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Serum lipidomics of bovine paratuberculosis: Disruption of choline-containing glycerophospholipids and sphingolipids

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Abstract

Objectives: Bovine paratuberculosis is a devastating infection with *Mycobacterium avium* subspecies *paratuberculosis* that ultimately results in death from malnutrition. While the infection is characterized by a long (2–4 years) subclinical phase with immune activation, ultimately host defense mechanisms fail and the bacteria spread from the small intestine to other organs. Since both the gastrointestinal tract and liver are essential for the biosynthesis of structural glycerophospholipids, we investigated the circulating levels of these lipids in field infections and experimentally infected cattle.

Methods: Serum lipidomics of control and *M. avium* subspecies *paratuberculosis*–infected cattle were performed utilizing high-resolution mass spectrometry.

Results: In *M. avium* subspecies *paratuberculosis*–positive cattle, demonstrating clinical signs, we monitored large decreases in the levels of circulating phosphocholine-containing lipids. These included phosphatidylcholines, choline plasmalogens, and sphingomyelins. Next, we monitored the time course of these lipid alterations in experimentally infected calves and found that altered lipid levels were only detected in cattle with clinical signs of infection.

Conclusions: Our data indicate that altered availability of choline-containing lipids occurs late in the disease process and is most likely a result of malnutrition and altered biosynthetic capacities of the liver and gastrointestinal tract. Alterations in the bioavailability of these critical structural lipids presumably contributes to the demise of *M. avium* subspecies *paratuberculosis*–infected cattle. In light of increasing concern that *M. avium* subspecies *paratuberculosis* may be a zoonotic bacterium that contributes to the development of Crohn's disease and multiple sclerosis, our data also have human clinical relevance.

Keywords

Bovine paratuberculosis, choline glycerophospholipids, sphingolipids, microflora, SLC11A1

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Paratuberculosis (PTB) is an enteritis of ruminants that involves infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) generally shortly after birth through ingestion of bacteria shed in the feces of infected animals or colostrum from and infected dam. Affected cattle have a long (>2 years) subclinical phase prior to the emergence of clinical signs including persistent diarrhea, weight loss, and decreased milk production.[1,2] The emergence of the clinical phase of the disease is characterized by a transition of Th1 cell-mediated immune responses to Th2 immune responses.[3] This shift in immune regulation results in alterations in the composition of the gastrointestinal (GI) flora,[4] severe granulomatous enteritis,[5] and liver damage.[6,7]

Proteomics,[8,9] metabolomics,[10] transcriptomics,[11,12] and microRNA[13,14] studies of PTB have supported an altered immune response in infected cattle. Proteomics studies have reported an induction of acute phase reactants in the serum of

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infected cattle[8] further supporting altered liver function in PTB. In contrast, there have not been any studies of the lipidome in PTB, except for a transcriptomics study that detected altered enzyme function regulating cholesterol metabolism.[15,16] Since both the liver and GI epithelium are important sources of glycerophospholipids[17,18] and are compromised in PTB, we undertook a non-targeted high-resolution mass spectrometric lipidomics analysis[19–21] of serum in MAP-infected cattle.

Materials and methods

Cattle: pilot study

Serum and fecal samples were submitted from clinical cases by the practitioners for diagnosis of MAP. Enzyme-linked immunosorbent assay (ELISA) (IDEXX MAP ELISA Ab Test kit, Westbrood, Maine) on serum samples and real-time polymerase chain reaction (PCR) assay (rt-PCR) (VetMAX™-Gold MAP Detection Kit, Thermofisher scientific, Waltham, Massachusetts) on fecal samples were carried out by using commercial kits. All testing was performed at the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL), a fully accredited laboratory by American Association of Veterinary Laboratory Diagnosticians (AAVLD). Serum and fecal samples from three MAP-negative and three MAP- positive cattle were stored at −80°C prior to analyses. The cycle threshold (Ct) of rt-PCR is used as an estimate of the amount of MAP DNA in the fecal material and gives a general idea about shedding status of the animal. The Ct was used as an estimate of the amount of MAP DNA in the fecal material. Generally, the lower the number, the more DNA in the fecal material. MAP DNA can then be correlated to the number of organisms shed in the fecal material as per the United States Department of Agriculture (USDA) guidelines: <25 Ct = Very Heavy Fecal Shedder; <30 Ct = Heavy Fecal Shedder; <33 Ct = Moderate Fecal Shedder; <36 Ct = Light Fecal Shedder; <40 Ct = Suspect Fecal Shedder. The three positive cattle were heavy shedders. They were angus cattle from different farms and were approximately 2 years of age.

While the pilot study included only three animals, these were confirmed MAP-positive cattle that also demonstrated overt clinical signs. Hence, while the N was small, it was anticipated to be able to detect any *robust* alterations in the serum lipidome.

Cattle: validation study

For the validation study, field samples from 12 MAP-negative cattle and 12 MAP-positive (by ELISA) cattle with overt signs (excessive diarrhea and weight loss) of Johne's disease (>2 years old) were examined. The samples came from different farms to the University Veterinary Diagnostic Laboratory. The cattle were mainly 2- to 2.5-year-old angus. Both serum and fecal MAP DNA analyses and clinical criteria were utilized as described above.

The N of 12 for the validation study was based on our prior experience with monitoring the effects of bacterial infections on the host lipidome. In addition, this was a real-world study in that the samples were submitted to a central Veterinary Diagnostic Laboratory from different farms.

Cattle: time course study (experimental infection)

To monitor the time course of lipid changes, six control calves and seven experimentally infected calves[9] were monitored from month 0 to month 16. Two of the calves developed clinical signs of Johne's disease by month 16. The samples we examined were from the high-dose animals in the metabolomics study conducted by De Buck et al.[9] The high-dose inoculum (5×10^9 CFU) was deposited at the root of the tongue with a syringe on 2 consecutive days. Animal care protocol M09083 covered the experimental infection of dairy calves with MAP for the purpose of discovering biomarkers of infection by metabolomic profiling and was approved by the Health Sciences Animal Care Committee of the University of Calgary.

Lipidomics

Serum samples were vortexed with 1 mL of methanol containing stable isotope internal standards.[19–21] Next, 1 mL of water and 2 mL of methyl-tert-butyl ether were added and the tubes vigorously shaken at room temperature for 30 min prior to centrifugation at 4000 xg for 10 min at room temp. The upper organic layer was isolated and dried by centrifugal vacuum evaporation and dissolved in isopropanol: methanol: chloroform (4:2:1) containing 7 mM ammonium acetate. The stable isotope internal standards included [$^2H_{31}$]PtdE 34:1, [$^2H_{54}$]PtdE 28:0, [$^2H_{31}$]PtdC 34:1, [$^2H_{54}$]PtdC 28:0, and bromocriptine as internal standards.

Direct infusion lipidomics utilized high-resolution data acquisition, with an orbitrap mass spectrometer (Thermo Q Exactive). In negative ion electrospray ionization (ESI), the anions of ethanolamine plasmalogens (PlsE), phosphatidylethanolamines (PtdE), lysophosphoethanolamines (LPE), lysophosphatidic acids (LPA), sphingosine 1-phosphate (S-1-P), phosphatidylglycerols (PG), phosphatidylinositols (PI), and phosphatidylserines (PS). In positive ion ESI, the cations of choline plasmalogens (PlsC), phosphatidylcholines (PtdC), lysophosphatidylcholines, and sphingomyelins were quantitated. The cations and anions of bromocriptine were used to monitor for potential mass axis drift. Between injections, the transfer line was washed with successive 500 µL washes of methanol and hexane/ethyl acetate/chloroform (3:2:1). The monitored ions and parts per million error for altered phosphocholine-containing lipids is presented in Table 1.

Statistical analysis

Semi-quantitative data are presented as R values which represent the ratio of endogenous lipid peak area to the peak

Table 1. Exact masses, calculated cations, and the ppm mass error for the cations monitored by high-resolution mass spectrometry for PtdC and SM.

Biomarker	Exact mass	$[M+H]^+$	ppm
PtdC 38:1	815.6404	816.6477	0.31
PtdC 38:2	813.6248	814.6320	0.84
PtdC 38:3	811.6091	812.6164	2.18
PtdC 38:4	809.5935	810.6007	4.85
PtdC 38:5	807.5778	808.5851	5.96
PtdC 38:6	805.5622	806.5694	3.04
PtdC 40:3	839.6404	840.6477	0.24
PtdC 40:4	837.6248	838.6320	0.70
PtdC 40:5	835.6091	836.6164	1.49
PtdC 40:6	833.5935	834.6007	1.49
PtdC 40:7	831.5778	832.5851	1.74
PtdC 40:8	829.5622	830.5694	0.92
PlsC 36:1	771.6141	772.6214	0.36
PlsC 36:2	769.5985	770.6058	0.52
PlsC 36:3	767.5829	768.5902	0.96
PlsC 36:4	765.5673	766.5746	1.48
PlsC 36:5	763.5517	764.5590	1.54
PlsC 36:6	761.5361	762.5434	0.98
PlsC 38:3	795.6142	796.6215	0.19
PlsC 38:4	793.5986	794.6059	0.48
PlsC 38:5	791.5829	792.5902	0.65
PlsC 38:6	789.5673	790.5746	0.60
*PlsC 38:7	787.5517	788.5589	0.58
PlsC 38:8	785.5360	786.5433	0.30
SM 16:1	700.5519	701.5592	0.54
SM 17:0	716.5832	717.5905	1.1
SM 18:0	730.5989	731.6062	0.99
SM 18:1	728.5832	729.5905	1.72
SM 24:0	814.6928	815.7001	0.78
SM 24:1	812.6772	813.6845	1.29

ppm: parts per million mass error; PtdC: phosphatidylcholines; PlsC: choline plasmalogens; SM: sphingomyelins.

area of an appropriate internal standard. A Student's *t*-test (Microsoft Excel) was used to determine significant differences in serum levels of metabolites between MAP-infected animals and controls.

Results

Pilot lipidomics study: preliminary determination of decrements in choline-containing lipids

In our pilot study of three control and three infected cattle, we found small to no changes in the serum levels of PG, PS, PI, PtdE, and PlsE (data not shown). However, we noted significant and consistent decreases in the circulating pools of PtdC, PlsC, and sphingomyelins. Plots of these decreases for 36:1 to 36:6 PlsC, 38:1 to 38:6 PtdC, and 16:1 to 24:1 sphingomyelins are presented in Figure 1.

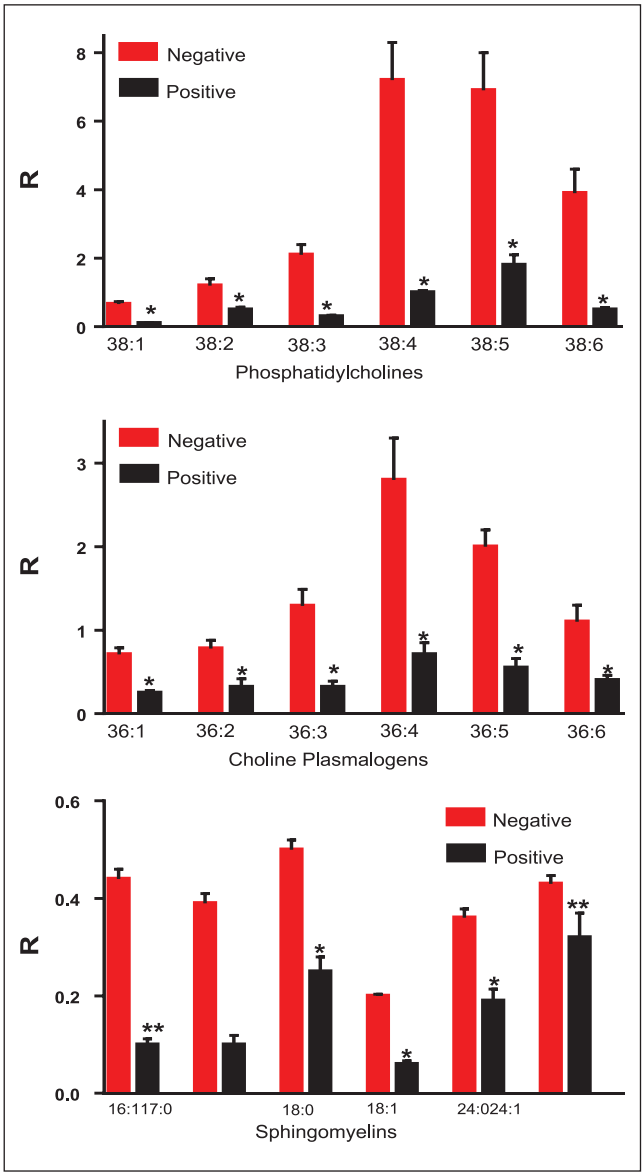

Figure 1. Pilot lipidomics observations for MAP-positive (N = 3) versus MAP-negative cows (N = 3). The MAP-positive cows had Ct values of 24.32, 25.06, and 30.26. Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SD).
*$p < 0.01$; **$p < 0.05$.

Validation lipidomics study: validation of decrements in choline-containing lipids

In a follow-up study of 12 control and 12 infected cattle, we similarly observed specific decrements in the levels of choline-containing glycerophospholipids and sphingolipids in infected cattle. Large decrements in circulating levels of PtdC (Figure 2) and PlsC (Figure 3) were monitored in cattle demonstrating clinical signs of MAP infection.

With regard to phosphatidylcholine metabolites, lysophosphatidylcholines were decreased while their phospholipase D

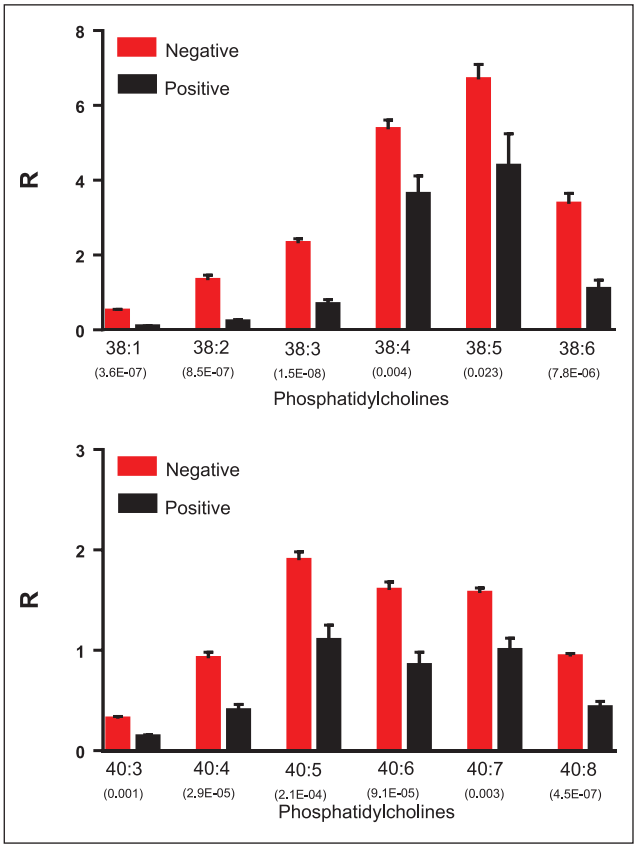

Figure 2. Plasma levels of phosphatidylcholines in PTB positive versus negative cows (N = 12 per group). Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SEM).
p values are presented in the brackets.

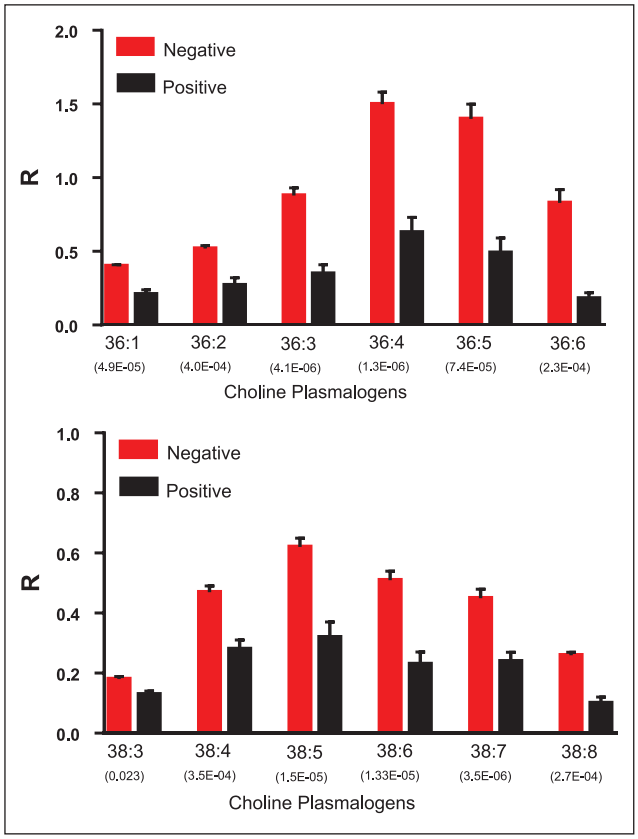

Figure 3. Plasma levels of choline plasmalogens in PTB positive versus negative cows (N = 12 per group). Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SEM).
p values are presented in the brackets.

(PLD) metabolites, LPA and alkyl-acyl phosphatidic acid, were increased (Figure 4) suggesting that PtdC are extensively degraded and/or their synthesis is decreased with MAP infection.

Large decrements in circulating levels of sphingomyelins (Figure 5), another class of choline-containing lipids, were monitored in our non-targeted lipidomics evaluation of infected cattle. In the case of sphingomyelin metabolism, increased levels of S-1-P were monitored (Figure 5). Since S-1-P is both a precursor and metabolite of sphingomyelins, it remains to be defined if augmented sphingomyelin metabolism and/or decreased sphingomyelin synthesis occurs with PTB infection.

Time course study: decrements in choline-containing lipids only in cattle demonstrating overt clinical signs

A study of experimentally infected calves was undertaken to monitor the time course of lipid alterations induced by infection with MAP. Of the seven infected calves, only two developed clinical signs of Johne's disease by 16 months. The group as a whole did not demonstrate any alterations in the levels of choline-containing lipids (Figure 6) with only one of the two cows demonstrating clinical signs having decrements in lipid levels at 16 months (35% decrease in PtdC, 50% decrease in PlsC, 31% decrease in sphingomyelins).

Discussion

Bacterial infection of the ileal mucosa and lymph nodes by MAP results in the development of a chronic granulomatous inflammation but only after a long (months to years) incubation period in cattle. After this long preclinical phase, compositional and functional changes in the gut microbiota,[4] along with a shift to a Th2-biased immune response results in lesions of the intestinal epithelium[5,22] and liver.[6,7] The expressions of clinical signs at this stage include treatment-resistant diarrhea, muscle wasting, and decreased milk production.[2,3] This phase of the infection has devastating effects on the health of dairy herds and significant negative economical impact.

A number of "omics" technologies have been utilized in an attempt to define biomarkers of early disease expression.

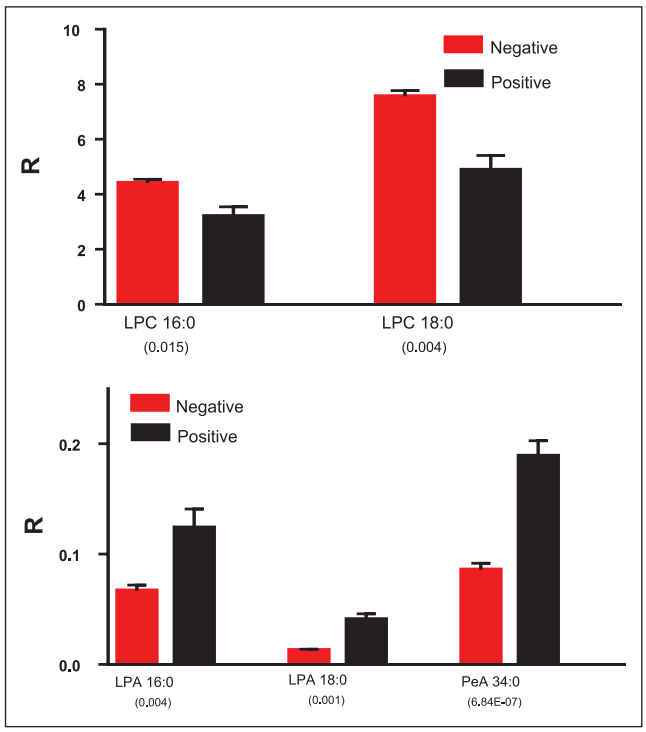

Figure 4. Plasma levels of lysophosphatidylcholines (LPC), alkyl-acyl phosphatidic acid 34:0 (PeA), and lysophosphatidic acids (LPA) in infected versus negative cows (N = 12 per group). Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SEM).
p values are presented in the brackets.

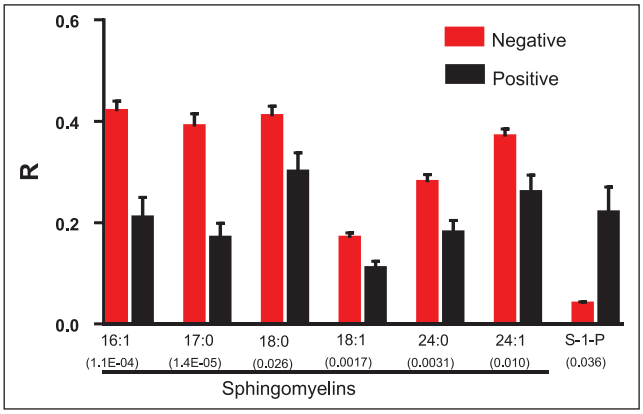

Figure 5. Plasma levels of sphingomyelins and sphingosine 1-phosphate (S-1-P) in PTB positive versus negative cows (N = 12 per group). Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SEM).
p values are presented in the brackets.

These include metabolomics studies[10] of amino acids and metabolic intermediates that have pointed to alterations in the gut microbiome while proteomics studies have observed a general hypoproteinemia, late in the disease process.[7–9] At

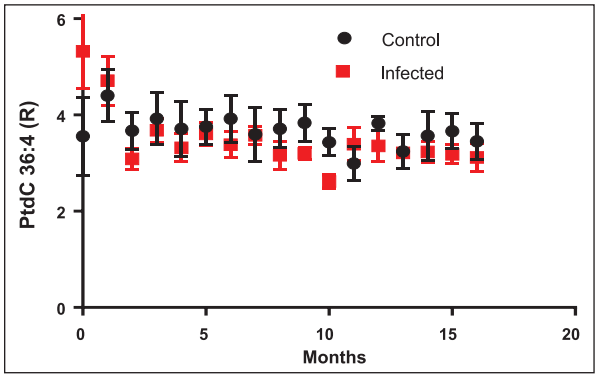

Figure 6. Serum levels of phosphatidylcholine (PtdC) 36:4 in experimentally infected (N = 7) and control cows (N = 6) from time zero and 1 to 16 months post-infection. Data (R) are presented as the ratio of the peak area of the endogenous lipid to the peak area of the internal standard (mean ± SEM).

this stage of the disease, there is widespread destruction of the mucosal wall of the small intestine and bacterial spread to the uterus, mammary gland, muscle, and liver.[22,23] Elevated liver and muscle enzymes[6,7] also are reflective of liver damage and muscle wasting, respectively. Altered liver immune function is further supported by elevations in the levels of circulating acute phase reactants.[8]

Our lipidomics data from cows demonstrating clinical signs reveal a profound decrease in the circulating levels of choline-containing glycerophospholipids and sphingolipids. The major anatomical sites that supply these lipids to the bloodstream are the liver and ileal epithelium.[17,18] Disruption of the gut wall will both decrease the absorption of glycerophospholipid and sphingolipid precursors and disrupt the synthetic capacity of the GI epithelium to synthesize these lipids. Similarly, with liver lesions in MAP-positive animals,[7] the biosynthesis of structural lipids will be negatively affected. The GI epithelium and the liver are the major sources of circulating glycerophospholipids and sphingolipids and the dysfunction at these anatomical sites can explain the decrements in choline-containing lipids that we monitored. In addition, the absorption of choline from the GI tract may be decreased and altered GI microflora may result in altered metabolism of choline within the gut.

It should also be noted that MAP has a cell wall rich in complex lipids and waxes containing mycolic acid.[15] It is possible that reduction of some of these lipids in the cattle could be due to the fact that the MAP cell wall requires high amount of these lipids for maintaining the cell wall and for replication.

In our time course study, no alterations in the circulating levels of choline-containing lipids were detected in the early phases of the disease and of the two cows demonstrating clinical signs at the end of the 17-month study, only one was advanced enough for us to monitor decreases in the choline-containing lipids. These data support the concept that

disruption of the gut mucosa and liver are responsible for the observed serum lipid changes in cows demonstrating clinical signs. However, our lipidomics data do not provide any indices for the early detection of infection.

With the lack of effective vaccines or therapy for late-stage infections, control of MAP infection involves herd management strategies. Therefore, it is imperative to increase our understanding of the risk factors for MAP infection if new therapies are to be developed. In this regard, polymorphisms in SLC11A1 represent a significant risk factor for MAP infection.[24–27] SLC11A1 is a divalent metal ion transporter family with polymorphisms having the potential to alter the levels of metal ions essential for the activity of a number of enzymes. In this regard, PLD which is activated by divalent metal ions[28] hydrolyzes PtdC and lysophosphatidylcholines to generate phosphatidic acids (PA) and LPA, respectively. In our lipidomics analyses, we did not monitor circulating levels of diacyl-phosphatidic acids in control or infected cows. However, we did monitor significant increases in alkyl-acyl phosphatidic acid 34:0 and LPA 16:0 and 18:0 (Figure 4). These data, in conjunction with the decreased levels of the direct precursors of each of these metabolites, suggest that abnormal SLC11A1 function may contribute to the monitored decrements in serum choline-containing lipids.

Our observations also may be of significance to human health. There is increasing concern that MAP may be a zoonotic bacterium[29] that contributes to the development of Crohn's disease[30,31] and multiple sclerosis.[32] Furthermore, multiple sclerosis patients with MAP infection[31] and Crohn's disease patients with MAP infection[33] possess polymorphisms in SLC11A1. While these may represent a small subset of patients with these diseases, it suggests that the contribution of MAP infections to the disease process in patients with SLC11A1 polymorphisms should be investigated further.

This pilot study is only limited by the small sample size. The next steps would be to investigate a larger cohort of cattle and to include analysis of SLC11A1 polymorphisms to evaluate any possible correlation with alterations in choline-containing lipids.

Conclusion

MAP infection in cows can result in dysfunctional host-GI flora interactions and frank lesions of the gut mucosa and liver. These dysfunctions ultimately result in the emergence of clinical signs. Our lipidomics data suggest that at this phase of the disease, tissue pathologies lead to large-scale decrements in the biosynthesis of essential choline-containing glycerophospholipids and sphingolipids. The contribution of SLC11A1 polymorphisms to altered lipid metabolism requires further investigation, including in Crohn's disease and multiple sclerosis patients with SLC11A1 polymorphisms. Our data also suggest that choline supplementation may be worthy of investigation in MAP-positive cows.

Acknowledgements

All authors were involved in the conception and design of the experiments. The lipidomics studies were conducted by P.L.W. The animal experiment was conducted by J.D. P.L.W. performed the data analysis and wrote the manuscript.

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation. The present study involved serum samples from client-owned animals submitted for diagnostic purposes; it demonstrated a high standard (best practice) of veterinary care and involved informed client consent.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Health Sciences Animal Care Committee of the University of Calgary, M090803.

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