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EXTENDED ABSTRACT

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Impact of prematurity on newborn urine: a metabolomics strategy to identify markers of organ maturity

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Introduction

Preterm birth (PTB), defined by the World Health Organization as birth occurring before 37 completed gestational weeks (g.w.) [1], is the leading cause of neonatal deaths [2]. It is also the major cause of death in children under 5 years-of-age, corresponding to *ca*. 1 million deaths in 2015 [3]. In Portugal, ca. 8.0% of all neonates were born prematurely in 2018, and 0.2% died before 28 days of life [4]. Depending on gestational age, PTB is divided into sub-categories, with different prognosis: extremely preterm (E-PTB, <28 g.w.), very preterm (V-PTB, 28-31 g.w.), moderate preterm (M-PTB, 32-33g.w.) and late preterm (L-PTB, 34-36 g.w.) [1]. Metabolomics, defined as the qualitative and quantitative analysis of metabolites present in a biological system [5], has been used to study PTB at pre- and post-natal levels [6,7] with the aim of identifying predictive markers and assessing PTB babies health status, respectively.

In this work, Nuclear Magnetic Resonance (NMR) spectroscopy was used to analyze the urine of PTB newborns and the resulting spectra were handled in order to: a) differentiate distinct PTB stages in terms of metabolic behaviour; and b) establish a urinary metabolic trajectory of PTB newborns until theoretical term-time, while searching for markers of organ maturity.

Methods

Samples and sample analysis: Urine was collected at the Maternity Bissaya Barreto, University Hospital Center of Coimbra, under the following ethical approvals (18/04, 29/09 and 0159/CES), for PTB babies: E-PTB (n=2), V-PTB (n=9), M-PTB (n=5), L-PTB (n=28) and healthy newborns (hNB, n=46). Informed parental consent was obtained for each infant and clinical information was obtained from neonatal medical records. Additionally, 18 PTB newborns were followed during probation in the Neonatal Intensive Care Unit (NICU) and urine samples were collected longitudinally. Sample collection procedures, preparation and NMR spectral acquisition conditions have been described elsewhere [8]. Unidimensional ^{1}H NMR spectrum was acquired for each sample and, for selected samples, 2D spectra were acquired to aid peak assignment.

Data analysis: NMR spectra were transformed into a matrix of n rows (samples) and m columns (data points). Prior to multivariate analysis (MVA), the water, urea and ethanol peaks were removed and the spectra were aligned [9] and normalized to total area. Unsupervised and supervised methods, namely Principal Component Analysis (PCA) and Partial Least-Discriminant Analysis (PLS-DA), respectively, were performed (SIMCA-P 11.5). Peaks were integrated (Amix 3.9.5) and comparison between groups was performed through effect size determination [10] and p-values (Wilcoxon test). In order to assess the predictive power of PLS-DA models, Monte Carlo Cross Validation was performed, with recovery of Q^2 values and confusion matrices.

Results and discussion

Typical newborn urine proton NMR spectra comprise information on several metabolites (Figure 1). 1D/2D NMR enabled the identification of *ca*. 60 compounds, including amino acids and derivatives, sugars, organic acids and other compounds.

Keywords:
preterm birth; multivariate statistical analysis; prematurity markers

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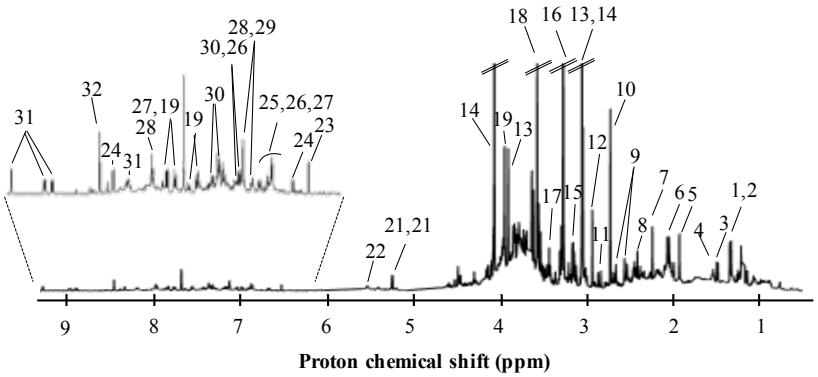

Figure 1 - Average ^{1}H NMR spectrum of urine of healthy newborns. Legend: 1, lactate; 2, threonine; 3, alanine; 4, adipate; 5, acetate; 6, N-acetylneuraminic acid; 7, acetone; 8, succinate; 9, citrate; 10, dimethylamine; 11, methylguanidine; 12, dimethylglycine; 13, creatine; 14, creatinine; 15, ethanolamine; 16, betaine; 17, taurine; 18, glycine; 19, hippurate; 20, glucose; 21, lactose; 22, sucrose; 23, fumarate; 24, *N*-methyl-2-pyridone-5-carboxamide; 25, 4-hydroxyphenylacetate; 26, tyrosine; 27, 4-hydroxy-hippurate; 28, 1-methylhistidine; 29, histidine; 30, phenylacetylglutamine; 31, *N*-methylnicotinamide; 32, formate.

To compare the metabolic profile of newborn urine between different sub-categories and hNBs at birth, a cross-sectional study was performed. Complementary selected pairwise comparisons were performed (VE-PTB (very and extreme) *vs* ML-PTB (moderate to late) and each one *vs* hNB. The PLS-DA quality parameters obtained (Table 1) revealed robust models only when extreme groups were compared (see Q^2 values > 0.5).

Table 1 - PLS-DA quality parameters for pairwise models obtained with two different scalings (centered and unit variance). N/A, not applicable: this model was not performed due to low sample numbers; CR, classification rate; Q^2, predictive power (values in bold indicate robust models); sens, % sensitivity; spec., %specificity; UV, unit variance.

	Scaling	CR	sens.	spec.	Q^2 median
step by step evaluation					
V (n=9) vs E (n=2)	centered	N/A	N/A	N/A	N/A
	UV	N/A	N/A	N/A	N/A
M (n=5) vs V (n=9)	centered	69	37	87	-0.16
	UV	69	37	87	-0.16
L (n=28) vs M (n=5)	centered	84	92	41	0.086
	UV	88	93	63	0.15
hNB (n=46) vs L (n=28)	centered	83	92	68	0.45
	UV	82	94	64	0.44
selected models					
ML (n=33) vs VE (n=11)	centered	69	8	89	-0.039
	UV	67	19	82	-0.0042
ML (n=33) vs hNB (n=46)	centered	52	32	65	-0.15
	UV	84	67	97	**0.54**
VE (n=11) vs hNB (n=46)	centered	96	89	98	**0.76**
	UV	94	78	97	**0.84**

This was confirmed by the PLS-DA scores plot comparing VE-PTBs and hNBs (Figure 2), which showed one of the highest Q^2 values. These results indicate that changes during probation occur gradually, so that statistically relevant differences are only noted when more extreme stages are compared.

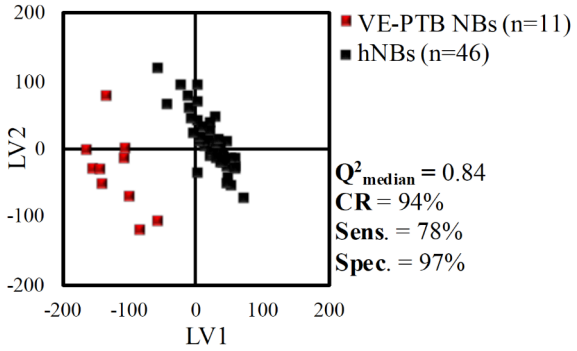

Figure 2 - Pairwise PLS-DA scores plot between extremely + very preterm newborns (n=11) and controls (n=46). CR, classification rate; hNBs, healthy newborns; Q^2, predictive power; Sens., % sensitivity; Spec., % specificity; VE-PTB

Metabolites which vary significantly between groups included amino acids (creatine, histidine, lysine, taurine and tyrosine), organic acids (3-aminoisobutyrate, 4-deoxythreonic acid, 4-hydroxyphenylacetate, 4-hydroxyhippurate, acetoacetate, adipate, cis-aconitate, citrate, formate, lactate, malonate, *N*-acetylneuraminic acid, pyruvate and succinate), sugars (galactose, glucose), other compounds (1-methylhistidine, *N*-methyl-2-pyridone-5-carboxamide, acetone, allantoin, carnitine, dimethylamine, dimethylglycine, hypoxanthine, methylguanidine, *m*-inositol, *N*-methylnicotinamide, phenylacetylglutamine, trigonelline and xanthine) and 11 still unknown signals.

Some of the above metabolites were selected for follow-up of the longitudinal cohort. In fact, some metabolites never reached healthy levels, even at theoretical term time (creatine,3-aminoisobutyrate, malonate, creatinine, ethanolamine, methylguanidine and taurine), whereas others, probably more-organ-maturity-dependent, (e.g. glucose, *N*-methylnicotinamide, lysine, carnitine, 4-hydroxyphenylacetate, succinate, xanthine, pyruvate, dimethylamine, 4-hydroxyhippurate, cis-aconitate, *N*-acetylneuraminic acid, 1-methylhistidine, *N*-methyl-2-pyridone-5-carboxamide) attained healthy levels. On the other hand, histidine was always at healthy levels. Other variations were found for tyrosine, 4-deoxythreonic acid, acetate, adipate, formate, lactate, acetone, allantoin, dimethylglycine, galactose, hypoxanthine, *m*-inositol, phenylacetylglutamine and trigonelline. This analysis may enable the follow-up of the specific development processes of the newborn lungs, liver, kidney and gut microflora during probation.

Conclusions:

Multivariate statistical analysis is useful for differentiating PTB subcategories and healthy newborns and identifying important varying metabolites, potentially enabling metabolic markers of organ maturity and immaturity to be advanced.

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