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Short Communication

The effect of haemolysis on the metabolomic profile of umbilical cord blood

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ABSTRACT

Objectives: Metabolomics is defined as the comprehensive study of all low molecular weight biochemicals, (metabolites) present in an organism. Using a systems biology approach, metabolomics in umbilical cord blood (UCB) may offer insight into many perinatal disease processes by uniquely detecting rapid biochemical pathway alterations. In vitro haemolysis is a common technical problem affecting UCB sampling in the delivery room, and can hamper metabolomic analysis. The extent of metabolomic alteration which occurs in haemolysed samples is unknown.

Design and methods: Visual haemolysis was designated by the laboratory technician using a standardised haemolysis index colour chart. The metabolomic profile of haemolysed and non-haemolysed UCB serum samples from 69 healthy term infants was compared using both ^{1}H-NMR and targeted DI and LC–MS/MS approach.

Results: We identified 43 metabolites that are significantly altered in visually haemolysed UCB samples, acylcarnitines (n = 2), glycerophospholipids (n = 23), sphingolipids (n = 7), sugars (n = 3), amino acids (n = 4) and Krebs cycle intermediates (n = 4).

Conclusion: This information will be useful for researchers in the field of neonatal metabolomics to avoid false findings in the presence of haemolysis, to ensure reproducible and credible results.

Introduction

Interest in the effect of early life status on lifelong health is increasing. As a result, many predictive, diagnostic and prognostic biomarker discovery studies have begun to biobank and use umbilical cord blood (UCB) as their investigative specimen of choice [1]. However, haemolysis is a common pre-analytical problem that arises in UCB sampling in the delivery room.

Haemolysis (or hemolysis) results when red blood cells are ruptured and their contents are released into the surrounding fluid, following damage to the cell membrane. Haemolysis of UCB can occur in vivo but more commonly in vitro due to a combination of high haematocrit [2] and specimen collection errors. For example, difficult collection or handling, incorrect needle size, unnecessary mixing, under fill of the sample tube and excessive force, can all result in the breakdown of red blood cells which contaminate the surrounding serum or plasma [3].

Metabolomics is the systematic study of temporal interactions between the compliment (metabolome) of low molecular weight (bio)-chemicals (metabolites) abundant within living organisms, tissues and cells. In UCB, metabolomics allows the measurement of rapid biochemical alterations and may offer early insight into many perinatal disease processes. Unfortunately haemolysis introduces high pre-analytical variability, resulting in potentially unreliable results, and recommendations have been made to avoid using haemolysed samples in metabolomic experiments [4]. However, neonatal samples for biomarker discovery are difficult and expensive to collect, and may be collected to examine rare or orphan diseases. Excluding all haemolysed samples is therefore not ideal, and may affect study feasibility.

In the present study, retrospective data from two previous metabolomic investigations [5,6], were combined to assess whether or not the visual haemolysis of UCB serum affects metabolite concentrations in a healthy control population. Metabolites must demonstrate robustness to haemolysis in order to translate to the clinical setting. Knowledge from this study will facilitate this translation by allowing

Abbreviations: UCB, umbilical cord blood; SOPs, standardised operating procedures; ^{1}H-NMR, proton nuclear magnetic resonance; DI, direct infusion; LC, liquid chromatography; MS/MS, tandem mass spectrometry; SD, standard deviation; CI, confidence interval; PC, phosphatidylcholines; lysoPC, lysophosphatidylcholines; SM, sphingolipids; IQR, interquartile range.

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researchers to avoid false results, and may enable correction for sample haemolysis.

Materials and methods

Patient selection

Retrospective data from an ongoing birth cohort study, the Cork BASELINE Birth Cohort Study, recruited between June 2010 and July 2011 was analysed [1]. Ethical approval was granted by the clinical research ethics committee of the Cork Teaching Hospitals, and written informed consent from all participants was obtained. Healthy control infants were selected based on the following criteria; Apgar scores ≥8 at 1 min, ≥9 at 5 min, duration of ruptured membrane <24 h, temperature in labour ≤37 °C, gestational age ≥36 weeks, cord arterial pH ≥7.2 and birth weight centile ≥10%. This population had no underlying medical issues and normal neonatal exams.

Sample collection and storage

UCB was drawn in all infants using standardised operating procedures (SOPs). In brief, 6 mL was collected in a plain serum tube (BD Vacutainer no. 366431) within 20 min of placental delivery and allowed clot for 30 min at 4 °C, before centrifugation (2400 ×*g*, 10 min, 4 °C). The serum was transferred to a second spin tube and centrifuged (3000 ×g, 10 min, 4 °C) before being aliquoted into lithium heparin microtubes (VWR no. 89179-704) and stored at −80 °C until analysis. Total time from birth to samples being frozen at −80 °C was always under 3 h.

Haemolysis

The haemolysis status was designated by laboratory technicians. In order to standardise visual assessment of the separated serum, a haemolysis index colour chart (Mayo Medical Laboratories, T 598) was used. If a sample was dark orange-red in colour (corresponding haemoglobin ≥ 100 mg/dL), it was deemed haemolysed.

Metabolomic analysis

Using both nuclear magnetic resonance (^{1}H-NMR) spectroscopy and combined targeted direct infusion (DI-) and liquid chromatography (LC-) tandem mass spectrometry (MS/MS) assay (AbsolutIDQ p180 kit, Biocrates Life Sciences AG, Innsbruck, Austria), we have described the metabolomic profile of UCB from healthy term infants [5,6] and evaluated the preanalytical effects of haemolysis to describe metabolite alterations. Quality control (QC) samples were employed to evaluate the precision and repeatability of the metabolite quantification. A detailed description of the metabolomic methods has been previously reported [5,6].

Statistical analysis

Statistical analysis was performed using SPSS version 21.0. Metabolite data was normalised using log transformation for parametric tests and the antilog of the mean transformed data was reported; otherwise median and non-parametric tests were used. Statistical comparison between haemolysis and clean serum groups was tested individually using Mann–Whitney *U* test or using the Student's *t*-test.

No correction for multiple comparisons was performed, as the aim of this study was not to reduce the probability of false discovery, but to create a non-conservative "watch" list of haemolysis sensitive metabolites that could potentially confound any future biomarker studies.

Results

UCB serum samples (n = 69) were classified as being with (haemolysed, n = 13) or without (clean, n = 56) haemolysis upon visual inspection post-centrifugation. All 69 samples underwent initial mass spectrometry metabolomic analysis using a combined targeted DI and LC–MS/MS approach. For ^{1}H-NMR spectroscopy, ten samples were excluded due to insufficient sample volume, leaving 59 samples for the analysis. See Table 1 for demographic details of the study cohort.

Using the targeted method 148 metabolites were identified and quantified; 35 metabolites were significantly altered between groups, see Table 2. Separately, 37 were identified and quantified using ^{1}H-NMR spectroscopy and eight unique metabolites were significantly altered between the clean (n = 48) and haemolysed (n = 11) groups, see Table 2.

Evident from the fold change values in Table 2, is the magnitude of metabolite concentration change with visual haemolysis. For example, acylcarnitines displayed an average fold change increase of 1.2 and glycerophospholipids an average fold change decrease of 1.3. Metabolites closely associated with Krebs cycle, acetate, formate and succinate, and the non-essential amino acid ornithine, all showed large fold increased concentrations of 1.5, 1.8, 1.9 and 1.5 in haemolysed samples respectively.

Table 1
Clinical and demographic details for the healthy control study populations of two separate metabolomic investigations.

	^{1}H-NMR spectroscopy cohort (n = 59)		Mass spectrometry cohort (n = 69)	
	Haemolysed (n = 11)	Clean (n = 48)	Haemolysed (n = 13)	Clean (n = 56)
Gestational age (weeks)	39.6 (1.2)	40.3 (0.9)	39.7 (1.1)	40.2 (0.9)
Gender (male/female)	7/4	32/16	12/12	38/18
Birth weight (gm)	3430 (595)	3667 (474)	3448 (484)	3627 (480)
Weight centile (%)	64 (10, 80)	62 (26, 86)	55.4 (32.0)	48.6 (31.0)
Method of delivery				
Spontaneous vaginal	5 (46%)	12 (25%)	5 (39%)	17 (30%)
Instrumental assisted	4 (36%)	25 (52%)	6 (46%)	26 (46%)
Emergency caesarean section	2 (18%)	10 (21%)	2 (15%)	12 (21%)
Elective caesarean section	–	1 (2%)	–	1 (2%)
Apgar score 1 min	9 (8,9)	9 (9,9)	9 (8,9)	9 (9,9)
Apgar score 5 min	9 (9,10)	10 (10,10)	9 (9,10)	10 (9,10)
Maternal ethnicity				
Caucasian	10 (91%)	47 (98%)	12 (92%)	55 (98%)
Indian	1 (9%)	–	1 (8%)	–
Asian	–	1 (2%)	–	1 (2%)
Maternal age (years)	30.7 (6.7)	29.6 (4.5)	31.0 (6.2)	29.2 (4.7)
Maternal BMI (kg/m^2)	23.7 (4.0)	23.9 (3.5)	24.4 (4.0)	24.2 (4.2)

Values are mean (SD), median (interquartile range), or n (%).

Discussion

This study examined the effect of visual haemolysis on UCB serum metabolites using quantitative metabolomic methods DI and LC–MS/MS and ^{1}H-NMR, and found that 43 metabolites were significantly altered when comparing haemolysed and clean serum. These metabolites belonged to several classes; acylcarnitines (n = 2), glycerophospholipids (n = 23), sphingolipids (n = 7), sugars (n = 3), amino acids (n = 4) and Krebs cycle intermediates (n = 4), see Table 2. The p-values presented in Table 2 may seem marginal, but it is important to note that this was a retrospective study with only a small number of visually haemolysed samples. However, the fold-change values do indicate potential confounding effects in any true disease biomarker discovery.

Concentration changes for each metabolite were examined as these may arise from the metabolite being either degraded or metabolized by enzymes released from haemolysed erythrocytes [4]. Besides haemologlobin, erythrocytes also contain several structural proteins, lipids and carbohydrates which could influence and interfere with the sample [7]. Out of a total of 185 quantified metabolites, 56 metabolites were found to have increased concentrations in visually haemolysed samples, 34 and 22 detected by DI and LC–MS/MS and ^{1}H-NMR respectively. Of the significantly altered metabolites, nine displayed concentration increases in haemolysed samples, while the remaining decreased. Increases occurred in both acylcarnitines; L-acetylcarnitine and hexanoylcarnitine, a sugar glycerol, an amino acid phenylalanine, a non-essential amino acid ornithine, and the kerb cycle intermediates, acetate, citrate, formate and succinate. A recent study evaluating the effect of pre-analytical variables in blood and plasma found 81 blood metabolites which were significantly altered by haemolysis, with an increase in 50 metabolites and a decrease in 31 [8].

The erythrocytes and monocytes released into the cytoplasm have the ability to convert arginine to ornithine [9], which is in agreement with our results. Though not significantly altered by haemolysis, arginine displayed a concentration decrease in haemolysed serum (78.9 IQR 67.4–97.7 μM) compared to clean (86.8 IQR 74.0–97.7 μM) and ornithine the corresponding increase in concentration. Increasing acetate and decreasing glucose has also been previously shown by Theil et al., examining plasma metabolites from bovine samples [10]. Likewise, false elevations in creatinine have been documented in haemolysed samples during clinical testing [3]. We found that creatinine concentrations increased from clean (23.0 IQR 17.9–27.2 μM) to visually haemolysed (25.4 IQR 21.5–27.9 μM) serum.

Table 2
Median or *mean metabolite concentration with 95% confidence intervals (CI), of metabolites significantly different when visually haemolysed and clean UCB serum samples were compared. The percentage relative standard deviation (%RSD) of the QC samples, for each metabolite is given. Fold change of increased ↑, or decreased ↓ concentration in haemolysed samples.

Metabolite	Clean serum median/*mean μM (95% CI)	Haemolysed serum median/*mean μM (95% CI)	%RSD QC	Fold change	Increase/Decrease	p-Value
Mass spectrometry						
L-Acetylcarnitine	3.8 (3.3, 4.2)	4.8 (3.7, 6.1)	3	1.2	↑	0.0205
Hexanoylcarnitine	0.07 (0.07, 0.08)	0.08 (0.07, 0.1)	14	1.2	↑	0.0287
PC aa C38:0	1.7 (1.5, 1.8)	1.3 (1.1, 2.0)	6	−1.3	↓	0.0355
PC aa C38:3	40.1 (38.1, 43.1)	32.2 (27.3, 42.5)	8	−1.2	↓	0.0355
PC aa C38:5	26.4 (24.4, 28.2)	20.7 (16.7, 25)	8	−1.3	↓	0.0097
PC aa C38:6	75.5 (68.9, 82.3)	57.3 (47.2, 76.7)	4	−1.3	↓	0.0246
PC aa C40:4	3.2 (2.9, 3.6)	2.3 (2.0, 3.1)	4	−1.4	↓	0.0071
PC aa C40:5	6.6 (5.6, 7.6)	4.8 (3.6, 5.8)	2	−1.4	↓	0.0060
PC aa C40:6	30.3 (26.7, 32.2)	21.8 (15.1, 30.1)	5	−1.4	↓	0.0089
PC aa C42:0	0.5 (0.4, 0.5)	0.4 (0.2, 0.7)	3	−1.2	↓	0.0288
PC ae C32:1	2.4 (2.1, 2.6)	1.8 (1.2, 2.5)	3	−1.4	↓	0.0209
PC ae C34:3	1.2 (1.1, 1.3)	0.9 (0.6, 1.4)	3	−1.3	↓	0.0193
PC ae C38:4	8.7 (8.0, 9.8)	6.9 (6.1, 9.7)	2	−1.3	↓	0.0255
PC ae C38:5	7.7 (7.1, 8.2)	6.4 (4.5, 9.3)	2	−1.2	↓	0.0478
PC ae C38:6	3.4 (3.2, 3.8)	2.7 (1.6, 3.8)	3	−1.3	↓	0.0266
PC ae C40:4	1.8 (1.7, 1.9)	1.3 (1.2, 1.9)	5	−1.3	↓	0.0135
PC ae C40:5	1.6 (1.4, 1.7)	1.3 (1.0, 1.8)	5	−1.2	↓	0.0222
PC ae C40:6	2.5 (2.3, 2.8)	2.0 (1.2, 3.1)	3	−1.3	↓	0.0420
PC ae C44:4	0.3 (0.3, 0.3)	0.2 (0.2, 0.3)	4	−1.4	↓	0.0362
PC ae C44:5	1.1 (1, 1.2)	0.8 (0.5, 1.1)	2	−1.3	↓	0.0342
PC ae C44:6	0.9 (0.8, 0.9)	0.7 (0.4, 1.)	2	−1.3	↓	0.0390
lysoPC a C16:0	59.75 (54.9, 62.9)	49.9 (37, 63.9)	5	−1.2	↓	0.0355
lysoPC a C16:1	4.5 (3.9, 4.9)	3.3 (2.5, 4.8)	2	−1.4	↓	0.0156
lysoPC a C18:0	10.3 (9.8, 10.7)	8.3 (6.3, 9.8)	6	−1.2	↓	0.0129
lysoPC a C18:1	13 (11.6, 14.6)	9.1 (6.3, 14.1)	5	−1.4	↓	0.0266
SM (OH) C16:1	1.5 (1.4, 1.6)	1.4 (1, 1.8)	12	−1.1	↓	0.0444
SM (OH) C22:2	3.5 (3, 3.7)	2.8 (2, 3.6)	4	−1.2	↓	0.0428
SM (OH) C24:1	1 (0.9, 1.1)	0.9 (0.6, 1.1)	3	−1.1	↓	0.0428
SM C16:1	10.2 (9.5, 11.1)	7.8 (7.4, 11.1)	4	−1.3	↓	0.0323
SM C22:3	0.4 (0.3, 0.4)	0.2 (0.1, 0.4)	2	−1.6	↓	0.0121
SM C24:0	16.4 (14.7, 17.4)	12.5 (10.3, 17.5)	3	−1.3	↓	0.0197
SM C24:1	27.7 (25.7, 29.1)	20.9 (12.2, 30)	4	−1.3	↓	0.0305
Hexose	4064 (3844, 4500)	3451 (3361, 4070)	3	−1.2	↓	0.0124
Glycine*	252.5 (236, 265)	220 (192, 262)	1	−1.1	↓	0.0021
Serine*	128.5 (124.1, 133.1)	116.8 (103.7, 131.5)	3	−1.1	↓	0.0359
^{1}H-NMR spectroscopy						
Acetate	14.7 (12.7, 17.5)	21.3 (12.4, 46.1)	4	1.5	↑	0.0504
Citrate	35.2 (23.4, 40.4)	44.5 (36.2, 52.9)	17	1.3	↑	0.0185
Formate	11.2 (10, 13.3)	20.7 (8.4, 42)	14	1.8	↑	0.0293
Glucose*	1666.7 (1575.7, 1763)	1488.7 (1411.8, 1569.8)	7	−1.2	↓	0.0037
Glycerol	16.7 (14.6, 19.1)	23.1 (9.8, 33.1)	16	1.4	↑	0.0460
Ornithine	17.6 (15.5, 20.2)	26.9 (15.3, 36.5)	8	1.5	↑	0.0265
Phenylalanine	26.6 (24.9, 30.9)	34.2 (23.6, 38.8)	7	1.3	↑	0.0482
Succinate	1.5 (1.2, 1.8)	2.8 (1.2, 6.9)	12	1.9	↑	0.0022

QC; quality control, RSD; relative standard deviation, PC; phosphatidylcholines, lysoPC; lysophosphatidylcholines and SM = sphingolipids.

All remaining metabolites, particularly the lipids (phosphatidylcholines, lysophosphatidylcholines and sphingolipids), had decreased concentrations in haemolysed samples, potentially being degraded by erythrocyte enzymes or diluted by chemical interference. A study by Yin et al. using a non-targeted LC metabolomic approach found 69 mass features significantly changed comparing haemolysed and control plasma samples. Two of these mass features were identified as the lysophosphatidylcholines (lyso-PCs) C16:0 and C18:0 which showed increased concentrations in haemolysed samples, while lyso-PC C16:0 showed a strong correlation ($p = 0.0003$; $r = 0.61$) with free haemoglobin [4]. Our results indicate that these lipids decrease with haemolysis in UCB.

In the present study, UCB was meticulously biobanked in adherence to strict SOPs [1], to ensure that samples were suitable for metabolomic analysis. The prevalence of haemolysis in routine venepuncture samples has been described as 3.3% [3], however, the prevalence in biobanked UCB is unknown. Visible haemolysis as an indicator of erythrocyte breakdown is only apparent when the serum or plasma has been separated; therefore, the opportunity to resample from the placenta has elapsed.

A limitation of this study was the subjective visual verification of haemolysis [11]. Instead a spectrophotometric technique could be used to quantify haemolysis using the haemolysis index.

Though our results seem biologically plausible, the sample size of our study is relatively small and may be illustrating population differences in metabolites which are highly variable, rather than analytical differences. However our samples were selected from a homogeneous population of normal healthy full term infants in an attempt to leave haemolysis as the sole discernible difference.

Conclusion

The results from our study have indicated potential differences in UCB serum metabolites in visually haemolysed samples. These differences may be the result of chemical interference or concentration changes of analytes, caused by the release of free haemoglobin and other intracellular contents from the erythrocyte. This is the first time that UCB metabolite levels have been compared in clean and haemolysed serum, from healthy term infants.

Haemolysis remains a major concern for both clinical and research laboratories, by contributing to erroneous results. Excluding haemolysed samples from biomarker discovery is not always feasible; therefore, future work in the field of metabolomics must concentrate on defining a list of peaks or metabolites that are unreliable to measure in haemolysed samples.

Conflict of interest statement

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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