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Gas chromatographic determination of fatty acid composition in breast milk of mothers with different health conditions

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PRELIMINARY COMMUNICATION

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ABSTRACT

Human milk (HM) of healthy, well-nourished, lactating mothers is a unique and ideal source of nutritive factors, like hormones, cytokines, chemokines, growth factors that ensures the proper growth and development of infants. Among the main components of HM, fat is an important energy source and a regulatory factor. The quality of milk fat depends on its fatty acid (FA) composition. Gas chromatography coupled with flame ionisation detection is one of the most common methods for analysis of the FA profile of HM. The aim of this study was to evaluate the FA composition of HM, collected from mothers with different health conditions (normal Body Mass Index (nBMI); overweight and obese) using GC-FID method. The results showed that saturated FAs were present in the highest amount in the HM samples, of which palmitic acid was the main representative. The major monounsaturated FA was oleic acid, while linoleic

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acid was the most abundant of the polyunsaturated FAs (PUFA). Overweight and obese women have lower levels of PUFA in their breast milk. The data were subjected to principal component and quadratic discriminant analysis (QDA). QDA classified nBMI and overweight and obese mother milk samples with 88.24% accuracy. Significant differences were found between normal and overweight and obese HM samples in case of C10:0 and C18:3 FAs. Higher maternal BMI was associated with a higher n-6/n-3 PUFA ratio.

1. INTRODUCTION

The World Health Organization recommends exclusive breastfeeding during the first six postnatal months, but breastfeeding may be an important source of different maternal factors for the infant throughout the first two years of life (WHO/UNICEF, 2003). Human milk (HM) is the ideal food for neonates and infants, which provides a variety of nutrients, growth factors, immune components, and energy (Ballard and Morrow, 2013). HM has a fat content of about 4%, triacylglycerols (TAG) account for 98% of HM lipids, and their properties are largely influenced by their fatty acid (FA) composition (Bobiński and Bobińska, 2020). HM contains more than 200 FAs, the majority of FAs are saturated fatty acids (SFA: 48.2%) or monounsaturated (MUFA: 39.8%) followed by polyunsaturated FAs (PUFA: 10.8%) (Guo, 2020). Within the SFAs there are short chain (carbon chain length<6), medium chain (carbon chain length 6–10), and long chain SFAs (carbon chain length >12) (EFSA, 2010).

The FAs composition of HM has essential physiological roles. The shorter chain SFAs are more easily absorbed than long chain SFAs, because they do not need carnitine transport for their metabolism (EFSA, 2010). Long chain PUFAs (LCPUFA), especially α-linolenic acid (ALA; C18:3) and linoleic acid (LA; C18:2), are considered essential for infants (0–24 months) since they cannot be synthesised by the human body. The other important LCPUFAs, docosahexaenoic acid (DHA; C22:6) and arachidonic acid (AA; C20:4), can be synthesised from ALA and LA (FAO/WHO, 2008).

The long chain SFAs trigger induced inflammation in the intestinal tract of infants (Kong et al., 2021). Among the LCPUFA, DHA contributes to the improvement of brain development and cognitive function (Kuszewski et al., 2017), it also decreases morbidity associated with atherosclerosis and cardiovascular disease (Yagi et al., 2017; Zehr and Walker, 2018). The AA was shown to be a pro-inflammatory factor and the ratio of total n-3/n-6 FA is a health-related indicator (Derbyshire, 2017). European Union regulations (2016) from February 2022 require that all infant formulas have to contain from 0.33 to 1.14% DHA. The FA composition of HM is variable and influenced by certain factors, such as maternal dietary habits, geographic region, obesity (Butts et al., 2018). Lindholm et al. (2013) found that obese mothers' milk contained fewer n-3 fatty acids than normal weight mothers' milk. This resulted in a 40% higher n-6/n-3 ratio in breast milk from obese mothers compared to breast milk from normal-weight mothers. Obesity affects public health and leads to complications worldwide,

causing both short- and long-term negative effects on the offspring's health (Galliano and Bellver, 2013).

Several analytical techniques for determining the FA composition of HM have been developed. Gas chromatography (GC) is the most routinely used separation method for FA analysis since the 1950s (Insull and Ahrens, 1959). GC coupled with flame ionisation detector (GC-FID) is one of the most popular combinations for FAs analysis in many sample types, including HM (Orata, 2012; Visentainer et al., 2018).

The present study aimed to evaluate the FA profile of HM of mothers with different BMI status by GC-FID.

2. MATERIALS AND METHODS

2.1. Breast milk samples

Seventeen mother milk samples were collected from mothers, recruited at the Department of Obstetrics and Gynaecology of the University of Pécs (Hungary) between the 10th and 12th postpartum week. During sample collection, the mothers were asked to completely empty the breast by using a mechanical milk pump (Philips Avent, Farnborough, United Kingdom) into disposable sterile polypropylene tubes. From the total expressed volume, 2 mL were taken with a sterile enteral syringe and aliquoted into microtubes (Eppendorf, Hamburg, Germany). Milk samples were immediately placed in a freezer at −20 °C. Then the samples were shipped on dry ice to the laboratory and stored at −80 °C until analysis. Based on the BMI of the mothers, the samples were divided into two groups: normal weight (BMI: 18.5–25.0; $n = 8$) and overweight and obese (BMI: >25.0; $n = 9$).

The research was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Regional and Local Research Ethics Committee of the University of Pécs, Pécs, Hungary (PTE KK 7072-2018).

2.2. Sample preparation for GC analysis

Sample preparation was based on a slightly modified ISO 16958:2015 reference method (ISO/IDF, 2015). On the day of analysis, the frozen breast milk samples were taken to room temperature and shaken vigorously to ensure homogeneity. 0.5 g of breast milk was pipetted into 50 mL centrifuge tubes and 2.5 mL of tert-butyl methyl ether was added to the samples. This step was followed by the transesterification procedure, where 2.5 mL of 5% (m/v) methanolic sodium hydroxide solution was added, then the sample was vortexed for 10 s using LABINCO L46 Power Mixer (Labinco B.V., Breda, The Netherlands). The tubes were opened after 180 s, and 1 mL of isooctane was added. After 30 s 5 mL neutralisation solution containing 10% (m/v) disodium hydrogen citrate and 15% (m/v) sodium chloride aqueous solution was added. The samples were carefully shaken again. The two-phase solution was separated by centrifuging at 1750 r.p.m. ($g = 375$) for 5 min in the Hettich Mikro 22R Refrigerated Centrifuge (Andreas Hettich GmbH andCo. KG, Tuttlingen, Germany). 150 μL of the supernatant was diluted 10-times with isooctane into a gas chromatography vial. Following dilution, GC-FID measurements were performed on a 1 μL volume of each sample. Due to the limited amount of the available sample, we were not able to perform parallel sample preparations.

2.3. Gas chromatography

The samples were analysed by an Agilent 6890 GC-FID (Agilent Technologies, Palo Alto, CA, USA) system equipped with an Agilent 7,683 autosampler. For separation, a Phenomenex Zebron ZB-FAME (60 m, 0.25 mm, 0.20 µm) column with a cyanopropyl stationary phase and hydrogen gas (1.2 mL min^{-1}) mobile phase was used. The inlet temperature was 250 °C and the detector temperature was 260 °C. A split ratio of 50:1 and 1 µl injection volume were used. The temperature program was as follows: the initial oven temperature 100 °C was held for 3 min, then the column was heated to 166 °C (held for 5 min) applying 20 °C min^{-1} temperature gradient rate, then 1 °C min^{-1} gradient was used to reach 180 °C, the final temperature of 240 °C (held for 3 min) was achieved with a temperature gradient of 10 °C min^{-1}. The total chromatographic time lasted around 40 min. Data acquisition and processing were performed with the GC-FID Mass Hunter software. The investigated fatty acid compounds were identified based on the FAME (fatty acid methyl ester) standard mixture solution (Supelco 37 component FAME Mix; Supelco, Bellefonte, PA, USA). Peak area of each FAME compound was integrated. The FA composition of the breast milk was expressed as a percentage of the peak area of total fatty acid components measured in the sample.

2.4. Statistical analyses

To investigate the differences between the two groups, an analysis of variance was performed. To confirm differences at the $P < 0.05$ significance level, Tukey's post-hoc test was used. These calculations were performed with SPSS software (Ver23, IBM, Armonk, NY, USA). Multivariate data analysis was used to evaluate the fatty acid profile of the samples, which were performed using The Unscrambler X 10.4 (CAMO, Oslo, Norway) software. Principal component analysis (PCA) was used for pattern recognition to reveal hidden structures among the samples. Quadratic discriminant analysis (QDA) was performed to classify the samples according to their FA profile.

3. RESULTS AND DISCUSSION

3.1. Fatty acid composition of HM samples

Our results confirmed that all major fatty acids were present in the breast milk samples analysed (Fig. 1.).

Saturated fatty acids (SFA) were found in the highest amount in HM (nBMI: 48.33%; O: 54.21%), followed by MUFAs (nBMI: 33.35%; O: 33.52%) and PUFAs (nBMI: 15.76%; O: 11.50%) (Table 1). The total SFA content of HM from the overweight and obese mother group was higher, while the PUFA content was lower compared to samples from the nBMI mother group. There was no significant difference in the MUFA content within two groups of HM samples (nBMI: 33.35%; O: 33.52%).

In comparison to other studies, the FA composition of breast milk of Hungarian mothers with nBMI contained high amounts of SFA (48.34%), similar to that of South Korean mothers (48.1%). MUFA content (33.35%) was similar to that of Greek women (35%), and PUFA content (15.76%) was lower than that of these two countries (21.5% and 18.6%) (Kim et al., 2017). The SFA content of breast milk from overweight and obese Hungarian mothers (54.21%) was
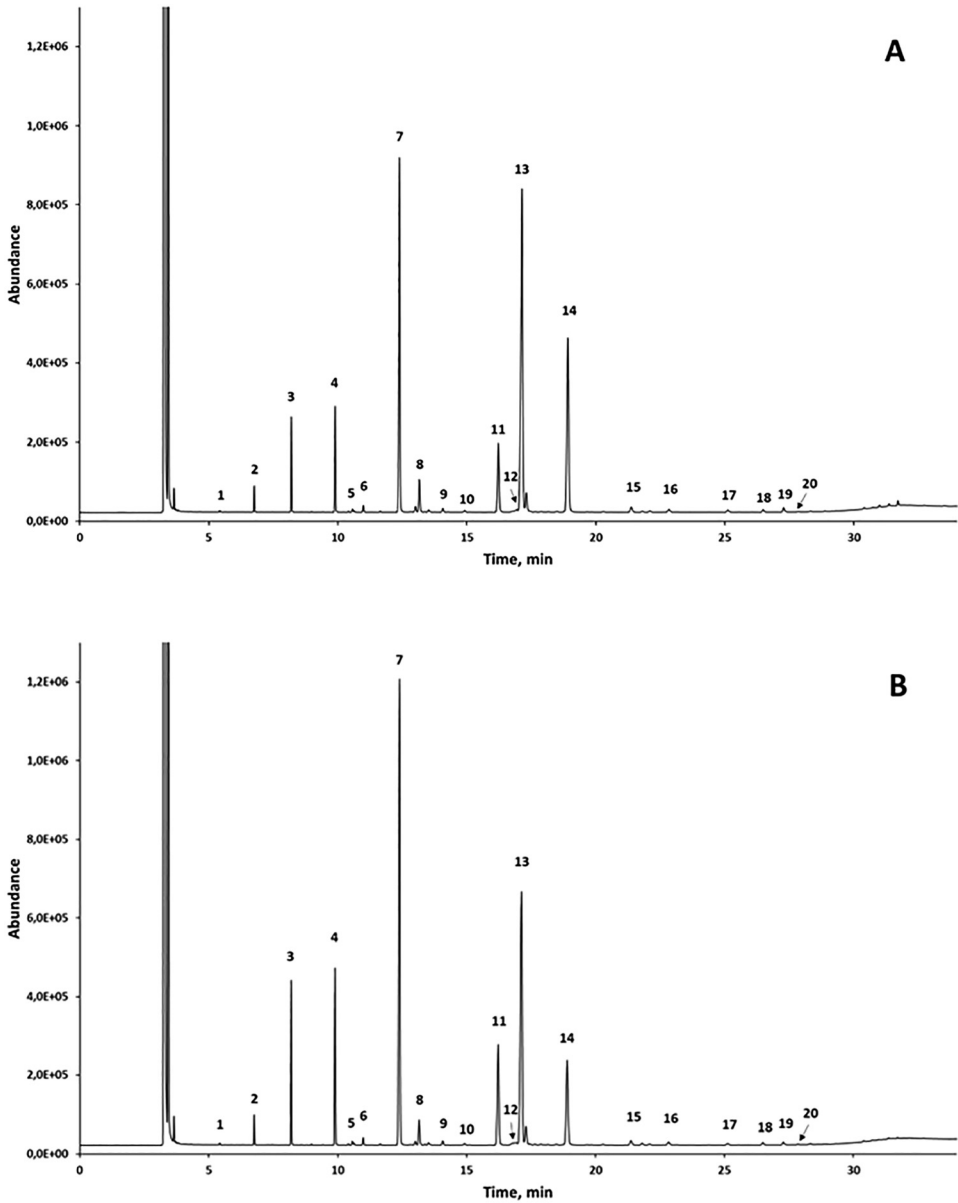
Fig. 1. GC-FID chromatograms of the fatty acid composition of human milk samples from mothers with normal BMI (A) and mothers with overweight and obesity (B). (1: C8:0 – caprylic acid; 2: C10:0 – capric acid; 3: C12:0 – lauric acid; 4: C14:0 – myristic acid; 5: C14:1 – myristoleic acid; 6: C15:0 – pentadecanoic acid; 7: C16:0 – palmitic acid; 8: C16:1 – palmitoleic acid; 9: C17:0 – heptadecanoic acid; 10: C17:1 – heptadecenoic acid; 11: C18:0 – stearic acid; 12: C18:1 tr (n9) – oleic acid; 13: C18:1 cis (n9) – oleic acid; 14: C18:2 – linoleic acid; 15: C18:3 – α-linolenic acid; 16: C20:1- eicosenoic acid; 17: C20:2 – eicosadienoic acid; 18: C20:3 – dihomo-gamma-linolenic acid; 19: C20:4 – arachidonic acid; 20: C22:0 – behenic acid)

Table 1. Fatty acid composition of breast milk samples from mothers with different health statuses

Parameter		Normal BMI (n = 8) Average (%)	SD	Overweight and obese (n = 9) Average (%)	SD
BMI data	body mass index	23.32[a]	1.80	27.34[b]	3.63
C8:0	caprylic acid	0.03	0.07	0.02	0.04
C10:0	capric acid	0.96[a]	0.42	1.13[b]	0.21
C12:0	lauric acid	4.57[a]	1.35	5.59[a]	1.33
C14:0	myristic acid	6.55[a]	2.22	7.75[a]	2.15
C14:1	myristoleic acid	0.11	0.08	0.08	0.10
C15:0	pentadecanoic acid	0.35	0.09	0.33	0.23
C16:0	palmitic acid	28.48[a]	4.27	31.06[a]	3.73
C16:1	palmitoleic acid	2.25[a]	0.37	1.98[a]	0.55
C17:0	heptadecanoic acid	0.31	0.05	0.19	0.19
C17:1	heptadecenoic acid	0.08	0.11	0.02	0.06
C18:0	stearic acid	7.06[a]	0.63	8.14[a]	1.47
C18:1 tr (n9)	*trans*-oleic acid	n.d.	n.d.	0.14	0.29
C18:1 cis (n9)	*cis*-oleic acid	30.64[a]	3.93	31.21[a]	4.80
C18:2	linoleic acid	14.59[a]	4.06	11.13[a]	2.46
C18:3	α-linolenic acid	0.45[a]	0.33	0.13[b]	0.22
C20:1	eicosenoic acid	0.28	0.18	0.09	0.14
C20:2	eicosadienoic acid	0.15	0.16	n.d.	n.d.
C20:3	dihomo-gamma-linolenic acid	0.25	0.12	0.06	0.11
C20:4	arachidonic acid	0.33	0.20	0.18	0.19
C22:0	behenic acid	0.02	0.04	n.d.	n.d.
$\sum$SFA	sum of saturated fatty acids	48.33[a]	6.92	54.21[a]	5.51
$\sum$MUFA	sum of monounsaturated fatty acids	33.35[a]	3.92	33.52[a]	4.77
$\sum$PUFA	sum of polyunsaturated fatty acids	15.76[a]	4.59	11.50[a]	2.29

SD: standard deviation; BMI: body mass index; *n*: number of samples; n.d.: not detected; different letters (a, b) show significant differences at $P < 0.05$ level.

significantly higher than that of obese Swedish (47.50%) and Spanish (27.80%) mothers (Lindholm et al., 2013; Garza Puentes et al., 2019).

The main FAs present in HM samples were oleic acid (C18:1 cis n-9; nBMI: 30.64%; O: 31.21%), palmitic acid (C16:0; nBMI: 28.48%; O: 31.06%), and linoleic acid (C18:2; nBMI: 14.59%; O: 11.13%), followed by stearic acid (C18:0; nBMI: 7.06%; O: 8.14%), myristic acid (C14:0; nBMI: 6.55%; O: 7.75%), lauric acid (C12:0; nBMI: 4.57%; O: 5.59%), palmitoleic acid (C16:1 n-7c: nBMI: 2.25%; O: 1.98%), and capric acid (C10:0: nBMI 0.96%; O 1.13%) (Fig. 2). All main FA contents were higher in obese samples, except for linoleic acid.

C16:0 (31.06%) and C18:0 (8.14%) levels in breast milk of overweight and obese Hungarian mothers were slightly higher than those of obese Swedish (C16:0: 26.9%; C18:0: 6.40%) and Spanish mothers. Elevated levels of C16:0 in Hungarian mothers' breast milk could be linked to their consumption of animal products, fast foods, processed foods, and high-fat dairy products, which are the primary dietary sources of SFA (Lindholm et al., 2013; Mihályi et al., 2015).
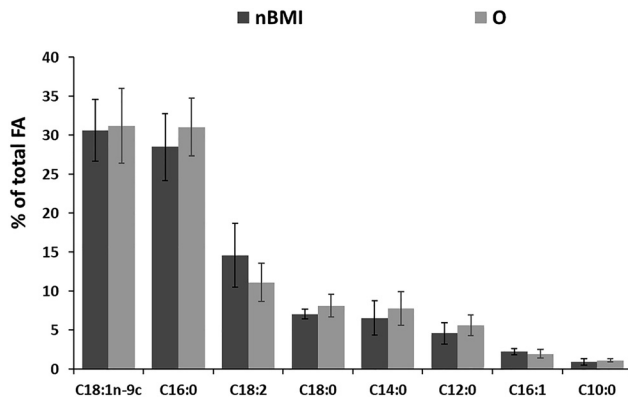
Fig. 2. The main fatty acid distribution in breast milk samples from mothers with different health statuses. (FA: fatty acid; nBMI: women with normal body mass index (BMI); O: overweight and obese women)

When the omega-3 and omega-6 fatty acid compositions of nBMI and O groups were compared, we found that O group mothers had a 53% higher n-6/n-3 ratio in their breast milk. This finding agreed with previous studies from Sweden (Lindholm et al., 2013) and Spain (Garza Puentes et al., 2019). In our study, breast milk samples from O group mothers had lower levels of n-3 PUFA than the normal group (nBMI: 0.4%; O: 0.1%), which was consistent with the findings of Chamorro et al. (2022).

3.2. Multivariate data analysis

PCA was used for statistical evaluation of data, and it was applied for pattern recognition purposes (Fig. 3). Data were mean-centred, and full cross-validation was used to test the model parameters. The first three principal component (PC) explained the 95% of the variance of the data (PC1: 69%; PC2: 14%; PC3: 12%). In the calibration and validation, the residual variances of PC1-3 were 0.85/1.14, 0.46/0.99, and 0.14/0.29, respectively. Considering the FA profiles, no clear separation between the nBMI and O groups could be detected. However, PC3 had the greatest influence, as the majority of samples from the overweight and obese groups were on the negative end of the scale when compared to the normal group.

The PCA biplot (Fig. 4) shows which fatty acids have the greatest influence on each sample's relative position in the PCA plot. The greater the effect, the further the components are from the centre. The biplot of sample scores and loadings clearly demonstrated that, due to the greater variation in the percentage of FAs, C14:0, C16:0, C18:1, and C18:2 FAs had the greatest influence on the relative position of the samples.

Beside PCA evaluation quadratic discriminant analysis (QDA) was also applied in order to classify the samples based on the FAs % ratio of total FAs (Fig. 5). The accuracy of the QDA model obtained 88.24%. Two samples, namely O6 and nBMI3, were misclassified with this statistical method. Nevertheless, the model is still highly accurate in prediction and classification with external samples. As a result of the discriminant analysis, samples belonging to the normal BMI group were separated from the samples in the obese BMI group based on their fatty acid content.
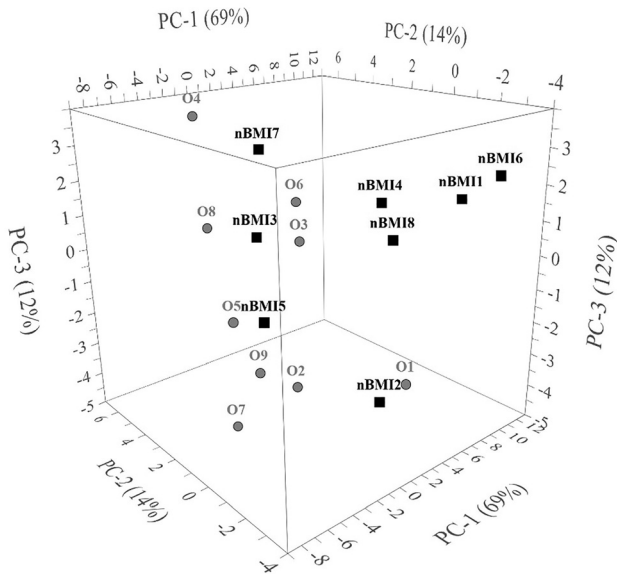
Fig. 3. The scores plot of principal component analysis (PCA) of the fatty acids in human milk samples (squares: normal BMI; dots: overweight and obese HM samples)
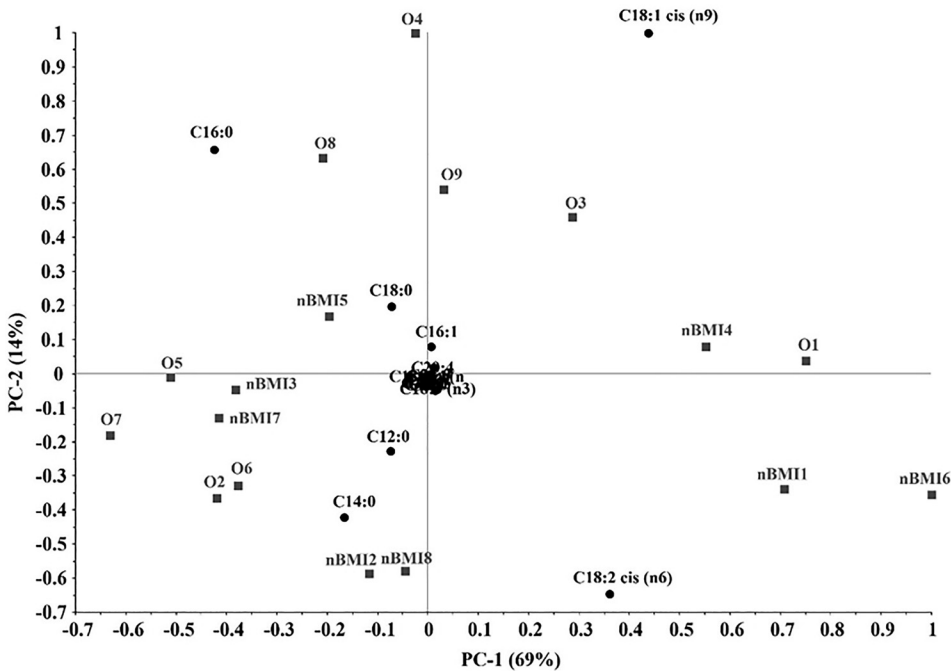

Fig. 4. Bi-plot of PCA loadings and scores of the fatty acids of human milk samples (nBMI: normal body mass index; O: overweight and obese samples)
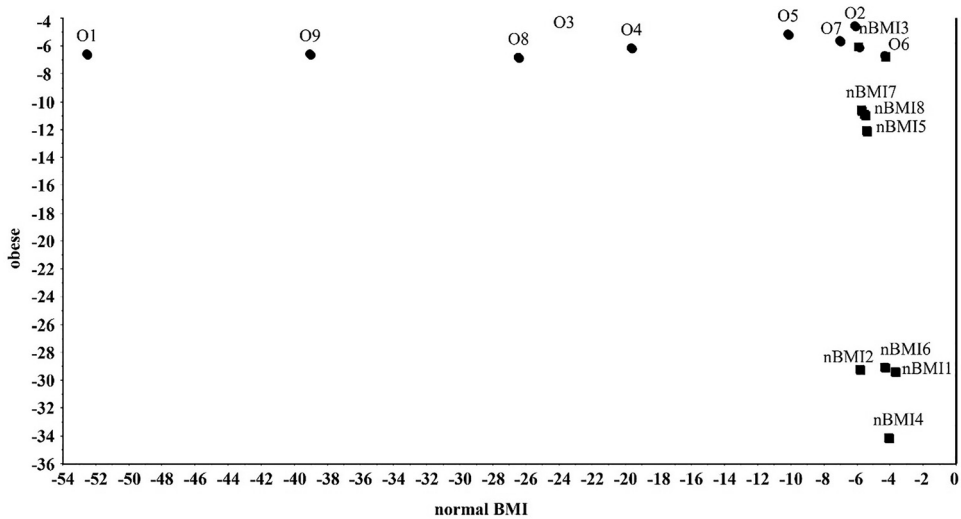
Fig. 5. Quadratic discriminant analysis (QDA) of human milk samples (squares: normal BMI; dots: obese samples)

4. CONCLUSIONS

During our research, we examined the relationship between maternal obesity and the fatty acid profile of the breast milk. Gas chromatography is an excellent technique for determining the fatty acid composition of human milk. Among the twenty FAs measured, C10:0 and C18:3 fatty acids showed significant differences between normal BMI and overweight and obese HM samples. Because of the high n-6/n-3 ratio, C18:2 and C18:3 FAs should be given more consideration in HM samples from overweight and obese mothers, as the n6/n3 ratio may have a negative impact on baby cognition function. Our findings show that maternal obesity has a significant impact on the fatty acid composition of breast milk. Adequate information about mothers' nutritional needs during pregnancy and breastfeeding appears to be one of the most important pieces of knowledge for ensuring their own and their newborns' future health.

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