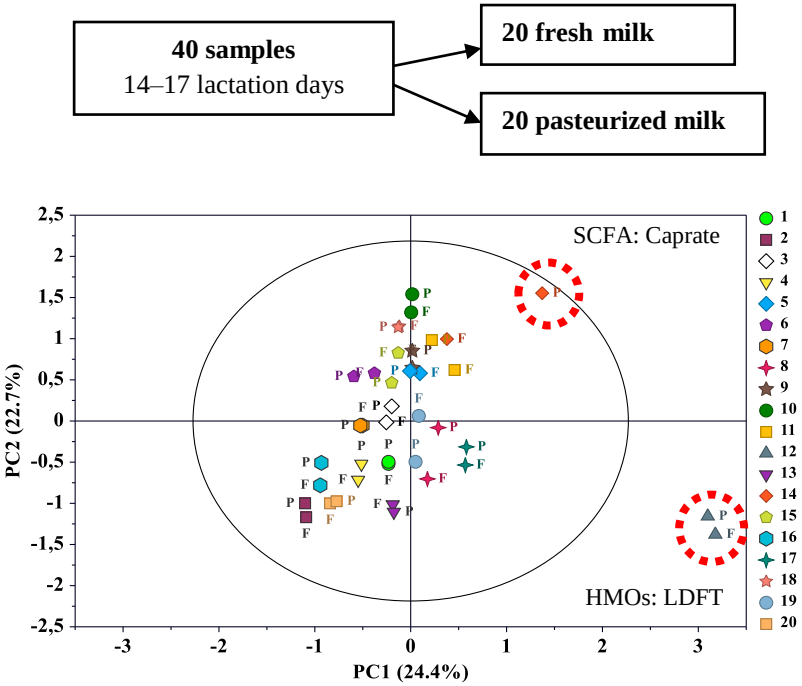


Fig. 5. Lactoferrin and lysozyme concentrations before and after Holder pasteurisation (n = 42).

In cooperation with scientists from Cagliari University (Italy), effect of Holder pasteurisation on human milk metabolome content was assessed. 40 paired samples of human milk were analysed using NMR spectroscopy. The examination of water-soluble metabolite fraction of human milk did not show significant differences between frozen fresh and pasteurised human milk (Figure 6).

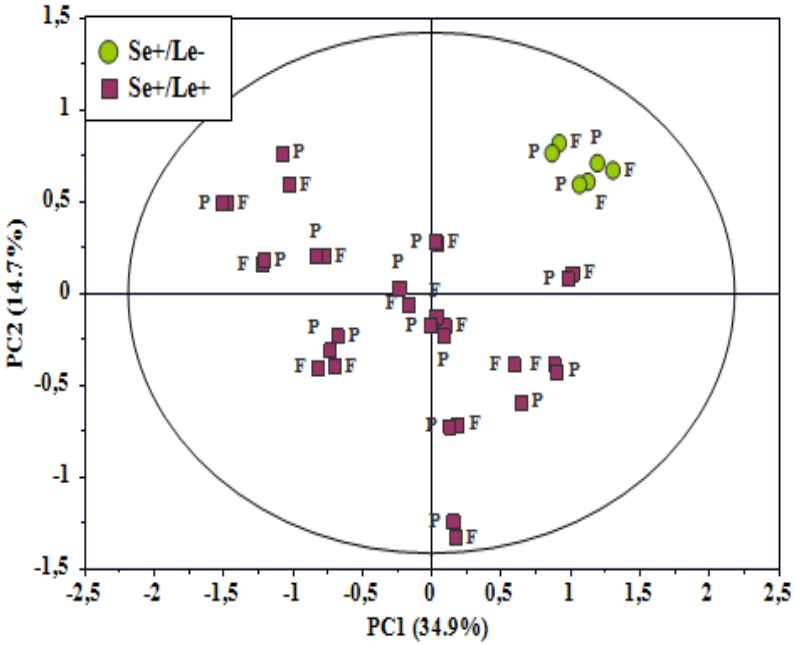


SCFA – short-chain fatty acids; HMOs: LDFT – human milk oligosaccharides: lactodifucotetraose; outliers are rimmed with a red dotted line

Fig 6. Human milk metabolome (water-soluble metabolite fraction) before and after Holder pasteurisation

A visual analysis of the spectral portion containing the signals of the human milk oligosaccharides (HMO) spectra enabled the establishment of two phenotypes of study participants (both secretory): Se+/Le+ (17 mothers) and Se+/Le– (3 mothers).

Following the analysis, samples of fresh human milk were graphically close to respective samples of pasteurised human milk – this shows that there are no differences in HMO content before and after Holder pasteurisation (Figure 7).



F – fresh milk, P – pasteurised milk; Se+/Le– and Se+/Le+ – secretory types according to dominant human milk oligosaccharides (Se – secretory gene, Le – Lewis gene)

Fig 7. Human milk oligosaccharides before and after Holder pasteurization

DISCUSSION

This is the first study of its kind which enabled a thorough assessment of the milk content of women living in Lithuania and diverse factors having effect on the said content. In order to analyse human milk composition and its changes from various points of view, the study was divided into three parts, and different methods of analysis were used. The findings have shown that human milk content constantly changes, adapting itself to changing nutrition needs of a newborn (infant). This study aimed not only to reveal physiological human milk changes, but also find out the changes in human milk content during freezing and pasteurisation.

Hereinafter a discussion of the findings of each part of the study is presented.

Study of human milk macronutrients and energy value from week 2 to week 7 after delivery

Like previous studies, our study has revealed that composition of human milk macronutrients changes depending on the time that elapsed after delivery [8, 31].

Colostrum contains relatively much protein, later on its amount gradually decreases until lactation week 5–6, and finally it remains more or less stable [8, 32]. Our findings coincide with the findings of previous studies; they have also disclosed certain specific features of human milk composition of premature newborns. During lactation weeks four and five, the milk of mothers of extremely premature newborns (GA of < 28 weeks) was found to contain more protein. Similar findings are presented by a metaanalysis comprising 41 studies [8] – significant changes in protein concentration between full-term and premature newborn milk were observed during weeks 2–4 and 7–9 after delivery. According to Bauer J and Gerss J [33], protein concentration in human milk was inversely proportional to a

newborn's gestational age. On the other hand, Maly J with the co-authors provide opposite results – they did not find significant differences in human milk protein concentration between groups of mothers of newborns of different gestational age (GA of weeks 24–30 and 31–35) [34]. Proteins, which contain many biologically active substances, are especially important for a newborn's growth, as well as his/her immune, digestive, central nervous and other systems. The findings obtained by us and other researchers make it possible to state that human milk corresponds to specific newborn's needs – higher protein content is necessary for maturation of systems of a premature newborn's various organs, immune protection against a higher risk of infectious diseases, as well as stable growth of weight.

Fat concentration in human milk showed the largest individual differences; however, no significant changes in human milk fat content were established from week two to week seven after delivery. Other authors also present similar results [8, 34].

According to our findings, carbohydrate concentration in transitional human milk was relatively lower than in mature human milk. Interestingly enough, this difference was more pronounced in the milk of mothers of premature newborns (GA of 22^{+0}–36^{+6} weeks). That could mean certain adaptation of the mother's body to individual needs of a newborn: lower carbohydrate content in human milk determines its lower osmotic concentration and possibly compensates for the insufficiency of enzyme lactase, thus creating more favourable conditions for better digestion of human milk in a newborn's intestine [35]. Our findings confirm the results of previous studies stating that carbohydrate concentration in human milk has a tendency to increase during the first weeks after delivery [34].

This study has also revealed other factors, which are hopefully not random, that make an impact on human milk composition. Interestingly enough, transitional milk of mothers who had borne boys had a higher fat content and calorific value than the milk of

mothers who had borne girls. Similar results are also presented by Fischer Fumeaux C. J. with the co-authors [36]. A previous study in Kenya disclosed that human milk differed not only depending on a newborn's gender, but also on the eco-social status: more fat was found in the milk of better-off mothers raising boys, while in the lower income segment, conversely, more fat was found in the milk of mothers breastfeeding girls [37]. These data enable guessing about possible "programming" of the breast gland with the foetus still in the womb.

Delivery mode also affected human milk content – in our study, transitional milk of mothers who gave birth naturally (via vaginal delivery) had a higher fat content and calorific value than that of mothers who delivered their newborns via caesarean section. Recent studies conducted in Turkey also revealed differences in human milk composition determined by the mode of delivery: colostrum of mothers who gave birth naturally contained more protein than that of mothers who underwent caesarean section [38]. These data make us pay special attention to weight gain by newborns, who were delivered via caesarean section, during the first weeks after delivery and look for signs of insufficient nutrition.

In conclusion, the factors influencing human milk composition were pregnancy duration, delivery mode, a newborn's gender, lactation duration. From week two to week seven post-delivery, the amount of protein in human milk gradually decreases, while the amount of carbohydrates increases. During this lactation period, there are no marked changes in the amount of fat and energy value in human milk. Differences in human milk composition of mothers who delivered newborns of different GA were observed: during certain periods of lactation, human milk of mothers of premature newborns contained more protein and fewer carbohydrates. Transitional milk of mothers who had delivered their newborns naturally had a higher calorific value due to a higher fat content as compared to the milk of mothers who had undergone caesarean section. It is likely that changes in human milk composition, which depend on various

factors, are not random and are of high significance for a newborn's growth, development and health.

Circadian changes in the composition of human milk macronutrient and energy content

There is yet no clear explanation but only suggestions and speculations of why the diurnal fluctuations of human milk composition exist and what impact they have on a newborn's health and development. Hormonal changes in lactating women, breastfeeding patterns, the influence of the degree of breast fullness, circadian dietary habits, ethnic differences, and different techniques for the measurement of milk macronutrients are among the factors that can influence circadian changes in milk composition [39–41]. However, the particular role of each factor and why and how these factors affect dynamic changes in specific macronutrients are unknown.

While most of the studies (as well as our study) show that the greatest circadian variation is in the fat content in breast milk [11, 40, 42], the data regarding other macronutrients remain controversial.

The DARLING study [43] showed that the macronutrient content in breast milk could be influenced by breast fullness; human milk protein and fat concentrations were negatively related to milk volume, while milk lactose concentration was positively related to milk volume at certain lactation periods. In our study, the majority of the women usually rested at night and did not empty their breasts early in the morning (3 AM), so their breasts were full for the morning sampling (6 AM) compared to the midnight sampling. In spite of this, we did not find any differences in fat and protein content in the midnight and morning samples, different from other researchers.

The results regarding circadian fluctuations in protein content are still conflicting. Sánchez López C. L. and colleagues [44] found

circadian changes in protein content during the mature milk stage with the highest protein concentrations during night (8 PM–8 AM) expression. In contrast to the Spanish study, we found the highest levels of protein during day expression (at 12 PM and 6 PM) and the lowest levels during night expression (at 6 AM and 12 AM). Keeping in mind that diurnal variations of the fat content of expressed milk had the same trend, we could only relate these fluctuations to the dietary habits of hospitalised women, i.e., day samples (at 12 PM and 6 PM) of milk were taken after breakfast and lunch meals, while night samples (at 12 AM and 6 AM) were taken on an empty stomach – relative to after a fasting period. Other investigators did not find any circadian changes in protein or nitrogen substance content, but the number of women participating in the studies was relatively small [11, 45, 46]. When analysing the preterm and full-term milk samples separately, we did not find significant differences in the circadian fluctuations of protein content in the two groups, although the preterm milk samples tended to show more apparent circadian variations of protein concentration than the full-term samples. We anticipate that the differences did not reach significance because of the small sample size of the preterm milk samples, despite the clear tendency of circadian variations.

Human milk carbohydrates showed the least variation in macronutrient concentrations over 24 h. Our results agree with those of previous studies showing no circadian variation in carbohydrate concentrations [11, 39, 46]. On the other hand, in our study, the preterm milk samples also tended to show more apparent circadian variations in carbohydrate content than the full-term samples.

Analysis of the human milk macronutrient components was performed only during a short lactation period (i.e., 14–16 days after delivery), representing a limitation of our study. There are data showing that the circadian variation in human milk composition has different patterns throughout the evolution of the whole breastfeeding period [44, 47]. Therefore, the results of our study can only be applied to the transitional phase of the lactation period and

cannot be adjusted for other phases of lactation. On the other hand, the analysis of the circadian variations of the macronutrient content of human milk of the mothers of preterm and full-term newborns at the same time after delivery could be the strength of our study. To the best of our knowledge, no other study has tried to find diurnal differences in human milk macronutrient composition after preterm and term childbirths during the same lactation period.

In summary, our study showed the circadian variability of human milk macronutrients, with the highest content of protein and fat during the day expressions (12 PM and 6PM) and the lowest content during the night expressions (12 AM and 6 AM). We speculate that these diurnal changes could be due to the ethnic peculiarities and dietary habits of the mothers. There were no significant fluctuations in carbohydrate content in the human milk during 24 h. Moreover, the circadian fluctuations of the macronutrient content in human milk were more prominent following premature childbirth. Further research is needed to clarify the circadian changes in maternal milk during the whole lactation period and to determine whether preterm babies could benefit from these changes in human milk composition.

The effect of freezing and Holder pasteurisation on the macronutrient, bioactive protein and metabolome compositions of Human Milk

The quality of human donor milk, as well as the maximum retention of its biological and nutritional properties during the treatment process, is a highly relevant issue today. Despite numerous studies carried out to date that have estimated the effects of freezing and Holder pasteurisation on the composition of macronutrients and bioactive components of human milk, the results are quite different or even contradictory. A summary of 44 papers [48] demonstrated that Holder pasteurisation has the highest effect on the composition of bioactive components in human milk. Inconsistent findings from

previous studies could be explained by the duration and temperature at which milk samples are stored before pasteurisation [49, 50], by the method of thawing [23], by the quantities of milk being pasteurised, by the choice of equipment for Holder pasteurisation [48, 51], and finally by the method used to prepare the milk samples for testing [52].

Our study has demonstrated that the storage of frozen human milk at –40 °C up to 10 months, followed by thawing and pasteurisation, have no significant effect on macronutrient (protein, fat and carbohydrate) or energy content of human milk. According to previous studies, Holder pasteurisation of human milk does not affect carbohydrate content but may lead to reductions in fat and protein, although the given results are discordant [48, 53]. These differences in findings could be partially explained by the pre-pasteurisation storage of human milk samples because not only pasteurisation but also freezing influence human milk composition [54].

Freezing and thawing can alter the fat globule structure in human milk, promoting fat aggregation and adhesion to the walls of the containers used. Moreover, proteins are likely to be absorbed into the membranes of disrupted fat globules [23]. It was also reported that human milk lipase activity is maintained at a milk storage temperature of –20 ºC, resulting in active lipolysis and lipid content reduction [55]. Orbach R. et al. described a relationship between lower freezing temperatures and reduced loss of fat content in human milk [49].

Furthermore, the methods of collection and preparing the milk samples for macronutrient analysis may affect the results [56]. Common problems faced when handling milk samples are creaming and adherence of fat to container walls, and it is influenced by the surface-to-volume ratio of containers used. It is more pronounced in smaller sample containers compared to larger ones [56]. There is also evidence that milk homogenisation before analysis ensures representative results [52, 56, 57].

Overall, however, even though some studies indicate that several macronutrients are altered by Holder pasteurisation, researchers rarely consider these nutritional changes to be clinically relevant [53]. In our study, the unchanged macronutrient content of thawed pasteurised milk compared to fresh human milk can be explained by the fact that the milk samples were stored frozen at low temperature (–40 ºC) and homogenised prior to Miris analysis.

According to the data in the literature, lactoferrin accounts for approximately 15–20 % of the total protein content in human milk, with lactoferrin concentrations being the highest in colostrum (~5.05 mg/ml) and then gradually decreasing thereafter (to 3.3 mg/ml in transitional milk and 1.44 mg/ml in mature milk on average) [58]. Our mean lactoferrin concentrations in unpasteurised transitional human milk (2.845 ± 1.07 mg/ml) are close to those reported by other sources [58, 59]. We found that lactoferrin was almost completely degraded after Holder pasteurisation (by > 99 %), whereas other studies reported a 35 to 90 % loss of this bioactive protein [48]. In our study, all the milk samples were Holder pasteurised simultaneously. This process was performed in strict accordance with the manufacturer's recommendations and was controlled by computer, and no temperature regime violations were reported. After pasteurisation, the samples were immediately delivered to the laboratory for enzyme-linked immunosorbent assay analysis. Accordingly, our findings revealed that lactoferrin loss caused by Holder pasteurisation may be even higher than reported by other studies.

According to previous studies, the average lysozyme concentration is 0.32 mg/ml in colostrum and ranges from 0.28 to 1.1 mg/ml in transitional and mature human milk [58]. We found significant individual variations in the lysozyme concentration in transitional human milk (19.568 ± 20.11 μg/ml), which are lower than those reported in some studies [58, 59] but similar to results obtained by Hsu Y. C. et al. [9]. We speculate that the reduction in the lysozyme concentration in the pre-pasteurised thawed human

milk samples could be caused by freezing the human milk and its storage time until pasteurisation. According to Chang J. C. et al., freezing human milk at –20 °C for a prolonged period results in a significant reduction in lysozyme content (by as much as 39.8 %), whereas freezing-induced lactoferrin loss in human milk was only 11.5 % [60]. On the other hand, we found a 35 % reduction in lysozyme as a result of Holder pasteurisation, which is consistent with results from previous studies [48].

Little has been known so far about the impact of Holder pasteurisation on human milk metabolome composition [61]. Our human milk metabolome study has revealed that Holder pasteurisation had no impact on the composition of the water-soluble metabolite fraction and human milk oligosaccharides. Our findings confirm the data of Hahn W. and co-authors that pasteurised human milk preserves very important biological functions determined by human milk oligosaccharides [62].

Our study is useful for supplementing the data described to date on the effects of freezing and Holder pasteurisation on the composition of human milk. Unfortunately, we cannot distinguish between the effects of freezing, the duration of storage and Holder pasteurisation on the macronutrient, lysozyme and lactoferrin contents in human milk. Understanding the contribution of each of these processes to these effects would allow the selection of optimal methods for human milk processing to minimise the loss of macronutrients and bioactive components.

In summary, freezing and Holder pasteurisation had no significant effects on the macronutrient content, oligosaccharides and water-soluble metabolite fraction of human milk, but caused considerable loss of biologically active proteins (lactoferrin and lysozyme). To improve the quality of human donor milk, new methods for human milk treatment and optimal conditions of milk storage need to be identified that ensure the destruction of pathogenic microorganisms while retaining the biological and nutritional value of the milk to the maximum extent possible.

CONCLUSIONS

1. Human milk macronutrient content undergoes changes post-delivery. Protein concentration was highest in the transitional human milk, then it gradually decreased. Comparing transitional and mature human milk, carbohydrate concentration had a tendency to increase. Fat content and human milk energy value showed no significant changes in the comparison of the transitional and mature human milk.

2. For the macronutrient content of human milk, significant factors are pregnancy duration, delivery mode and a newborn's gender. No link between the mother's age or her body mass index and human milk macronutrient content was established.

3. Significant circadian fluctuations of human milk protein and fat concentration were established. Human milk carbohydrate concentration did not show significant circadian variations.

4. Human milk freezing and Holder pasteurisation determine marked changes in the content of biologically active proteins (lactoferrin and lysozyme); however, they have no effect on the concentration of macronutrients, oligosaccharides and water-soluble metabolites in human milk.

5. Human milk metabolome study has revealed that all studied mothers belonged to the secretory type (Se+) according to oligosaccharides dominant in their milk; 17 out of 20 mothers (i.e. 85 %) had the type depending on the *Lewis* gene.

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PUBLICATIONS AND PRESENTATIONS

Published articles

1. Paulavičienė IJ, Liubšys A, Usonis V. Motinos pienas neišnešiotam naujagimiui: ar tik maistas, ar ir vaistas? Lietuvos akušerija ir ginekologija. 2017;20(4):278–85.

2. Paulaviciene IJ, Liubsys A, Molyte A, Eidukaite A, Usonis V. Circadian changes in the composition of human milk macronutrients depending on pregnancy duration: a cross-sectional study. Int Breastfeed J. 2020;15(1):49.

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4. Paulavičienė IJ, Liubšys A, Tamulienė L, Vaitkevičienė R, Molytė A, Usonis V. Laktacijos laikotarpis ir kiti veiksniai, turintys įtakos motinos pieno maistinių medžiagų sudėčiai. Lietuvos akušerija ir ginekologija 2020;23(3): 240–7.

Oral presentations

1. Paulaviciene IJ, Liubsys A, Tamuliene L, Vaitkeviciene R, Gudaitiene R, Strupiene L, Usonis V. Using a human milk analyser in clinical practice: circadian changes of human milk composition. XIV Baltic Congress of Laboratory Medicine. Vilnius, May 10–12, 2018.

2. Paulaviciene IJ, Liubsys A, Usonis V. Dynamics of human milk macronutrient composition during a 24-hour period. IV International Conference “Evolutionary Medicine: Health and diseases in changing environment”. Vilnius universitety Faculty of Medicine, June 5–8, 2018.

3. Paulavičienė I. Motinos pieno ir donorinio motinos pieno vertė neišnešiotam naujagimiui. Nacionalinė konferencija "Vaikų sveikatos aktualijos 2018. Sergančių vaikų maitinimas". Vilnius, 2018 m. birželio 01 d.

4. Paulaviciene IJ.The Influence of Circadian Rhythm and Holder Pasteurization on Human Milk Composition. 4th Baltic Paediatric Congress & European Academy of Paediatrics (EAP/UEMS-SP) Spring Meeting. Vilnius, May 16–18, 2019.

Poster presentations

1. Tamuliene L, Paulaviciene IJ, Liubsys A. Just maternal milk for sick and premature babies: first experience of donor milk bank at Vilnius Perinatal Centre". 4th EMBA International Milk Banking Congress, Glasgow (Scotland), October 5–6, 2017.

2. Paulaviciene IJ, Liubsys A, Tamuliene L, Molyte A, Usonis V. Macronutrient Composition of Human Milk Depending On Different Gestational Age at Birth. 8th International Congress of UENPS**.** Bucharest (Romania), October 3–5, 2018.

3. Tamuliene L, Paulaviciene I.J., Liubsys A. First – Year Experience of Donor Milk Bank at the Vilnius Perinatal Centre. 8th International Congress of UENPS**.** Bucharest (Romania), October 3–5, 2018.

4. Paulaviciene IJ, Liubsys A, Usonis V. Circadian changes of human milk macronutrients composition depending on pregnancy duration. 4th Baltic Paediatric Congress & European Academy of Paediatrics (EAP/UEMS-SP) Spring Meeting. Vilnius (Lithuania), May 16–18, 2019.

5. Paulaviciene IJ, Liubsys A, Vaitkeviciene R, Gudaitiene R, Usonis V. Does pregnancy duration or Holder pasteurization influence macronutrient or bioactive protein content in human milk? 3rd jENS Congress of joint European Neonatal Societies**.** Maastricht (Netherlands), September 16–21, 2019.

6. Paulaviciene IJ, Tamuliene L, Liubsys A, Usonis V. The influence of freezing and Holder pasteurisation on human milk composition. 5th international EMBA Congress. Turin (Italy), October 10–11, 2019.

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