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NMR based serum metabolomics for monitoring newborn preterm calves' health

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Abstract

It was aimed to detect the novel future biomarkers using a metabolomics approach in premature calves. Calves born previous to 270 days' pregnancy are at risk, and the earlier the calving is, the higher the risk. More trials are needed in neonatology field as it little known almost the generally metabolic status of preterm neonates. To date, this is the first NMR (nuclear magnetic resonance) based study on serum metabolomics at set intervals in premature calves. Biochemical health profiles and NMR based metabolomic analysis were performed in twenty-five premature dairy calves. The whole animals partly recovered following 72h. Clinical data were compatible with those of premature animals. Increased levels of AST and CPK may be attributed to subclinic trauma at birth. Alterations in metabolites, increases in 3-hydroxybutyrate, citrate, leucine and isoleucine at 48th and 72h; choline, formate, fatty acids and polyunsaturated fatty acids at 72h, and valine at 48h; and decreases in myo-inositol at 48h and 72h were meaningful for monitoring the recovery at a molecular level in premature calves. Metabolomics became an important tool for identification of premature calves' clinical pathology and monitoring therapeutic picture.

Key Words: Premature calf, Omics, Metabolomics, NMR.

Introduction

Although the preterm birth rate of calves is not known in the world, premature calving remains a major participant in neonatal calf mortality and morbidity. While studying biomarkers in premature calves [30,1] their utility has not been accepted widely, because a stronger signature of varying metabolites, suggestive of disturbances in nucleotide metabolism, lung surfactants biosynthesis and renal function, along with enhancement of tricarboxylic acid cycle activity, fatty acids oxidation, and oxidative stress are features of premature newborns because a stronger signature of varied metabolites, implicational disturbances in nucleotid metabolism, respiratory organ surfactants biogenesis and excretory organ perform,

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beside improving of tricarboxylic acid cycle activity, fatty acids reaction, and oxidative stress are options of premature newborns [11]. Hence, more understanding approximately the metabolic and improvement forms of neonates which can be assist valuable within the change of their clinical administration is required. Metabolomics offers the capability for identifying the variations in metabolite profiles that can be applied for disease discrimination. This is notably necessary for neonatal and pediatric studies particularly for severely sick patient diagnosing and early identification [21]. In previous studies [3,4] we took advantage of metabolomics to the way better understanding of extreme sepsis in neonatal calves, and bronchopneumonia in calves. Metabolomics may be useful for understanding the longer-term effects of biomarkers of preterm birth [14]. In spite of metabolomic studies in human medicine for premature infants, a metabolomic approach is not encountered in premature calves. This study aimed at evaluating the NMR based metabolomics at set intervals in serum of neonatal premature calves.

Materials and Methods

Ethical Committee: The Committee on Animal Research and Ethics of Faculty of Veterinary Medicine at Selcuk University, approved this experimental design (Protocol Number: 2018/18). All therapy protocol carried out following the Committee rules was needed for supporting by the European Commission.

Calves and Prematurity Criteria: Twenty-five neonatal premature calves, gestation length of around 7 to 8 months, were used. General weakness, difficulty or unable to stand, weakening or absence of sucking reflex, soft nails, an incomplete eruption of the incisor teeth from gums, short and silky hair coat, low body weight, dyspnea, and hypothermia made up inclusion criteria. The calves were treated by oxygen (administered at 10 L/min.) and antibiotic (aerosolized ceftiofur sodium, 1 mg/kg, IM, for five days), vitamins (vitamin A 1.000.000 IU, vitamin E 100 mg) and minerals (Ca 0.5 g, P 0.25 g, Se 1 mg), and critical care (setting and including dextrose, amino acid and lipid emulsion solutions without electrolytes) was performed, as needed.

Blood Sampling: Serum samples were obtained by centrifuging (1000 ×g for 15 min at 4°C) the blood collected at 24, 48, and 72 hours.

Biochemical Analyses: Biochemical parameters that reflect health profile (glucose, cholesterol, triglyceride, total protein, albumin, bilirubin, urea nitrogen, creatinine, enzymes (AST, ALT, ALP, GGT) and minerals (Ca, P, Mg) in serum samples was performed by using commercial kits (Mindray Chemistry Reagents) via an automated analyzer in our clinical laboratory.

Nuclear Magnetic Resonance Analysis: Thawing serum samples on ice and extracting them eliminate macromolecules (e.g., proteins) and establish a fused metabolic profile for water-soluble and lipid-soluble metabolites [25]. After dissolving water-soluble extracts in 700 µL of $^{2}H_2O$ and homogenization by vortexing for 1 min., and centrifugation (3000 rpm at 4°C for 15 min), each supernatant (630 µL) was added to 70 µL of potassium phosphate buffer (1.5 M K_2HPO_4, 100% (v/v) $^{2}H_2O$, 2 mM NaN_3, 5.8 mM deuterated trimethylsilyl propanoic acid (TMSP); pH 7.4). After stirring, a total of 600 µL from each mixture was transferred into 5 mm NMR tubes. After dissolving lipid extracts in 700 µL of $CDCl_3$, and homogenization by vortex-ing for 1 min., an aliquot (600 µL) from each sample was transferred into 5 mm NMR tubes.

Using a Bruker 600 MHz spectrometer operating at 600.13 MHz proton Larmor frequency 1D ^{1}H-NMR spectra were acquired and equipped with a 5 mm PATXI ^{1}H-^{13}C-^{15}N and ^{2}H-decoupling probe including a-z axis gradient coil, an automatic tuning and matching (ATM),

and an automatic and refrigerated sample changer. For temperature stabilization at the level of approximately 0.1K at the sample, a BTO 2000 thermocouple was used. After keeping the samples for at least 5 minutes inside the NMR probe head for temperature equilibration at 310K, they were measured.

All experiments are based on the use of a fully automated and standardized 600 MHz platform which is developed ensuring high reproducibility in metabolomic studies (https://www.bruker.com/products/mr/nmr/avance-ivdr.html). This platform includes the "B.I. Methods" which comprises all the automatisms necessary to standardize the generation of metabolomic data from biofluids. It also includes efficient quality control procedures ensuring the transferability of spectral information generated. In detail, for each water and lipid-soluble sample, a 1D ^{1}H-NMR spectrum was acquired using a standard Nuclear Overhauser Effect Spectroscopy pulse sequence (NOESY 1D presat) (noesygppr1d.com; Bruker BioSpin) using 98,304 data points, a spectral width of 18,028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, a mixing time of 0.01 s and 128 scans.

Also, for all the extracts, using a standard spin echo Carr-Purcell-Meiboom-Gill pulse sequence (CPMG) another ^{1}H-NMR spectrum was obtained [19] (cpmgpr1d.comp; Bruker Bio-Spin) with 128 scans, 73,728 data points, a spectral width of 12,019 Hz and an acquisition time of 3.1 s.

Spectral Processing: Before practising Fourier transform, free induction decays were multiplied by an exponential function equivalent to 0.3 Hz line-broadening factor. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (anomeric glucose doublet at 5.24 ppm for serum water-soluble extracts and chloroform singlet at 7.20 ppm for lipid-soluble samples) using TopSpin 3.2.

For water-soluble samples, each 1D spectrum (0.2 and 10.0 ppm range) was segmented into 0.02 ppm chemical shift bins and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Through the binning technique, the number of total variables is reduced and small shifts in the signals are compensated, making the analysis more robust and reproducible [16]. Then, regions between 4.62 and 4.77 ppm containing residual H_2O signals were removed.

Instead of serum lipid-soluble extracts, only regions between 0.2 and 6.70 ppm were considered and due to the presence of various shifts in the NMR signals of lipids, many regions were removed in this range of ppm.

On remaining bins, Probabilistic Quotient Normalization [12] (PQN) was applied before patter recognition both for water-soluble and lipid-soluble fractions.

Statistical Analysis: To explain whether all data are parametric or nonparametric normality test was performed for biochemical data. For comparing parametric values which calculated as mean ± SD, ANOVA and Tukey tests were used. Mann–Whitney U test was performed and presented as median (SPSS 21.0) for comparing the nonparametric ones.

Using R an open-source software for statistical analysis of data, all metabolomic analyses were performed [17]. On processed data, multivariate analysis and Principal Component Analysis (PCA) [27] were used as a first exploratory approach. To perform supervised data reduction and classification, multilevel PLS (M-PLS) [28] was employed.

For all classifications, the global accuracy was evaluated utilizing 100 cycles of Monte Carlo cross-validation scheme (MCCV, R scrip in-house developed). To build the model, for this method, 90% of data are randomly chosen as the training set, at each iteration. After testing the remaining 10% of data and establishing sensitivity, specificity, and accuracy for the classification, for deriving a mean discrimination accuracy in each group this procedure was repeated 100 times.

After performing univariate statistical

Table 1. Biochemical profile in premature calves.

Parameters	Hours		
	24	48	72
AST (U/L)	79.60±34.82[a]	66.76±32.06[ab]	57.92±28.54[b]
ALP (U/L)	322±339.84	244.48±239.44	217.08±125.65
ALT (U/L)	18.92±13.06	21.64±15.64	21.64±17.95
CPK (U/L)	503 (70/2626)[a]	242 (31/2874)[ab]	162 (31/2268)[b]
GGT (U/L)	14 (7/883)	90 (6/842)	74 (8/798)
Glucose (mg/dL)	83.52±38.10	97.04±43.54	82.60±29.40
Protein (g/dL)	4.53±1.16	4.64±1.34	4.56±0.97
Albumin (g/dL)	2.16±0.33	2.29±0.28	2.31±0.24
Total Bilirubin (mg/dL)	1.80±0.74	2.11±1.19	2.01±1.52
BUN (mg/dL)	11.48±5.76	13.28±10.47	14.80±10.04
Creatinine (mg/dL)	1.16±0.67	0.9±0.42	0.84±0.33
Ca (mg/dL)	9.48±1.34	9.48±1.08	9.83±1.13
Mg (mg/dL)	2.10±0.47	2.14±0.45	1.98±0.36
P (mg/dL)	6.34±1.15	6.90±1.32	7.10±0.99

[a, b, ab] Means with different superscripts in the same row differ significantly (P<0.05).

analysis on ^{1}H-NMR spectra, normalized with PQN method, well defined and resolved spectral regions related to the different metabolites were particularly assigned by using matching routines of AMIX 3.8.4 (Bruker BioSpin) and published literature data. To obtain concentrations of metabolites in arbitrary units, after integration of the same regions these concentrations were analyzed to determine discriminating metabolites among the groups. When two groups are constituted by subjects that could be compared in pairs (e.g the same individual before and after a specific treatment) a pairwise analysis can be particularly performed. What has just been said, justifies the previous use of multilevel PLS analysis and the pairwise Wilcoxon signed-rank test for the determination of meaningful metabolites in the context of univariate statistical analysis.

After the pairwise Wilcoxon test, false discovery rate correction was particularly used applying the Benjamini-Hochberg method (FDR) [6], and adjusted P-value < 0.05 was considered statistically significant. Then, changes in metabolites levels between two groups of NMR spectra at a time were calculated as the log2 fold change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups (fold change).

Results

Partly clinical recovery was observed at the 72h in all the calves. Biochemical parameters between the hours were meaningful, AST and CPK were decreased gradually with statistical significance (Table 1).

^{1}H-NMR spectra of serum samples have been acquired. For serum water-soluble fractions, five subjects were removed from the statistical analysis because of the bad quality of related spectra and three subjects have been removed in the analysis of serum lipid-soluble samples for the same reasons.

To have an overview of the main differences between the three-time of blood collection, processed 1D spectra including all groups that differ in the collection time of serum sample (after 24h, 48h, and 72h) were analyzed firstly using

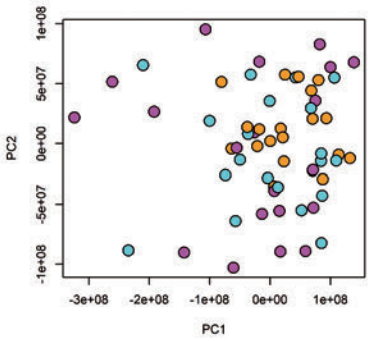

Figure 1a.

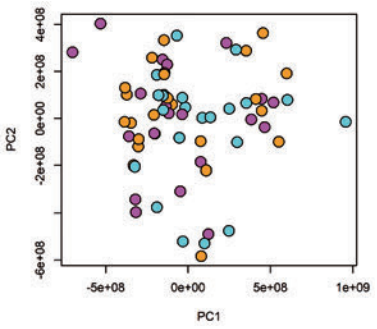

Figure 1b.

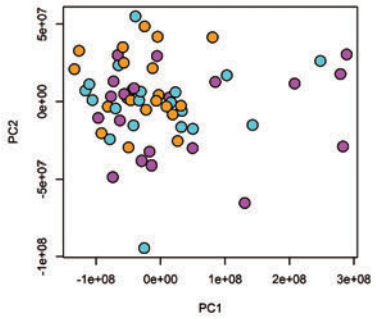

Figure 1c.

Fig. 1 (a – c). Principal component analysis (PCA) score plots. Each dot represents a single ^{1}H-NMR spectrum and each colour represents a group of premature calves that differ in the time of blood sample collection: 24h samples (magenta dots), 48h samples (cyan dots) and 72h samples (orange dots). a PCA on 1D NOESY spectra of serum water-soluble samples; b PCA on 1D NOESY spectra of serum lipid-soluble samples; c PCA on 1D CPMG spectra of serum water-soluble samples.

the unsupervised multivariate method (PCA). PCA score plots on 1D NOESY of serum water and lipid-soluble extracts and 1D CPMG of serum water-soluble samples are shown in Fig. 1 (a-c).

To explore changes in the metabolic profile after 24h, 48h and 72h, serum samples were compared in groups of two: first of all, we performed a comparison between samples collected at 24h and 48h, then between samples collected at 48h and 72h and in the end, samples at 24h and 72h have been compared to evaluate global metabolic changes during the entire period of blood collection.

To characterize the within-subject changes presented in the personal metabolic profile the M-PLS approach was used for all comparisons. Between-subject varieties were evaluated and as it were the within-subject varieties were considered with this approach. For serum water-soluble samples, M-PLS models on 1D NOESY spectra and 1D CPMG spectra (Fig. 2(a–f)) were built and a different number of components were retained in the model depending on the type of samples under analysis.

For serum water-soluble extracts, all the built models, as shown by prediction accuracies of cross-validation analyses in Fig. 2(a–f), were able to discriminate premature calves in the different time of blood collection with greater accuracies that 70% and in particular, we obtained higher accuracy when samples at 24h were compared both with 48h and 72h samples, suggesting a probable change in the metabolic profile in the first phase of blood collection.

Lower prediction accuracies than 70% have been obtained when M-PLS models were built on 1D NOESY spectra from serum lipid-soluble fractions (Fig. 3(a–c)). This can be justified considering that the presence of various shifts in the NMR signals of lipids led to consider, for the statistical analysis, limited portions of the NMR spectra, mainly related to alkyl chains of fatty acids.

To identify discriminating metabolites among the groups, NMR spectra were also analyzed. The complete list of identified and quantified metabolites from each type of sample (lipophilic and hydrophilic fractions) is in Table 2; adjusted P values are reported only for significantly different metabolites (P-value <0.05) among the various comparisons performed. In particular, for both types of extract, samples collected at 24h were compared with 48h samples and this second group was compared also with 72h samples.

To explore metabolites being significantly different between the beginning and the end of the blood collection, the comparison between serum samples collected from premature calves at 24h and 72h was performed too.

From the analysis of serum water-soluble samples, it appeared that the 24h group showed higher levels of myo-inositol both for

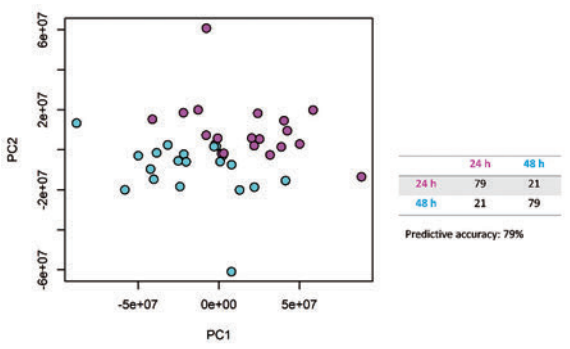

Figure 2a.

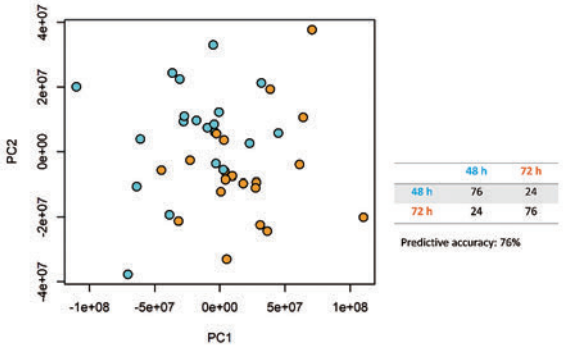

Figure 2b.

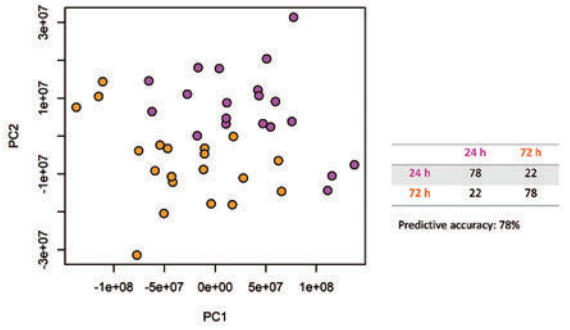

Figure 2c.

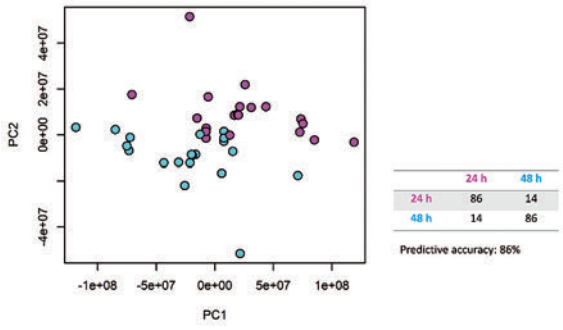

Figure 2d.

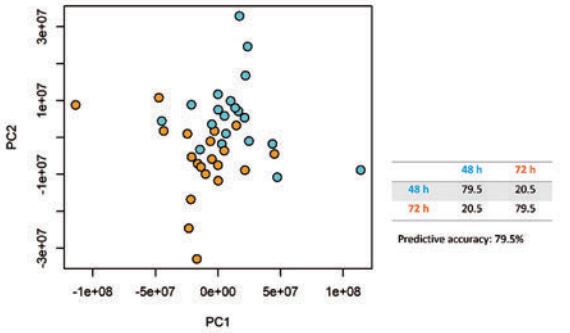

Figure 2e.

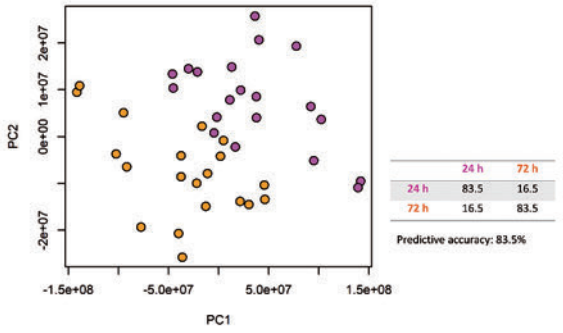

Figure 2f.

Fig. 2 (a – f). M-PLS score plots of serum water-soluble extracts. Each dot represents a single ^{1}H-NMR spectrum and each colour represents a group of premature calves that differ in the time of blood sample collection: 24h samples (magenta dots), 48h samples (cyan dots) and 72h samples (orange dots). **a** M-PLS on 1D NOESY spectra comparing 24h vs 48h samples; **b** M-PLS on 1D NOESY spectra comparing 48h vs 72h samples; **c** M-PLS on 1D NOESY spectra comparing 24h vs 72h samples; **d** M-PLS on 1D CPMG spectra comparing 24h vs 48h samples; **e** M-PLS on 1D CPMG spectra comparing 48h vs 72h samples; **f** M-PLS on 1D CPMG spectra comparing 24h vs 72h samples. Confusion matrices and related predictive accuracy of cross-validation analysis are reported for each model.

the comparison with the group of samples collected at 48h and 72h. Instead, higher levels of 3-hydroxybutyrate, citrate, leucine, and isoleucine were reported both for 48h and 72h groups when they were compared to 24h group.

Higher levels of myo-inositol have been reported also in the 48h group when it was compared with serum samples collected at 72h.

From the analysis of serum lipids, it resulted that signals of alkyl chains of fatty acids arising from $CH_3(CH_2)_n$, $-CH_2-CO$, $=CH-CH_2-CH_2$ protons were higher in the 72h group compared with the one whose samples have been collected at 24h. For the same comparison, also signals arising from

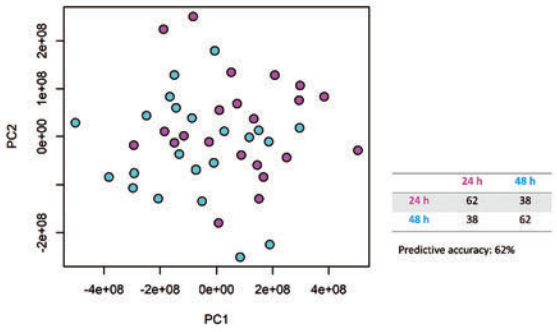

Figure 3a.

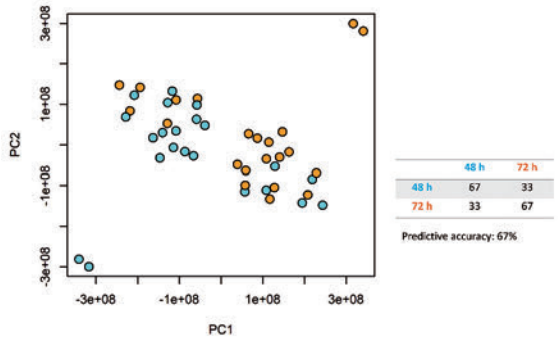

Figure 3b.

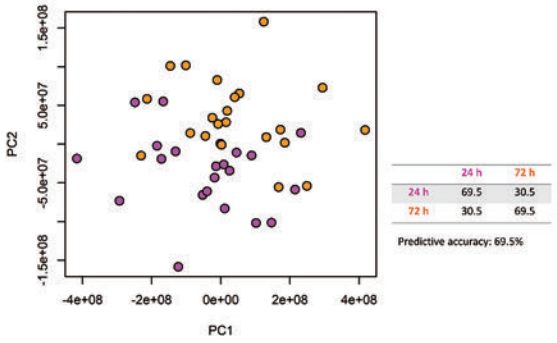

Figure 3c.

Fig. 3 (a – c). M-PLS score plots of serum lipid soluble extracts. Each dot represents a single ^{1}H-NMR spectrum and each colour represents a group of premature calves that differ in the time of blood sample collection: 24h samples (magenta dots), 48h samples (cyan dots) and 72h samples (orange dots). **a** M-PLS on 1D NOESY spectra comparing 24h vs 48h samples; **b** M-PLS on 1D NOESY spectra comparing 48h vs 72h samples; **c** M-PLS on 1D NOESY spectra comparing 24h vs 72h samples. Confusion matrices and related predictive accuracy of cross-validation analysis are reported for each model.

cholesterol C(18)H_3 and polyunsaturated fatty acids (18:2) bis allylic protons resulted higher for the 72h group.

Discussion

In premature calves, metabolomics was assessed for the first time. Our data showed that metabolomics was a favorable mean for metabolite identification and meaningful biomarker quantification for indicating the recovery at a molecular level for premature calves. Calves born alive with a gestation length of fewer than 270 days are considered as premature. Infections, poisonings, acute rumen acidosis, fatty liver syndrome, nutritional deficiencies, hereditary factors, induction of premature parturition, situations that cause compulsory slaughtering and procedures applied in that cows exposed to in the last period of pregnancy plays a role in the etiology of premature calving. Diagnosis is based on a short gestation period, clinical and laboratory findings [2]. For premature calves with respiratory difficulty, blood lactate and pCO_2 are prognostic [30]. In this study, clinical data were compatible with most references. Difficult births lead to elevated indicators of subclinical trauma and decreased vigour in the neonate, which can be quantified by measuring serum CPK, AST, and vigour parameters, respectively [24]. In the present study, however AST changes were between reference ranges CPK levels were decreased gradually. This may be attributed to subclinical trauma at birth, and indicates the recovery with therapy.

Neonatal health care using all the present resources to increase the survival rate of all especially those with critical conditions is carried out for the primary target of neonatology [21]. Treatments of premature calves should be an immediate and powerful treatment and be made for hypothermia, hypoglycemia, metabolic and respiratory acidosis, opportunistic infections, respiratory distress syndrome and failure of passive transfer [2]. In the current study, all the

Table 2. Concentrations in arbitrary units (median ± Median Absolute Deviation (MAD)) of the metabolites assigned in serum samples (both hydrophilic and lipophilic fractions). Statistically significant adjusted (13) *P* value < 0.05 from all comparisons are also reported.

	Metabolites	24 h (arbitrary units)	48 h (arbitrary units)	72 h (arbitrary units)	*P* value
Serum water soluble (SWS)	1,2-propandiol	143.1 ± 126.0	195.4 ± 101.4	203.6 ± 75.2	
	2-hydroxybutyrate	117.4 ± 42.2	145.0 ± 39.8	132.8 ± 50.0	
	3-hydroxybutyrate	1013.1 ± 365.5	1545.3 ± 445.9	1623.3 ± 485.9	<0.05 (24h versus 48h) <0.05 (24h versus 72h)
	3-hydroxyisobutyrate	225.4 ± 62.7	249.2 ± 65.1	264.2 ± 79.0	
	Acetate	6377.6 ± 2189.4	7315.6 ± 2210.4	6664.9 ± 1285.8	
	Alanine	3172.8 ± 856.5	3667.9 ± 1228.9	3420.9 ± 776.0	
	Carnitine	1700.4 ± 761.2	1466.5 ± 604.4	1309.0 ± 443.8	
	Citrate	1303.0 ± 364.1	1857.6 ± 815.8	2024.7 ± 642.8	<0.05 (24h versus 48h) <0.05 (24h versus 72h)
	Choline	639.4 ± 200.7	862.8 ± 423.1	963.4 ± 197.3	<0.05 (24h versus 72h)
	Creatine	2476.2 ± 851.6	2354.6 ± 621.7	2672.4 ± 1043.8	
	Creatinine	861.6 ± 223.5	824.7 ± 180.2	790.5 ± 216.4	
	Dimethylsulfone	184.7 ± 137.1	223.9 ± 161.0	262.4 ± 164.4	
	Formate	507.2 ± 183.2	618.9 ± 151.0	592.3 ± 135.4	<0.05 (24h versus 72h)
	Fructose	1068.0 ± 699.6	1182.9 ± 558.9	1021.4 ± 305.9	
	Glycine	942.5 ± 652.0	1120.7 ± 369.6	1200.1 ± 454.6	
	Glucose	2736.3 ± 1349.4	2799.4 ± 1039.3	2953.0 ± 739.8	
	Hippurate	140.0 ± 68.4	143.7 ± 57.2	96.6 ± 68.6	
	inosine	8.3 ± 8.3	24.2 ± 10.9	21.4 ± 14.5	
	Isoleucine	201.5 ± 74.3	280.6 ± 105.3	339.6 ± 115.5	<0.05 (24h versus 48h) <0.05 (24h versus 72h)
	Lactate	5998.8 ± 2543.2	5775.0 ± 1997.9	4996.2 ± 1276.3	
	Leucine	477.1 ± 155.7	589.4 ± 178.0	675.6 ± 167.6	<0.05 (24h versus 48h) <0.05 (24h versus 72h)
	Lysine	848.0 ± 118.4	851.6 ± 125.2	857.5 ± 95.1	
	Mannose	154.8 ± 82.3	177.9 ± 82.5	130.4 ± 48.3	
	Myo-inositol	1911.9 ± 504.7	967.3 ± 303.3	619.0 ± 210.7	<0.05 (24h versus 48h) <0.05 (48h versus 72h) <0.001 (24h versus 72h)
	Phenylalanine	415.5 ± 141.1	482.9 ± 95.8	602.2 ± 106.3	
	Proline	111.5 ± 43.9	134.1 ± 23.8	116.2 ± 23.5	
	Succinate	710.9 ± 283.5	946.9 ± 307.4	774.5 ± 172.6	
	Threonine	79.0 ± 19.7	69.9 ± 33.2	82.9 ± 23.5	
	Trimethylamine N-oxide	8872.2 ± 3160.9	7375.3 ± 1869.8	7167.7 ± 1631.0	
	Tyrosine	362.3 ± 149.3	414.4 ± 114.5	492.6 ± 111.6	
	Unknow	181.7 ± 48.2	183.3 ± 44.9	154.2 ± 36.9	
	Valine	931.6 ± 418.8	1169.3 ± 324.4	1324.0 ± 393.1	<0.05 (24h versus 48h)
Serum lipid soluble (SLS)	Cholesterol C(18)$\mathbf{H}_3$	19585.5 ± 7180.7	19353.0 ± 4525.2	22563.4 ± 3462.6	<0.05 (24h versus 72h)
	Fatty acid-CH=CH-C$\mathbf{H}_2$-CH=CH-	11995.0 ± 4849.9	12106.2 ± 2319.8	11202.4 ± 2563.2	
	Fatty acid-(C$\mathbf{H}_2$)n-	669798.0 ± 81373.8	684309.2 ± 43494.3	669677.9 ± 52028.9	
	Fatty acid-C$\mathbf{H}_3$	34133.1 ± 12367.9	33866.0 ± 6847.8	36961.7 ± 5745.7	<0.05 (24h versus 72h)
	Fatty acid-C$\mathbf{H}_2$-CO	7580.0 ± 2945.9	9145.3 ± 1700.6	8497.5 ± 2151.2	<0.05 (24h versus 72h)
	Fatty acid=CH-CH$_2$-C$\mathbf{H}_2$	14142.1 ± 2787.1	15335.8 ± 1375.5	15840.2 ± 1907.8	<0.05 (24h versus 72h)
	Polyunsatured fatty acids (18:2, bis allylic protons)	4382.9 ± 1081.4	5530.3 ± 680.4	5872.1 ± 1079.3	<0.05 (24h versus 72h)
	Unsaturated fatty acid -C$\mathbf{H}$=C$\mathbf{H}$-	40993.4 ± 11224.4	45087.2 ± 5496.9	44814.6 ± 9431.5	

calves recovered clinically without monitoring the picture of clinical pathology. Knowledge obtained in this respect would be significant for planning an effective prevention and treatment strategy for the future because the pathogenesis is not yet completely understood [9]. Metabolomics is applying for monitoring of postnatal metabolic maturation, and for identification of biomarkers as early predictors of outcome and the diagnosis, and the "tailored" management of neonatal disorders [13]. Metabolomics is a promising approach to identify novel specific biomarkers [25]. Novel roads for biomarkers revelation totally different branches of medicine, containing perinatology are opened

utilizing metabolomics [26]. In human medicine, metabolomics significantly enhances the relation to 7-day survival in premature infants [22]. In this current study, alterations in metabolites, increases in 3- hydroxybutyrate, citrate, leucine and isoleucine at 48th and 72nd hours; choline, formate, fatty acids and poly-unsaturated fatty acids at 72nd hour, and valine at 48th hour; and decreases in myoinositol at 48th and 72nd hours were meaningful for monitoring the recovery at a molecular level in premature calves. The metabolites and their functions in the present study are the following: *citrate* is an intermediate in the krebs cycle, a central metabolic pathway for animals; *leucine*: protein turnover regulation through cellular mammalian target of rapamycin signaling and gene expression; glutamate dehydrogenase activator; branched-chain amino acid balance; flavor enhancer; *isoleucine*: glutamine and alanine synthesis; balance among branched-chain amino acid; *valine*: glutamine and alanine synthesis; balance among branched-chain amino acid [29]. The 3 main branched-chain amino acids (leucine, isoleucine, and valine) are initially catabolized by common pathways and later diverge into complex pathways [7]. *Formate* is important in embryonic development. Increased formate levels may have a role in fetal development and suggest that extracellular formate may affect the interorgan distribution of one-carbon groups [8]. *Myo-inositol* is available in totally different sorts of cells in free form or is consolidated into inositol phospholipids, additionally other inositol subordinates and in this way plays an imperative administrative part [10], and seems to be involved in lung maturation during antenatal life [20] and could be implicated in glucose homeostasis [8]. For fetal development especially of the brain, *choline* is essential [18]. In spite of adequate macronutrient supply and weight gain, insufficient choline may contribute to the impeded incline body mass development and respiratory and neurocognitive advancement of preterm newborn children. In this setting a reevaluation of current proposals for choline supply to preterm newborn children is required [5]. Long-chain PUFAs, similar to choline, are critical for brain improvement [15]. Obtained metabolites above in this present study suggest that metabolomics have therapeutic importance in premature calves, and that premature calves need energy, especially amino acid and myo-inositol supplementation. After a premature birth, parenteral amino acid nutrition is needed to be insure sufficient growth and neurodevelopment [7].

In conclusion this first study showed the metabolomics could indeed be a strong mean providing information on the factors in charge for the metabolic changes in premature calves. The metabolites in the present study may become potential biomarkers for monitoring therapy, however, the predictive and prognostic value extents of this given set of metabolites will be needed for more clinical trials.

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