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Minimal variation of the plasma lipidome after delayed processing of neonatal cord blood.

John M Wentworth[1,2,5], Naiara G Bediaga[1,2], Megan AS Penno[3], Esther Bandala-Sanchez[1,2], Komal N Kanojia[4], Konstantinos A Kouremenos[4], Jennifer J Couper[3], Leonard C Harrison[1,2] and the ENDIA Study Group.

1. Walter and Eliza Hall Institute of Medical Research, Parkville, Australia
2. Department of Medical Biology, University of Melbourne, Parkville, VIC 3010, Australia.
3. Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide, South Australia, Australia
4. Metabolomics Australia, Bio21 Institute, University of Melbourne, Parkville, Australia
5. Corresponding author; wentworth@wehi.edu.au

Abstract

Background
Cord blood lipids are potential disease biomarkers. We aimed to determine if their concentrations were affected by delayed blood processing.

Method
Refrigerated cord blood from six healthy newborns was centrifuged every 12 hours for four days. Plasma lipids were analysed by liquid chromatography/mass spectroscopy.

Results
Of 262 lipids identified, only 8 varied significantly over time. These comprised three dihexosylceramides, two phosphatidylserines and two phosphatidylethanolamines whose relative concentrations increased and one sphingomyelin that decreased.

Conclusion
Delay in separation of plasma from refrigerated cord blood has a minimal effect overall on the plasma lipidome.

Birth cohort studies have incorporated cord blood collection and storage for disease biomarker discovery (1-4). Targeted lipidomics is a promising tool for biomarker discovery (5) but whether delay in blood processing, which is common when samples are shipped to a central laboratory (6), is a source of variation is not well documented. We aimed therefore to determine whether delays in cord blood processing affected the observed concentrations of plasma lipids.

With ethical approval from Walter and Eliza Hall Institute Human Research Ethics Committee and written informed consent from mothers of newborns, cord blood samples of 55-120mL in 21mL of citrate anticoagulant were obtained from six healthy infants (4M, 2F) born at between 35 and 41 weeks' gestation. Samples were transferred to the laboratory within 4h of collection, aliquoted into 1.5mL Eppendorf tubes and placed at 4˚C. Approximately every 12h for 4 days the tubes were inverted 5 times, centrifuged and the plasma stored at -80˚C.

Lipid was extracted by adding 100μL 1:1 butanol:methanol spiked with internal lipid standards (PC19:0/19:0, PE-d31, PG17:0/17:0, DG-d5 and TG-d5) to 10μL plasma, mixing for 1h at room temperature then sonicating and centrifuging at 17000xg for 10 minutes (7). A small amount of each sample supernatant was pooled to create 16 internal quality controls for loading at regular intervals during the run. Each supernatant was injected into an Agilent (Santa Clara, CA) 2.7μm Poroshell column with isopropanol:acetonitrile:water solvent and analysed by LC ESI-MS/MS using an Agilent 1290 liquid chromatography system and 6490 triple quadrupole mass spectrometer as previously described (8). Data processing using Agilent Mass Hunter software identified 265 lipids. Raw data for each lipid are provided as supplemental data. The coefficient of variation for each of the five internal standards within 5 external adult plasma quality control samples was less than 8%.

Three lipids whose CVs exceeded 20% in the internal quality control samples were excluded. Peak areas for the remaining 262 lipids were then normalised to internal standards (lipid-standard pairs provided as supplemental information) and $\log_2$-transformed and scaled to mean and standard deviation. Principal component analysis (PCA) to determine variation due to individual and processing delay was performed using the Phyloseq package within R software (v1.18.0; www.r-project.org). The paired LIMMA test with 5% false discovery rate (9) was used to identify significant differences in lipid abundance relative to the baseline sample, defined as an adjusted p value<0.05.

PCA demonstrated that refrigerated storage for more than 90 hours had minimal impact on intra-individual lipidomic reproducibility (Figure 1a). Individual differences between the six newborns accounted for 97.6% of the variability whereas delay in processing accounted for just 0.6%. Eight of the 262 lipids changed significantly from baseline at one or more of the 8 subsequent time points. All three dihexosylceramide (DHC) and both phosphatidylserine (PS) lipids progressively increased by at least 2-fold over baseline after 90 hours. Smaller changes were observed for two of the five plasmenylethanolamine (PE (P)) lipids and one of the 29 sphingomyelin (SM) lipids (Figure 1b).

Thus, only a minority of the 262 cord plasma lipids identified by targeted lipidomic profiling showed significant variation after delayed processing and storage at 4°C. This is consistent with a prior study that described delayed processing of EDTA blood stored on ice for 6 hours did not alter concentrations of 15 lipids that were also identified using our method (10). Nonetheless, storage of blood at room temperature for more than 2 hours increases lysophospholipid concentrations and, to a much greater extent, changes the concentrations of amino acids, carbohydrates and signaling molecules (10, 11).

The progressive changes in the concentrations of all measured PS and DHC lipids are likely to reflect specific biological processes. For example, PS is a marker of platelet activation (12) and apoptotic cell death (13), which are expected to increase over storage time. DHC, existing mostly as lactosylceramide in humans, can be produced by the action of lactose synthase in blood (14), potentially explaining the increase in DHC over time.

In summary, delayed processing of refrigerated cord blood has minimal impact overall on its plasma lipidomic profile. Nevertheless, time-dependent changes in the 8 variable lipids may have implications for biomarker discovery. The increases in PS and DHC are also potentially relevant for cord blood-based immune studies because PS is a broad-spectrum immunosuppressant (15) and lactosylceramide (DHC) promotes cell proliferation, migration and adhesion (16).

Conflict of interest
No author has a conflict of interest to declare

Author contributions
The study was devised by JMW, MASP and LCH, who with EB-S arranged sample collection and storage. Lipid data were generated by KK and analysed by KK and NGB. JMW and LCH drafted the manuscript and all authors contributed to the final version.

**Compliance with ethical requirements**
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Figure. Prolonged storage of cord blood at 4˚C affects a minority of plasma lipids identified by targeted lipidomics.
**a**. Principal components analysis of lipidomes of cord plasma collected from six healthy newborns and stored for up to 96h at 4˚C before processing and storage.
b. Changes over time in the relative abundance of lipids that varied significantly at one or more time point compared to baseline. The mean±SD processing delay for the six samples is indicated. PS, phosphatidylserine; PE (P), plasmenylethanolamine; DHC, dihexosylceramide; SM, sphingomyelin.

a.
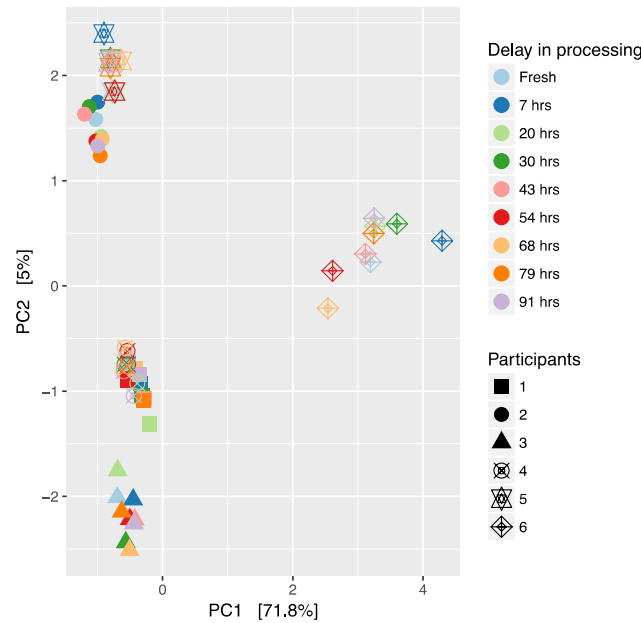
b.
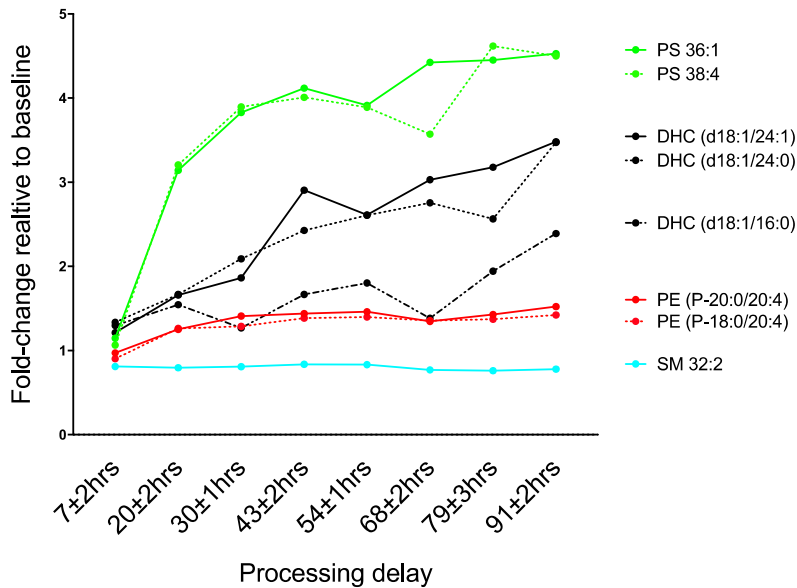
References

1. Janssen BG, Madlhoum N, Gyselaers W, Bijnens E, Clemente DB, Cox B, et al. Cohort Profile: The ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study. Int J Epidemiol. 2017.

2. Penno MA, Couper JJ, Craig ME, Colman PG, Rawlinson WD, Cotterill AM, et al. Environmental determinants of islet autoimmunity (ENDIA): a pregnancy to early life cohort study in children at-risk of type 1 diabetes. BMC Pediatr. 2013;13:124.

3. Vuillermin P, Saffery R, Allen KJ, Carlin JB, Tang ML, Ranganathan S, et al. Cohort Profile: The Barwon Infant Study. Int J Epidemiol. 2015;44(4):1148-60.

4. Lee HS, Burkhardt BR, McLeod W, Smith S, Eberhard C, Lynch K, et al. Biomarker discovery study design for type 1 diabetes in The Environmental Determinants of Diabetes in the Young (TEDDY) study. Diabetes Metab Res Rev. 2014;30(5):424-34.

5. Meikle PJ, Wong G, Barlow CK, Kingwell BA. Lipidomics: potential role in risk prediction and therapeutic monitoring for diabetes and cardiovascular disease. Pharmacol Ther. 2014;143(1):12-23.

6. Penno MA, Thomson RL, Couper JJ. Bunbury to Bundaberg, Darwin to Dover: establishing a successful Regional Participation Program for the ENDIA type 1 diabetes cohort study. Med J Aust. 2016;205(10):486.

7. Alshehry ZH, Barlow CK, Weir JM, Zhou Y, McConville MJ, Meikle PJ. An Efficient Single Phase Method for the Extraction of Plasma Lipids. Metabolites. 2015;5(2):389-403.

8. Huynh K, Pernes G, Mellett NA, Meikle PJ, Murphy AJ, Lancaster GI. Lipidomic Profiling of Murine Macrophages Treated with Fatty Acids of Varying Chain Length and Saturation Status. Metabolites. 2018;8(2).

9. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5(10):R80.

10. Kamlage B, Maldonado SG, Bethan B, Peter E, Schmitz O, Liebenberg V, et al. Quality markers addressing preanalytical variations of blood and plasma processing identified by broad and targeted metabolite profiling. Clin Chem. 2014;60(2):399-412.

11. Kamlage B, Neuber S, Bethan B, Gonzalez Maldonado S, Wagner-Golbs A, Peter E, et al. Impact of Prolonged Blood Incubation and Extended Serum Storage at Room Temperature on the Human Serum Metabolome. Metabolites. 2018;8(1).

12. Lentz BR. Exposure of platelet membrane phosphatidylserine regulates blood coagulation. Prog Lipid Res. 2003;42(5):423-38.

13. Schutters K, Reutelingsperger C. Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis. 2010;15(9):1072-82.

14. Yamato K, Yoshida A. Biosynthesis of lactosylceramide and paragloboside by human lactose synthase A protein. J Biochem. 1982;92(4):1123-7.

15.	Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell Death Differ. 2016;23(6):962-78.
16.	Chatterjee S, Pandey A. The Yin and Yang of lactosylceramide metabolism: implications in cell function. Biochim Biophys Acta. 2008;1780(3):370-82.