A Lipidomic Approach Reveals Colostrum and Milk Biomarkers Predictive of Production-Related Metabolic Disease in Dairy Cows

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Abstract

Production-related metabolic diseases (PRMDs) are debilitating disorders in dairy cows during early lactation. PRMDs might be prevented if at-risk animals could be identified. Our study sought predictive PRMD biomarkers in colostrums or milk from dairy cows. Colostrums and postpartum day 4 milk were collected from asymptomatic cows and heifers. Animals developing PRMD were frequency matched with controls of similar calving date, age, and parity. Lipids were extracted from specimens and analyzed by a global lipidomics approach that employed electrospray-ionization, time-of-flight mass spectrometry. Peak abundance was analyzed statistically and quantitatively different lipid species were modeled into panels. Many lipids were significantly different between groups. A panel of 3 colostrums biomarkers predicted PRMDs with 90.0% sensitivity at 86.4% specificity. Similar results were obtained for milk or combinations of colostrums plus milk biomarkers. One group of triacylglycerol's were profoundly changed in PRMD specimens. The results suggest easily obtained, novel lipid biomarkers predict future PRMDs.

Keywords: Production-related metabolic diseases; lipidomics; mass spectrometry; colostrums; milk; predictive biomarkers.

1. Introduction

1.1.1 Production-related metabolic diseases (PRMDs) in dairy cows continue to be a costly problem for the dairy industry [1]. Different phenotypes of PRMDs have been documented, including hypocalcaemia (commonly known as milk fever), left displaced abomasums (LDA), ketosis, obturator nerve paresis (OP), fatty liver syndrome, and retained placenta among the most common disorders [2].

These health problems may present with decreased milk production, altered milk composition, reduced reproductive capacity, shortened life expectancy and/or lower cull value, resulting in both animal and economic loss. Interventions for many PRMDs are currently available after onset of clinical signs, but result in additional economic cost associated with medication and additional labor.

1.1.2 Identifying animals that will have these complications early enough to prevent onset of PRMDs has not been possible previously. PRMDs are multifactorial and the causes are complex. The different phenotypes of PRMDs have been documented based on the clinical signs manifested.

1.1.3 However, although a variety of health problems have been identified, they are currently thought to be interrelated physiologically, and not distinct, independent entities [3,4]. Acute milk fever results from hypocalcaemia and is characterized by reduced ionized blood calcium levels. It is treated as a medical emergency [5]. Displaced abomasums, in contrast, has been shown to be preceded by ketosis, and is often accompanied by subclinical hypocalcaemia [6]. Ketosis is a metabolic process that occurs when the body does not have enough glucose to maintain normal cellular energy production [7]. In consequence, stored fats are broken down and mobilized to meet energy needs, resulting in elevated fatty acids and a build-up of organic acids, termed ketone bodies, in the circulation. Fatty liver syndrome in dairy cows has been shown to occur when the synthesis of triacylglycerols (TG) is higher than their export and appears to be associated with hormone deregulation [8] and not surprisingly, disease states associated with ketosis may have varying degrees of hepatic lipidosis. Any of the aforementioned PRMDs may also occur with concurrent mastitis, metritis, retained placenta, or other health problems, prompting the question as to whether or not affected animals have a genetic predisposition for these diseases and/or specific phenotypes. Since PRMDs are not limited to a single phenotype, and represent an array of disorders having interrelated causes, including dietary and environmental management practices, biomarkers that can identify downstream problems in milk production associated with these metabolic disorders would be a valuable tool to detect dairy cows at risk, thereby allowing for appropriate interventions.

1.2 Lipid metabolism has been reported to be abnormal in certain metabolic diseases in dairy cows [9]. For example, the liver triglyceride to glycogen ratio has been used to predict susceptibility of cows to ketosis [9]. Increases in plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) were both significantly associated with development of peripartum diseases [10]. Milk fatty acids, such as C18:1 cis-9, have been proposed as possible biomarkers able to diagnose elevated concentrations of plasma NEFA early in dairy cows [11]. However, there has not been a comprehensive study of lipid biomarkers with the intent of their predicting PRMDs. Most current research has focused on dietary management and how diet and/or environmental interactions (e.g., over-conditioning at calving, low nutrient intake, environmental stress) can lead to peripartum diseases. Therefore, it is still highly desirable to discover novel biomarkers that are able to predict PRMDs with adequate sensitivity and specificity.

In other previous studies, lipids have been shown to be related to metabolic disorders, including ketosis [12] and fatty liver disease [13] which are also associated with development of LDA and milk fever. Most PRMDs occur shortly after calving during early lactation, and are believed to be due to difficulty adapting to the high demands of lactation, resulting in physiological imbalances in susceptible cows [14].

1.3 ‘Shotgun’, i.e. global, in-depth or comprehensive lipidomics can survey thousands of unique lipids in a single biological specimen. One such lipidomic approach using direct injection, electrospray ionization coupled with highly mass accurate, mass spectrometers (ESI-MS) represents a powerful tool for cataloguing and quantifying lipids [15] in tissue, cells or body fluids. Lipidomics can complement peptidomic and proteomic methods. Indeed, colostrums and milk samples can be readily fractionated into lipid- and protein-rich layers. Lipidomics, especially direct injection lipidomics, is substantially less involved than top-down, global proteomic methods, which typically require enzyme digestion and multiple separation steps prior to MS. Because there are serum lipid abnormalities associated with PRMDs in animals with established disease and because colostrum and milk are both rich in lipids and readily accessible for collection, we hypothesized that lipid expression differences exist between dairy cows later developing PRMDs and dairy cows that remain healthy and that these could be potential predictive biomarkers.

Consequently, a prospective animal study was undertaken using a lipidomics approach to find useful predictive lipid biomarkers for PRMDs. Having such markers could allow for earlier interventions to reduce animal losses [16]. Moreover, while colostrum biomarkers would provide earlier risk prediction, both colostrum and milk were

analyzed in an effort to potentially improve PRMD predictive capability and also to more broadly study altered lipid metabolism in anticipation of PRMDs, thus increasing our understanding of PRMDs.

2. Materials and Methods

2.1 Materials and lipid standards

Reproducibility and quantitative comparisons using direct injection lipidomics are dramatically improved with the introduction of known, consistent amounts of one or more synthetic lipid standards to the specimen to be analyzed. Given that this is a discovery study, markers have yet to be found and identified, consequently a synthetic lipid standard, archeol, not found in animals, was added in known quantity to each specimen as part of these studies. Other reagents used to perform extractions or used in the analyses of the lipids included chloro form, methanol, isopropyl alcohol (suitable for LC and UV-spectrophotometer) and ammonium acetate and were of the highest commercial grade.

2.2 Animal population and sample collection:

This study met the Brigham Young University Institutional Animal Care and Use Committee's criteria for an exempt study. The animals were maintained, fed and milked uniformly per dairy protocols and standards.

2.2.1 Animals were enrolled in the study prospectively. Enrollment was by calendar date of calving occurrence (05 February 2014 through 08 May 2014) to provide an unbiased method of animal selection, and to obtain a population of clinically normal primiparous (n = 101) and multiparous (n=109) Holstein cows at a university affiliated dairy. The dairy maintained a herd size of ~5,300 milk cows, had a calving interval of 13.3 months and milk production rolling herd average of 28,000 pounds. After calving and enrollment the animals were followed for 60 days. All heifers were ~2 years old and cows were all 3-6 years old with parity and lactation numbers of 3-5. Primiparousand multiparous cows with similar predicted calving dates were housed alike in separate pens. Total mixed rations formulated for each parity were fed ad libitum, with cows consuming a dry cow diet pre-partum and a fresh cow, early lactation diet postpartum after calving. We note that only 5 cows of the 210 study animals had retained placenta (RP); 4 resolved with gentle traction to assist expulsion and had adequate milk production, but were excluded as controls and not included in the PRMD CS and MK lipid analyses. Only one designate PRMD case cow with an LDA also had a RP 3 days post-partum.

2.2.2 Of these, 21 developed PRMDs. Diagnosis was made by a research veterinarian using well established standard criteria. The specific phenotypes observed were ketosis, left displacement abomasum (LDA), milk fever (MF), fatty liver, and/or hind limb weakness attributed to obturator nerve paresis (OP) (See Table 1).

These PRMD animals were then frequency matched to cows without any medical or lactation problems in this same cohort to form the control group. Matching criteria were calving date, age, and lactation number (parity) to insure that observed lipid differences were due to PRMD pathology and not due to feed, season, animal age bias or care.

2.2.3 Composite aliquots of colostrum secreted on the day of parturition and milk produced on the fourth day of lactation were collected from the four quarters of each cow's udder as it pooled in a milking claw and sampled via the exit milk tube. Samples were collected in 50 mL conical vials, then buried immediately and completely in crushed ice for transport to the analysis laboratory at Brigham Young University approximately 45 min away. Each sample was divided into 10 mL aliquots before being flash frozen in liquid N_2 and stored in a -80^{o}C freezer until later analysis. Cows 19010, 21389 and 23567 did not have milk collected on postpartum day 4 due to having already established PRMD or no longer being in the herd. Cow 23608 and 23971 did not have milk samples analyzed due to specimen loss or the specimen being compromised.

2.3 Lipid extraction:

Colostrum and milk samples were processed for lipidomic analysis in the same way. Samples were kept frozen in a -80^{o}C freezer prior to analysis and thawed completely at room temperature. The lipid layer and the aqueous protein-rich sublayer of colostrum (or milk) were separated by centrifugation for 20 min at 650 x g at 4^{o}C. After separation, 10 mg of the upper, lipid containing layer of the sample were taken and mixed with 3.8 mL of a solution of 2:1:1.25 (v/v/v) chloroform: methanol: isopropanol [17].

After shaking the mixture for ~30 sec until complete lipid dissolution, 1.2 mL of double distilled deionized water was added and the mixture was shaken again, then allowed to sit for organic and aqueous layer separation. After incubating at 37^{o}C overnight, the bottom organic phase was collected for complete lipid extraction and diluted

500 times with a 2:1:1.25 (v/v/v) solution of chloroform: methanol: isopropanol containing 15 mM of ammonium acetate and containing the lipid standard archaeol (6 nM), then submitted to instrumental analysis. Because of the somewhat longer extraction protocol, lipid stability was assessed for both milk and colostrum biomarkers. Aliquots of the organic extract from colostrum and from milk containing the lipid markers were removed at 0, 8 and 24 hr and were assayed in triplicate. Those lipids considered as biomarkers were evaluated by mass spectrometry as part of a single run. Multiple runs of the same biologic specimen were also analyzed for quantitative reproducibility of the biomarkers of interest without normalization to internal standard to determine the relative standard deviation as an estimate of measurement reproducibility.

2.4 Lipid profiling by time-of-flight mass spectrometry:

Mass spectrometric analysis was initiated by direct injection through an electrospray ionization (ESI) needle directed into a time-of-flight mass spectrometer (6230 ESI-TOFMS, Agilent Technologies, Santa Clara, CA). The ionization voltage was set to 3.5 kV, gas pressure to 15 psi and the source was controlled by instrument software (Mass Hunter Workstation Data Acquisition software, Agilent). All lipid samples were infused at the flow rate of 10 µL/min by a syringe pump (New Era Pump Systems, Farmingdale, NY, USA) and analyzed in the positive ion mode for 3 min with MS data collected over the range of m/z 100 to 1500. Technical replicates (n = 2) were performed for each sample and the values averaged in order to reduce instrumental variability.

2.5 Data analysis:

Instrument software (Mass Hunter Qualitative Analysis B.07.00 software, Agilent) was used to generate a peak list with the abundance of each lipid (in ion counts) recorded for each peak for each specimen. The abundances of the 2 instrumental replicates were exported as CSV files using MS software. Two data columns were generated for each file that included the m/z values and their corresponding abundances. The peaks were aligned between each run by a homemade macro program within an Excel file through Visual Basic Programming Language according to the instrument assigned m/z values and the associated abundances recorded. Correctness of peak mass alignment across samples was further checked manually. The intensity of the lipid standard in each run was determined and used for data normalization, i.e. a specific lipid's abundance was divided by the standard's abundance in that run. Then, the normalized abundances of the 2 instrumental replicates were averaged.

2.6 Statistical analysis:

2.6.1 This study was a nested case-control, discovery study that sought to demonstrate the feasibility of finding and the potential for using colostrum or milk lipid biomarkers to predict PRMDs. This employed a comprehensive or global lipidomics analysis approach that measures hundreds of previously unmeasured lipids, to find those that were present in significantly altered concentrations in PRMD versus normal postpartum specimens. Then, the goal was to statistically reduce these quantitatively different candidate markers into more highly predictive sets comprised of a few markers (<10), i.e. thereby generating hypothetically important PRMD predictive sets for further evaluation. To accomplish this, an initial, two-tailed Student's t-test was carried out on the normalized abundances for the two comparison groups, animals remaining healthy and animals developing a PRMD days to weeks after calving. Colostrum and milk samples were analyzed separately. Some candidates were significantly different in both colostrum and milk. These were referred to as 'shared' markers. Furthermore, for these shared markers quantitative differences between the normalized abundances for each peak were calculated by subtraction (the quantity in colostrum minus the quantity in milk), and the differences further considered in the statistical analyses.

2.6.2 Candidate lipid biomarkers, i.e. those having significantly different quantitative abundances between the two groups, were then submitted to linear discriminative analysis to model combinations or panels of milk and/or colostrum biomarkers (SAS 9.3, SAS Institute Inc., Cary, NC, USA) and optimized for area under the curve. Linear discriminant analysis has been described previously [18]. In brief, each statistically different lipid was evaluated in a step-wise discriminant statistical analysis with it being the independent variable of lipid abundance in samples from cows with a health score (HSC) ranking of 0 (healthy) or 1 (PRMD diagnosed--treated/culled/died within 60 days postpartum) as the dependent variables. A significance level of $p < 0.05$ was considered significant for all tests. Discriminant analysis, grouping variable HSC, was performed for each colostrum (CS) and milk (MK) biomarker, including 'shared' markers.

2.6.3 Additionally, after reviewing the results in milk, it was observed that all lipid biomarkers of a particular m/z range, very likely representing a common lipid type, were increased in the milk of animals that later developed PRMDs, whereas all the lipid biomarkers in a higher m/z range, also likely representing a common lipid type, were reduced in the animals later developing PRMDs.

To test the significance of this, a 2x2 contingency table was created that looked at the classification of diseased animals with the consistency of an elevated or decreased abundance observed for lipid biomarkers based on their m/z category. A Fisher Exact test was performed and a p-value <0.05 was considered significant.

2.7 Lipid identification:

2.7.1 Three panels of high performing predictive biomarkers were generated by statistical modeling. All markers from the three panels were then chemically characterized by ESI-tandem mass spectrometry (MS/MS). Targeted MS/MS was applied to extracts in an effort to identify or substantially characterize each useful biomarker. A combination of 'exact' mass studies, together with MS/MS fragmentation studies using collisionally-induced dissociation, were used to chemically characterize candidate biomarkers. Fragmentation data was acquired on both a QSTAR Pulsar I quadrupole orthogonal time-of–flight mass spectrometer through an Ion Spray Source (Applied Biosystems, Foster City, CA, USA) and on an Agilent 6530 accurate-mass quadruple/time-of flight mass spectrometry (Agilent Technologies, Santa Clara, CA, USA). While both instruments are comparable tandem mass spectrometers and fragmentation patterns were similar, there was sometimes additional information gained by using both. Specific colostrum and milk samples were selected for characterization based on their having a higher abundance of the targeted lipid of interest being present. The sample was extracted using 2:1:1.25 (v/v/v) chloroform: methanol: is opropanol with 15 m M ammonium acetate present (that facilitated adducts being formed with otherwise neutral compounds).

2.7.2 One tandem MS instrument, the QSTAR, used the following settings: voltage potential of 4800 V for the ion spray needle coupled with a flow rate of 4 µL/min using a syringe pump. The drying gas was N_2 as was the collision gas. Optimal collision energies varied depending on the size, structure, and abundance of each lipid, but were typically between 15 and 40 eV. Analyst QS software was used for operating the instrument, and a 50 - 2000 mass/charge range was used for each scan.

For the second instrument, the Agilent tandem Q-TOF, direct infusion with the syringe pump was used for sample injection at a flow rate of 10 µL/min. The ion selection quadrupole Q1 was operated under unit resolution and fragments were detected within the m/z range of 100-1000 in the positive ion mode. Each MS/MS spectrum was acquired for 30 sec with a collision energy range of 15-40 eV. An isolation window of 1.3 was used.

2.7.3 Chromatograms were extracted from ESI total ion chromatograph (TIC) at a MS/MS level using a instrument based software program (Mass Hunter Qualitative Analysis B.07.00, Agilent). The exported product ion mode was broadly used for lipids identification. Predicted identities of target lipids were searched for using the on-line reference site LIPID MAPS[19] and the Elemental Composition Calculator programmed by Frank Antolasic (School of Applied Sciences, RMIT University, Melbourne, Victoria, Australia) was used in conjunction with the experimentally determined accurate lipid mass after determining the adduct present. Fragmentation information was further manually evaluated in product ion mode through review of neutral loss species, or scanned fragment information. The LIPID MAPS MS fragment prediction tool (http://www.lipidmaps.org/tools/index.html) was also applied to determine predicted product ion peak lists, which often represented sn1 and sn2 acyl losses mainly for glycerolipids.

3. Results

3.1 Descriptive Data:

This was a nested case-control discovery study. Day 1 colostrums and where possible day 4 milk specimens were collected from 101 primiparous and 109 multiparous asymptomatic postpartum Holstein cows. All cows were prospectively enrolled and their health history tracked for 60 days postpartum. Approximately 10% of these cows developed PRMDs within 1-27 days of calving. PRMD phenotypes included left displaced abomasum (LDA), hypocalcaemia (commonly known as milk fever (MF)) and obdurate nerve paresis (OP) and a summary of the phenotype and the timing of occurrence is provided in Table 1.

Table 1.Animal Population Characteristics. Postpartum first day colostrum and milk secreted on day 4 were collected from 210 Holstein cows; cows that developed PRMDs were selected as case animals and compared with age-, parity-, and lactation-matched controls. CASE: developed PRMD; CTL: control; MF: hypocalcaemia, commonly known as milk fever; LDA: left displaced abomasum; OP: obturator nerve paresis; CS: postpartum day 1 colostrums; MK: postpartum day 4 milk; PROB: probable

Animal Status	ID Number	Diagnosis	Lipids
CTL	19048	NA	CS+MK
CTL	20505	NA	CS+MK
CTL	20804	NA	CS+MK
CTL	20873	NA	CS+MK
CTL	21132	NA	CS+MK
CTL	21155	NA	CS
CTL	21247	NA	CS+MK
CTL	21859	NA	CS+MK
CTL	22219	NA	CS+MK
CTL	22598	NA	CS+MK
CTL	22877	NA	CS+MK
CTL	23609	NA	CS+MK
CTL	23772	NA	CS+MK
CTL	26566	NA	CS+MK
CTL	26558	NA	CS+MK
CTL	26678	NA	CS+MK
CTL	26776	NA	CS+MK
CTL	26852	NA	CS+MK
CTL	26998	NA	CS+MK
CTL	29241	NA	CS+MK
CTL	29552	NA	CS+MK
CTL	29554	NA	CS
CTL	29610	NA	CS
CASE	14112	DIED	CS+MK
CASE	16320	MF	CS+MK
CASE	17829	OP, DIED	CS+MK
CASE	17841	MF, DIED	CS+MK
CASE	19010	MF	CS
CASE	20594	LDA	CS+MK
CASE	20712	LDA	CS+MK
CASE	21389	LDA	CS+MK
CASE	22377	LDA	CS+MK
CASE	23567	MF, LDA	CS
CASE	23608	LDA	CS
CASE	23762	LDA	CS+MK
CASE	23971	LDA	CS
CASE	25249	LDA	CS+MK
CASE	25853	LDA	CS+MK
CASE	26035	MF, OP	CS+MK
CASE	26832	LDA	CS+MK
CASE	29337	LDA	CS+MK
CASE	29551	LDA	CS+MK
CASE	29685	LDA	CS+MK
CASE	23374	PROB MF	CS+MK

Once PRMD animals had been identified, a comparable number of healthy control animals were then selected that shared similar calving date, age and lactation number (parity). The PRMD and selected control samples were submitted to lipidomic analysis.

3.2 Lipids quantitatively different between cows later developing PRMDs and controls using a 'global' lipidomic approach:

A total of ~2000 ion peaks, representing approximately that number of different lipids, were listed from MS runs of separately analyzed colostrums and milk samples.

Peak abundances were normalized to an internal control and tested statistically for apparent quantitative differences between the comparison groups. Candidate markers with statistical differences (p-value <0.05) between cows that went on to develop PRMDs and their frequency matched controls are listed in Tables 2 and 3.

Table 2.PRMD predictive colostrum lipid biomarkers comparing animals with later PRMD to resistant dairy cows. Means are normalized to endogenous controls.

Marker (m/z)	Mean CTL	Mean Cases	Higher In	P-Value
344.22	0.104	0.115	CASES	0.042
388.25	0.113	0.125	CASES	0.048
489.10	0.291	0.374	CASES	0.049
570.46	0.112	0.176	CASES	0.013
586.54	0.266	0.203	CTL	0.0014
598.50	0.081	0.113	CASES	0.029
612.55	0.078	0.066	CTL	0.044
615.56	0.088	0.069	CTL	0.043
648.46	0.115	0.085	CTL	0.049
652.55	0.074	0.098	CASES	0.047
654.56	0.653	0.939	CASES	0.015
659.52	0.143	0.198	CASES	0.0042
668.58	0.116	0.166	CASES	0.015
675.50	0.100	0.126	CASES	0.034
680.58	0.349	0.513	CASES	0.0042
682.59	1.405	2.591	CASES	0.00095
687.55	0.224	0.381	CASES	0.00041
694.60	0.068	0.107	CASES	0.0032
695.59	0.138	0.192	CASES	0.0077
696.60	0.148	0.225	CASES	0.0025
703.53	0.163	0.262	CASES	0.0014
704.55	0.081	0.134	CASES	0.00055
706.59	0.140	0.218	CASES	0.0027
708.61	0.366	0.732	CASES	0.00047
710.63	0.753	1.198	CASES	0.0028
723.62	0.243	0.400	CASES	0.0011
731.56	0.085	0.128	CASES	0.0019
732.57	0.082	0.113	CASES	0.0055
734.61	0.078	0.098	CASES	0.040
736.64	0.168	0.250	CASES	0.0031
749.64	0.068	0.126	CASES	0.00033
751.65	0.134	0.197	CASES	0.0030
752.66	0.105	0.139	CASES	0.011
848.77	0.750	0.935	CASES	0.017
850.78	2.001	2.587	CASES	0.0079
855.74	0.396	0.491	CASES	0.0062
858.75	0.075	0.101	CASES	0.00065
861.57	0.099	0.071	CTL	0.035
862.79	0.119	0.166	CASES	0.0049
864.79	0.263	0.325	CASES	0.0075
872.74	0.187	0.223	CASES	0.023
873.74	0.128	0.154	CASES	0.017
874.78	0.330	0.478	CASES	0.00041
876.80	0.693	1.366	CASES	0.00025
878.81	0.494	0.961	CASES	0.00047
881.76	0.175	0.304	CASES	0.000062
883.76	0.112	0.202	CASES	0.00011
889.79	0.119	0.143	CASES	0.016
890.81	0.111	0.151	CASES	0.0016
891.81	0.273	0.339	CASES	0.0074
897.73	0.098	0.158	CASES	0.00037
899.76	0.089	0.140	CASES	0.00022
900.79	0.139	0.221	CASES	0.00027
902.81	0.176	0.366	CASES	0.00045
904.82	0.156	0.333	CASES	0.00073
906.84	0.101	0.181	CASES	0.00097
917.82	0.099	0.170	CASES	0.00022
919.83	0.067	0.124	CASES	0.00015
964.59	0.105	0.073	CTL	0.016
965.70	0.064	0.107	CASES	0.00066
967.71	0.102	0.123	CASES	0.037

Table 3. PRMD predictive milk lipid biomarkers comparing animals with later PRMD to control dairy cows. Means are normalized to endogenous controls.

Marker (m/z)	Mean CTL	Mean CASES	Higher In	P-Value
572.48	0.489	0.227	CTL	0.000057
600.51	0.918	0426	CTL	0.000018
626.53	0.531	0.333	CTL	0.00013
633.50	0.266	0.151	CTL	0.0000093
640.54	0.134	0.105	CTL	0.0059
642.58	0.452	0.270	CTL	0.00000028
652.55	0.218	0.164	CTL	0.0014
654.26	2.098	1.573	CTL	0.00095
656.58	6.780	4.802	CTL	0.000024
657.58	2.549	1.821	CTL	0.000026
658.58	0.562	0.415	CTL	0.000045
659.52	0.333	0.271	CTL	0.0053
661.53	0.620	0.450	CTL	0.000037
668.58	0.390	0.315	CTL	0.0031
669.58	0.476	0.311	CTL	0.0000020
670.59	0.733	0.530	CTL	0.000011
675.50	0.197	0.142	CTL	0.0022
677.51	0.440	0.296	CTL	0.0024
684.61	5.376	4.039	CTL	0.00045
689.56	0.525	0.404	CTL	0.00042
695.49	0.349	0.289	CTL	0.010
697.61	0.974	0.762	CTL	0.00019
705.54	0.389	0.280	CTL	0.0082
712.64	1.880	1.282	CTL	0.000060
717.58	0.219	0.155	CTL	0.000080
721.50	0.234	0.194	CTL	0.048
725.63	0.700	0.552	CTL	0.0011
732.58	0.163	0.132	CTL	0.028
733.57	0.173	0.118	CTL	0.0020
734.62	0.156	0.116	CTL	0.00065
738.65	1.049	0.744	CTL	0.00016
740.67	0.834	0.493	CTL	0.00037
752.66	0.274	0.230	CTL	0.0075
753.66	0.266	0.190	CTL	0.000055
754.67	0.185	0.123	CTL	0.000015
764.67	0.302	0.234	CTL	0.00075
766.69	0.723	0.478	CTL	0.00016
768.70	0.590	0.396	CTL	0.020
780.69	0.133	0.094	CTL	0.000026
792.70	0.282	0.221	CTL	0.0016
794.72	0.701	0.538	CTL	0.019
808.73	0.145	0.112	CTL	0.00046
809.74	0.126	0.096	CTL	0.014
810.74	0.140	0.112	CTL	0.028
848.76	0.971	1.206	CASES	0.0015
850.78	2.310	3.140	CASES	0.000026
855.74	0.353	0.459	CASES	0.0000069
862.78	0.206	0.262	CASES	0.0036
863.78	0.229	0.262	CASES	0.0056
864.79	0.344	0.445	CASES	0.00031
865.79	0.232	0.288	CASES	0.00024
872.79	0.204	0.241	CASES	0.025
874.78	0.602	0.884	CASES	0.00031
876.80	2.283	3.975	CASES	0.000045
878.81	1.794	2.900	CASES	0.000020
879.81	0.894	1.398	CASES	0.000021
881.76	0.382	0.623	CASES	0.0000060
883.76	0.286	0.430	CASES	0.0000074
888.79	0.087	0.123	CASES	0.00085
889.79	0.144	0.193	CASES	0.00024
890.81	0.213	0.318	CASES	0.00015
891.81	0.313	0.453	CASES	0.000013
896.75	0.075	0.090	CASES	0.023
897.74	0.223	0.303	CASES	0.013
898.76	0.176	0.233	CASES	0.0063
900.79	0.269	0.393	CASES	0.00029
902.81	0.637	1.199	CASES	0.000022
904.52	0.720	1.308	CASES	0.000013
906.84	0.365	0.588	CASES	0.000019
908.80	0.111	0.157	CASES	0.000086
909.78	0.133	0.222	CASES	0.0000056
915.80	0.075	0.108	CASES	0.00033
917.82	0.223	0.375	CASES	0.000056
919.83	0.188	0.292	CASES	0.000049
939.68	0.241	0.188	CTL	0.00016
967.71	0.197	0.168	CTL	0.0060

In total, 61 statistically significant, quantitatively different lipids were discovered in colostrum (Table 2), and 77 significant, quantitatively different lipids were discovered in milk (Table 3). Of these, 31 lipids were found to be significantly different in both colostrum and milk when comparing cases versus controls. Additionally, for these 31 'shared' markers, differences between colostrum values and milk values of the same peak were calculated by using the normalized abundance of that lipid in colostrum minus its normalized abundance in milk (Table 4).

Table 4. Statistical analysis of 'shared' peak differences, i.e. for lipids significantly different between diseased and control animals in both colostrum and milk. The differences of shared biomarkers were calculated by using the normalized abundance of colostrum minus the normalized abundance of milk. Mean CTL: mean differences of controls; Mean CASES: mean differences of animals with disease; 31 shared peaks were found as shown, p-values in the rightmost column were obtained through SAS 9.3.

Marker (m/z)	Mean CTL	Mean CASES	Difference	P-value
652.55	-0.145	-0.0636	-0.081+0.016	<0.0001
654.56	-1.45	-0.612	-0.841+0.148	<0.0001
659.52	-0.191	-0.067	-0.124+0.024	<0.0001
668.58	-0.275	-0.145	-0.130+0.028	<0.0001
675.50	-0.0973	-0.0107	-0.087+0.021	0.0003
695.59	-0.212	-0.0903	-0.122+0.023	<0.0001
732.57	-0.080	-0.014	-0.066+0.018	0.0009
734.61`	-0.078	-0.016	-0.062+0.014	0.0001
752.66	-0.170	-0.088	-0.082+0.018	<0.0001
848.76	-0.243	-0.285	0.041+0.093	0.6585
850.78	-0.372	-0.600	0.227+0.266	0.3993
855.74	0.039	0.027	0.012+0.049	0.8059
862.79	-0.091	-0.094	0.002+0.027	0.9297
864.79	-0.085	-0.116	0.031+0.038	0.4263
872.75	-0.020	-0.019	-0.001+ 0.026	0.9777
874.78	-0.278	-0.405	0.126+0.072	0.0915
876.80	-1.60	-2.56	0.961+0.316	0.0046
878.81	-1.308	-1.900	0.591+0.201	0.0058
881.76	-0.207	-0.299	0.093+0.043	0.0375
883.76	-0.173	-0.220	0.046+0.030	0.1346
889.79	-0.027	-0.049	0.022+0.015	0.1629
890.81	-0.103	-0.163	0.060+0.025	0.0210
891.81	-0.046	-0.112	0.066+0.036	0.0774
897.73	-0.125	-0.140	0.015+0.035	0.6680
900.79	-0.131	-0.164	0.033+0.035	0.3417
902.81	-0.463	-0.816	0.353+0.099	0.0011
904.82	-0.565	-0.961	0.396+0.099	0.0003
906.84	-0.265	-0.401	0.136+0.040	0.0018
917.82	-0.126	-0.198	0.072+0.029	0.0194
919.83	-0.120	-0.163	0.042+0.022	0.0614
967.71	-0.098	-0.044	-0.053+0.013	0.0003

Note: Those markers that were directionally opposite in both colostrum and milk provided dramatic differences and very low p-values. All of the non-significant differences were for markers that were directionally the same in both colostrum and milk.

3.3 Predictive PRMD lipid biomarker panels:

3.3.1 Markers found in colostrum have the advantage of an earlier time point with greater opportunity for an intervention in at-risk animals. The best colostrum biomarker panel, as found for these data using linear discriminative analysis, contained three lipids as summarized in Table 5. The panel provided 90.0% sensitivity at 86.4% specificity.

Table 5. Identification/classification of predictive PRMD lipid markers that were part of the optimized panel for colostrum. These provided 90.0% sensitivity at 86.4% specificity. This panel predicted 19 out of 22 control cows and 18 out of 20 cows with later PRMDs. DG: diacylglycerol; TG: triacylglycerol

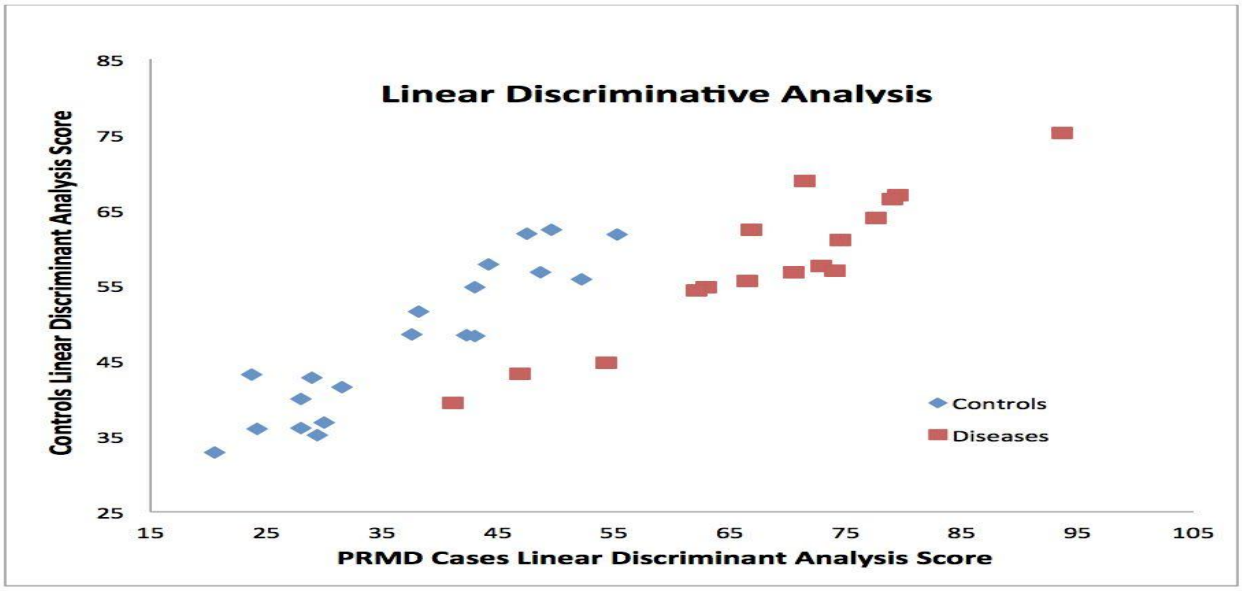
Markers (m/z)	Elemental composition	Classification (constituents)	Abundance	P-value
570.46	$C_{40}H_{56}O+NH_4^+$	Unknown lipid class	Higher in disease	0.013
586.54	$C_{35}H_{68}O_5+NH_4^+$	DG(16:0/16:0)	Higher in control	0.0014
855.75	$C_{55}H_{98}O_6+H^+$	TG(16:0/18:1/18:3)	Higher in disease	0.00065

3.3.2 Milk markers may likewise still be predictive of animals at risk for PRMD development after day 4. Identifying changes in the lipid composition in colostrum and additionally milk might reveal altered metabolism that underlies and potentially contributes to PRMDs. The best milk biomarker panel contained two lipids as summarized in Table 6 that provided a sensitivity of 75.0 % at a specificity of 90.0%.

3.3.3 Having colostrum and milk from the same animals allowed for an optimized combined colostrum and milk biomarker panel. This included 7 lipid markers: 2 lipids were from the colostrums data set, 2 lipids were from the milk data set, and 3 markers represented calculated differences between colostrum and milk for the 'shared' marker set. This combined biomarker panel demonstrated 87.5% sensitivity at 100.0% specificity (Table 7, Figure 1).

Figure Legend Figure 1

Figure 1. Plot of linear discriminative analysis results for the optimal set of lipid biomarkers demonstrating separation between modeled biomarker values for animals developing PRMDs later (red) and healthy unaffected animals (blue). The x-axis and y-axis represent the discriminant function scores.



3.3.4 All of the candidate lipid biomarkers were completely stable during the extraction step, i.e. their abundances did not decrease (<5%) as a function of time for up to 24 hr. The MS reproducibility, as suggested by relative standard deviation (RSD), of colostrum biomarkers without normalization was 17.4%. For milk biomarkers the RSD of replicates was 11.8%.

3.4 Characterization of PRMD biomarker candidates:

Lipid marker composition was chemically characterized or in some instances likely identified by means of targeted tandem MS/MS analyses on QqTOF-MS instruments using collisionally-induced dissociation (CID), i.e. fragmentation. The fragmentation studies were limited to the markers that made up the three panels that were later submitted to this further characterization.

This represented 10 unique lipid biomarkers. Of these, 5 markers were successfully classified as triacylglycerols (TG), including m/z 855.75 which appeared to represent the protonated TG (16:0/18:1/18:3) based on 2 abundant fragments at m/z 573.49 and m/z 599.48 representing neutral losses of fatty acid constituents. The third fatty acid was predicted based on mass differences. The marker m/z 906.84 was determined to be an ammoniated TG because of a peak at $[M+NH_4-17]^+$. Utilizing this same approach, marker m/z 740.67 was very likely $[TG (12:0/14:0/16:0)+NH_4]^+$. Characterization studies on the marker m/z 919.83 yielded two possibilities, including an oxidized TG $[(18:0/18:0/19:1)+OH]^+$ or an oxidized TG $[(18:0/18:1/19:0)+OH]^+$. As for the marker m/z 682.59, a NH_3 neutral loss was observed in the spectra and the exact mass of that fragment at m/z 664.56 suggested its elemental composition to be $C_{41}H_{76}O_6$, which indicates strongly that the marker is a TG. Three of the 10 markers were categorized as diacylglycerols (DG) through the same approach with determinations based on the fragments and neutral loss peaks in the fragmentation spectra. The fragmentation studies were most consistent with these 3 markers being DG (16:0/16:0), DG (18:0/18:0) and DG (18:2/19:0).The elemental composition of the final two lipid markers having m/z 570.46 and m/z 344.22 were determined as $[C_{40}H_{56}O+NH_4]^+$ and $[C_{21}H_{26}O_3+NH_4]^+$. However, these two markers were not classified into a specific lipid group due to a lack of identifiable headgroups or recognizable constituent species in the fragmentation data.

4. Discussion

4.1 Production-related metabolic diseases (PRMDs) continue to be a major problem for dairy cows during milk production. Their incidence is high in herds worldwide, e.g. milk fever occurs in up to 7% of all animals after calving and displaced abomasums in ~5% of postpartum cows even in well-managed dairy herds [1,3-5]. The treatment of PRMDs, once the disease is established, can require intensive dietary supplementation, drug therapy or even surgery but there may still be loss of the animal depending on how late the intervention or how severe the disease at the time of diagnosis. Additionally, there is reduced milk production, fertility issues, and sometimes culling. Prevention of PRMDs is likely possible and would be a far more effective and cheaper strategy than any medical treatment of active disease. This, however, would require a reliable and easy way of predicting cows at risk for PRMDs in advance of clinical signs. If developed, such a test would help significantly in conserving animal health, enhancing overall herd production and lowering economic losses.

4.2.1 Previous attempts to predict cows at risk for PRMDs have focused on levels of lipids in plasma or serum. For example, animals with an elevated serum NEFA concentration (more than 0.3 mEq/L) between 14 to 2 days prior to calving or animals with an elevated serum β-hydroxybutyrate concentration (more than 10 mg/dL) and NEFA concentration (more than 0.6 mEq/L) 3 to 14 days postpartum, have been shown to be at somewhat greater risk of transition PRMDs in dairy cows [10]. However, the sensitivity and specificity of these existing markers were inadequate to be useful. Moreover, collection of blood samples by venipuncture is labor intensive, expensive and impractical as a screening tool.

4.2.2 Our hypothesis was that a global lipidomic approach could overcome many of the current limitations of biomarker testing and would find early lipid biomarkers in samples that were easy to obtain and that were effective in predicting PRMDs in asymptomatic postpartum dairy cows. Our studies suggest strongly that this has been accomplished. We recognize that this is a discovery study and that more samples from geographically distinct regions and other breeds of cattle are needed for analysis to validate our results to build confidence that biomarkers from our study perform similarly in other cows from unrelated herds. Still our biomarkers substantially outperformed currently published biomarkers, and even showed biologic consistency and redundancy, suggesting that the changes we found are real. At a minimum these results can be viewed as providing a hypothesis or set of hypotheses that can be tested hereafter.

4.2.3 It is noted that 4 cows developed milk fever within 24 hr of calving. In these animals there would be no value in having positive lipidomic results predicting disease even if analysis could be done that quickly. However, in the majority of other animals having day 1 colostrum results or even day 4 milk results would precede the disease by several days to a few weeks, and as such could provide valuable information regarding still asymptomatic animals at risk.

4.2.4 This study appears to represent the first application of a comprehensive, global lipidomics approach to identifying lipid biomarkers for PRMDs. It is also the first effort to define biomarkers prior to appearance of PRMDs using day 1 postpartum colostrum and day 4 postpartum milk. A similar ESI-MS lipid method has been used for other medical indications [20,21].

In this approach the original organic lipid extract be diluted 500 times before ESI-MS to allow the great majority of the observed lipids to fall within the linear dynamic concentration range of the instrument, i.e. the range over which ion signal is directly proportional to the analyte concentration. Nonetheless, a total of 1800-2000 different ion peaks were observed using the positive ion mode alone (abundance >600 ion count).

4.3 Statistical linear discriminative analysis has been used to combine and assess the performance of sets or panels of discriminating biomarkers previously [18,22]. The results of such modeling here provided promising biomarker signatures for colostrum, for milk and for a combination of milk, colostrum and 'shared' biomarkers, i.e. biomarkers that were significantly different in both biological specimens. Many combinations provided useful prediction of PRMDs but we have reported on only marker sets having optimized AUCs. Predictive sensitivities and specificities were in general at or above 80%. The combined set was able to completely discriminate (See Figure 1) between asymptomatic animals developing PRMDs later and animals that remained healthy.

4.4 After developing these optimized predictive biomarker panels, targeted MS/MS analyses were performed to chemically characterize the 10 relevant lipid biomarkers obtained from the three panels. As summarized in Table 2, among the 61 statistically significant biomarker candidates in colostrum, 55 of them were present in higher concentration based on their normalized abundances in dairy cows that subsequently developed PRMDs. Other investigators have previously demonstrated that a number of compounds, including IgG, nutrients (e.g., vitamins, lipids) and other serum proteins, are secreted into colostrum as it accumulates in the mammary gland over several weeks before calving under the influence of various lactogenic hormones, including prolactin, 17β-estradiol, and insulin [23,24]. Consequently, some of the differences observed in colostrum lipid biomarkers may exist prior to calving, but that was not tested and would be difficult to test given the absence of colostrum and milk expression. Until now, there have been few if any research studies carried out even on day 1 colostrum as part of lipid biomarker discovery.

4.5.1 Our findings in the post-partum day 4 milk lipid biomarkers suggested a profound and significant change in the biological composition of triacylglycerols between animals destined to develop PRMDs and those animals that remained healthy. Among the 77 statistically significant biomarker candidates in milk listed in Table 3, those having m/z values from 572.48 to 810.74 (n = 45) were all higher in controls, whereas those having m/z values from 848.76 to 919.83 (30 of 32) were higher in animals that later developed disease. The remaining 2 statistically significant biomarker candidates having m/z values of 939.68 and 967.71 were both higher in controls.

4.5.2 Overall these lipid 'class' distinctions in milk were highly significant ($p=2.3x10^{-19}$). All of these particular milk lipid biomarkers appear to be triacylglycerols (TG). Such sweeping changes in one particular subclass of compounds likely denotes substantial metabolic changes present in these asymptomatic animals that later developed PRMDs. It has been previously established that the mammary gland of the cow synthesizes *de novo* fatty acids with an even number of carbons ranging in chain length from 4 to 14 carbons (i.e. fatty acids 4:0 to 14:0) together with production of about half of the 16:0 fatty acids. These short fatty acids account for approximately 60% of the total fatty acids present in milk on a molar basis, respectively [25]. The remaining 40% of milk fatty acids are longer-chain (16 carbons and longer), predominantly C_{18} fatty acids derived mainly from dietary lipids, but are also from lipolysis of adipose tissue triacylglycerols, which make their way into the circulation, mainly as plasma NEFA and triglyceride-rich lipoproteins[25]. Thus, fatty acids incorporated into milk fat TGs are provided either from mammary *de novo* synthesis or from the uptake of pre-formed fatty acids from the peripheral circulation. Although we did not determine the structure of all the milk lipid biomarkers, those lipids with m/z values from 848.76 to 919.83 almost all represent these longer chain TGs based on their m/z values and on the analysis of selected lipids in this range. Indeed, those milk biomarkers that were included in the optimal panel, then fragmented and characterized having m/z values of 906.83 and m/z 919.83 were found to be TGs having longer chain fatty acid components. These were higher in clinically normal cows that later developed PRMDs (see Table 7). Milk lipids in the range of m/z 510 to ~810 were very likely TGs composed of shorter chain fatty acids, based on size and selected tandem MS identifications. For example, the biomarker at m/z 740.67 was characterized as a TG having shorter chain fatty acids present, i.e., 12:0/14:0/16:0 and was found in higher levels in controls (or reduced levels in cows with later PRMDs). Since the dairy cows that developed PRMDs and the matched control group cows selected for this study were provided with the same feed, environment, care the differences in the lipid biomarker concentrations between later affected and healthy animals cannot be attributed to diet or other environmental factor.

Table 6. Identification/classification of predictive PRMD milk biomarkers. An optimized predictor panel of milk lipids provided 75.0% sensitivity at 90.0% specificity, by predicting 12 out of 16 cows that later developed PRMDs and 18 out of 20 cows that remained healthy that were used as controls. DG: diacylglycerol; TG: triacylglycerol

Markers (m/z)	Elemental composition	Classification (constituents)	Abundance	P Value
642.56	$C_{39}H_{76}O_5+NH_4^+$	DG(18:0/18:0)	Higher in controls	2.84E-07
906.84	$C_{57}H_{108}O_6+NH_4^+$	TG(18:0/18:0/18:1)	Higher in diseases	1.86E-05

Table 7. Identification/classification of predictive PRMD biomarkers combining colostrum (CS), milk (MK) and shared (DF, difference) markers. An optimized predictor panel using a combination of colostrum, milk and the differences between milk and colostrum for 'shared' lipids found in both of these secretions showing significant differences in both colostrum and milk, provided 87.5% sensitivity at a specificity of 100.0%, by predicting 14 out of 16 cows that later developed PRMDs and 20 out of 20 cows that remained healthy and served as controls. The difference between the normalized abundance of the biomarker for lipids with m/z 652.55, 906.84 and 919.83 indicated it was higher in milk than in colostrum. DG: diacylglycerol; TG: triacylglycerol

Markers (m/z)	Elemental composition	Classification (constituents)	Abundance	P-value
MK 642.56	$C_{39}H_{76}O_5+NH_4^+$	DG(18:0/18:0)	Higher in controls	2.84E-07
CS 682.56	$C_{41}H_{76}O_6+NH_4^+$	TG	Higher in diseases	0.00095
CS 344.23	$C_{21}H_{26}O_3+NH_4^+$	Unknown lipid class	Higher in diseases	0.0421
DF 906.84	$C_{57}H_{108}O_6+NH_4^+$	TG(18:0/18:0/18:1)	Higher changes in diseases	0.0614
DF 919.83	$C_{58}H_{110}O_6+OH$	TG 18:0/18:0/19:1)+OH or TG 18:0/18:1/19:0)+OH	Higher changes in diseases	0.0018
DF 652.55	$C_{40}H_{74}O_5+NH_4^+$	DG(18:2/19:0)	Higher changes in controls	<0.0001
MK 740.67	$C_{45}H_{86}O_6+NH_4^+$	TG(12:0/14:0/16:0)	Higher in controls	0.00037

4.5.3 The long chain fatty acid C18:1 cis-9 has been previously proposed as a possible biomarker whose increased circulating concentration was broadly indicative of elevated concentrations of plasma NEFA[11]. As for hyperketonemia, 90% of non-hyperketonemic controls showed a milk fat C18:1 cis-9-to-C15:0 ratio of 40 or lower, whereas 70% of cows suffering from hyperketonemia showed milk fat C18:1 cis-9-to-C15:0 ratios exceeding 40, which is consistent with our findings of potential different trends between longer chain TGs and shorter chain TGs [26]. Perhaps the higher expression of longer chain TGs in diseased animals is an early compensatory response to reduced *novo* maternal short chain fatty acid production by these cows.

4.5.4 As to the biological relevance of reduced levels of shorter chain fatty acid TGs seen in the animals destined to develop PRMDs, this suggests that those particular cows have an inability to generate adequate amounts of their own TGs when they have to meet high production demands after the onset of lactation. These differences were dramatic, remarkably consistent and to some degree uniform (i.e. several similar lipids all part of the same class changed in concert) suggesting marked differences in milk biology between animals that will develop PRMDs and those that remain healthy, and a clear harbinger of later PRMDs.

4.5.5 In colostrum the results were quite different. The statistically significant biomarkers consisting of both short chain and longer chain TGs were elevated for 55 out of 61TG species in the colostrum of asymptomatic animals later developing PRMDs. Clearly, colostrum production and milk production are representative of different stages in lactation but also somewhat different underlying biology.

It has been observed that the colostrum secreted within 24 hr after calving has a distinctive fat composition compared with the secretion produced on day 4 after calving in dairy cows [27]. Nevertheless, in colostrum all lipid biomarkers representative of both short chain and long chain fatty acid TGs were significantly increased in those animals that later developed PRMDs. Given the timing of colostrum versus milk production, the data strongly suggests that there are preexisting metabolic problems prior to calving in those animals that later develop PRMDs leading to increased TGs in colostrum.

The data suggest that the demands of milk production by day 4 postpartum deplete endogenously produced TGs in animals destined to develop PRMDs. Collectively, the data suggest that there are profoundly compromised metabolic pathways that lead to or reflect PRMDs, but these remain to be fully identified.

4.5.6 Of those markers that were part of the optimized panel, 8 out of 10 lipid biomarkers were successfully identified. Even though it was challenging to determine confidently the lipid class of colostrum biomarker m/z 344.22, based on the most likely elemental composition determined as $C_{21}H_{26}O_3+NH_4^+$ predicted by exact mass studies, it would suggest that it is an oxidized lipid. This marker showed a higher normalized intensity in the colostrum of case animals compared with healthy controls.

4.6 The colostrum-specific, milk-specific and mixed panels of lipid markers optimized for their ability to classify at-risk and healthy animals may be useful biomarkers for later routine application. All have shown predictive abilities with greater than 75% sensitivity and specificity. Clearly, this was a discovery study; further confirmation needs to occur in properly sized and powered studies. In addition, it will be important to develop cutoffs or a specific risk index, i.e., a numeric likelihood of developing PRMDs based on specific quantities of the several biomarkers as part of one or more panels. Broader application of these markers as a test will also require evaluating assay performance characteristics and more accurate quantization using specific standards. Ideally, a single collection of colostrum (and potentially milk) with lipidomic measurement of targeted lipids could be used to determine the percent likelihood of an animal developing a PRMD. Also, as mentioned above, additional studies will need to be done surveying broader geographic regions and different dairy breeds to confirm the effectiveness of the current biomarkers across all dairy cows or to the development of biomarkers specific to different dairy breeds. Finally, the marked alterations in TGs, especially in milk, now provide a previously unrecognized change that may yield important insights into PRMD pathology.

5. Conclusions

These studies suggest that colostrums and day 4 milk have significantly altered lipid compositions that predate and predict the development of PRMDs in most affected dairy cows. These quantitative lipid changes were interrogated and documented using a novel lipidomic mass spectrometric approach applied for the first time to animals developing PRMD, specifically to the analysis of their colostrum and milk. Some of these differences denote marked changes in lipid biosynthesis as part of the run up to PRMDs. Though the data are preliminary, the optimized panels of these biomarkers show high promise as a means to identify most of the dairy cows developing PRMDs early enough to allow for dietary or other interventions to preserve these animals.

List of abbreviations:

PRMD: production-related metabolic diseases; MF: milk fever or hypocalcemia; LDA: left displaced abomasum; OP: obdurate nerve paresis; CS: colostrums; MK: milk; MS: mass spectrometer; ESI: electrospray ionization; Q-TOF: quadrupole-time-of-flight; TIC: total ion chromatogram; CID: collisionally-induced dissociation; DG: diacylglycerols; TG: triacylglycerols; NEFA: non-esterifies fatty acids; BHBA: beta-hydroxybutyric acid; HSC: health score; MS/MS: tandem mass spectrometry.

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