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# Preterm human milk analysis confirms that all examined components change within four weeks of life

## Analiza mleka kobiecego matek wcześniaków wykazała zmianę w składzie wszystkich analizowanych składników odżywczych w pierwszych czterech tygodniach życia

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Abstract

**Introduction and objective:** Human milk contains all the nutrients and bioactive components required for the optimal growth and development of newborns. It is also an optimal source of nutrition in premature infants, however it does not fully meet their nutritional demands. Studies have shown that infants fed exclusively human milk have better enteral nutrition tolerance, lower risk of necrotising enterocolitis, and improved neurodevelopmental outcomes. The aim of the study was to analyse preterm content of human milk collected from women as part of a randomised controlled trial assessing tailored nutrition. **Materials and methods:** We studied 726 samples from 39 mothers of premature infants during their first postnatal four weeks. Infants were assigned to standard or tailored enteral nutrition, but all mothers were asked to pool milk samples from full breast expression at each feed across a 24-hour period twice a week. Glucose, fat, protein, and energy levels were measured using the Miris Human Milk Analyser and compared to published values. We assessed the relationship between postnatal week and human milk content. **Results:** Protein content decreased over time. Median protein at four weeks of postnatal age was 1.37 and 1.32 per 100 mL (tailored vs. standard group, respectively). Lipid content increased over time. Median lipid content at four weeks of postnatal age was 4.06 and 3.82 per 100 mL (tailored vs. standard group). Glucose remained stable. Energy content increased over time only in the tailored group. Median energy at four weeks of postnatal age was 73.35 per 100 mL. **Conclusions:** Variations in human milk content exist between populations and should be considered when prescribing enteral nutrition in preterm infants.

**Keywords:** nutrition, preterm, human milk

Streszczenie

**Wprowadzenie i cel:** Pokarm kobiecy zawiera wszystkie niezbędne składniki odżywcze potrzebne do prawidłowego przyrostu masy ciała i rozwoju noworodka. Jest optymalnym źródłem żywienia dla noworodków urodzonych przedwcześnie, jednakże nie zaspokaja w pełni potrzeb odżywczych wcześniaków. Badania wykazały, że wcześniaki karmione wyłącznie pokarmem kobiecym lepiej tolerują żywienie enteralne, uzyskują wyższe wyniki w badaniach neurologicznych i rzadziej zapadają na martwicze zapalenie jelit. Celem badania była analiza wartości odżywczych zawartych w pokarmie kobiecym pobranym od matek wcześniaków. **Materiał i metody:** Autorki przebadały 726 próbek pobranych od 39 matek wcześniaków w pierwszych 4 tygodniach po porodzie. Niemowlęta przydzielono do standardowego lub zindywidualizowanego żywienia dojelitowego. Wszystkie matki poproszono o zebranie próbek mleka z pełnego odciągnięcia piersi przy każdym karmieniu przez okres 24 godzin dwa razy w tygodniu. Zbadano stężenie glukozy, tłuszczu, białka i energii. Pomiary wykonywano przy pomocy analizatora mleka kobiecego Miris i porównywano z opublikowanymi wartościami. Dodatkowo oceniono związek pomiędzy tygodniem po porodzie a zawartością mleka kobiecego. **Wyniki:** Zawartość białka zmniejszała się w kolejnych tygodniach po urodzeniu. Mediana białka w 4. tygodniu po porodzie wynosiła 1,37 i 1,32 2 100 ml (grupa zindywidualizowana *vs* standardowa). Z kolei zawartość lipidów wzrastała w kolejnych tygodniach. Mediana zawartości lipidów w 4. tygodniu życia po porodzie wyniosła 4,06 i 3,82 na 100 ml (grupa zindywidualizowana *vs* standardowa). Stężenie glukozy było stabilne. Zawartość energii wzrastała w kolejnych tygodniach obserwacji w grupie zindywidualizowanej. Mediana energii

w 4. tygodniu życia po porodzie wyniosła 73,35 na 100 ml. **Wnioski:** Wyniki wskazują, że istnieją różnice w zawartości substancji odżywczych w mleku kobiecym, co należy wziąć pod uwagę podczas przepisywania żywienia enteralnego wcześniakom.

**Słowa kluczowe:** żywienie, wcześniak, mleko kobiece

## INTRODUCTION

Human milk (HM) contains all the nutrients and bioactive components required for the optimal growth and development of newborns. The World Health Organization recommends that all infants be exclusively breastfed until six months of age[1]. Breast milk is also an optimal source of nutrition in premature infants[2]. In this group of infants, HM does not fully meet the nutritional demands, but provides crucial ingredients, such as immunoglobulins, hormones, growth factors, and enzymes[3]. Studies have shown that infants fed exclusively HM have better enteral nutrition tolerance, lower risk of necrotising enterocolitis (NEC), and improved neurodevelopmental outcomes[3,4]. Overall, HM reduces morbidity and mortality in all infants born prematurely[5,6]. HM content is highly variable and changes depending on the time of day and stage of lactation, as well as between women and populations[7,8]. In the preterm population, HM varies depending on the gestational and postnatal ages of infants. Fortifiers can be added to HM to enrich it with proteins, energy, or vitamins[9].

The time and mode of sample collection also influence HM composition, including fat, protein, lactose, and other bioactive factors[7,8].

Mothers who deliver extremely low birth weight (ELBW) infants must express milk for an extended period until their babies develop an effective sucking reflex and can be put to the breast. Different collection methods are available, such as hand and breast pump expressions. Breast milk lipids and energy components vary from the start to the end of the collection session and are influenced by the collection method in both term and preterm milk[7,10].

Furthermore, HM content variability is a result of different laboratory methods used to estimate breast milk composition. A recent study has shown that differences between devices are clinically significant and can impact daily dietary prescriptions and the results of clinical trials[11]. One way to overcome this variability is to use infrared (IR) HM analysers (HMA), which are quick and safe to use in clinical settings[12–14]. However, it is important to note that in most studies to date, these devices were used without calibration, validation, or quality assurance[15]. This may lead to significant errors in the determination of protein and fat content[16,17].

Nutritional exposure, especially in preterm infants, affects an individual's lifelong health outcomes, both short-term infant growth/body composition and future risks of obesity and metabolic disorders[18,19].

Previous studies have reported milk composition in populations around the world, but none have focused on HM milk content recorded within a set time period from the same mothers who delivered prematurely. Hence, we are the first to report HM content from a set group of ELBW mothers collected in accordance with the golden standard and analysed using IR HMA in an Eastern European setting.

## MATERIALS AND METHODS

### Participants

This study evaluated the biochemical composition of milk samples obtained from Polish women who delivered prematurely. HM milk analysis data were obtained from mothers of ELBW recruited in a randomised controlled trial (RCT). The trial, which evaluated the effect of tailored HM fortification on growth and development, was conducted between June 2019 and June 2022 at the Neonatal and Intensive Care Department of the Medical University of Warsaw[20]. Detailed information about the study protocol and results of the RCT has been published previously[20,21]. The study protocol was approved by the local ethics committee, and the trial was registered at clinicaltrials.gov (NCT03775785).

#### Eligibility criteria

All parents of infants born at less than 32 weeks of gestation and admitted to the Neonatal Intensive Care Unit (NICU) were approached by one of the research team members within the first week of life (as full enteral feeding is usually reached at a minimum of seven days of life).

#### Inclusion criteria

Patients eligible for the trial had to comply with the following criteria at randomisation:

1. gestational age at birth ≤32 weeks;
2. enteral feeding of at least 80 mL/kg/day;
3. 50% donor or maternal milk-based enteral feeding;
4. parenteral/legal guardian informed consent.

### Sample collection

All mothers provided HM samples (expressed 24 hours prior to the analyses) three times per week (Monday/Wednesday/Friday) and after protocol amendment twice per week (Tuesday/Thursday). Each mother received written guidelines explaining how to perform a 24-hour collection based on the gold standard[22,23]. Women were asked to express milk



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from a single breast every three hours from the previous expression, at least five times a day (including one night between midnight and 6:00 am). A minimum of 2 mL of HM was required per sample. At the end of each collection, all samples were pooled, mixed in one transport container, and delivered to the unit. The samples were stored at 4°C until analysis.
One of the researchers (JSS) performed milk analysis in the NICU research laboratory at Princess Anna Mazowiecka Hospital three times per week (Monday/Wednesday/Friday) at 10:00 am and after protocol amendment twice per week (Tuesday/Thursday) from batches collected on the two previous days. Unpredictably, our study was conducted in the initial COVID-19 period, hence mothers had limited transport possibilities and restricted access to their babies. This turned out to be an additional burden on already highly stressed mothers and was one of the main reasons for poor protocol attrition. Based on analysed samples, we decided that biweekly (Tuesday/Thursday) milk sampling would be sufficient to estimate milk composition. A 10 mL aliquot from each batch of native breast milk was used for macronutrient analysis per protocol[24]. We analysed three samples per 2 mL from each batch according to the manufacturer's instructions. First, HM was thawed and heated to 37°C. Each sample was then homogenised using a sonicator (Miris® ultrasonic processor). Next, each sample was analysed using IR HMA (Miris® HMA) calibrated with HM standards. MIR spectroscopy was specifically designed for HM composition analyses (ISO 9622:1999). The device was calibrated and validated prior to each measurement session as per the manufacturer's recommendations. We measured the levels of fat, protein, lactose, and total solids simultaneously.

## Data management

All data collection was conducted electronically.

## Confidentiality

Complete patient and study information was stored on a secure, password-protected web-based platform. Only researchers involved in the study were provided with a personalised login and password to access the study information. The statistical team did not have access to any sensitive data such as date of birth, address, or contact details. All records containing patient details and relevant medical history were stored separately in a locked file cabinet. There were no plans for collection, laboratory evaluation, or storage of biological specimens for genetic or molecular analysis in this trial/future use. We did not plan to perform any genetic or molecular analysis in this trial.

# RESULTS

## Demographics

Between 2019 and 2022, a total of 392 infants born at <32 weeks' gestation were admitted to the NICU and screened for eligibility. Initially, 344 infants were excluded from the study for the following reasons: declined consent ($n$ = 200), paused recruitment ($n$ = 100), and failure to meet inclusion criteria ($n$ = 44). Fifty-five infants were initially randomised; however, 16 did not receive the allocated intervention. Thirty-nine singleton ($n$ = 25) and twin ($n$ = 7) births at a median age of 29 (range, 26–31) weeks and a mean birth weight of 1,306 (±454.3) g were randomly assigned to standard fortification ($n$ = 21) or tailored fortification ($n$ = 18). Siblings from multiple pregnancies were randomly assigned to different treatment groups.

## Milk samples-nutritional composition

We measured 726 samples from 39 mothers of premature infants during the first postnatal week using the Miris HMA. As per the protocol, infants were assigned to standard or tailored enteral nutrition, but all mothers were asked to pool milk samples from full breast expression at each feed across a 24-hour period twice a week.

### Protein content

We measured HM samples from the mothers of infants born between 24 + 0/7 and 31 + 6/7 weeks of gestation. At one week of postnatal age, the median HM protein content was 2.00 and 1.91 per 100 mL, for the tailored and standard groups, respectively. The mean deviation was ±0.09 with a 95% confidence interval (CI) of [−0.10, 0.29]. The protein content decreased over time, and the median protein at four weeks of postnatal age was 1.37 and 1.32 per 100 mL, for the tailored and standard groups, respectively. The mean deviation was ±0.05 with a 95% CI of [−0.14, 0.24] (Tab. 1).

### Energy content

At one week of postnatal age, the median lipid content of HM was 3.48 and 3.65 per 100 mL for the tailored and standard groups, respectively. The mean deviation was ±8.00 with a 95% CI of [−17.0, 8.00]. The lipid content increased over time, and the median lipid content at four weeks of postnatal age was 4.06 and 3.82 per 100 mL for the tailored and standard groups, respectively. The mean deviation was ±0.05 with a 95% CI of [−0.80, 0.80]. The median glucose content of HM was 7.60 and 7.40 per 100 mL, and the mean deviation was ±0.2 with a 95% CI of [−0.10, 0.50]. The glucose content did not change over time. The median energy content of HM was 71.06 and 71.21 per 100 mL, and the mean deviation was ±6.15 with a 95% CI of [−1.27, 13.57]. Energy content increased over time only in the tailored group, and the median energy at four weeks of postnatal age was 73.35 per 100 mL (Tab. 1).

## Changes in nutritional composition depending on lactation week

Tab. 2 summarises the macronutrient values measured during the first four weeks of lactation.



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| Variable | Tailored | Standard | MD [95% CI] | *p* |
|---|---|---|---|---|
| **Milk volume [mL]** | | | | |
| Day 0 | - | - | - | - |
| Week 1 | 241.60 ± 78.80 | 220.39 ± 65.79 | 21.21 [−30.10, 72.52] | 0.406 |
| Week 4 | 305.60 ± 106.79 | 264.00 ± 114.45 | 41.60 [−62.39, 145.59] | 0.412 |
| Last measurement | 323.29 ± 114.93 | 313.60 ± 127.25 | 9.69 [−71.86, 91.25] | 0.811 |
| **Protein [g/100 mL]** | | | | |
| Day 0 | 2.02 ± 0.34 | - | - | - |
| Week 1 | 2.00 ± 0.34 | 1.91 ± 0.22 | 0.09 [−0.10, 0.29] | 0.328 |
| Week 4 | 1.37 ± 0.24 | 1.32 ± 0.15 | 0.05 [−0.14, 0.24] | 0.582 |
| Last measurement | 1.34 ± 0.26 | 1.38 ± 0.25 | −0.04 [−0.21, 0.13] | 0.640 |
| **Glucose [g/100 mL]** | | | | |
| Day 0 | 7.50 [7.10, 7.80] | - | - | - |
| Week 1 | 7.60 [7.07, 7.80] | 7.40 [7.05, 7.60] | 0.20 [−0.10, 0.50][1] | 0.219[2] |
| Week 4 | 7.60 [7.53, 7.77] | 7.90 [7.67, 8.00] | −0.30 [−0.50, 0.00][1] | **0.039[2]** |
| Last measurement | 7.70 [7.60, 7.80] | 7.80 [7.60, 7.90] | −0.10 [−0.20, 0.10][1] | 0.348[2] |
| **Fat [g/100 mL]** | | | | |
| Day 0 | 3.36 ± 1.44 | - | - | - |
| Week 1 | 3.48 ± 1.40 | 3.65 ± 1.20 | 8.00 [−17.00, 8.00][1] | 0.417[2] |
| Week 4 | 4.06 ± 1.56 | 3.82 ± 0.56 | −0.10 [−0.80, 0.80][1] | 0.940[2] |
| Last measurement | 4.19 ± 1.48 | 3.52 ± 1.05 | 0.68 [−0.17, 1.53] | 0.113 |
| **Energy [kcal/100 mL]** | | | | |
| Day 0 | - | - | - | - |
| Week 1 | 71.06 ± 12.38 | 71.21 ± 12.04 | −0.15 [−8.57, 8.27] | 0.972 |
| Week 4 | 73.50 ± 12.20 | 73.10 ± 4.93 | 0.40 [−8.35, 9.15] | 0.925 |
| Last measurement | 75.35 ± 12.18 | 69.20 ± 10.06 | 6.15 [−1.27, 13.57] | 0.101 |

Data are presented as mean ± standard deviation (except for glucose, which is presented as median and interquartile range).
**MD** – mean or median[1] difference (tailored vs. standard); **CI** – confidence interval. Groups were compared using Student's independent test or Mann–Whitney *U* test[2], as appropriate, depending on the distribution normality.

*Tab. 1. Milk composition during supplementation period*

| Variable | Week 1 | Week 4 | MD [95% CI] | *p* |
|---|---|---|---|---|
| **Tailored group** | | | | |
| Protein [g/100 mL] | 2.10 ± 0.19 | 1.41 ± 0.21 | −0.69 [−0.88, −0.50] | **<0.001** |
| Glucose [g/100 mL] | 7.49 ± 0.40 | 7.49 ± 0.59 | 0.00 [−0.56, 0.56] | >0.999 |
| Fat [g/100 mL] | 3.77 ± 0.81 | 4.20 ± 1.59 | 0.34 [−0.61, 1.48] | 0.367 |
| Energy [kcal/100 mL] | 74.22 ± 7.07 | 75.00 ± 11.93 | 0.78 [−7.61, 9.17] | 0.836 |
| **Standard group** | | | | |
| Protein [g/100 mL] | 1.83 ± 0.25 | 1.34 ± 0.13 | −0.49 [−0.71, −0.27] | **0.001** |
| Glucose [g/100 mL] | 7.40 ± 0.41 | 7.89 ± 0.21 | 0.49 [0.21, 0.77] | **0.004** |
| Fat [g/100 mL] | 3.14 ± 1.03 | 3.74 ± 0.54 | 0.60 [−0.27, 1.47] | 0.149 |
| Energy [kcal/100 mL] | 66.89 ± 10.28 | 72.67 ± 5.02 | 5.78 [−2.83, 14.39] | 0.160 |

Data for paired observations are presented as the mean ± standard deviation.
**MD** – mean difference (week 4 vs. week 1); **CI** – confidence interval.
Groups were compared using the paired *t*-test.

*Tab. 2. Milk composition development over time*

## DISCUSSION

Research on breastfeeding and HM is significantly complicated by the fact that HM composition is highly variable, follows a diurnal pattern, and can change between women and populations[7,8,25,26]. Our study reports higher values of energy, fat, protein, and glucose than those previously described[9]. However, we found that the same HM content changed over time. The mean protein levels were higher in early milk, but the fat and glucose levels increased with postnatal age.

A recent meta-analysis by Gidrewicz and Fenton revealed that preterm milk content data were mostly available from studies conducted in the late 1980s, 1990s, and early in 2000[9]. Health awareness has increased significantly over the last 30 years. In our study, we were able to follow the changes in HM composition in samples taken from the



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same mother for four weeks. We analysed samples collected from 39 women longitudinally during the first postnatal week 4. The energy content did not decrease significantly, as reported in previous studies[9]. This may be related to the different calculation factors used, which leads to under- or overestimation of the HM energy content[10,27].

## Protein

The mean protein level was higher during the first week and decreased steadily over time, which is consistent with the findings reported by other authors. Amino acid analysis is the most accurate method for determining true protein content, but it is also time-consuming and costly[28]. Other methods were also reported in the systematic review by Boyce et al.[29]. However, it is difficult to compare the exact estimates, as some researchers have reported protein content assuming all nitrogen is protein, some both protein estimates, and some only total protein[9,13]. IR spectroscopy used in our study varies in accuracy depending on the calibration, reference method, and number of samples; however, protein values reported were consistent with those found by more direct studies[29].

## Lipids

As reported by others, lipid concentrations increased over four weeks[29]. The preferred modalities for estimating the total lipid concentration include the Folch and Roese-Gottlieb methods[29]. Corvaglia et al. found that IR spectroscopy was comparable to the Roese-Gottlieb method[12]. However, 24- hour sample collection, mixing at 38°C (homogenisation), and calibration are essential to achieve representative samples for analysis[29,30]. Neither study reported whether the researchers followed all the essential steps before performing the measurements and reported higher lipid concentrations than others[29].

## Glucose

The glucose levels did not change throughout the observation period, which is inconsistent with the results of other studies. HM contains lactose, free glucose, galactose, and oligosaccharides[29]. However, available studies have reported lactose and total carbohydrate concentrations, which might explain why our results differ from those reported by other authors. The total carbohydrate content was higher than lactose, and both increased gradually over weeks 1–4[18]. Faerk et al. used IR spectroscopy and reported values for multiple weeks[acc. to 29]. However, the authors did not provide details of the calibration method; therefore, we were unable to compare our results. Nonetheless, it is important to note that from a clinical perspective, the total carbohydrate value is preferable.

## Strengths and limitations

We are the first to report HM content from a large sample of 726 measurements collected prospectively from mothers who delivered prematurely. Mothers were asked to pool milk samples from full breast expression at each feed over a 24-hour period twice a week. This method is considered the "gold standard" for HM collection, as it is believed to be the most representative of milk consumed by infants[22,23]. Furthermore, we collected samples from the same group of mothers for four weeks, which has not been reported previously. It is important to note that collection of full breast expressions from all feedings over a 24-hour period is not practical for large population-based studies and may have the potential to interfere with normal breastfeeding[29]. This approach places a significant burden on mothers, and it is difficult to achieve in a research setting; hence, in our study, we had the unique opportunity to collect this data, as mothers were already participating in a RCT[20].

Our study was limited to a single Eastern European population with specific dietary regimes; hence, our results may not be applicable to other settings. Furthermore, we did not measure total carbohydrate levels, which are the main determinants of energy levels. Hence, the energy levels of the reported samples may have been underestimated.

## Further work

To determine the precise composition of preterm human milk, a large multi-population study should be performed. The study should include 24-hour collections from the same women over a specific time and undertake all macronutrient analyses using the best methodologies available.

## CONCLUSIONS

Given the high energy demand of preterm infants, adequately analysing preterm HM would help us understand the level of fortification required to support the nutritional needs of this vulnerable population.

**Conflict of interest**

*The authors report no financial or personal relationships with other individuals or organisations that could adversely affect the content of the publication and claim ownership of this publication.*

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**Institutional review board statement**

*The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of the Medical University of Warsaw (protocol code: KB/89/2018, date of approval: May 21, 2018).*

**Author contributions**

*Original concept of study; collection, recording and/or compilation of data; analysis and interpretation of data; writing of manuscript; critical review of manuscript; final approval of manuscript: JSS, AC.*

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