



Serum metabolomics identifies metabolite panels that differentiate lame dairy cows from healthy ones

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Abstract

Introduction Although much is known about lameness application of metabolomics technologies to better understanding its etiology and pathogenesis is of utmost interest.

Objectives The objective of this study was to investigate serum metabolite alterations in pre-lame, lame and post-lame dairy cows in order to identify potential screening serum metabolite biomarkers for lameness and better understand its pathobiology.

Methods A combination of direct injection and tandem mass spectrometry (DI–MS/MS) with a reverse-phase liquid chromatography and tandem mass spectrometry (LC–MS/MS) analysis was performed in the serum of six cases of lameness and 20 healthy control cows (CON) at – 8 and – 4 weeks prepartum, at lameness diagnosis week, and at +4 and +8 weeks postpartum.

Results Data indicated that pre-lame, lame, and post-lame cows experienced altered concentrations of multiple metabolites. It is interesting to note that throughout the 16-weeks of the study, 7 serum metabolites [e.g., diacyl-phosphatidylcholine (PC aa) C30:0, phosphatidylcholine acyl-alkyl (PC ae) C40:2, sphingomyelin (SM) (OH) C14:1, SM C18:0, isoleucine (Ile), leucine (Leu), and lysine (Lys)] differentiated CON cows from the lame ones. Furthermore, 4 metabolic pathways (i.e., Lys degradation, biotin metabolism, tryptophan (Trp) metabolism, and valine [(Val)-Leu-Ile degradation) were altered in cows with lameness during the onset and progression of the disease.

Conclusion Multiple metabolite and pathway alterations were identified in the serum of pre-lame, lame, and post-lame cows that through light into the pathobiology of the disease and that can be used as potential biomarker sets that can predict the risk of lameness in dairy cows.

Keywords Lameness · Dairy cows · Metabolomics serum biomarkers · DI/LC–MS/MS

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1 Introduction

Lameness is one of the most common diseases of dairy cows (Ametaj et al. 2010). It is a costly condition in terms of reduced reproductive success, milk yield, and treatment costs (Kujala 2010). The current diagnostic method is based on a lameness locomotion scoring system developed by Sprecher et al. (1997). It should be noted that there are no available metabolite-based screening tests for lameness in dairy cows.

Lameness may go unnoticed in its early stages and it is often difficult to detect it before appearance of clinical signs including alterations in the gait of the animal; and by the time it displays clinically it might be too late to treat it and often cows are culled from the herd (Kujala 2010). Detection of lameness as early as possible, in its

subclinical stages, could give dairy producers and veterinarians enough time to apply preventive measures.

It should be pointed out that the etiology and pathogenesis of lameness are not completely understood. A recent research from our group indicates that pre-lame cows experience elevated concentrations of multiple innate immunity variables including several cytokines and acute phase proteins (APP) as well as lactate during the dry off period as well as around parturition, which gives insights into the presence of potential inflammatory triggers before and during lameness (Zhang et al. 2015). Additionally, it would be of interest to study metabolites and metabolic pathways involved during pre-lame, lame and post-lame events.

Metabolomics, one of the four basic sciences of systems biology approach, is an emerging field of “omics” research involving high throughput technologies for identification and quantification of small molecule metabolites (< 1500 Da) (Psychogios et al. 2011). Metabolites are a very diverse group of small molecules including but not limited to amino acids, lipids, sugars, organic acids, and peptides (Wishart 2008). Metabolomics is increasingly being used to study the pathobiology of complex diseases as well as for identification of biomarkers for screening, diagnosis, and prognosis purposes (Martin et al. 2012; Xia et al. 2013). For instance, nuclear magnetic resonance (NMR)-based metabolomics was used to distinguish between cows with clinical and subclinical ketosis from healthy controls (Sun et al. 2013). Furthermore, NMR metabolomics analysis of dairy cows revealed that milk glycerophosphocholine to phosphocholine ratio could be used as a screening biomarker for the risk of ketosis (Klein et al. 2012). Recently, Hailemariam et al. (2014a) reported that a 3-metabolite plasma biomarker profile (carnitine, propionyl carnitine, and lysophosphatidylcholine acyl C14:0) could predict which cows would develop periparturient diseases, up to – 4 weeks prior to occurrence of clinical symptoms. In a recent study from our group, DI/LC-MS/MS-based metabolomics revealed altered amino acids and sphingolipids in sick cows during the transition period, which gives insights into the pathobiology of the disease in transition dairy cows (Hailemariam et al. 2014b).

To the best of our knowledge, comprehensive metabolomics analysis prior to, during, and after lameness event have not been conducted. We hypothesized that transition dairy cows affected by lameness might have blood alterations prior to appearance of clinical signs of lameness as well as during and after the event of lameness. Therefore, the objectives of the current study were to: (1) determine whether there are alterations in the blood metabolites related to amino acid, lipid, and carbohydrate metabolism in transition dairy cows, before, during and after clinical appearance of lameness; (2) identify the top most important metabolite biomarkers in the blood that might be useful for screening

dairy cows for risk of lameness; (3) better understand the etiopathology of lameness.

2 Materials and methods

This study was part of a project targeting the identification of screening biomarkers for multiple periparturient diseases of dairy cows. All procedures applied to animals were approved by the University of Alberta Animal Policy and Welfare Committee for Livestock, and cows were cared for in agreement with the guidelines of the Canadian Council on Animal Care (Olfert et al. 1993).

2.1 Animals and diets

One hundred pregnant Holstein dairy cows housed in a tie-stall barn were included in this nested case-control study. The experimental period lasted for 16 weeks starting from – 8 weeks prior to parturition until + 8 weeks after parturition. All results related to dry matter intake (DMI), milk composition and yield as well as those related to evaluation of innate immunity were published previously in a companion research article by Zhang et al. (2015). Briefly, cows were fed a close-up diet, prior to calving, and then immediately after calving were offered a ration containing all the required nutrients for lactation cows with increasing amounts of grain during the first week of parturition to provide high energy for high milk yielding cows. Feed was offered ad libitum, as a Total Mixed Ration (TMR), once daily at 0800 h to allow approximately 5% orts. Detailed diet information was reported previously (Zhang et al. 2015). The TMR was formulated to provide all the required nutrients for a 680 kg lactating dairy cow as per the guidelines of NRC (2001).

All cows in the study were monitored for their health status on a daily basis. Monitoring parameters included daily DMI and milking yield as well as locomotion scoring (LCS) for the diagnosis of lameness. The protocol used for locomotion scoring was based on procedures developed by Sprecher et al. (1997). This protocol applies a 5-point scale scoring system where cows under LCS-1 are classified as normal, LCS-2 as mildly lame, LCS-3 as moderately lame, LCS-4 as lame, and LCS-5 as severely lame. Six pregnant multiparous cows (parity: 3.0 ± 0.6 , mean \pm SEM) with a locomotion score of ≥ 3 , showing an arched-back and short strides with one or more legs or favoring one or more limbs or reluctant to move were diagnosed with lameness [at week + 1, + 2, + 2, + 3, + 3, and + 3, respectively (i.e., 5–21 day; mean: 11 day)]. It should be noted that the incidence of lameness was greater than 6 cows; however, all lame cases associated with another periparturient disease including mastitis, metritis, ketosis, retained placenta, or

milk fever were excluded from further evaluation. The reason for that was to characterize lameness from the metabolomics perspective and compare those metabolotypes with the metabolotypes of healthy cows. All cows affected by lameness were treated by trimming, and medicated IM once a day with Excenel RTU (Zoetis Canada, Kirkland, QC, Canada) at 1 mL per 50 kg for a period of 3 days. If necessary, Procaine Penicillin Gr (Dominion Veterinary Laboratories Ltd., Winnipeg, MB, Canada) was used IM, at 2 mL per 45 kg, twice a day for 3 days, as preventive treatment against potential infections. All lame cows were recovered after medication and restored the normal gait, except for 1 cow that continued to be lame until +4 weeks after calving. As per requirements of a nested case–control study, lame cows in the study were matched with 20 healthy control cows with a LCS of 1 (normal gait), that were similar in parity (3.1 ± 0.4 , mean \pm SEM) and body condition score (BCS; CON: 2.87 vs lam cows: 2.62).

2.2 Blood sample collection

Blood samples were collected from the coccygeal vein from each cow at 0700 before feeding. Blood samples for metabolomics analysis were obtained at 5 time points within the 16 weeks from each cow. Specifically, sampling took place at –8 (53–59 days) and –4 (25–31 days) weeks prior to parturition, at lameness diagnosis week (5–21 days, mean: 11 days) as well as at +4 (25–31 days) and +8 (53–59 days) weeks after parturition. Blood samples were collected into 10-mL vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to clot. Then, tubes were centrifuged at $2090 \times g$ at 4 °C for 20 (Rotanta 460 R centrifuge, Hettich Zentrifugan, Tuttlingen, Germany) and the separated serum was aspirated into a sterile 10-mL plastic test tube (Fisher Scientific, Toronto, ON, Canada). Samples were split into multiple vials and frozen immediately at –80 °C until the metabolomics analysis to prevent loss of bioactivity and contamination and were thawed on ice bath for approximately 2 h before use.

2.3 DI/LC–MS/MS compound identification and quantification

A direct injection and tandem mass spectrometry (DI–MS/MS) coupled with a reverse-phase liquid chromatography and tandem mass spectrometry (LC–MS/MS) was used to quantify amino acids (AAs), acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and hexose in the serum. We used a commercial kit (AbsoluteIDQ p180) provided by BIOCRATES Life Science AG (Innsbruck, Austria) for analyses (For more information see: <https://biocrates.com/absoluteidq-p180-kit/>). The kit assay was used with an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex,

Foster City, CA) mass spectrometer to identify and quantify a total of 186 metabolites species including AAs, acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and hexose. Biogenic amines and AAs were quantified by LC–MS/MS, whereas the rest of metabolites were analyzed by DI–MS/MS.

Briefly, the protocol included derivatization and extraction of metabolites and detection with selective mass spectrometry, using multiple reaction monitoring (MRM) pairs. For quantification of metabolites, internal standards were integrated into a kit plate filter. The AbsoluteIDQ 180 kit used included a 96 deep-well plate with a filter plate, along with sealing tape as well as reagents and solvents necessary to prepare the plate assay. The first 14 wells were used for quality control and standardization including 1 blank, 3 zero samples, 7 standards, and 3 quality control wells; the remaining 82 wells were used for analysis of serum samples. Serum samples were thawed on ice, vortexed, and centrifuged at $13,000 \times g$ for 3 min, at 4 °C. Ten microlitre of each serum sample was added onto the center of the filter on the upper 96-well kit plate and dried with a stream of nitrogen using Zantek Analytical Evaporator (Glas-Col, Terre Haute, IN, USA). Then, 20 μ L of a 5% phenylisothiocyanate solution was pipetted for derivatization. Immediately after incubation, the filter spots were dried again under the nitrogen stream of the same evaporator.

For extraction of metabolites 300 μ L methanol, containing 5 mM ammonium acetate was added to samples. Then, samples were centrifuged using Sorvall Evolution RC Super-speed Centrifuge (Fisher Scientific, Toronto, ON, Canada) and extracts were collected from the lower 96 deep-well plate. Then, extracts were diluted with 600 μ L of the MS running solvent, included in the kit. Mass spectrometric analyses were conducted by ABI 4000 Q-trap tandem mass spectrometry (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with a solvent delivery system. For sample registration, automated calculation of metabolite concentrations, and export of data into other data analysis programs, a Biocrates MetIQ software, included in the kit, was used. Additionally, for quantification of all metabolites, a targeted profiling scheme including MRM, neutral loss, and precursor ion scans, were used.

2.4 Statistical analysis

All statistical analyses followed recommended procedures as described in previously published protocols (Xia et al. 2009). In brief, univariate analysis was used to process all continuous data by Wilcoxon–Mann–Whitney (rank sum) test with R package (Version 3.0.3, R Development Core Team 2008). A p-value less than 0.05 was used as the cut-off for statistical significance. The MetaboAnalyst

software (<https://www.metaboanalyst.ca>) was applied for metabolomics analyses (Xia et al. 2009).

If metabolites were found to be more than 20% below the limit of detection or with > 20% missing values they were removed from calculations. Otherwise, missing values of one metabolite were replaced by a value of 1/2 of the minimum positive value of the same metabolite in the original data. Before statistical analysis and pathway analysis, data were normalized using log-transformation and pre-processed using auto-scaling to achieve Gaussian distribution, as described and recommended by Xia et al. (2009).

For comparisons between the group of healthy cows (CON) and the group of cows with lameness a standard cross-sectional 2-group analysis was conducted separately at each time point (− 8, − 4, disease diagnosis, + 4, and + 8 weeks around calving). MetaboAnalyst was used to conduct principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), metabolite quantitative enrichment analysis (MSEA), and metabolic pathway analysis. To rank the metabolites based on their importance in discriminating the lame group of cows from CON cows the PLS-DA model with a variable importance in the projection (VIP) plot was used. Metabolites with the highest VIP values are the most powerful discriminators between the lame and CON cows. It should be noted that, VIP values higher than 1 are considered as significant and VIP values higher than 2 are considered as highly significant. To validate the reliability of the model, a 2000-permutation test was implemented using random resampling of CON and lame cows to determine the probability that separated clusters of CON and lame cows were a result of chance (Xia and Wishart 2011).

Receiver-operator characteristic (ROC) curves, calculated by MetaboAnalyst 3.0 (Xia et al. 2015), were used for identification of biomarker profiles and determination of the quality of biomarker panels. Besides, the software calculates the paired sensitivity and false-positive ratios (1—specificity) at different classification decision boundaries. A ROC curve was created with sensitivity values (i.e., the true positive rates) on the Y-axis and the corresponding false-positive rates (1—specificity) on the X-axis. ROC curves are graphical plots also known as the area under the ROC curve (AUC), that illustrate the diagnostic ability or the accuracy of a test for correctly distinguishing one group from another group (e.g., lame cows vs CON ones). The AUC has a value of 1.0, if all positive samples are ranked before the negative ones, which suggests a perfect discriminating test. The p values and 95% confidence interval (CI) were also calculated. A permutation test (n = 1000) was conducted for each ROC curve at 5 time points, respectively. A general guide for evaluating the utility of a biomarker panel, based on its AUC value, is 0.9 ~ 1.0 = excellent; 0.8 ~ 0.9 = good;

0.7 ~ 0.8 = fair; 0.6 ~ 0.7 = poor; 0.5 ~ 0.6 = fail (Xia et al. 2015).

3 Results

DI/LC–MS/MS-based metabolomics was performed on 6 cows diagnosed with lameness and 20 CON cows at − 8, − 4, disease diagnosis (week 1–3; days 5–21; mean: days 11), + 4, and + 8 weeks. A total of 128 metabolites were identified and quantified using an in-house mass-spectrometry library. The metabolites measured are classified into 5 groups: AAs (29), glycerophospholipids (77), sphingolipids (14), ACs (7), and hexose (1). By a combination of univariate and multivariate analyses, we compared the lame group of cows with CON cows at 5 time points, separately. A total of 39, 62, 37, 35, and 28 metabolites were found to differentiate the two groups of cows in the serum at − 8, − 4, lameness diagnosis week, and at + 4, and + 8 weeks around the expected day of parturition between the two groups. The mean ± SEM concentration values, p values along with fold change and direction of change in lameness cases relative to CON cows are provided in Table 1 and Supplementary Table 1.

3.1 Serum metabolotypes prior to disease diagnosis

Results of the univariate analysis indicated that a total of 39 and 62 metabolites in the serum were significantly different at − 8 and − 4 weeks between the two groups. Specifically, 1 acylcarnitine, 9 glycerophospholipids, 10 sphingolipids, 13 AAs and hexose were elevated, whereas 2 glycerophospholipids, 1 sphingolipid, and 2 AAs were lowered in the serum of pre-lame cows at − 8 weeks prepartum (Table 1). The same alterations were observed at − 4 weeks prior to parturition of lameness with 1 acylcarnitine, 29 glycerophospholipids, 9 sphingolipids, 17 AAs and hexose increased, and 3 glycerophospholipids, 1 sphingolipid, and 1 AA decreased in pre-lame cows compared with CON ones (Table 1).

Multivariate analysis showed that when data from CON cows were compared with those of pre-lame cows at − 8 and − 4 weeks, both PCA and PLS-DA had two separate clusters at the two time points (Figs. 1a, b; 2a, b). Permutation testing (p < 0.05) revealed that the observed separation was not by chance and the results of cross-validation were reliable. A VIP plot of the PLS-DA from − 8 to − 4 weeks in which the metabolites were ranked based on their contribution to discriminating the pre-lame cows from CON ones are shown in Figs. 1c and 2c. The top 15 important metabolites are shown in the respective VIP plots. The greater the distance from the Y-axis (i.e., the greater the VIP value), the greater the contribution of a particular metabolite in distinguishing pre-lame cows from the CON ones. The VIP plots indicated that lysine (Lys), lysophosphatidylcholine acyl C28:0 (lysoPC a

Table 1 Concentrations of serum metabolites (mean (SEM)) in healthy control (CON) and lame (L:AM) cows at 3 time points (– 8 week, – 4 week, and the week of diagnosis of disease) as determined by DJ/LC–MS/MS

Metabolite ^a , μM	8 weeks before parturition				4 weeks before parturition				L:AM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM	CON	P-value	Fold change	LAM/CON	
Number of cases	6	20	–	–	6	20	–	–	–	6	20	–	–	–	
C10	0.05 (0.003)	0.02 (0.01)	0.003*	2.5	Up	0.03 (0.003)	0.03 (0.01)	0.003*	1	Up	0.04 (0.004)	0.03 (0.01)	0.003*	1.33	Up
C16	0.02 (0.004)	0.02 (0.004)	0.709	1	Down	0.01 (0.002)	0.03 (0.003)	0.324	– 3	Down	0.03 (0.01)	0.02 (0.004)	0.387	1.5	Up
C18	0.02 (0.003)	0.03 (0.003)	0.564	– 1.5	Down	0.02 (0.004)	0.04 (0.01)	0.196	– 2	Down	0.07 (0.02)	0.04 (0.01)	0.191	1.75	Up
C2	1.31 (0.28)	1.30 (0.10)	0.869	1.01	Up	0.90 (0.10)	1.43 (0.22)	0.176	– 1.59	Down	1.62 (0.31)	1.22 (0.11)	0.268	1.33	Up
C3	0.12 (0.01)	0.14 (0.01)	0.488	– 1.17	Down	0.13 (0.02)	0.14 (0.02)	0.836	– 1.08	Down	0.20 (0.06)	0.15 (0.05)	0.157	1.33	Up
C4	0.07 (0.004)	0.08 (0.01)	0.575	– 1.14	Down	0.08 (0.01)	0.07 (0.01)	0.26	1.14	Up	0.07 (0.01)	0.07 (0.003)	0.457	1	Up
C5	0.06 (0.01)	0.08 (0.01)	0.096	– 1.33	Down	0.06 (0.01)	0.06 (0.01)	0.656	1	Down	0.05 (0.01)	0.06 (0.002)	0.421	– 1.2	Down
lysoPC a	60.30 (7.07)	27.89 (2.68)	0.002*	2.16	Up	44.45 (3.02)	24.44 (2.64)	0.001*	1.82	Up	40.90 (12.66)	23.78 (2.09)	0.573	1.72	Up
lysoPC a	1.76 (0.24)	1.58 (0.17)	0.408	1.11	Up	1.29 (0.10)	1.26 (0.13)	0.976	1.02	Up	1.53 (0.18)	1.26 (0.11)	0.268	1.21	Up
C16:1	8.63 (1.06)	3.03 (0.36)	0.001*	2.85	Up	6.64 (0.76)	2.00 (0.24)	0.001*	3.32	Up	2.37 (0.47)	1.85 (0.23)	0.387	1.28	Up
C17:0	66.53 (6.39)	25.79 (3.09)	0.001*	2.58	Up	54.39 (5.06)	19.49 (2.33)	0.001*	2.79	Up	31.48 (8.30)	17.67 (1.70)	0.123	1.78	Up
lysoPC a	19.06 (2.30)	16.09 (1.54)	0.243	1.18	Up	15.33 (1.33)	14.14 (1.58)	0.573	– 1.08	Up	18.16 (2.64)	13.37 (1.38)	0.157	1.36	Up
lysoPC a	14.17 (2.30)	18.49 (1.87)	0.408	– 1.3	Down	10.11 (1.25)	17.12 (2.09)	0.054	– 1.69	Down	16.45 (2.96)	17.43 (2.01)	0.79	– 1.06	Down
lysoPC a	2.34 (0.42)	3.25 (0.33)	0.243	– 1.39	Down	1.55 (0.18)	1.67 (0.17)	0.744	– 1.08	Down	1.31 (0.30)	1.82 (0.22)	0.268	– 1.39	Down
C20:3	2.20 (0.28)	3.61 (0.36)	0.012*	– 1.64	Down	1.73 (0.24)	2.24 (0.18)	0.196	– 1.29	Down	1.66 (0.33)	2.30 (0.26)	0.242	– 1.39	Down
C20:4	0.31 (0.03)	0.83 (0.05)	0.001*	– 2.68	Down	0.27 (0.02)	0.61 (0.05)	0.001*	– 2.26	Down	0.24 (0.03)	0.63 (0.05)	0.001*	– 2.63	Down
lysoPC a	0.52 (0.08)	0.75 (0.10)	0.129	– 1.44	Down	0.37 (0.03)	0.51 (0.04)	0.095	– 1.38	Down	0.48 (0.06)	0.53 (0.04)	0.533	– 1.1	Down
C28:1	2.35 (0.34)	1.86 (0.18)	0.192	1.26	Up	1.41 (0.09)	1.54 (0.19)	0.976	– 1.09	Down	2.58 (0.38)	1.66 (0.15)	0.028*	1.55	Up
PC aa	3.85 (0.37)	2.54 (0.25)	0.029*	1.52	Up	2.95 (0.20)	2.17 (0.26)	0.033*	1.36	Up	4.48 (0.44)	2.28 (0.21)	0.001*	1.96	Up
C30:0															

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM	CON	P-value	Fold change	LAM/CON	
PC aa C30:2	0.80 (0.11)	0.33 (0.05)	0.006*	2.42	Up	0.59 (0.07)	0.23 (0.04)	0.001*	2.57	Up	0.90 (0.21)	0.28 (0.05)	0.001*	3.21	Up
PC aa C32:0	7.68 (0.76)	8.61 (0.75)	0.575	-1.12	Down	5.70 (0.62)	7.06 (0.78)	0.268	-1.24	Down	8.22 (1.22)	6.68 (0.61)	0.295	1.23	Up
PC aa C32:1	5.75 (0.47)	7.88 (0.71)	0.06	-1.37	Down	4.30 (0.31)	6.74 (0.81)	0.095	-1.57	Down	8.29 (1.25)	6.70 (0.59)	0.219	1.24	Up
PC aa C32:2	7.91 (0.92)	8.44 (0.89)	0.869	-1.07	Down	6.47 (0.79)	5.99 (0.69)	0.573	1.08	Up	7.78 (1.17)	6.33 (0.59)	0.196	1.23	Up
PC aa C32:3	14.11 (1.68)	15.78 (1.97)	1	-1.12	Down	9.59 (1.33)	10.64 (1.30)	0.882	-1.11	Down	12.19 (2.02)	11.33 (1.12)	0.836	1.08	Up
PC aa C34:1	80.53 (8.18)	97.65 (8.90)	0.169	-1.21	Down	66.43 (4.89)	99.96 (14.13)	0.295	-1.5	Down	132.04 (23.68)	94.61 (10.26)	0.176	1.4	Up
PC aa C34:2	100.38 (7.46)	144.82 (13.13)	0.051	-1.44	Down	70.90 (5.68)	154.36 (23.31)	0.046*	-2.18	Down	170.26 (29.97)	147.47 (15.38)	0.355	1.15	Up
PC aa C34:3	17.64 (2.35)	28.61 (2.98)	0.06	-1.62	Down	11.85 (0.98)	23.14 (2.75)	0.013*	-1.95	Down	23.95 (4.01)	24.09 (2.44)	1	-1.01	Down
PC aa C34:4	7.09 (1.00)	7.44 (0.91)	0.869	-1.05	Down	4.47 (0.48)	3.86 (0.41)	0.614	1.16	Up	4.43 (1.13)	4.39 (0.55)	0.836	1.01	Up
PC aa C36:0	8.72 (2.00)	6.74 (0.77)	0.447	1.29	Up	8.15 (0.84)	4.40 (0.52)	0.002*	1.85	Up	8.03 (1.52)	4.89 (0.64)	0.083	1.64	Up
PC aa C36:1	108.96 (13.57)	105.11 (9.08)	0.818	1.04	Up	96.50 (7.73)	90.50 (10.75)	0.494	1.07	Up	130.20 (17.38)	87.64 (8.59)	0.072	1.49	Up
PC aa C36:2	150.86 (15.00)	166.65 (14.53)	0.447	-1.1	Down	117.45 (8.92)	157.08 (19.70)	0.242	-1.34	Down	198.99 (23.82)	149.78 (14.23)	0.083	1.33	Up
PC aa C36:3	70.18 (9.79)	79.46 (7.61)	0.575	-1.13	Down	47.37 (3.08)	69.68 (8.48)	0.157	-1.47	Down	92.13 (13.54)	69.02 (6.53)	0.139	1.33	Up
PC aa C36:4	33.37 (4.35)	39.05 (3.45)	0.336	-1.17	Down	22.77 (1.88)	30.96 (3.49)	0.242	-1.36	Down	41.77 (6.21)	31.91 (2.98)	0.268	1.31	Up
PC aa C36:5	9.12 (1.61)	10.24 (0.94)	0.303	-1.12	Down	5.76 (0.67)	7.96 (0.79)	0.196	-1.38	Down	9.92 (1.74)	8.14 (0.82)	0.533	1.22	Up
PC aa C36:6	3.06 (0.44)	3.30 (0.33)	0.717	-1.08	Down	2.31 (0.30)	2.13 (0.20)	0.744	1.08	Up	2.40 (0.55)	2.49 (0.24)	0.355	-1.04	Down
PC aa C38:0	2.05 (0.54)	1.87 (0.20)	0.921	1.1	Up	2.35 (0.31)	1.28 (0.16)	0.009*	1.84	Up	1.80 (0.53)	1.34 (0.17)	0.656	1.34	Up
PC aa C38:1	5.39 (1.76)	4.24 (0.62)	0.818	1.27	Up	5.65 (0.78)	2.84 (0.39)	0.003*	1.99	Up	4.02 (1.27)	3.22 (0.44)	0.656	1.25	Up
PC aa C38:3	61.95 (10.10)	55.71 (5.47)	0.575	1.11	Up	41.80 (3.37)	30.27 (3.09)	0.054	1.38	Up	40.37 (8.70)	34.70 (4.31)	0.533	1.16	Up

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM/CON	P-value	Fold change	LAM	CON	P-value	Fold change	LAM/CON		
PC aa C38:4	54.99 (5.21)	56.15 (4.79)	0.974	-1.02	Down	42.61 (3.43)	36.75 (3.51)	0.421	1.16	Up	51.24 (8.71)	39.73 (4.36)	0.242	1.29	Up
PC aa C38:5	27.65 (4.60)	25.35 (2.17)	0.921	1.09	Up	19.31 (1.89)	21.00 (2.22)	0.605	-1.09	Down	32.68 (5.06)	21.56 (2.14)	0.083	1.52	Up
PC aa C38:6	4.35 (0.62)	4.27 (0.35)	0.717	1.02	Up	3.15 (0.30)	3.97 (0.52)	0.573	-1.26	Down	5.60 (0.96)	4.18 (0.45)	0.219	1.34	Up
PC aa C40:2	0.48 (0.09)	0.35 (0.05)	0.192	1.37	Up	0.41 (0.04)	0.20 (0.02)	0.001*	2.05	Up	0.32 (0.09)	0.23 (0.03)	0.533	1.39	Up
PC aa C40:3	5.95 (1.93)	4.41 (0.64)	0.447	1.35	Up	4.77 (0.80)	1.97 (0.35)	0.009*	2.42	Up	2.85 (1.36)	2.17 (0.46)	0.614	1.31	Up
PC aa C40:4	17.01 (3.82)	11.72 (1.16)	0.192	1.45	Up	14.22 (1.43)	6.47 (0.84)	0.001*	2.20	Up	10.39 (3.67)	7.40 (1.13)	0.744	1.4	Up
PC aa C40:5	23.09 (3.09)	18.80 (1.69)	0.336	1.23	Up	18.35 (1.31)	14.34 (1.55)	0.196	1.28	Up	21.38 (3.74)	15.78 (1.65)	0.196	1.35	Up
PC aa C40:6	3.93 (0.42)	3.49 (0.27)	0.336	1.13	Up	3.49 (0.21)	3.17 (0.35)	0.457	1.1	Up	4.77 (0.61)	3.67 (0.40)	0.196	1.3	Up
PC aa C42:1	0.11 (0.01)	0.07 (0.01)	0.015*	1.57	Up	0.11 (0.01)	0.05 (0.00)	0.001*	2.2	Up	0.08 (0.01)	0.05 (0.00)	0.023*	1.6	Up
PC aa C42:2	0.16 (0.02)	0.12 (0.01)	0.112	1.33	Up	0.15 (0.01)	0.08 (0.01)	0.001*	1.88	Up	0.13 (0.03)	0.08 (0.01)	0.171	1.63	Up
PC aa C42:4	0.33 (0.10)	0.25 (0.03)	0.447	1.32	Up	0.31 (0.05)	0.13 (0.02)	0.002*	2.38	Up	0.19 (0.08)	0.14 (0.03)	0.882	1.36	Up
PC aa C42:5	1.08 (0.40)	0.81 (0.09)	0.818	1.33	Up	1.00 (0.16)	0.54 (0.08)	0.028*	1.85	Up	0.64 (0.28)	0.50 (0.09)	0.882	1.28	Up
PC aa C42:6	0.40 (0.05)	0.36 (0.02)	0.435	1.11	Up	0.41 (0.04)	0.28 (0.03)	0.023*	1.46	Up	0.34 (0.06)	0.27 (0.02)	0.219	1.26	Up
PC ae C30:0	0.88 (0.07)	0.72 (0.06)	0.216	1.22	Up	0.65 (0.05)	0.56 (0.05)	0.457	1.16	Up	0.80 (0.10)	0.60 (0.06)	0.139	1.33	Up
PC ae C30:1	1.89 (0.29)	1.32 (0.18)	0.148	1.43	Up	1.51 (0.19)	0.83 (0.10)	0.002*	1.82	Up	1.90 (0.36)	0.90 (0.11)	0.001*	2.11	Up
PC ae C32:1	2.78 (0.35)	3.82 (0.37)	0.169	-1.37	Down	2.15 (0.26)	3.10 (0.33)	0.083	-1.44	Down	3.05 (0.38)	3.07 (0.27)	0.836	-1.01	Down
PC ae C32:2	5.34 (0.60)	7.13 (0.80)	0.408	-1.34	Down	4.01 (0.40)	5.26 (0.68)	0.457	-1.31	Down	5.54 (0.79)	5.48 (0.55)	1	1.01	Up
PC ae C34:0	3.59 (0.36)	3.66 (0.36)	1	-1.02	Down	3.05 (0.36)	2.35 (0.24)	0.295	1.3	Up	2.87 (0.84)	2.49 (0.34)	0.7	1.15	Up
PC ae C34:1	13.69 (1.80)	15.63 (1.42)	0.447	-1.14	Down	11.24 (0.92)	11.80 (1.12)	0.7	-1.05	Down	14.79 (2.24)	11.87 (1.15)	0.324	1.25	Up

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM/CON
PC ae C34:2	16.25 (2.56)	18.55 (1.95)	0.53	-1.14	Down	12.71 (1.04)	14.92 (1.74)	0.7	-1.17	Down	19.31 (2.64)	14.90 (1.48)	0.219	1.3	Up
PC ae C34:3	17.41 (3.40)	19.30 (2.50)	0.974	-1.11	Down	11.67 (1.44)	15.06 (2.07)	0.457	-1.29	Down	19.06 (2.64)	15.57 (1.71)	0.295	1.22	Up
PC ae C36:0	2.11 (0.27)	2.45 (0.24)	0.53	-1.16	Down	1.98 (0.17)	1.62 (0.18)	0.242	1.22	Up	1.61 (0.45)	1.75 (0.20)	0.7	-1.09	Down
PC ae C36:1	20.47 (2.89)	17.77 (1.47)	0.53	1.15	Up	17.88 (1.66)	12.83 (1.18)	0.083	1.39	Up	18.81 (3.40)	12.63 (1.23)	0.108	1.49	Up
PC ae C36:2	23.70 (2.92)	23.00 (2.23)	0.767	1.03	Up	16.79 (1.99)	17.17 (1.59)	0.882	-1.02	Down	22.48 (3.46)	16.98 (1.62)	0.157	1.32	Up
PC ae C36:3	10.09 (1.73)	10.60 (1.17)	0.869	-1.05	Down	6.63 (0.61)	7.58 (0.74)	0.457	-1.14	Down	9.48 (1.52)	7.83 (0.75)	0.457	1.21	Up
PC ae C36:4	9.43 (1.83)	9.19 (1.09)	0.767	1.03	Up	5.82 (0.41)	6.13 (0.68)	0.976	-1.05	Down	8.08 (1.18)	6.37 (0.59)	0.295	1.27	Up
PC ae C36:5	7.61 (1.48)	7.47 (0.81)	0.818	1.02	Up	4.99 (0.45)	5.25 (0.49)	0.744	-1.05	Down	6.99 (1.26)	5.56 (0.52)	0.533	1.26	Up
PC ae C38:0	1.79 (0.26)	1.59 (0.14)	0.668	1.13	Up	1.65 (0.16)	1.14 (0.09)	0.016*	1.45	Up	1.63 (0.26)	1.27 (0.11)	0.242	1.28	Up
PC ae C38:1	4.58 (0.94)	3.11 (0.31)	0.169	1.47	Up	3.92 (0.39)	1.76 (0.23)	0.001*	2.23	Up	3.59 (1.02)	1.85 (0.28)	0.108	1.94	Up
PC ae C38:2	4.72 (0.68)	3.66 (0.35)	0.148	1.29	Up	3.44 (0.41)	2.31 (0.22)	0.054	1.49	Up	3.55 (0.74)	2.37 (0.27)	0.219	1.5	Up
PC ae C38:3	8.47 (1.40)	6.06 (0.63)	0.169	1.40	Up	5.48 (0.44)	3.21 (0.33)	0.002*	1.71	Up	5.12 (1.37)	3.50 (0.49)	0.324	1.46	Up
PC ae C38:4	8.62 (1.23)	7.48 (0.71)	0.488	1.15	Up	5.97 (0.71)	4.71 (0.47)	0.355	1.27	Up	6.26 (1.73)	5.06 (0.68)	0.614	1.24	Up
PC ae C38:5	5.99 (1.17)	5.31 (0.49)	0.767	1.13	Up	3.82 (0.35)	3.70 (0.34)	0.79	1.03	Up	4.96 (0.92)	4.01 (0.40)	0.573	1.24	Up
PC ae C38:6	3.92 (0.79)	4.02 (0.39)	0.974	-1.03	Down	2.84 (0.27)	3.00 (0.32)	0.831	-1.06	Down	4.01 (0.53)	3.47 (0.33)	0.573	1.16	Up
PC ae C40:1	0.52 (0.11)	0.38 (0.04)	0.336	1.37	Up	0.47 (0.06)	0.28 (0.03)	0.016*	1.68	Up	0.34 (0.08)	0.29 (0.04)	0.836	1.17	Up
PC ae C40:2	1.52 (0.19)	0.94 (0.10)	0.007*	1.62	Up	1.25 (0.08)	0.65 (0.06)	0.001*	1.92	Up	1.12 (0.15)	0.71 (0.08)	0.039*	1.58	Up
PC ae C40:3	2.19 (0.24)	1.47 (0.16)	0.042*	1.49	Up	1.61 (0.09)	0.78 (0.08)	0.001*	2.06	Up	1.22 (0.27)	0.94 (0.15)	0.421	1.3	Up
PC ae C40:4	2.74 (0.40)	2.08 (0.20)	0.169	1.32	Up	1.90 (0.13)	1.18 (0.14)	0.016*	1.61	Up	1.64 (0.46)	1.39 (0.21)	0.614	1.18	Up

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM/CON	P-value	Fold change	LAM	CON	P-value	Fold change	LAM/CON		
PC ae C40:5	3.46 (0.54)	2.71 (0.24)	0.303	1.28	Up	2.49 (0.23)	1.94 (0.21)	0.157	1.28	Up	2.60 (0.61)	2.12 (0.24)	0.656	1.23	Up
PC ae C40:6	1.31 (0.23)	1.05 (0.08)	0.408	1.25	Up	0.92 (0.10)	0.84 (0.07)	0.79	1.1	Up	1.20 (0.19)	0.98 (0.09)	0.387	1.22	Up
PC ae C42:1	0.19 (0.02)	0.20 (0.02)	0.818	-1.05	Down	0.15 (0.01)	0.13 (0.01)	0.614	1.15	Up	0.15 (0.03)	0.14 (0.02)	0.879	1.07	Up
PC ae C42:2	0.27 (0.04)	0.22 (0.02)	0.303	1.23	Up	0.28 (0.02)	0.14 (0.01)	0.001*	2	Up	0.21 (0.05)	0.14 (0.02)	0.242	1.5	Up
PC ae C42:3	0.33 (0.04)	0.23 (0.02)	0.051	1.43	Up	0.29 (0.02)	0.13 (0.01)	0.001*	2.23	Up	0.22 (0.05)	0.15 (0.02)	0.242	1.47	Up
PC ae C44:3	0.06 (0.00)	0.05 (0.00)	0.06	1.20	Up	0.05 (0.00)	0.03 (0.00)	0.001*	1.67	Up	0.05 (0.01)	0.03 (0.00)	0.062	1.67	Up
PC ae C44:4	0.11 (0.01)	0.08 (0.00)	0.019*	1.38	Up	0.10 (0.01)	0.06 (0.00)	0.002*	1.67	Up	0.09 (0.02)	0.05 (0.01)	0.123	1.8	Up
PC ae C44:5	0.09 (0.01)	0.07 (0.01)	0.336	1.29	Up	0.08 (0.01)	0.05 (0.00)	0.019*	1.6	Up	0.06 (0.02)	0.05 (0.00)	0.882	1.2	Up
SM (OH) C14:1	17.34 (1.32)	10.95 (0.87)	0.002*	1.58	Up	12.57 (1.49)	8.94 (0.90)	0.039*	1.41	Up	15.42 (1.58)	9.73 (0.83)	0.004*	1.58	Up
SM (OH) C16:1	13.72 (1.15)	8.48 (0.81)	0.007*	1.62	Up	11.10 (0.91)	6.26 (0.56)	0.001*	1.77	Up	10.67 (1.53)	6.84 (0.65)	0.026*	1.56	Up
SM (OH) C22:1	27.92 (2.22)	12.57 (1.34)	0.001*	2.22	Up	19.57 (2.21)	11.97 (1.76)	0.023*	1.63	Up	33.73 (2.86)	12.23 (1.26)	0.001*	2.76	Up
SM (OH) C22:2	10.13 (1.17)	5.55 (0.55)	0.004*	1.83	Up	8.04 (0.69)	4.36 (0.46)	0.001*	1.84	Up	10.90 (1.15)	4.45 (0.41)	0.001*	2.45	Up
SM (OH) C24:1	2.83 (0.22)	1.24 (0.12)	0.001*	2.28	Up	2.07 (0.13)	1.00 (0.10)	0.001*	2.07	Up	2.31 (0.22)	1.03 (0.09)	0.001*	2.24	Up
SM C16:0	120.83 (11.69)	83.03 (7.45)	0.042*	1.45	Up	95.27 (8.56)	70.51 (8.21)	0.072	1.35	Up	137.01 (11.03)	74.72 (7.00)	0.001*	1.83	Up
SM C16:1	13.37 (1.59)	9.75 (0.86)	0.071	1.37	Up	10.33 (0.99)	6.85 (0.70)	0.016*	1.51	Up	12.76 (1.23)	7.54 (0.69)	0.003*	1.69	Up
SM C18:0	15.57 (1.87)	9.37 (0.85)	0.016*	1.66	Up	12.71 (1.46)	8.17 (0.87)	0.023*	1.56	Up	17.81 (1.75)	8.48 (0.81)	0.001*	2.1	Up
SM C18:1	5.33 (0.79)	3.97 (0.37)	0.192	1.34	Up	4.35 (0.45)	3.27 (0.34)	0.083	1.33	Up	5.48 (0.52)	3.43 (0.34)	0.007*	1.6	Up
SM C20:2	0.08 (0.03)	0.28 (0.05)	0.019*	-3.5	Down	0.04 (0.02)	0.17 (0.02)	0.005*	-4.25	Down	0.06 (0.03)	0.16 (0.02)	0.031*	-2.67	Down
SM C24:0	33.21 (5.95)	17.37 (2.01)	0.012*	1.91	Up	27.29 (1.74)	12.94 (1.35)	0.001*	2.11	Up	29.07 (3.00)	13.85 (1.53)	0.001*	2.1	Up

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM/CON
SM C24:1	13.28 (0.48)	8.08 (0.79)	0.004*	1.64	Up	10.61 (1.10)	7.65 (0.78)	0.062	1.39	Up	16.81 (1.52)	7.45 (0.80)	0.001*	2.26	Up
SM C26:0	0.57 (0.11)	0.24 (0.02)	0.001*	2.38	Up	0.50 (0.04)	0.19 (0.02)	0.001*	2.63	Up	0.43 (0.07)	0.19 (0.02)	0.001*	2.26	Up
SM C26:1	0.36 (0.16)	0.10 (0.02)	0.234	3.60	Up	0.26 (0.11)	0.13 (0.02)	0.503	2	Up	0.23 (0.03)	0.12 (0.02)	0.011*	1.92	Up
Hexose	4.063 (247)	3.093 (232)	0.019*	1.31	Up	3.561 (199)	2.458 (202)	0.003*	1.45	Up	3.377 (121)	2.354 (179)	0.001*	1.43	Up
Alanine	259.00 (23.38)	195.40 (10.64)	0.038*	1.33	Up	242.50 (15.96)	158.36 (10.07)	0.003*	1.53	Up	180.33 (14.89)	135.39 (12.93)	0.094	1.33	Up
Arginine	212.40 (14.06)	108.14 (7.43)	0.001*	1.96	Up	195.33 (5.76)	103.05 (5.91)	0.001*	1.9	Up	137.05 (16.02)	96.03 (7.56)	0.024*	1.43	Up
Asparagine	12.68 (0.86)	30.59 (3.78)	0.001*	-2.41	Down	12.54 (1.90)	26.45 (2.54)	0.003*	-2.11	Down	20.42 (5.39)	26.11 (2.56)	0.421	-1.28	Down
Aspartic acid	19.04 (1.97)	16.06 (3.49)	0.185	1.19	Up	23.48 (2.96)	14.60 (3.92)	0.031*	1.61	Up	16.22 (2.39)	12.33 (2.49)	0.073	1.32	Up
Citrulline	69.56 (10.48)	53.75 (3.47)	0.112	1.29	Up	72.33 (8.27)	56.30 (4.31)	0.083	1.28	Up	94.48 (14.85)	51.87 (4.50)	0.001*	1.82	Up
Glutamine	247.40 (25.06)	286.15 (21.62)	0.262	-1.16	Down	284.67 (30.78)	247.88 (25.27)	0.563	1.15	Up	297.33 (41.13)	218.18 (21.60)	0.144	1.36	Up
Glutamate	185.60 (19.81)	90.53 (7.67)	0.002*	2.05	Up	185.83 (14.49)	71.90 (7.54)	0.001*	2.58	Up	96.28 (22.15)	68.42 (6.18)	0.191	1.41	Up
Glycine	259.40 (19.01)	208.55 (16.17)	0.083	1.24	Up	277.83 (30.31)	269.50 (26.26)	0.605	1.03	Up	416.00 (84.43)	252.83 (34.64)	0.055	1.65	Up
Histidine	69.50 (5.12)	51.23 (3.37)	0.024*	1.36	Up	69.33 (5.44)	39.51 (3.03)	0.001*	1.75	Up	52.18 (3.33)	38.33 (2.69)	0.011*	1.36	Up
Isoleucine	293.40 (11.69)	113.87 (10.02)	0.001*	2.58	Up	279.00 (20.30)	99.88 (9.23)	0.001*	2.79	Up	315.67 (14.04)	100.92 (8.94)	0.001*	3.13	Up
Leucine	375.20 (47.35)	150.69 (15.62)	0.001*	2.49	Up	335.67 (19.17)	134.57 (12.29)	0.001*	2.5	Up	467.17 (38.98)	129.56 (11.81)	0.001*	3.61	Up
Lysine	511.20 (71.63)	90.61 (5.80)	0.001*	5.64	Up	433.00 (64.89)	74.14 (4.78)	0.001*	5.84	Up	563.67 (32.07)	69.24 (6.51)	0.001*	8.14	Up
Methionine	29.97 (13.90)	26.94 (3.19)	0.435	1.11	Up	38.72 (13.43)	21.86 (1.91)	0.583	1.77	Up	24.47 (7.45)	19.19 (2.08)	0.447	1.28	Up
Ornithine	27.28 (12.16)	40.48 (2.60)	0.336	-1.48	Down	38.89 (13.08)	30.55 (2.94)	0.976	1.27	Up	27.26 (9.33)	29.23 (3.42)	0.656	-1.07	Down
Phenylalanine	64.00 (4.98)	50.22 (3.84)	0.067	1.27	Up	70.78 (9.29)	43.73 (3.87)	0.011*	1.62	Up	48.73 (9.54)	41.71 (3.33)	0.692	1.17	Up

Table 1 (continued)

Metabolite ^b , μM	8 weeks before parturition				4 weeks before parturition				LAM diagnosis week ^a						
	LAM	CON	P-value	Fold change	LAM/CON	LAM	CON	P-value	Fold change	LAM	CON	P-value	Fold change		
Proline	82.92 (6.39)	72.70 (5.72)	0.408	1.14	Up	79.77 (6.74)	63.36 (4.22)	0.095	1.26	Up	61.92 (12.43)	57.62 (4.77)	0.324	1.07	Up
Serine	105.96 (11.20)	66.84 (6.58)	0.006*	1.58	Up	102.58 (4.52)	66.76 (6.96)	0.004*	1.54	Up	85.00 (11.78)	59.38 (5.25)	0.039*	1.43	Up
Threonine	87.46 (6.91)	78.58 (8.16)	0.097	1.11	Up	86.17 (8.74)	70.66 (7.01)	0.073	1.22	Up	70.30 (5.21)	64.22 (6.47)	0.324	1.09	Up
Tryptophan	22.58 (1.78)	41.67 (3.33)	0.004*	-1.85	Down	22.25 (2.25)	29.48 (3.29)	0.465	-1.32	Down	18.17 (1.34)	29.90 (3.47)	0.083	-1.65	Down
Tyrosine	57.72 (3.67)	44.12 (3.12)	0.019*	1.31	Up	49.27 (5.62)	35.55 (3.30)	0.072	1.39	Up	43.68 (4.79)	35.00 (3.41)	0.301	1.25	Up
Valine	317.40 (20.08)	218.05 (17.60)	0.017*	1.46	Up	271.00 (30.95)	185.31 (14.62)	0.019*	1.46	Up	240.33 (26.32)	183.46 (16.27)	0.095	1.31	Up
Acetylornithine	6.46 (1.39)	3.73 (0.47)	0.083	1.73	Up	5.45 (0.86)	3.72 (0.59)	0.059	1.47	Up	7.63 (2.10)	3.32 (0.47)	0.016*	2.3	Up
Asymmetric dimethylarginine total	0.96 (0.03)	0.79 (0.06)	0.035*	1.22	Up	0.91 (0.07)	0.60 (0.06)	0.021*	1.52	Up	0.62 (0.09)	0.55 (0.06)	0.761	1.13	Up
Dimethylarginine	1.09 (0.01)	0.77 (0.06)	0.015*	1.42	Up	1.11 (0.08)	0.68 (0.06)	0.001*	1.63	Up	0.99 (0.04)	0.64 (0.05)	0.002*	1.55	Up
Carnosine	7.68 (1.18)	5.60 (0.42)	0.083	1.37	Up	6.82 (0.79)	4.96 (0.33)	0.039*	1.38	Up	6.37 (0.91)	4.25 (0.37)	0.048*	1.5	Up
Creatinine	83.30 (2.61)	77.12 (6.00)	0.174	1.08	Up	87.95 (4.54)	68.88 (3.98)	0.026*	1.28	Up	77.32 (5.18)	57.76 (4.36)	0.036*	1.34	Up
Kynurenine	14.42 (1.44)	8.34 (0.69)	0.004*	1.73	Up	15.40 (1.43)	6.54 (0.60)	0.001*	2.35	Up	13.30 (0.94)	6.58 (0.77)	0.001*	2.02	Up
Sarcosine	4.50 (0.68)	2.74 (0.44)	0.062	1.64	Up	4.60 (1.23)	3.51 (0.65)	0.268	1.31	Up	4.70 (0.55)	2.62 (0.64)	0.013*	1.79	Up
Taurine	50.92 (8.80)	37.70 (2.49)	0.169	1.35	Up	54.23 (5.76)	36.44 (3.11)	0.014*	1.49	Up	51.93 (5.87)	33.93 (4.02)	0.039*	1.53	Up

^aCows were diagnosed with lameness (n = 6) ranging from week + 1 to + 3 (i.e., 5–21 days, mean 13 days)

^bC10: decanoyl-L-carnitine; C16: hexadecanoyl-L-carnitine; C18: octadecanoyl-L-carnitine; C2: acetyl-L-carnitine; C3: propionyl-L-carnitine; C4: butyryl-L-carnitine; C5: valeryl-L-carnitine; lysoPC a: lysophosphatidylcholine acyl; PC aa: phosphatidylcholine diacyl; PC ae: phosphatidylcholine acyl-alkyl; SM (OH): hydroxysphingomyelin; SM: sphingomyelin; lysoPC, PC aa, and PC ae are glycerophospholipids; SM (OH) and SM are sphingolipids

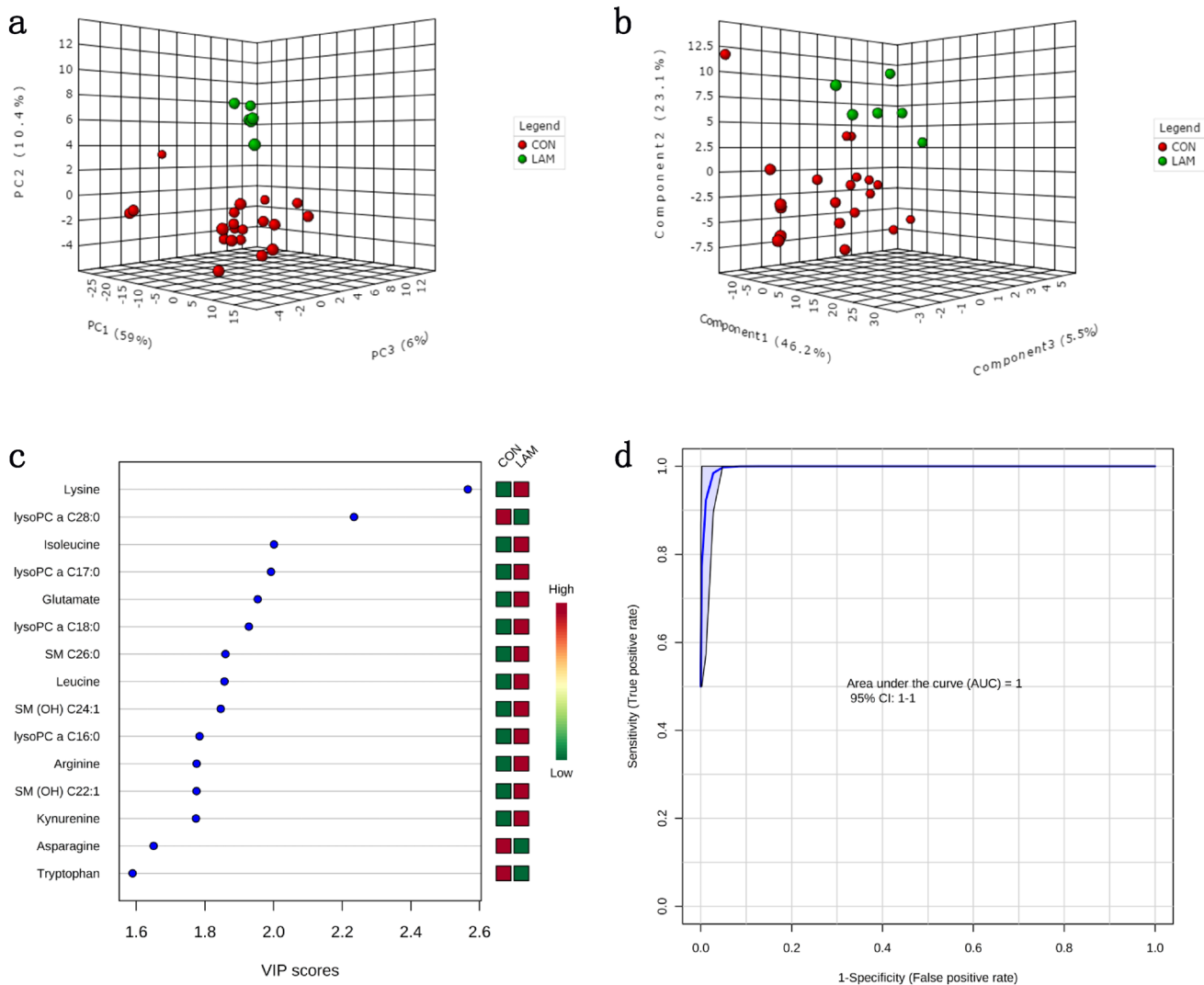


Fig. 1 **a** Principal component analysis (PCA) and **b** Partial least squares-discriminant analysis (PLS-DA, permutation test: $P < 0.05$) of 20 control (CON) and 6 pre-lame cows at -8 weeks before parturition showing 2 separated clusters for 2 groups. **c** Variables ranked

by variable importance in projection (VIP), and **d** Receiver-operator characteristic (ROC) curve of 20 CON and 6 pre-lame cows at -8 weeks before parturition for the top 5 serum variables (i.e., Lys, lysoPC a C17:0, Ile, lysoPC a C17:0, and Glu; empirical $P = 0.001$)

C28:0), isoleucine (Ile), lysophosphatidylcholine acyl C17:0 (LysoPC a C17:0), and glutamate (Glu) at -8 week and Lys, arginine (Arg), ile, leucine (Leu), and Glu at -4 weeks were the strongest discriminating metabolites for separating pre-lame cases from CON cows. The heat map on the right side of the two VIP plots indicated that most metabolites were increased except 3 metabolites [i.e., Lyso PC a C28:0, asparagine (Asn), and tryptophan (Trp)] that were decreased in pre-lame cows relative to CON ones. A ROC curve plot showing the performance of the top 5 VIP metabolites in predicting which cows will develop lameness at -8 and -4 weeks pre-calving using a PLS-DA model are shown in Figs. 1d and 2d.

The AUC for the two curves were 1.0 (95% CI 1-1) at -8 weeks (empirical $p = 0.001$) and 0.997 (95% CI 0.945-1)

at -4 weeks (empirical $p = 0.001$), respectively, which indicates that those serum biomarkers have strong predictive abilities. Alterations of serum metabolites preceded lameness (with a locomotion score 4) by 5-11 weeks (lameness was diagnosed between +1 and +3 weeks postpartum). Moreover, the results also demonstrated that the biomarker models developed at -8 and -4 weeks could be used to monitor the risk or susceptibility of cows to lameness.

3.2 Serum metabolites during the week of lameness

Univariate analysis indicated that a total of 37 metabolites in the serum were different during the week of diagnosis of lameness between the two groups of cows. Cows with

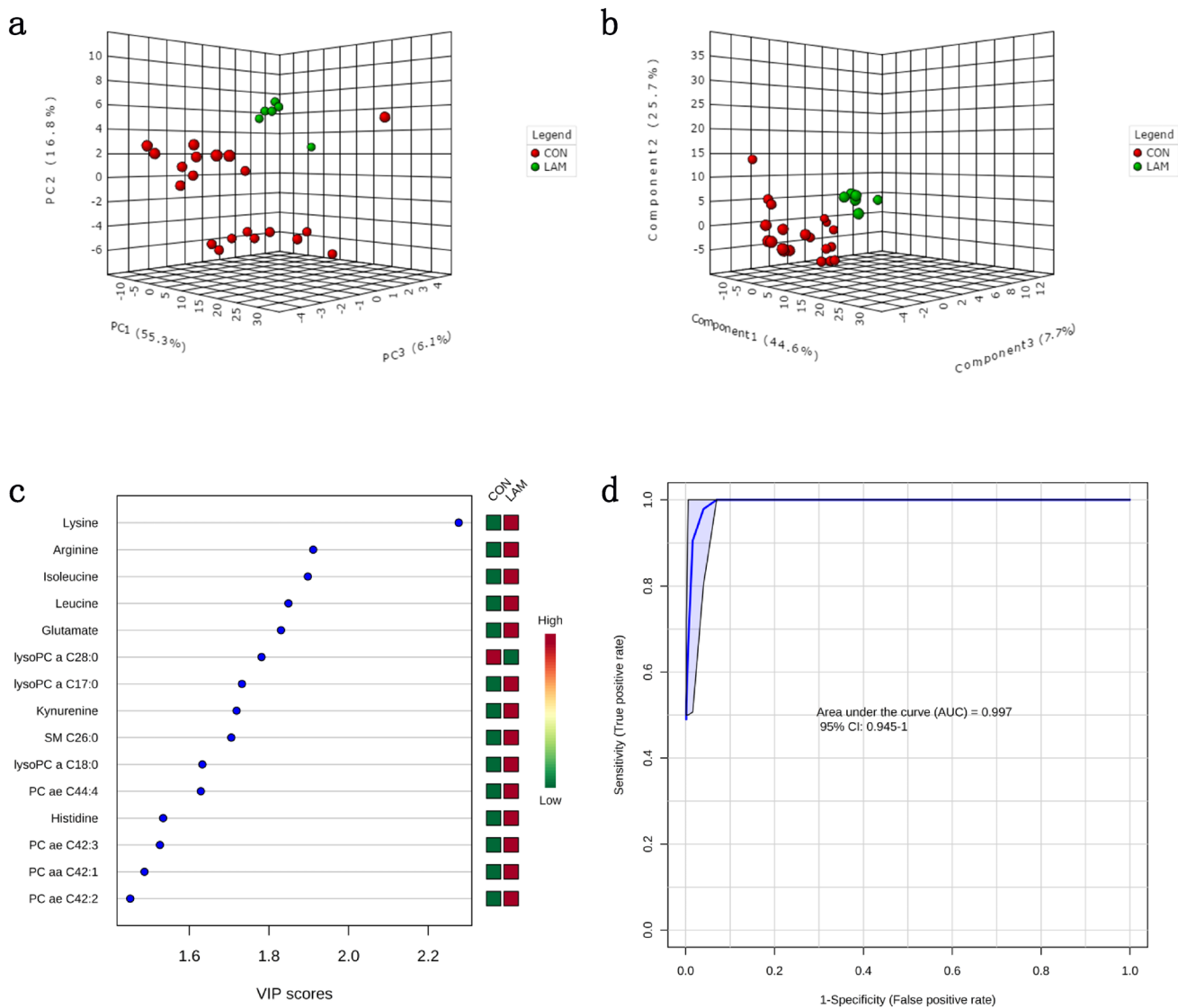


Fig. 2 **a** PCA and **b** PLS-DA (Permutation test: $P < 0.05$) of 20 CON and 6 pre-lame cows at -4 weeks before parturition showing 2 separated clusters for 2 groups, **c** VIP, and **d** ROC curve of 20 CON and

6 pre-lame cows at -4 weeks before parturition for the top 5 serum variables (i.e., Lys, Arg, Ile, Leu, and Glu; empirical $P = 0.001$)

lameness experienced elevated concentrations of 1 AC, 6 glycerophospholipids, 13 sphingolipids, 14 AAs, and hexose in the serum (Table 1). On the other hand, 1 glycerophospholipid and 1 sphingolipid were decreased in lame cows (Table 1).

When cows with lameness were compared with CON cows at lameness week, PCA and PLS-DA (permutation test: $p < 0.05$) multivariate analysis revealed a distinctive separation between the two groups of cows (Fig. 3a, b). In this case, 5 metabolites (i.e., Lys, Leu, Ile, hydroxysphingomyelin C22:1 (SM (OH) C22:1), and LysoPC a C28:0) with greatest VIP scores contributed most significantly to the observed separation (Fig. 3c). The ROC curve (Fig. 3d; empirical $p = 0.001$) indicated that this metabolite-combination was a

highly significant biomarker of lameness with AUC, 0.997 (95% CI 0.976–1).

3.3 Serum metabolotypes after lameness week

Results showed that at both $+4$ and $+8$ weeks after calving cows affected by lameness still had alterations in the serum metabolites in comparison with CON cows (Supplementary Table 1). At $+4$ weeks after parturition, lame cows had 35 metabolites altered in the serum compared to CON cows. In particular, all of the 35 metabolites (i.e., 2 ACs, 13 glycerophospholipids, 1 sphingolipid, and 8 AAs) were elevated in lame cows at $+4$ weeks postpartum. Intriguingly, during the $+8$ weeks after parturition, cows affected by lameness (free

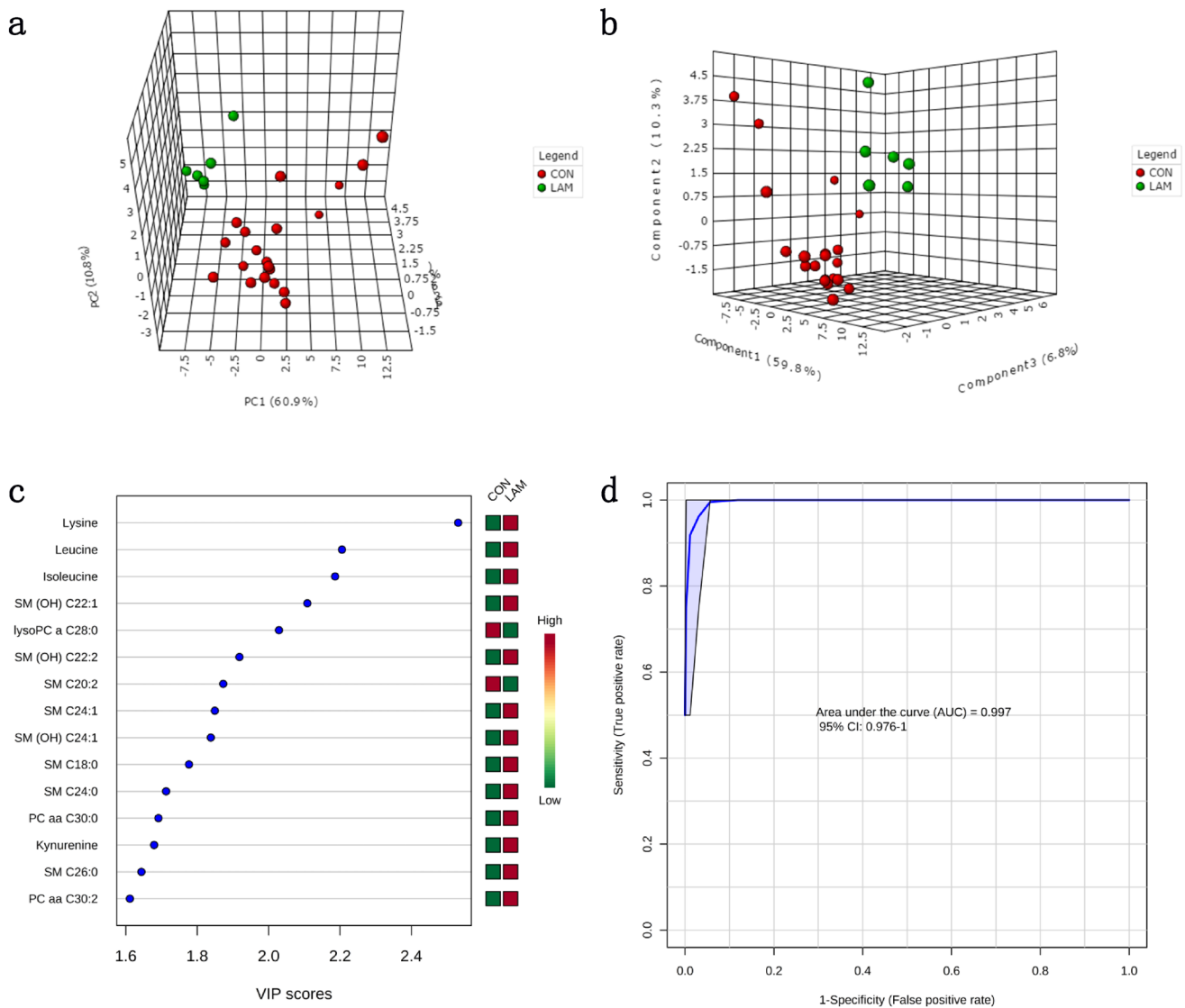


Fig. 3 **a** PCA and **b** PLS-DA (Permutation test: $P < 0.05$) of 20 CON and 6 lame cows at disease week showing 2 separated clusters for 2 groups, **c** VIP, and **d** ROC curve of 20 CON and 6 lame cows at dis-

ease week for the top 5 serum variables (i.e., Lys, Leu, Ile, SM (OH) C22:1, and lysoPC a C28:0; empirical $P = 0.001$)

of clinical signs of lameness) continued to have alterations of 28 metabolites in the serum, including 27 (i.e., 7 glycerophospholipids, 10 sphingolipids, and 10 AAs) enhanced and 1 BA (i.e., asymmetric dimethylarginine—ADMA) decreased.

Multivariate analyses [i.e., PCA and PLS-DA (permutation test: $p < 0.05$)] revealed that CON and lame cows were clearly separated at +4 and +8 weeks postpartum (Supplementary Figs. 1a, b, 2a, b). The corresponding VIP plots for those two time points are shown in Supplementary Figs. 1c and 2c, which indicated that Lys, Ile, Leu, acetylnithine, and hexadecanoyl-L-carnitine (C16) were the most discriminating metabolites at +4 weeks, and Lys, Ile, Leu, acetylnithine, and hydroxysphingomyelin C24:1 (SM (OH) C24:1) were the

top 5 metabolites for the separation of clusters at +8 weeks postpartum. Multivariate models (ROC curves) combining 5 discriminating metabolites (i.e., Lys, Ile, Leu, acetylnithine, and C16) at +4 weeks (empirical $p < 0.05$) and 5 metabolites (i.e., Lys, Ile, Leu, acetylnithine, and SM (OH) C24:1) at +8 weeks (empirical $p < 0.05$) produced an AUC of 0.985 (95% CI 0.806–1, Supplementary Fig. 1d) and 0.96 (95% CI 0.556–1, Supplementary Fig. 2d), respectively.

3.4 Metabolic pathways related to onset and progression of lameness

Quantitative enrichment analyses and metabolic pathway analyses were conducted between the two groups of cows

at 5 time-points, separately. Significant metabolic pathways and related metabolites that were involved at the 5-time points around parturition are presented in Table 2. Results showed that the main metabolic alterations in pre-lame cows at – 8 weeks prior to parturition were related to Lys degradation, biotin metabolism, protein biosynthesis, phospholipid metabolism, Trp metabolism, aspartate (Asp) metabolism, urea cycle, valine (Val)-Leu-Ile degradation, and Arg and proline (Pro) metabolism (Table 2 and Fig. 4a). At – 4 weeks prepartum, it was observed that the main metabolic pathways affected were those of Lys degradation, biotin metabolism, protein biosynthesis, Trp metabolism, Asp metabolism, Val-Leu-Ile degradation, ammonia recycling, and histidine (His) metabolism (Table 2 and Fig. 4b).

Metabolic pathway analysis also indicated that during the week of diagnosis of lameness, affected cows experienced altered concentrations of serum metabolites related to Lys degradation, biotin metabolism, Trp metabolism, and Val-Leu-Ile degradation (Table 2 and Fig. 4c). Interestingly, cows with lameness still encountered alterations of 3 metabolic pathways at + 4 weeks, and 7 pathways at + 8 weeks postpartum, respectively. This indicates that metabolic alterations of key pathways in lame cows were still present even at + 2 to + 6 weeks after lameness event (Table 2 and Fig. 4d, e).

Overall, throughout the 16-weeks of the study period, 13 serum metabolites (i.e., phosphatidylcholine diacyl C30:0 (PC aa C30:0), phosphatidylcholine diacyl C30:2 (PC aa C30:2), phosphatidylcholine diacyl C42:1 (PC aa C42:1), phosphatidylcholine diacyl C40:2 (PC ae C40:2), hydroxysphingomyelin C14:1 (SM (OH) C14:1), hydroxysphingomyelin C16:1 (SM (OH) C16:1), SM (OH) C22:1, SM (OH) C24:1, sphingomyelin C18:0 (SM C18:0), sphingomyelin C26:0 (SM C26:0), Ile, Leu, and Lys) were up-regulated and appeared to play a consistent role in distinguishing between the CON and pre-lame or lame cows (Table 1 and Supplementary Table 1). Furthermore, 4 metabolic pathways (i.e., Lys degradation, biotin metabolism, Trp metabolism, and Val-Leu-Ile degradation) were altered in cows prior to, during, and after lameness during the onset and progression of the disease.

4 Discussion

We hypothesized that DI/LC–MS/MS-based serum metabolomics would be able to identify and quantify metabolites that are perturbed in pre-lame dairy cows during the dry-off period, diagnosis of lameness as well as after lameness week. Indeed, our data show that there were multiple metabolite and pathway alterations in all time points considered

Table 2 Significant metabolic pathways involved in the onset and progression of laminitis in dairy cows from quantitative enrichment analysis

Metabolite set	Total cmpd	Hits	Significant cmp
Lysine degradation ^{a-c}	13	1	L-Lysine
Biotin metabolism ^{a-e}	4	1	L-Lysine
Protein biosynthesis ^{a,b}	19	12	L-Tyrosine; L-Phenylalanine; L-Proline; L-Threonine; L-Asparagine; L-Histidine; L-Lysine; L-Arginine; L-Glutamine; L-Leucine; L-Methionine; L-Valine
Phospholipid biosynthesis ^a	19	1	LysoPC(16:0)
Tryptophan metabolism ^{a-c}	34	1	L-Kynurenine
Aspartate metabolism ^{a,b}	12	3	L-Asparagine; Citrulline; D-Aspartic acid
Urea cycle ^a	20	4	Ornithine, L-Arginine, Glutamine, Citrulline
Valine, leucine and isoleucine degradation ^{a-c}	36	2	L-Leucine; L-Valine
Arginine and proline metabolism ^a	26	4	L-Proline, Ornithine, L-Arginine, Citrulline
Ammonia recycling ^b	18	5	Glycine, L-Asparagine, L-Histidine, L-Serine, L-Glutamine
Histidine metabolism ^b	11	2	Carnosine; L-Histidine
Bile acid biosynthesis ^e	49	2	Glycine; Taurine
Glutathione metabolism ^e	10	1	Glycine
Porphyrin metabolism ^e	22	1	Glycine
Taurine and hypotaurine metabolism ^e	7	1	Taurine

^aSignificant metabolic pathways at – 8 weeks before parturition; Holm $P < 0.05$

^bSignificant metabolic pathways at – 4 weeks before parturition; Holm $P < 0.05$

^cSignificant metabolic pathways at the week of diagnosis of disease; Holm $P < 0.05$

^dSignificant metabolic pathways at + 4 weeks after parturition; Holm $P < 0.05$

^eSignificant metabolic pathways at + 8 weeks after parturition; Holm $P < 0.05$

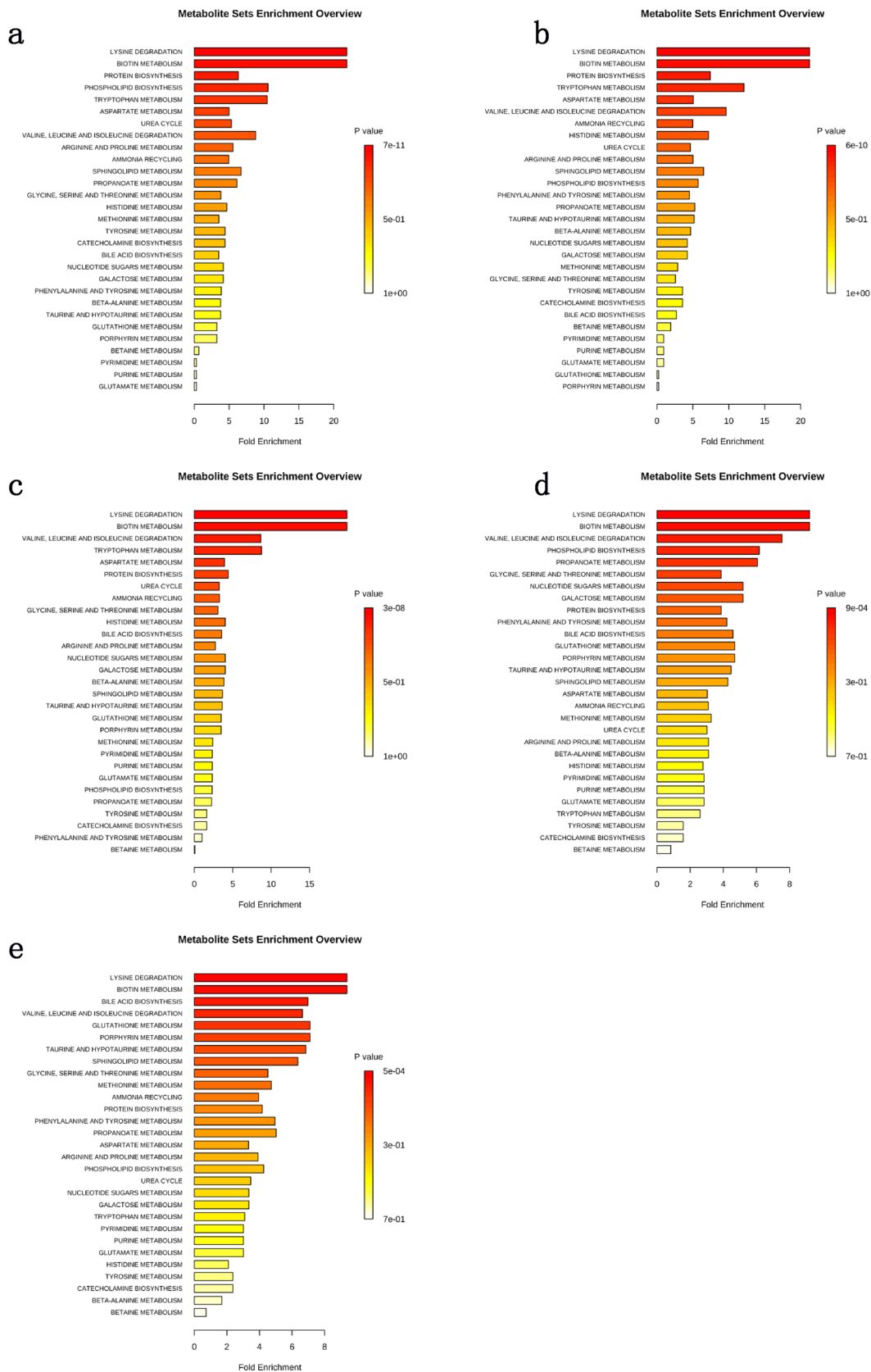


Fig. 4 Summary plots for quantitative enrichment analysis at **a** – 8 weeks, **b** – 4 weeks, **c** week of the diagnosis of disease, **d** +4 weeks, and **e** +8 weeks relative to parturition

in this study. The main metabolites identified as altered belonged to AAs, glycerophospholipids, sphingolipids, acyl-carnitines, and hexose.

We also aimed at identifying potential metabolite panels in the serum that might be useful for screening cows for the risk of developing lameness at the start and during the dry-off period. Indeed, the data showed that combination of 5 serum metabolites (i.e., lys, lysoPC a C28:0, ile, lysoPC a C17:0, and Glu at – 8 weeks and lys, arg, ile, leu, and Glu at – 4 weeks prepartum) appeared to be highly accurate predictors of the onset of lameness during the first 3 weeks of lactation.

In a companion article, we reported that pre-lame cows experienced alterations of serum variables related to innate immunity, carbohydrate, and lipid metabolism. Intriguingly, elevated concentrations of interleukin (IL)-6, tumor necrosis factor (TNF), haptoglobin (Hp), serum amyloid A (SAA), and lactate were observed in pre-lame cows starting at – 8 and – 4 weeks before calving in comparison with CON cows (Zhang et al. 2015). These alterations of pro-inflammatory cytokines and APP suggested that pre-lame cows experience a systemic inflammation prior to the clinical appearance of the disease. Since innate immunity related variables are non-specific indicators of an inflammatory state, the present article aimed to identify more specific screening panels of biomarkers for lameness in the serum of transition dairy cows.

One of the identified metabolite groups that was altered during pre-lame and lame periods was that of ACs. The latter are important screening biomarkers for disorders of fatty acid oxidation and organic acid metabolism (Rinaldo et al. 2008). Our findings are in agreement with Rutkowsky et al. (2014) who reported that ACs have the potential to activate pro-inflammatory signal pathways. Several other metabolomics reports have also shown that ACs are useful biomarkers for immune activation, organ dysfunction, and inflammatory disease (Frye et al. 2013; Hailemariam et al. 2014a; Sampey et al. 2012). Additionally, medium-chain ACs [i.e., decanoyl-L-carnitine (C10)], which are by-products of incomplete β -oxidation of long-chain fatty acids, contribute to dysregulated fatty acid oxidation in mitochondria by activation of pro-inflammatory signaling pathways (Adams et al. 2009; Rutkowsky et al. 2014). In a recent study by our lab cows affected by more than one of the four diseases including metritis, mastitis, lameness, and retained placenta also showed elevated concentrations of carnitine and ACs in the serum of sick cows compared with healthy ones (Hailemariam et al. 2014a). Enhanced concentrations of C10 in pre-lame cows at – 8 weeks and – 4 weeks prior to parturition, and the week of diagnosis of lameness confirms our previous report that pre-lame cows experience a state of inflammation during the dry-off period before the occurrence of disease with increased concentrations of pro-inflammatory cytokines

(i.e., IL-6 and TNF) and APP (i.e., Hp and SAA) in the serum (Zhang et al. 2015).

Results of the present study showed that there were elevated concentrations of several LysoPC (i.e., LysoPC a C16:0, LysoPC a C17:0; LysoPC a C18:0) in the serum of pre-lame cows at – 8 and – 4 weeks prior to parturition. Lysophosphatidylcholine (LysoPC), a pro-inflammatory phospholipid, is the most abundant lysophospholipid in the blood and tissues. LysoPC originates from hydrolysis of phosphatidylcholine (PC) by the enzyme lecithin-cholesterol acyltransferase, hepatic secretion, or action of phospholipase A2 (Liebisch et al. 2002). LysoPC has been suggested to play a functional role in the pathogenesis of various diseases in humans and animals alike (Hailemariam et al. 2014a; Liebisch et al. 2002). Particularly, LysoPC can induce a variety of pro-inflammatory actions in leukocytes, smooth muscle cells, and endothelial cells and its effects vary depending on the length of acyl chain (Ojala et al. 2007). LysoPCs' role in the regulation of immune functions and its properties in the process of inflammation have been previously reported (Kabarowski et al. 2002; Koh et al. 2000). Specifically, palmitic acid (16:0) and stearic acid are the primary fatty acid species that LysoPC associates with in plasma (Croset et al. 2000). Lysophosphatidylcholine acyl (LysoPC a) C16:0 is present in high density lipoproteins (HDL), whereas the LysoPC a C18:0 is mainly associated with apolipoprotein-B-containing lipoproteins [i.e., very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL)]. This association may explain acyl chain-dependent effects of LysoPC on immune functions and the individual role of LysoPC species on distinct metabolic pathways (Kontush et al. 2013; Ojala et al. 2007). Increased concentrations of LysoPC at – 8 and – 4 weeks prior to parturition might be related to an increased demand for metabolites to mount a chronic low-grade inflammatory response during the early stages of preclinical lameness.

Phosphatidylcholine (PC) is the most abundant phospholipid, about 60–80%, depending on species (Pison et al. 1994). It has been documented as a promising innate immune modulator of host responses to infection (Stremmel et al. 2010). Hazen and Chisolm (2002) reported that oxidized PCs are pattern recognition ligands for multiple pathways of innate immune response. Oxidized lipids (i.e., PCs) and lipoprotein by-products can induce leukocyte recruitment, activation, and apoptosis (Hazen and Chisolm 2002). Specifically, accumulation of these oxidation products (i.e., PCs) at the sites of inflammation can have severe pathological consequences, such as in the case of developing arterial lesions (Chisolm and Steinberg 2000; Glass and Witztum 2001). Most serum PCs increased in our cows with lameness at – 4 weeks prepartum, which suggest that the onset of pathological events of lameness might occur at least 6–7 weeks prior to diagnosis of lameness. Enhanced

concentrations of PCs in the serum of pre-lame cows also supports previous suggestions of histamine involvement in the development of lameness (Bergsten 2003). During the early stages of the disease, subclinical inflammation is accompanied with activation of basophils and mast cells in the claw area and release of histamine from those cells. In the meantime, phospholipid metabolism of basophils and mast cells changes dramatically, which is characterized by an increased turnover of PC, phosphatidylinositol, and phosphatidic acid (Crews et al. 1980). Therefore, it is speculated that inflammation associated with lameness could lead to lipid alterations in the serum that might help in the healing process and at the same time can be used to monitor cows for the risk of lameness.

In a previous study, we demonstrated that concentrations of various sphingolipids in the plasma were greater in cows with more than one periparturient disease at -4 (2 sphingolipids) and -1 weeks (8 sphingolipids) prior to parturition (Hailemariam et al. 2014b). In the current study, 13 detectable SM species in the serum were up-regulated, in cows affected by lameness, and most of them were already elevated during -8 and -4 weeks prior to parturition, in the pre-lame cows. Together with cholesterol and glycerophospholipids, sphingolipids (i.e., sphingomyelins (SMs) and glycosphingolipids) are ubiquitous building blocks of membranes of eukaryotic cells (Kolter and Sandhoff 2006). Sphingolipids are characterized by presence of a sphingoid base within the hydrophobic part of the molecule. In SM, a phosphorylcholine is bound to the terminal hydroxyl group of ceramide (*N*-acylsphingosine) (Kolter and Sandhoff 1999). Ceramide and other intermediates like sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), sphingosine, and sphingosylphosphorylcholine are released from the molecule of SM by de-novo synthesis, in response to proinflammatory cytokines like TNF, IL-1, and interferon (IFN)- γ (Kolter and Sandhoff 2006). More specifically, ceramide, S1P, C1P, sphingosine, and sphingosylphosphorylcholine are signaling molecules that regulate a diverse range of cellular processes that are pivotal in inflammation, immunity, inflammatory disorders, and various disease states (Brodesser et al. 2003; Kolter and Sandhoff 2006; Maceyka and Spiegel 2014). Our data suggest that increased concentrations of sphingolipids in the serum of cows before and during the occurrence of lameness might be attributed to the mobilization of sphingolipids to mount a response against inflammation that associates lameness.

The importance of AAs in mounting an immune response and their enhanced utilization during inflammatory responses have been reviewed previously (Li et al. 2007; McGaha et al. 2012). An increasing body of evidence indicates that AAs play essential roles in the synthesis and production of antibodies, cytokines, APP, and other cytotoxic substances (Li et al. 2007). Specifically, alanine (Ala)

is a major substrate for glucose synthesis in the liver, and a significant energy substrate for leukocytes (Newsholme and Newsholme 1989). Elevated serum Ala in lame cows during the disease week can be attributed to activation of innate and acquired immunity for clearance of inflammatory agents. On the other hand, Arg has been reported to be a potent secretagogue for several hormones such as insulin, insulin-like growth factor-I, growth hormone, and prolactin, all of which contribute to regulation of NO-independent effect of Arg on immune functions (Newsholme et al. 2005). Arginine has been shown to enhance immune responses including the release of cytokines, production and maturation of T-lymphocytes, and clearance of bacteria (Li et al. 2007). Histidine (His) is a precursor of histamine, which is a mediator of various biological processes including inflammation and regulation of immune functions (Maintz and Novak 2007). Increased concentrations of His in the serum of pre-lame cows at -8 and -4 weeks prior to parturition suggest that histamine might have been enhanced at early stages of lameness and potentially contributed to lameness.

Branched-chain amino acids (BCAA) including Leu, Ile, and Val play important roles in the endogenous synthesis of Ala and glutamine (Gln), primarily in skeletal muscles (Newsholme and Calder 1997). In particular, Leu is an important activator of the mTOR signaling pathway that regulates protein synthesis (such as pro-inflammatory cytokines, APP, and antibodies) and degradation in immune cells (Meijer and Dubbelhuis 2004). Isoleucine is necessary for the synthesis of hemoglobin, blood clotting proteins, and also stabilizes and mediates blood glucose and energy levels (Booth and Wettstein 2008). Lysine is an essential building block for the synthesis of all proteins (including cytokines, antibodies, APP, hormones, and enzymes). It also contributes to collagen formation and tissue repair (Spallota et al. 2013). Therefore, elevated concentrations of Lys in lame cows might be favorable to healing the lesions of the claw. The third BCAA, serine (Ser), is involved in the synthesis of glucose in the liver and kidney, especially in ruminants (Li et al. 2007). Moreover, Ser participates in the synthesis of ceramide, phosphatidylserine, and glycine (Gly) as elements of T and B cell structures and signaling (Kim et al. 2007; Li et al. 2007). Wu et al. (2006) reported that adequate amounts of Ser are essential for functioning of immune cells in ruminants, especially during late pregnancy.

Dimethylarginine (DMA), particularly asymmetric DMA (ADMA) has been demonstrated as an early predictor of sepsis-associated acute liver failure in humans (Brenner et al. 2012). In a study involving lipopolysaccharide (LPS)-induced endotoxemia, Bekpınar et al. (2013) demonstrated that administration of LPS increased levels of ADMA in the plasma. Enhanced concentration of total DMA in the serum of pre-lame cows in the present study, and during lameness week suggests a potential

involvement of endotoxin in the development of lameness. The kynurenine system and its role in immune function and modulation of the immune responses have been reported previously (Mándi and Vécsei 2012). Mounting evidence indicates an association between the kynurenine pathway and various cytokines including TNF, IFN- α , IFN- γ , IL-4, IL-23, and transforming growth factor (TGF)- β (Mándi and Vécsei 2012). In the current study, the 7 AAs (i.e., Ala, Arg, His, Leu, Ile, Lys, Ser) were up-regulated in pre-lame cows at - 8, - 4 weeks prepartum, and during the week of lameness diagnosis. These findings are consistent with our previous published data that showed that cows affected by more than one periparturient disease experience increased concentrations of 10 metabolites including 4 AAs at - 4 and - 1 weeks prepartum (Hailemariam et al. 2014b). Other AAs including aspartic acid (Asp), Glu, phenylalanine (Phe), tyrosine (Tyr), Val, ADMA, carnosine, creatinine, and taurine in the serum were also up-regulated in pre-lame cows at - 8 and - 4 weeks before parturition. The immune functions of these metabolites have been reviewed or studied by several authors elsewhere (Brenner et al. 2012; Li et al. 2012; McGaha et al. 2012; Teixeira et al. 2013).

Quantitative enrichment analyses and metabolic pathway analyses revealed that pre-lame and lame cows encountered 15 significantly altered metabolic pathways during the whole experimental period. It is notable that 4 metabolic pathways (i.e., Lys degradation, biotin metabolism, Trp metabolism, and Val-Leu-Ile degradation) were altered in pre-lame cows at - 8 and - 4 weeks prepartum and during the lameness event. It is of interest to discuss briefly about biotin metabolism involvement in lameness.

Biotin also known as vitamin B7 is essential for normal functioning of animal body. Biotin acts as a cofactor for five carboxylases that are necessary for the metabolism of glucose, fatty acid, and amino acid metabolism. Deficiency of biotin is related to various diseases, and mice deficient in this vitamin display enhanced inflammation. Several studies have demonstrated that biotin influences the functions of adaptive immune T and NK cells. Dendritic cells (DCs) grown in a medium deficient in biotin and activated with lipopolysaccharide (LPS) increased the release of the pro-inflammatory cytokines TNF- α and IL-1 β compared with control DCs cultured in biotin-sufficient media and stimulated by LPS (Agrawal et al. 2016). These findings are in agreement with a report that showed that supplementation of biotin for 5 months to dairy cows was associated with a decline in the heel erosions and sole avulsions along with total disappearance of white line fissures and double soles in the biotin supplemented dairy cows (Randhawa et al. 2008).

Besides alterations of the aforementioned metabolites in the serum before and during diagnosis of disease, cows with lameness continued to experience fluctuation of several

AAs, glycerophospholipids, sphingolipids, and ACs at +4 and +8 weeks after parturition. These findings suggest that cows affected by lameness were in an altered metabolic state within this 16 weeks period, which might severely affect their overall performance and milk yield, and have other detrimental effects including reproductive performance and increased involuntary culling rates. Therefore, it would be of interest to develop new preventive strategies during the dry-off period to avoid the occurrence of lameness postpartum.

It should be noted that although the number of lame cases was only 6, the accuracy of metabolomics technology gives an excellent metabolic characterization of what happens prior to, during, and after occurrence of lameness. However, it is desirable that the metabolites identified be validated first and then developed into a pen-side technology to help dairy producers and veterinary practitioners to identify the cows that are at risk of lameness and take preliminary actions to prevent development of lameness.

5 Conclusion

Overall, results of this study showed that pre-lame and lame cows experienced altered concentrations of AAs, glycerophospholipids, sphingolipids, ACs, and hexose in the serum during all 5 time points considered in this study. It is interesting to note that throughout the 16-weeks of the study, 13 serum metabolites (i.e., PC aa C30:0, PC aa C30:2, PC aa C42:1, PC ae C40:2, SM (OH) C14:1, SM (OH) C16:1, SM (OH) C22:1, SM (OH) C24:1, SM C18:0, SM C26:0, Ile, Leu, and Lys) were consistently greater than those in the CON cows and appeared to play an important role in differentiating between the CON and lame cows. Furthermore, 4 metabolic pathways (i.e., Lys degradation, biotin metabolism, Trp metabolism, and Val-Leu-Ile degradation) were altered in cows with lameness during the onset and progression of the disease. These findings give insights into the pathobiology of lameness in dairy cows, in characterization of lameness from the metabolic point of view, and in better understanding the etiopathology of lameness. Finally, biomarker analysis showed that AUCs for 5 ROC curves based on top 5 metabolites with greatest VIP values were 0.995 (95% CI 0.945–1) at - 8 weeks, 0.992 (95% CI 0.938–1) at - 4 weeks prior to calving, 0.988 (95% CI 0.913–1) at disease week, 1.00 (95% CI 1–1) at +4 weeks postpartum, and 0.99 (95% CI 1–1) at +8 weeks postpartum, respectively, which indicate that serum biomarkers identified have very high screening potential to be used for identifying cows at risk or greater susceptibility to lameness. It should be noted that the number of replicates in this study is small and further studies need to be conducted to validate the screening biomarkers identified.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical approval All experimental procedures were approved by the University of Alberta Animal Policy and Welfare Committee for Livestock, and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

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