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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 (%).