Lipid composition of human milk: robust LC-MS method for fat profiling

Mariusz Radkiewicz[1], Emilia Samborowska[1], Agnieszka Bzikowska-Jura[2], Aleksandra Molas[2], Johannes B. van Goudoever[3], C.H.P. van Akker[3], Enrico Bertino[4], Paola Tonetto[4], Serena Gandino[4], Stefano Sottemano[4], Elena Sinkiewicz-Darol[5], Izabela Drazkowska[6], Paulina Szczygiol[7], Barbara Krolak -Olejnik[7], Malgorzata Gawrońska[8] and Aleksandra Wesolowska[2,8]

1 Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics PAS
2 Laboratory of Human Milk and Lactation Research, Medical University of Warsaw, Warsaw, Poland,
3 Department of Pediatrics - Neonatology, Amsterdam UMC - Emma Children's Hospital, University of Amsterdam, Vrije Universiteit Amsterdam, The Netherlands
4 Neonatal Care Unit of the University, City of Health and Science Hospital, 10126 Turin, Italy
5 Human Milk Bank in Torun, Ludwik Rydygier' Provincial Polyclinical Hospital in Torun, Poland
6 Huma Milk Bank in Gdańsk, Division of Neonatology, Medical University of Gdańsk, 80-210 Gdańsk, Poland
7 Huma Milk Bank in Wroclaw, Department and Clinic of Neonatology, Wroclaw Medical University, Wroclaw, Poland.
8 Foundation of Human Milk Bank in Poland, Poland

Abstract

Women across Europe live in different conditions and are exposed to contrasting diets. These and many more affect the nutritional composition of human milk (HM) which is crucial to infant's wellbeing. Lipids are well characterised molecules with great biological potential. They not only act as energetic reactants but affect membranes integrity, participate in metabolism modulation, signalling pathways and importantly, are substrates for synthesis of eicosanoids – molecules controlling inflammation. Our project aimed at finding how dietary, environmental and lifestyle conditions affect nutritional values of HM.

Results

We have found out wide time-wise and country-wise dispersion of milk fatty acids. Interestingly Italian women had on average least of all fatty acids in all 3 time points, especially of the n-3 PUFAS. In comparison, both Dutch and Polish women had high levels of beneficial DHA and EPA across all 3 lactation time points which then gradually lowered.

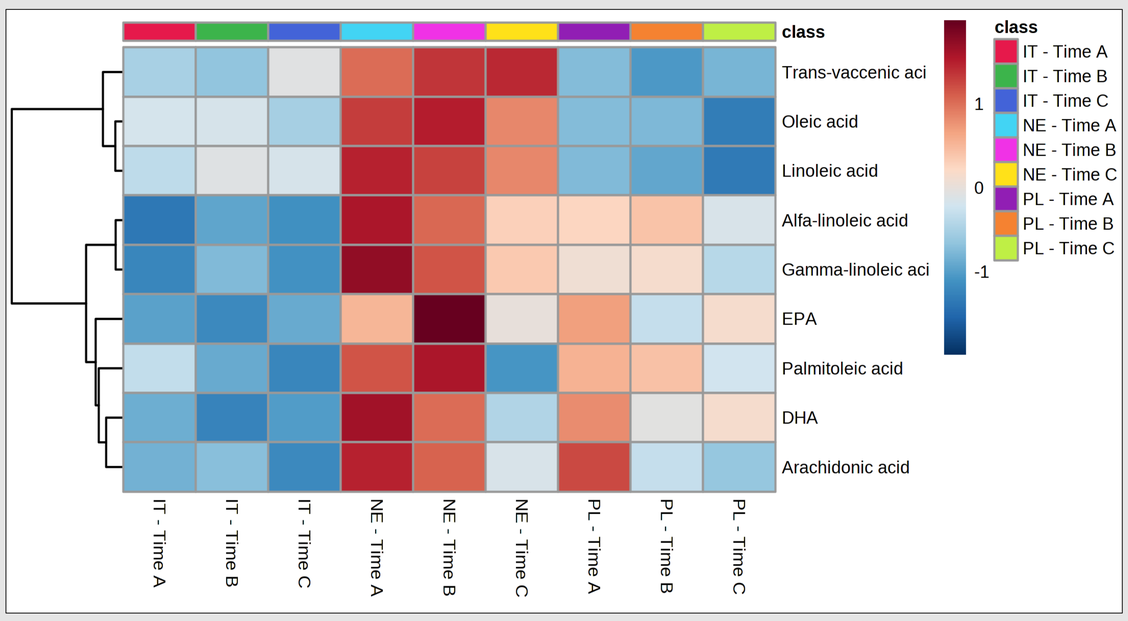

Figure 2. Heatmap presents relative differences in abundance of 9 quantified fatty acids among three populations (IT-Italy, NE-Netherlands and PL-Poland) in three time points (A, B and C)

Development of untargeted lipidomics reveals how High Fat Diet (HFD) and Low Fat Diet (LFD) influence lipid profiles of human milk. This analysis combines collection of spectra for hundreds of molecules coupling High Resolution Mass Spectrometry (HRMS). Use of sophisticated software enables identification of compounds based on their parental ions (MS1 spectra) and respective daughter ions (MS2 spectra), and classification to various lipid groups based on their retention time (RT). Using *Lipidex* software, we were capable to identify ≥500 lipids and differentiate between 5 patients with HFD and 6 with LFD. We have found 39 lipid molecules significantly differing those two group ($p<0.01$) with 29 triglycerides, 4 diglycerols, 1 phosphatidylserine, 1 sphingomyelin and 1 alkenyl triglyceride phosphate

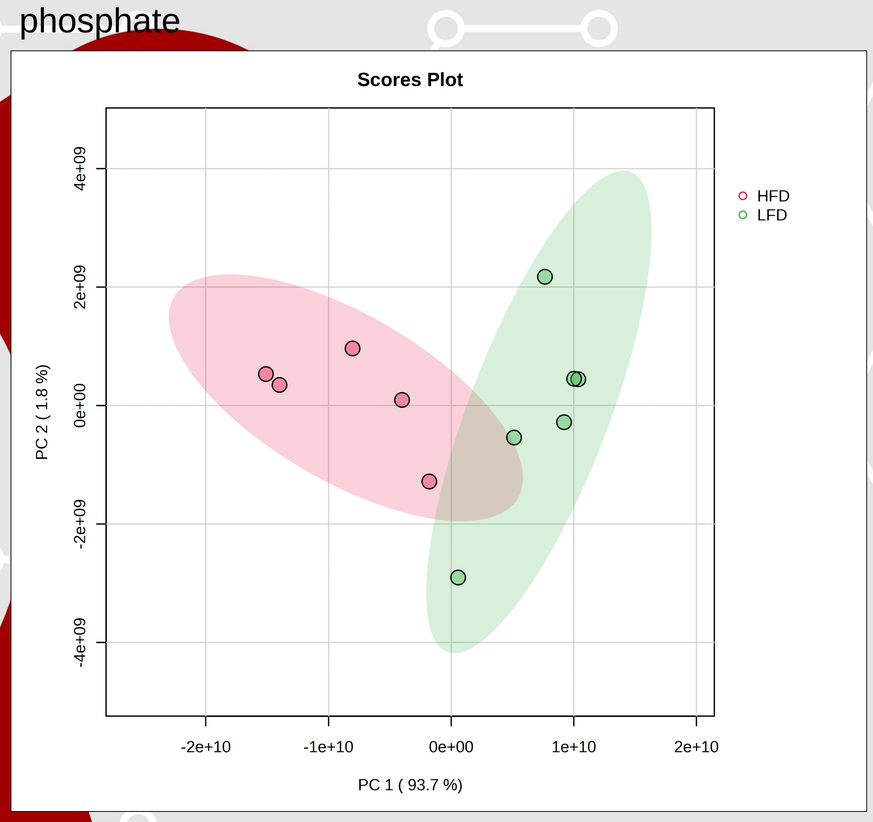

Figure 3. PCA graph shows patients clustered within HFD and LFD group based on the results for >400 lipids

Methodology

Samples were collected between 2019 - 2021 from representatives of the population of honorary breastmilk donors from one of three countries (Poland, Italy, Netherlands) as part of a multi-center project "The health-promoting importance of human milk donation" funded by the National Agency of Academic Exchange led by Medical University of Warsaw

Quantitative fatty acid analysis (Doyle method) using LC-MS/MS
50 µL of human milk sample was mixed internal standard solution in ACN. FAs hydrolysis based on Doyle protocol was performed using 6N HCl and 10N NaOH. FAs were extracted using hexane and stored at -80 °C for 30 mins. Organic phase was transferred to a new tube and evaporated to dryness under nitrogen flow in a water bath held at 50 °C. Lipid extract was reconstituted in 5% NH4OH in 65% MeOH solution and injected on UPLC coupled with Tandem Mass Spectrometer.

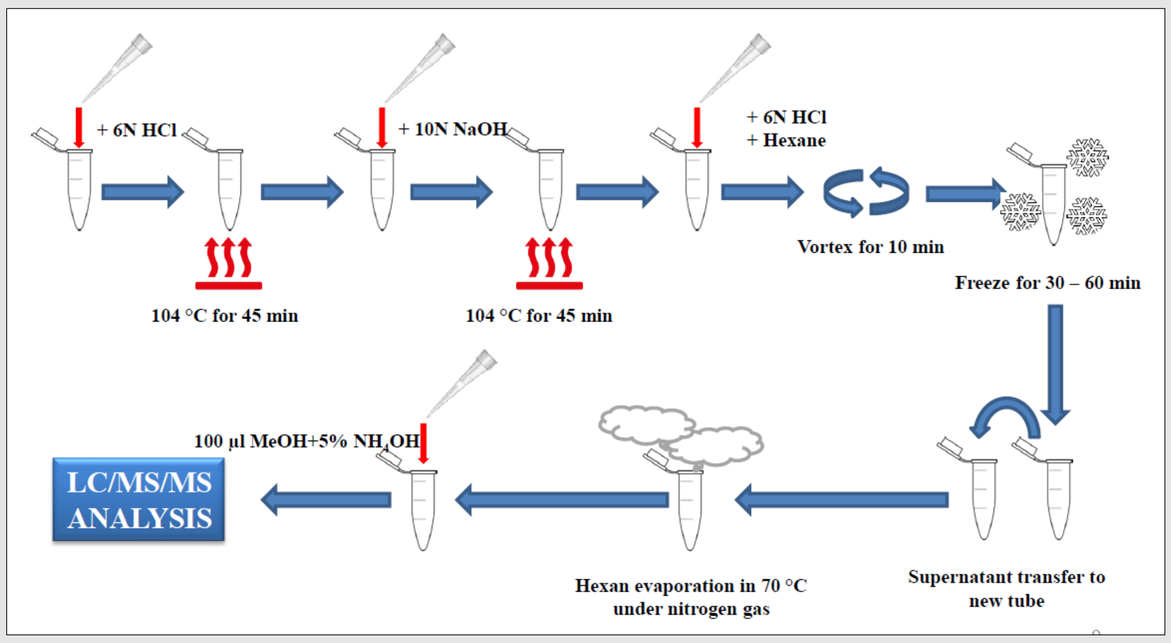

Figure 1. Graphical representation of the procedure steps in total fatty acid quantification using Doyle protocol for lipid extraction

Untargetted lipidomic analysis (Matyash method) using HRMS
30 µl of milk sample was mixed with cold MeOH and vortexed. Lipids were extracted with MTBE. Next cold MQ grade water was added to separate phases. After vortexing, samples were spun down. 700 µL of organic phase was transfered to a new tube, evaporated under nitrogen to dryness and reconstituted in Toluen:MeOH mixture (1:9).

Conclusions

1. Quantification of 9 fatty acids was possible thanks to validated LC-MS method to unveil lipidome profile changes in HM composition based on diet, lactation period and patients nationality
2. Results prove a time-wise change in milk's lipid composition and significant lipid profiles among three locations
3. Study found out lower abundance of fatty acids among Italians compared to Dutch and Polish
4. Study proves great influence of dietary choices on milk composition and possible implications of neonatal development
5. Untargeted lipidomics reveals differences in milk composition between women on HFD and LFD diets, proving this method to be a great tool of discovery new potential biomarkers of dietary effect on HM composition

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INSTITUTE OF BIOCHEMISTRY AND BIOPHYSICS POLISH ACADEMY OF SCIENCES

UNIVERSITAS MEDICA VARSOVIENSIS SALUTI PUBLICAE 1809

NAWA NARODOWA AGENCJA WYMIANY AKADEMICKIEJ

Contact me at:
m.radkiewicz@ibb.waw.pl
polim@ibb.waw.pl

Ms LAB IBB PAN