Identification of biomarkers associated with the onset and progression of ketosis in transition dairy cows

by

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Abstract

Ketosis is a common metabolic disorder of transition dairy cows during the early lactation period. Cows with ketosis have lower milk yield and reproductive performance, greater risk of other periparturient diseases, and higher culling rate. Although ketosis has been extensively studied, not many investigators have focused on the pathobiology of the disease over time. Previous work has been mainly concentrated on the diagnosis, epidemiology, and implications of ketosis around the transition period. There is a grey area with regards to the agents that initiate ketosis and the metabolic pathways involved in the pathobiology of the disease. Moreover, not very much is known about how to prevent ketosis. Most work has been focused on the treatment of ketotic cows. It would be of great interest to both the dairy industry and health specialists to detect ketosis as early as possible and to take appropriate preventive measures. Therefore, the principal objectives of this thesis are: 1) to find evidence of the involvement of innate immunity in the pathobiology of ketosis as well as changes of in carbohydrate and lipid metabolism in pre-ketotic, ketotic, and post-ketotic cows; 2) to determine blood and urine metabotypes of transition dairy cows before, during, and after occurrence of ketosis; and 3) to identify new screening or predictive metabolite biomarkers of ketosis in the serum and urine of dairy cows as early as 8 weeks before the expected day of parturition.

To achieve these objectives, an enzyme linked immunosorbent assay (ELISA) was utilized to quantify and compare selected pro-inflammatory cytokines [i.e.,

inerleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF)], as well as acute phase proteins [(APP), i.e., haptoglobin (Hp) and serum amyloid A (SAA)] in the serum of cows diagnosed postpartum with ketosis and healthy controls (CON) starting at -8 and -4 wks before parturition, during the week of disease diagnosis as well as at +4 wks after calving. We also used several metabolomics tools including direct injection/liquid chromatography-tandem mass spectrometry (DI/LC-MS/MS), proton nuclear magnetic resonance (^{1}H-NMR), gas chromatography-mass spectrometry (GC-MS), and inductively coupled plasma-mass spectrometry (ICP-MS) to quantify and compare metabolites from various analyte groups including amino acids (AAs), acylcarnitines, biogenic amines, glycerophospholipids [i.e., phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC)], sphingolipids [i.e., hydroxysphingomyelin: SM (OH) and sphingomyelin (SM)], hexose, organic acids, saccharides, ketones, alcohols, mineral elements, and others, in the serum/urine of pre-ketotic (-8 and -4 wks), ketotic (disease wk), post-ketotic (+4 and +8 wks), and CON cows.

Results of this study indicate that cows affected by ketosis display alterations of multiple variables of innate immunity as well as amino acid, carbohydrate and lipid metabolism several weeks prior to the diagnosis of the disease. Two sets of predictive biomarker models and one diagnostic biomarker model for ketosis with high sensitivity and specificity were identified in serum and urine, respectively. These newly identified sets (two from serum and two from urine) of ketosis biomarkers can predict the disease much earlier than measurement of ketone bodies during early

lactation. Results also demonstrated that cows with ketosis experienced lower dry matter intake (DMI), elevated milk somatic cell count (SCC), and a tendency for lower milk production, and lower milk fat.

Preface

This thesis is an original work by Guanshi Zhang based on an original idea and experimental design by my supervisor Dr. Burim N. Ametaj. This research project was led by Dr. Burim N. Ametaj in collaboration with Dr. David S. Wishart from the Departments of Biological Sciences and Computing Science at University of Alberta. All experiments and research activities of the study were conducted at Dr. Ametaj's Lab, Dr. Wishart's Lab - The Metabolomics Innovation Centre (TMIC), and the Dairy Research and Technology Centre (DRTC). The research project, of which this thesis is a part, received research ethics approval from University of Alberta Animal Care and Use Committee for Livestock (Animal use protocol No. 146) entitled "Identification of biomarkers associated with the onset and progression of major metabolic and infectious diseases of transition dairy cows", February 15th, 2011.

Dr. Ametaj supervised the training and conduction of the experiment, laboratory analyses, statistical processing as well as writing and editing of this thesis. Dr. Elda Dervishi and Dr. Dagnachew Hailemariam contributed to evaluation of clinical diseases, maintenance of barn records, and collection of samples. Dr. Suzanna M. Dunn provided assistance with part of laboratory analyses and collection of samples. Seyed Ali Goldansaz, Dr. Qilan Deng, and Dr. John F. Odhiambo assisted fully or partly in sample collection. Dr. Rupasari Mandal was in charge of arranging the metabolomics analysis at TMIC, and did the profiling of raw DI/LC-MS/MS data. The role of Dr. Wishart was in development of the research proposal and in providing assistance and suggestions in the laboratory analyses and statistical analyses as well as

in editing of the manuscripts and this thesis. I was responsible for collecting the samples, preparing and maintaining the databases, performing statistical analyses (e.g., raw GC-MS spectra profiling, raw NMR spectra profiling, and all metabolomics data analyses), conducting the major part of laboratory analyses (i.e., ELISA, GC-MS, and NMR) as well as writing of the manuscripts.

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book chapters, and this thesis made the progress of my PhD more efficient and smooth. Moreover, the weekly individual meetings we had were very effective to keep me on the right track and to keep my research knowledge up-to-date.

I am also sincerely grateful to my supervisory committee member Dr. David S. Wishart, for allowing me to work in his lab (TMIC) and giving me the chance to learn about new metabolomics techniques. He was always willing to help and give his best advice, especially about how to properly do laboratory analyses and data analyses, and present the data. Dr. Wishart also contributed by editing this thesis and his comments helped improve its quality further. Many thanks to my supervisory committee member, Dr. Richard Uwiera, who has guided me through the course "Basic Bovine Immunology and Metabolic Diseases" and given valuable advice and comments. Special thanks are extended to my candidacy examiners Dr. Judd Aiken and Dr. Martin Zuidhof and my PhD final defense examiners Dr. Wenzhu Yang and Dr. Martin Zuidhof for their time and valuable suggestions on my doctoral project.

Special thanks also goes to our lab manager, Dr. Suzanna M. Dunn, who guided my training in lab safety and SOPs and taught me how to prepare ELISA kits and use instruments in the lab. She was also involved in collection of samples and laboratory analyses. Suzanna has a very optimistic personality, which affected me in both my work and life. Her patience and continuous encouragement and positive suggestions helped me go through countless difficulties during my PhD studies. I owe her a big thank you.

I also would like to thank Dr. Rupasari Mandal, the manager of Dr. Wishart's lab.

Without her help in booking equipment and making schedules for our samples, the enormous amount of metabolomics analyses could not be finished in a timely manner. I have been aided by several coworkers from both Dr. Ametaj and Dr. Wishart's labs. I wish to express my gratitude to Dr. Elda Dervishi, Dr. Dagnachew Hailemariam, Seyed Ali Goldansaz, Dr. Qilan Deng, and Dr. John F. Odhiambo for their generous help in sample collection. Edison Dong for his help in sample preparation of NMR and GC-MS analysis. Philip Liu for his help in preparing the DI/LC-MS/MS kit. Dr. Beomsoo Han for his advice on data analysis. Dr. Trent Bjorndahl for his guidance and support in NMR techniques. Dr. Souhaila Bouatra and Dr. Farid Aziat for their training in raw NMR spectra profiling (i.e., the Chenomx NMR Suite). Dr. Ramanarayan Krishnamurthy for his guidance and support in GC-MS techniques. Hong Lin for her training in raw GC-MS spectra profiling (i.e., AMDIS GC-MS Processing Software and GC-MS Auto-Profiling). My sincere thanks are extended to undergraduate student volunteers and all the staff at DRTC for their help with the animal trial and care.

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May 26, 2016

Guanshi Zhang, Edmonton, Canada

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List of abbreviations

AAs	Amino acids
AB	Arginine biosynthesis
Ac	Acetone
AcAc	Acetoacetate
ACC	Acetyl-CoA carboxylase
ACP	Acyl-carrier-protein
ACs	Acylcarnitines
ADMA	Asymmetric dimethylarginine
Al	Aluminum
Ala	Alanine
AM	Aspartate metabolism
APM	Arginine and proline metabolism
APP	Acute phase proteins
APR	Acute phase response
AR	Ammonia recycling
Arg	Arginine
As	Arsenic
ASGM	Alanine, aspartate, and glutamate metabolism
Asn	Asparagine
ASNSM	Amino sugar and nucleotide sugar metabolism
Asp	Aspartic acid

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUC	The area under the ROC curve
B	Boron
BAM	Beta-alanine metabolism
BCAAs	Branched-chain amino acids
BHBA	β-hydroxy butyric acid
BM	Biotin metabolism
BMDB	Bovine Metabolome Database
BUM	Butyrate metabolism
BSC	Body condition score
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
C0	DL-Carnitine
C1P	Ceramide-1-phosphate
C2	Acetyl-L-carnitine
C3	Propionyl-L-carnitine
C3:1	Propenyl-L-carnitine
C3-DC (C4-OH)	Malonyl-L-carnitine / Hydroxybutyryl-L-carnitine
C3-OH	Hydroxypropionyl-L-carnitine
C4	Butyryl-L-carnitine
C4:1	Butenyl-L-carnitine
C5	Valeryl-L-carnitine

C5:1	Tiglyl-L-carnitine
C5:1-DC	Glutaconyl-L-carnitine
C5-DC (C6-OH)	Glutaryl-L-carnitine / Hydroxyhexanoyl-L-carnitine
C5-M-DC	Methylglutaryl-L-carnitine
C5-OH (C3-DC-M)	Methylmalonyl-L-carnitine/Hydroxyvaleryl-L-carnitine
C6:1	Hexenoyl-L-carnitine
C6 (C4:1-DC)	Fumaryl-L-carnitine / Hexanoyl-L-carnitine
C7-DC	Pimelyl-L-carnitine
C8	Octanoyl-L-carnitine
C9	Nonayl-L-carnitine
C10	Decanoyl-L-carnitine
C10:1	Decenoyl-L-carnitine
C10:2	Decadienyl-L-carnitine
C12	Dodecanoyl-L-carnitine
C12:1	Dodecenoyl-L-carnitine
C12-DC	Dodecanedioyl-L-carnitine
C14	Tetradecanoyl-L-carnitine
C14:1	Tetradecenoyl-L-carnitine
C14:1-OH	Hydroxytetradecenoyl-L-carnitine
C14:2	Tetradecadienyl-L-carnitine
C14:2-OH	Hydroxytetradecadienyl-L-carnitine
C16	Hexadecanoyl-L-carnitine

C16-OH	Hydroxyhexadecanoyl-L-carnitine
C16:1	Hexadecenoyl-L-carnitine
C16:1-OH	Hydroxyhexadecenoyl-L-carnitine
C16:2	Hexadecadienyl-L-carnitine
C16:2-OH	Hydroxyhexadecadienyl-L-carnitine
C18	Octadecenoyl-L-carnitine
C18:1	Octadecenoyl-L-carnitine
C18:1-OH	Hydroxyoctadecenoyl-L-carnitine
C18:2	Octadecadienyl-L-carnitine
Ca	Calcium
CACT	Carnitine-acylcarnitine translocase
CB	Catecholamine biosynthesis
CD	Caprolactam degradation
CDCA	Chenodeoxycholic acid
CI	Confidence interval
CK	Clinical ketosis
Co	Cobalt
CON	Healthy control
CP	Crude protein
CPT	Carnitine palmitoyltransferase
Cr	Chromium
CS	Cell signaling

Cu	Copper
CV	Coefficients of variation
Cys	Cysteine
DCA	Deoxycholic acid
DHA	1,3-dihydroxyacetone
DIM	Days in milk
DI-MS/MS	Direct injection - tandem mass spectrometry
DMI	Dry matter intake
DSS	Disodium-2,2-dimethyl-2-silapentane-5-sulphonate
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FFAM	Free fatty acid metabolism
FAO	Fatty acid oxidation
FFT	Fatty acid transport
GAM	Galactose metabolism
GC-MS	Gas chromatography - mass spectrometry
GL	Glycolysis
GLM	Glycerolipid metabolism
Gln	Glutamine
Glu	Glutamate
Gly	Glycine

GM	Glutamate metabolism
GNG	Gluconeogenesis
GPL	Glycerophospholipid metabolism
GSH	Glutathione
GSTM	Glycine, serine, and threonine metabolism
GTM	Glutathione metabolism
HCN	Hydrogen, carbon, and nitrogen
HDL	High density lipoproteins
His	Histidine
HM	Histidine metabolism
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
^{1}H-NMR	Proton nuclear magnetic resonance
Hp	Haptoglobin
Hs	Health status
ICP-MS	Inductively coupled plasma - mass spectrometry
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IKK-NFκB	IKB kinase nuclear factor κB
IL	Interleukin
Ile	Isoleucine
IM	Inositol metabolism
JNK-AP-1	c-*jun* N-terminal kinase activator protein-1

K	Potassium
KBM	Ketone body metabolism
KEGG	Kyoto Encyclopedia of Genes and Genomes
KTG	Ketogenesis
LC	Lipid catabolism
LCAD	Long-chain acyl-CoA dehydrogenase
LCFA	Long-chain fatty acids
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LD	Lysine degradation
LDL	Low-density lipoproteins
Leu	Leucine
LPS	Lipopolysaccharide
LSM	Least squares means
Lys	Lysine
lysoPC a	Lysophosphatidylcholine acyl
MA	Mineral absorption
MAS	Malate aspartate shuttle
MC	Membrane component
MCAD	Medium-chain acyl-CoA dehydrogenase
MEM	Methionine metabolism
Met	Methionine
MM	Methane metabolism

Mn	Manganese
Mo	Molybdenum
MRM	Multiple reaction monitoring
mTOR	Mammalian target of rapamycin
MUN	Milk urea nitrogen
Na	Sodium
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NOS	Nitric oxide synthase
NSM	Nucleotide sugars metabolism
OS	Oxidative stress
P	Phosphorus
PDA	Protein digestion and absorption
Pb	Lead
PB	Protein biosynthesis
PC	Principle component
PC	Phosphatidylcholine
PCA	Principal component analysis
PCB	Pantothenate and CoA biosynthesis
PC aa	Phosphatidylcholine diacyl

PC ae	Phosphatidylcholine acyl-alkyl
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase
PFG	Pulsed-field gradient
Phe	Phenylalanine
PLS-DA	partial least squares - discriminant analysis
PNM	Propanoate metabolism
PPM	Purine/pyrimidine metabolism
PPP	Pentose phosphate pathway
Pro	Proline
PTM	Phenylalanine and tyrosine metabolism
PYM	Pyruvate metabolism
Rb	Rubidium
RIs	Retention indices
ROC curve	Receiver-operator characteristic curve
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SARA	Sub-acute ruminal acidosis
SCAD	Short-chain acyl-CoA dehydrogenase
SCC	Somatic cell count
SCK	Subclinical ketosis

SD	Standard deviation
SDMA	Symmetric dimethylarginine
Se	Selenium
SEM	Standard error of the mean
Ser	Serine
SM	Sphingomyelin
SM	Sphingolipid metabolism
SM (OH)	Hydroxysphingomyelin
SMPDB	The Small Molecule Pathway Database
Sr	Strontium
SUM	Sulfur metabolism
TCA cycle	Tricarboxylic acid / citric acid cycle
TG	Triglycerides
Thr	Threonine
TMR	Total mixed ration
TNF	Tumor necrosis factor
Trp	Tryptophan
TS	Total solid
Tyr	Tyrosine
UC	Urea cycle
UMD	The Urine Metabolome Database
UPR	Unfolded protein response

Val	Valine
VIP	Variable importance in projection
VLCAD	Very long-chain acyl-CoA dehydrogenase
VLDL	Very-low-density lipoproteins
VLID	Valine, leucine and isoleucine degradation
Zn	Zinc

Chapter 1 Literature review

1.1 Ketosis and its impact on the dairy industry

1.1.1 Conventional understanding of ketosis

Transition dairy cows undergo major physiological changes from late gestation to early lactation. Ketosis is a common metabolic disorder that frequently occurs during the so called transition period. Ketosis is characterized by increased concentrations of ketone bodies including acetoacetate (AcAc), acetone (Ac), and β-hydroxybutyric acid (BHBA) in the blood, urine, and milk (Oetzel, 2004; Tehrani-Sharif et al., 2011).

The incidence rate of ketosis has surpassed those of ruminal acidosis and milk fever and has emerged as one of the most important metabolic perturbations of dairy cows in North America since the late 1990's (Oetzel, 2007). Indeed, almost 40% of dairy cows in North America have different degrees of ketosis within a few weeks after calving, with the incidence varying widely between farms and reaching as high as 80%, in some dairy herds (Duffield, 2000). The negative impacts of ketosis may include lowered milk yield, impaired reproductive performance (e.g., infertility), greater risk of other periparturient diseases including displaced abomasum, lameness, mastitis, metritis, and retained placenta, and a greater culling rate (Duffield et al., 2009; McArt et al., 2011, 2012; Ospina et al., 2010; Raboisson et al., 2014; Suthar et al., 2013). The cost of a single case of ketosis has been estimated to be around CAD

$50 to $100 (Duffield, 2000).

Although ketosis has been extensively reviewed over time (Duffield et al., 2000; Gordon, 2013a; McArt et al., 2011; Ospina et al., 2010), the precise causes and pathobiology of ketosis remain unknown. Dairy cows generally experience a state of negative energy balance (NEB) around calving characterized by excessive mobilization of fatty acids from adipose tissue due to low dry matter intake (DMI) (Herdt, 2000). Fatty acids follow four pathways in the liver: 1) complete oxidation via the tricarboxylic acid cycle to generate H_2O, CO_2 and energy; 2) incomplete oxidation resulting in the release of ketone bodies and less energy; 3) export from the liver as part of very low density lipoproteins (VLDL); and 4) esterification of non-esterified fatty acids (NEFA) to triglycerides (TG) and accumulation within the hepatocytes (Goff and Horst, 1997; Grummer, 2008).

The exact pathobiology of hyperketonemia is not fully understood (Herdt, 2000). The most dominant hypothesis is that there is an energy (i.e., glucose) deficit immediately postpartum. Apparently, the gluconeogenic ability of the liver cannot meet the demands of the postpartum cow for glucose, because 60 to 85% of the available glucose is utilized by the mammary gland for milk synthesis (Knowlton et al., 1998). Hypoglycemia may result from either inadequate gluconeogenic precursors or a limited rate of gluconeogenesis (Lyle, 1983; Gordon, 2013a). Low concentrations of blood glucose are associated with hypoinsulinemia, which subsequently triggers mobilization of fatty acids from adipose tissue, thereby increasing ketone body

formation. The process of hepatic ketogenesis and ketone body utilization in extrahepatic organs is illustrated in Figure 1-1. In the liver, fatty acids are either oxidized to carbon dioxide and ketone bodies or re-esterified into TG for storage and incorporation into very low density lipoproteins (VLDL) (Lyle, 1983). Excess accumulation of TG leads to fatty liver, which further impairs liver functions, limiting the process of gluconeogenesis.

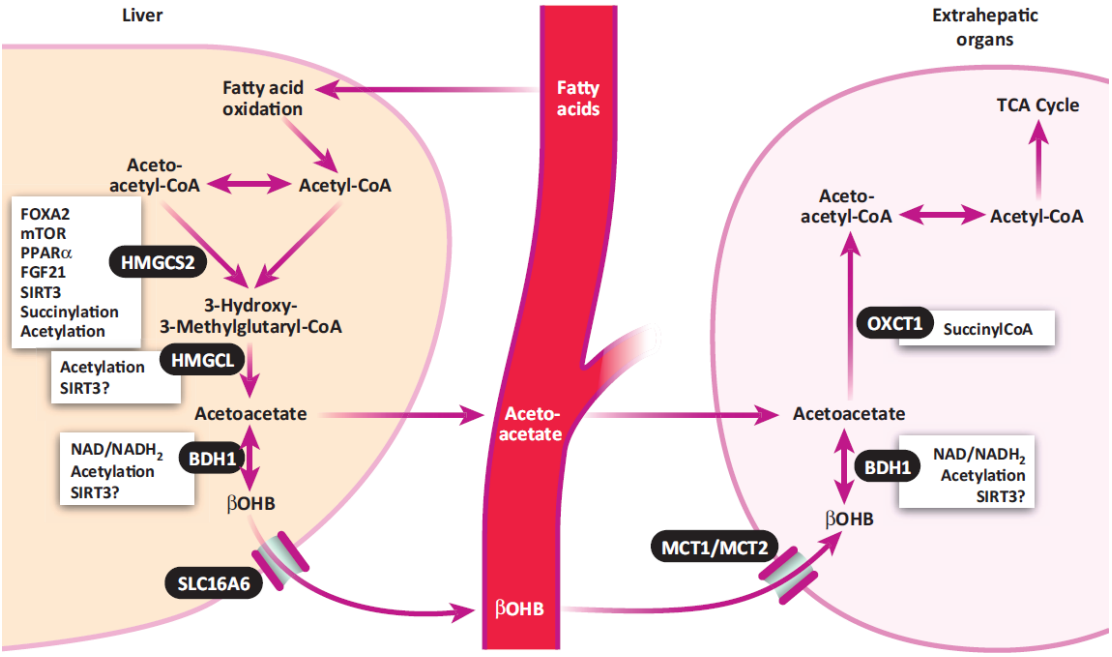

Figure 1-1. Outline of hepatic ketogenesis and ketone body utilization in extrahepatic organs. Abbreviations: BDH1, β-hydroxybutyrate dehydrogenase; βOHB, β-hydroxybutyratic acid (BHBA); FGF21, fibroblast growth factor 21; FOXA2, forkhead box A2; HMGCS2, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 2; HMGCL, HMG-CoA lyase; MCT1/2, monocarboxylic acid transporters 1/2; mTOR, mechanistic target of rapamycin; OXCT1, succinyl-CoA:3-ketoacid coenzyme A transferase; PPARα, peroxisome proliferator activated receptor α; SIRT3, sirtuin 3; SLC16A6, solute carrier family 16 (monocarboxylic acid transporter), member 6; TCA cycle, tricarboxylic acid cycle. (Source: Newman and Verdin, 2014)

It was reported that fat accumulation in the liver during the dry-off period or even during the previous lactation period may contribute to the development of

ketosis (Drackley et al., 2001; Hayirli, 2006). However, most investigations have been focused on the concentrations of ketone bodies in the body fluids postpartum for evaluating ketosis, although a few studies have attempted to assess the association between ketones or other variables in body fluids (i.e., blood and urine) prepartum and ketosis diagnosed after parturition. It is obvious that more research is warranted to better understand the pathobiology of ketosis and to determine more details about the metabolic alterations in the blood and urine during the prepartum period.

1.1.2 Classification of ketosis

Several schemes for classifying ketosis have been reported and two different approaches are commonly used by various researchers. The first categorization is based on concentrations of β-hydroxybutyric acid (BHBA) in the blood and the lack or presence of clinical signs of disease. Accordingly, ketosis is classified into two forms including subclinical ketosis (SCK) and clinical ketosis (CK). SCK is defined as an increase of ketone bodies in the blood, urine, or milk, in absence of obvious clinical signs of disease. Concentrations of BHBA in the serum between 1,200 to 1,400 μmol/L are generally used for diagnosis of SCK (Suthar et al., 2013). CK is characterized by hyperketonemia, hypoglycemia, and the presence of clinical symptoms including lower appetite, loss of body weight, decreased milk production, and dry manure (Gordon et al., 2013b). The clinical form of ketosis is usually recognized at greater concentrations of BHBA in the blood ranging between 2,600 to 3,000 μmol/L (Duffield, 2000; Oetzel, 2007).

The second classification scheme categorizes ketosis into three general types including type I ketosis, type II ketosis, and butyric acid silage ketosis based on the etiology and timing of hyperketonemia (Herdt, 2000; Holtenius and Holtenius, 1996; Oetzel, 2007). Type I, or primary ketosis is the classic form of ketosis that occurs between 3 and 6 weeks postpartum when milk energy outflow reaches its peak (Holtenius and Holtenius, 1996). It is defined as type I ketosis because it is aligned with type I diabetes mellitus in humans. Cows with type I ketosis experience hypoinsulinemia at the time of diagnosis of hyperketonemia because of chronic hypoglycemia due to a shortage of glucose precursors for milk production. In this case, glucose precursors are absorbed from the diet (mostly propionate) or from muscle proteins in the form of amino acids (AAs, i.e., gluconeogenesis). However, the capacity of the gluconeogenic process is limited due to host protection of muscle proteins. On the other hand, lipolysis and ketogenesis are enhanced and as a result, fatty acids and ketone bodies are utilized to spare glucose and meet energy requirements (Holtenius and Holtenius, 1996).

Type II, or secondary ketosis usually occurs immediately after parturition and is concurrent with other diseases such as fatty liver (Holtenius and Holtenius, 1996). This form of ketosis is named as type II ketosis based on its metabolic counterpart - type II diabetes mellitus. Cows with type II ketosis have high concentrations of both blood insulin and glucose at the diagnosis of hyperketonemia (Holtenius and Holtenius, 1996). Insulin resistance may also exist during type II ketosis (Oetzel, 2007). Obesity and overfeeding during the dry period are critical for the development

of this type of ketosis. Mobilization of body fat from adipose tissues and accumulation of triglycerides (TG) may occur prior to or at parturition (Oetzel, 2007). Excessive accumulation of TG in the liver not only impairs gluconeogenesis, but also suppresses immune functions of hepatocytes.

Ketosis related to consuming of silage enriched with butyric acid is the third type of ketosis under the second categorization scheme. This type of ketosis has been attributed to intake of feed high in ketogenic precursors (i.e., butyric acid) (Tveit et al., 1992). The reasons for accumulation of butyric acid in the silage are related to, for example, preparation of silage with hay that contains low water-soluble carbohydrates or silage that is chopped too wet favoring growth of bacteria from *Clostridium sp.* (Oetzel, 2007). Some carbohydrates in the silage are fermented into butyric acid rather than the desired lactic acid. Cows develop butyric acid silage ketosis when they ingest large quantities of silages that have undergone *clostridial* fermentation. However, whether or not a cow develops dietary butyric acid ketosis also depends on the amount of silage consumed and the presence of other risk factors (e.g., early lactation, ruminal acidosis, high milk production, low dietary energy, and high dietary protein) for ketosis (Oetzel, 2007).

1.1.3 Impact of ketosis on dairy cows and the dairy industry

Numerous investigators have demonstrated detrimental effects of ketosis on a cow's health, reproductive performance, milk production, milk composition, and the dairy industry profitability (Duffield, 2005; LeBlanc et al., 2005; Duffield et al., 2009;

Gordon, 2013a; McArt et al., 2011, 2012; Ospina et al., 2010). With regard to the association between ketosis and subsequent disease occurrence, most studies have been focused on SCK due to its widespread prevalence. Cows with SCK have been reported to be at a greater risk for disorders like displaced abomasum (DA), metritis, endometritis, mastitis, lameness, retained placenta, cystic ovarian disease, impaired fertility, reduced reproductive efficiency, and impaired immune functions (Duffield et al., 2006, 2009; Hammon et al., 2006; McArt et al., 2011, 2012; Ospina et al., 2010; Raboisson et al., 2014; Suthar et al., 2013). The relationship between CK and other periparturient diseases was also reported previously. Ketosis and associated periparturient diseases may increase the likelihood for early culling of cows from the herd during early lactation.

Deleterious effects of ketosis on milk production are well documented. Dohoo and Martin (1984) demonstrated that ketotic cows had 1.0 to 1.4 kg (4.4 to 6.6 %) lower daily milk yield compared with non-ketotic cows. Findings from another study were very similar, which estimated milk production loss in cows with ketosis at approximately 1 to 2 kg/d (Duffield et al., 2009). In addition, McArt et al. (2012) reported that each additional 0.1 mmol/L increase in the serum BHBA (beyond 1.2 mmol/L) at the first diagnosis of SCK is associated with 0.5 kg/d more milk loss for the first 30 day in milk (DIM). Milk loss due to hyperketonemia or ketosis consequently lowers the economic profitability of the entire dairy industry. No explanations as to how ketonemia affects milk production have been given by researchers involved in those studies.

The effect of ketosis on milk composition has also been reported previously. For example, Kauppinen (1984) reported that cows affected by SCK or CK have greater average annual milk fat yield compared with healthy cows. Another study by Miettinen (1994) demonstrated that milk fat percent was increased in cows with SCK. Moreover, milk protein percentage lowered in SCK cows (Miettinen and Setala, 1993). More research is warranted to identify other milk components affected or to assess the overall quality of milk produced by ketotic cows.

The cost of a single case of SCK has been estimated to be between CAD $50 to $100 (Duffield, 2000). A similar estimation was also reported by Geishauser et al. (2001), which indicated the cost of SCK at CAD $78 per case. The overall cost of ketosis in a dairy herd can be estimated based on the reported incidence of SCK at about 26.4 to 55.7% (Oetzel, 2013) and that of CK between 2 to 15% in the first month of lactation (Gordon et al., 2013b). The total ketosis (both CK and SCK) cost was estimated at US $55.19 to $123.94 per case (Liang, 2013). Due to the high incidence rate of ketosis, even a moderate cost per case can result in big losses to the dairy industry.

1.2 Current diagnostic approaches and biomarkers for ketosis

1.2.1 Ketone bodies in the blood, urine, and milk

Concentrations of ketone bodies in the blood, urine, and milk have been used for the diagnosis of ketosis in dairy cows for many years. Concentrations of BHBA in the blood have been used as the “gold standard” test for ketosis primarily because BHBA

is the predominant ketone body in ruminants, and it is more stable than Ac and AcAc (Työppönen and Kauppinen, 1980). The most commonly used cut-off value for SCK is ≥ 1,400 μmol/L of blood BHBA, while the CK is generally diagnosed by much greater levels of blood BHBA ≥ 3,000 μmol/L (Oetzel, 2004). There are no special sampling requirements for blood samples. However, a blood BHBA test should not be performed on blood samples collected from the mammary vein. It was reported that the concentration of BHBA in mammary vein blood is lower because the mammary gland tends to extract BHBA and releases AcAc (Kronfeld et al., 1968).

Various approaches for diagnosing ketosis based on blood metabolites including BHBA have been reviewed by several authors (Oetzel, 2004; Zhang et al., 2012). Quantification of serum BHBA can be performed using a colorimetric method by a commercial kit and detected by a microplate reader (Zhang et al., 2015). Other methods including fluorometric determination, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy have also been used to test blood levels of BHBA and Ac (Larsen and Nielsen, 2005; Klein et al., 2010).

Laboratory based tests of milk variables for diagnosis of ketosis have been widely used by researchers. Concentrations of milk BHBA and Ac were measured by Fourier transform infrared spectrometry to detect SCK (de Roos et al., 2007; Heuer et al., 2001). Besides ketone bodies, other milk components have been also used for detection of SCK in dairy cows. The ratio of milk fat percentage to milk protein percentage was used as a tool to evaluate the prevalence of SCK (Duffield et al., 1997;

Gantner et al., 2009). Milk fat/protein ratios greater than 1.5 suggests that the cow is affected by SCK (Čejna and Chládek, 2005). In a study conducted by van Haelst et al. (2008), long-chain fatty acids (e.g., C18:1 *cis*-9) in milk fat were elevated in cows with SCK during the first nine weeks of lactation.

1.2.2 Cow-side tests for ketosis

A variety of validated and practical tools for cow-side tests have been developed for detection of ketosis in dairy herds. Cow-side tests have several advantages including lower cost, immediate results, and less labor, when compared to laboratory analysis. All three main body fluids (i.e., blood, urine, and milk) are feasible for rapid cow-side ketosis tests. A hand-held biosensor for measuring blood ketones (i.e., Precision Xtra^{TM} meter) is now widely used for cow-side blood BHBA testing to diagnosis of ketosis in postpartum dairy cows (Heuwieser et al., 2007).

Urine, as an easily accessible body fluid, can be used for evaluation of ketosis by a cow-side test. The most widely used cow-side urine test for monitoring of ketosis is a semi-quantitative dipstick (Ketostix urine strip; Bayer Corp. Diagnostics Division, Elkhart, IN) that measures AcAc (Oetzel, 2004, 2007). Nitroprusside tablets (Acetest; Bayer Corp. Diagnostics Division, Elkhart, Indiana) were reported as an approach for quantitative evaluation of urine AcAc for cow-side diagnosis of ketosis (Nielen et al., 1994).

Cow-side milk tests for ketosis have a considerable advantage over both blood and urine tests for ease of milk collection and for assurance that all cows in the herd

can be tested at the time of milking. The milk BHBA test strip (also known as KetoTest, Ketolac BHBA, and Sanketopaper in different countries) is the most promising cow-side milk test for ketosis (Oetzel, 2004; Zhang et al., 2012). Milk AcAc can be qualitatively measured by Nitroprusside powders (Utrecht powder, KetoCheck powder) (Oetzel, 2004). A study conducted by Geishauser et al. (2000) reported that milk AcAc could be measured semi-quantitatively by Pink test liquid. A dipstick designed for the measurement of milk BHBA also has been utilized for evaluating urine BHBA (Osborne et al., 2002). The limitations of milk cow-side tests are that generally they are not as sensitive as urine tests in monitoring of ketosis.

Despite the advantages or disadvantages of urine or milk tests, none of them have perfect sensitivity and specificity compared to the "gold standard" test of blood BHBA. Comprehensive comparisons of sensitivity and specificity of different cow-side ketosis tests have been previously reviewed (Oetzel, 2004, 2007). Therefore, it is important that new cow-side tests with greater sensitivity and specificity need to be developed in the future.

1.2.3 Some newly identified diagnostic biomarkers

Besides elevated concentrations of ketone bodies in various body fluids, other variables might also be perturbed during ketosis. Introduction of systems biology approaches and the development of genomics, transcriptomics, proteomics, and metabolomics suggest that it is likely that new biomarkers of ketosis can be identified.

Recently, proteomics analysis of liver, urine, and serum of ketotic cows have

been reported. A comparative proteomic assessment of liver proteins and 5 enzymes (i.e., acetyl-coenzyme A acetyltransferase 2, 3-hydroxyacyl-CoA dehydrogenase type-2, elongation factor Tu, alpha-enolase and creatine kinase) revealed that they were differentially expressed in ketotic cows compared with healthy controls (Xu et al., 2008). Moreover, urine concentrations of 11 proteins including amyloid precursor protein, apolipoprotein (Apo) C-III, cystatin C, fibrinogen, hepcidin, human neutrophil peptides, osteopontin, VGF (non-acronymic) protein, serum amyloid A (SAA), the C1 inhibitor (C1INH), and transthyretin were decreased in ketotic cows (Xu et al., 2015a). The same authors (Xu et al., 2016) reported that serum fibroblast growth factor-21 might be a promising biomarker for detecting ketotic cows. It should be noted that these novel biomarkers have yet to be validated.

1.3 Metabolomics: an emerging systems veterinary approach for biomarker discovery

1.3.1 NMR spectroscopy and MS - based metabolomics

Metabolomics is a relatively new “omics” science, defined as the comprehensive and fully quantitative analysis of all detectable metabolites (low molecular weight molecules, MW < 1,500 Daltons) within a particular biological sample. Metabolomics permits a complete overview of an organism’s metabolic status, which can provide new insights into pathophysiologic mechanisms in diseases (Xia et al., 2013). Metabolomics has been widely-used as a powerful tool for elucidating disease etiologies, developing biomarkers for detection and characterization of biological

pathways as well as monitoring and predicting complex diseases (Martin et al., 2012; Nguyen et al., 2012; Xia et al., 2013).

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) techniques are the most commonly applied analytical platforms for metabolomics studies, although other analytical tools have also been utilized. MS detection is usually integrated with other chemical separation techniques such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE). Utilization of these platforms has helped researchers investigate metabolic profiles employing biological fluids such as blood serum/plasma, urine, milk, saliva, and cerebrospinal fluid. Metabolomics analysis is generally classified into two experimental strategies (i.e., targeted and non-targeted metabolomics) based on the objective of the investigation (Dunn et al., 2011). Targeted metabolomics involves quantification of defined groups of biochemically known and chemically characterized metabolites, while non-targeted metabolomics involves the global unbiased qualitative analysis of as many measurable small-molecule metabolites as possible, present within a biological sample including unknown compounds (Naz et al., 2014; Roberts et al., 2012).

The basic principles and concepts of the two major metabolomics platforms (i.e., NMR and MS) and their individual application in metabolomics research have been described previously (Dettmer et al., 2007; Lane, 2012; Saleem, 2013). The theory of NMR was originally proposed in 1924 by Wolfgang Pauli and NMR spectroscopy was first developed and utilized to measure NMR phenomena in 1946 by researchers

at Stanford and M.I.T., in the USA (Bloch et al., 1946; Purcell et al., 1946). Since then, chemists began to apply the technology to solve chemistry problems such as the characterization of the detailed chemical structure of both large and small molecules. In recent years, NMR technology has been widely used in metabolomics studies for metabolite structural determinations and quantification (Lane, 2012; Larive et al., 2014).

The NMR phenomenon is based on the fact that certain nuclei of atoms (e.g., ^{1}H,^{13}C, ^{31}P, ^{15}N, ^{19}F, etc.) have magnetic properties (i.e., a spin that generates a small, local magnetic field) that can generate chemical information. For a more comprehensive understanding of the principle behind NMR measurement, please refer to previously published book chapter by Lane (2012). An NMR spectrum can provide a great deal of information about the chemical structure of pure substances or the chemical composition of complex sample mixtures. Proton (^{1}H) NMR Spectroscopy is most popularly used in metabolomics researches, since hydrogen is naturally abundant and is present in almost all organic molecules. One-dimensional (1D) and 2D NMR approaches are most frequently applied for determining known, unanticipated, or even unknown metabolites. However, the commonest, quickest, and easiest NMR experiments in metabolomics studies involve collecting 1D NMR spectra.

NMR spectroscopy is recognized as one of the premier approaches for the analyses of multi-component mixtures due to its many advantages including simple sample preparation, essentially universal detection (wide spectrum of multiple classes

of metabolites), along with the facts that it is non-destructive, non-invasive, relatively rapid (>100 samples/day is attainable), cheap (after the initial costs of purchase and installation), and highly reproducible results. The major disadvantages of NMR are the high initial cost (over one million dollars) of the instrument and relatively poor sensitivity compared to MS.

As a complementary technique to NMR, a variety of MS approaches have been used to qualitatively and quantitatively measure metabolites from minimal amounts of biological material in numerous metabolomics investigations (Dettmer et al., 2007). The basic principles and applications of MS have been elaborated in many excellent books and review articles (de Hoffmann and Stroobant, 2007; Dettmer et al., 2007). Briefly, the first step in the MS analysis of compounds is the generation of gas-phase ions of the molecule of interest (i.e., charged molecules) by an ionization source [e.g., electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), and matrix assisted laser desorption (MALDI)]. After that, gas-phase ions need to be separated according to their mass-to-charge (m/z) ratio and detected by a mass analyzer. As there are different ionization sources, several types of mass analyzers such as magnetic sector analyzers (MSA), quadrupole analyzers (Q), time-of-flight analyzers (TOF), Fourier-transform ion cyclotron resonance (FT-ICR) analyzers as well as ion trap, orbitrap, and triple quad (de Hoffmann and Stroobant, 2007). Each mass analyzer has its advantages and disadvantages. All mass analyzers apply dynamic or static magnetic and electric fields that can be used alone or combined.

Tandem MS (MS/MS) even n*MS approaches are more powerful (e.g., higher specificity) and can be used to validate the identity of unknown molecules.

Mass spectrometry is commonly used in combination with prior separation modalities such as GC, LC and CE to generate hyphenated GC-MS, LC-MS, and CE-MS analysis platforms in metabolomics studies. Direct injection (DI) of serum and urine samples into tandem MS without prior chromatographic separation has also been reported (Boernsen et al., 2005; Bouatra et al., 2013; Dettmer et al., 2007; Psychogios et al., 2011). In comparison with NMR spectroscopy, the major advantage of MS is the higher selectivity and sensitivity, as mass spectrometers can measure analytes routinely in the femtomolar to attomolar range. One of the major weaknesses of MS techniques in metabolomics is that a sample preparation steps such as extraction and derivatization are often required. This procedure can cause metabolite losses and specific metabolite classes may be ignored or discriminated.

In MS - based metabolomics, GC-MS, DI-MS/MS, and LC-MS/MS are the most frequently used platforms. Specifically, GC-MS offers structural information, high throughput, reasonable quantitative precision, relatively high reproducibility and resolution as well as sensitivity that is at least 2 orders of magnitude higher than NMR. However, one limitation of GC-MS is its inability to study metabolites that are not volatile and thermally stable. The greatest advantage of DI-MS/MS is that ideally no metabolites are lost during the sample preparation. However, samples with high-salt or high-protein content can cause ion suppression and adduct formation in the electrospray process, poor chromatographic metabolite separation and degradation of

column performance (Dettmer et al., 2007; Pham-Tuan et al, 2003). The main advantage of LC chromatographic separation based MS for metabolomic studies is its flexibility. Different combinations of columns and mobile phases make it possible to tailor separations to the compounds of interest.

1.3.2 Metabolomic databases and bioinformatics tools for metabolomics data analyses and interpretation

Like other "omics" technologies, metabolomics deals with very large datasets. Besides recent innovations in instrumentation, bioinformatics software tools and metabolomic databases are vital for the unbiased, high-throughput and comprehensive analysis of metabolites. In metabolomics studies, both NMR and MS data are composed of thousands of signals derived from the many hundreds of small molecules. Analysis of such complex data is extremely challenging and time-consuming. In parallel with the development of analytical techniques, various metabolomic databases and computational tools have been developed by researchers in metabolomics.

The Human Metabolome Database (HMDB, http://www.hmdb.ca) is currently the world's most complete and comprehensive database covering most known human metabolites (Wishart et al., 2007; 2009; 2012). The most recent release of HMDB (version 3.6) has been significantly enhanced and expanded over previous versions. Specifically, the number of annotated metabolite entries has grown from 6,500 to 41,993, which include both water-soluble and lipid-soluble metabolites as well as

metabolites that are either abundant (> 1 μM) or relatively rare (< 1nM).

Several resources focusing on specific biofluids or species were derived and developed by Dr. Wishart's Research Group at University of Alberta, Canada. For example, the CSF Metabolome database (http://www.csfmetabolome.ca) (Wishart et al., 2008), the Serum Metabolome database (http://www.serummetabolome.ca) (Psychogios et al., 2011), the Urine Metabolome database (http://www.urinemetabolome.ca) (Bouatra et al., 2013), the Saliva Metabolome database (http://www.salivametabolome.ca) (Dame et al., 2015), the CyberCell Database (CCDB, http://ccdb.wishartlab.com/CCDB) (Sundararaj et al., 2004), the Yeast Metabolome Database (YMDB, http://www.ymdb.ca) (Jewison et al., 2012), and the *E. coli* Metabolome Database (ECMDB, http://ecmdb.ca) (Guo et al., 2012; Sajed et al., 2016).

The Bovine Metabolome Database (BMDB) (http://www.cowmetdb.ca/cgi-bin/browse.cgi) is another specific database for dairy cows and beef cattle, which is still in the development process, and is the result of a collaboration between Drs. Ametaj and Wishart at the University of Alberta, Canada. The goal of the BMDB is to establish a comprehensive, freely available electronic database of all the detectable metabolites in dairy and beef cattle. The data in the database includes literature and experimentally derived information on bovine serum, bovine urine, bovine milk, bovine ruminal fluid, and bovine meat. Furthermore, the BMDB links each metabolite to a MetaboCard that contains specific information acquired from the literature and other databases such as tissue location, biofluid

location, NMR, and MS spectra, average concentrations and associated metabolic pathways [e.g., Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways] and metabolic enzymes. In the following link, the metabolite glycine (http://www.cowmetdb.ca/cgi-bin/show_metabolite.cgi?METABOCARD=BMDB00123) has been taken as an example to illustrate the type of information that is available.

Besides the HMDB and BMDB, there are also other databases frequently used by metabolomics scientists. In general, six types of databases have been developed for metabolomics studies. These include: (i) comprehensive, organism-specific metabolic databases [e.g., HMDB (Wishart et al., 2007, 2009, 2012), BiGG (Duarte et al., 2007), SYSTONOMAS (Choi et al., 2007), and MetaboLights (Haug et al., 2012)]; (ii) metabolomics pathway databases [e.g., SMPDB (Frolkis et al., 2010; Jewison et al., 2014), KEGG (Kanehisa et al., 2006), MetaCyc (Caspi et al., 2014), HumanCyc (Romero et al., 2004), BioCyc (Caspi et al., 2014), and Reactome (Joshi-Tope et al., 2005)]; (iii) compound or compound-specific databases [e.g., PubChem (Wheeler et al., 2007), ChEBI (Degtyarenko et al., 2008), ChemSpider (Pence and Williams, 2010), KEGG Glycan (Kanehisa et al., 2006), Lipid Maps (Fahy et al., 2007), and Toxin, Toxin Targeted Database (T3DB) (Wishart et al., 2015)]; (iv) spectral databases [e.g., BMRB (Ulrich et al., 2008), MassBank (Taguchi et al., 2007), MMCD (Cui et al., 2008), Golm Metabolome Database (Kopka et al., 2005), Metlin (Smith et al., 2005), Fiehn GC-MS Database (Kind et al., 2010), and mzCloud (https://www.mzcloud.org)]; (v) disease/physiology databases [e.g., METAGENE (Frauendienst-Egger et al., 1999), OMIM (Hamosh et al., 2005), and OMMBID

(http://ommbid.mhmedical.com)]; and (vi) drug databases [e.g., DrugBank (Wishart et al., 2006), Therapeutic Target DB (TTD) (Chen et al., 2002), PharmGKB (Whirl-Carrillo et al., 2012), STITCH (http://stitch.embl.de), SuperTarget (Günther et al., 2008)].

The lack of bioinformatic tools, especially software for improved automation have been widely recognized as the bottleneck in metabolomics research. In the past decade, a number of efforts have been made to accelerate or automate compound identification and/or quantification in NMR spectroscopy, GC-MS, and LC-MS. Both raw NMR and MS data require extensive data pre-processing. Major progress has been made in the past decade such that the ability to measure the peaks in global metabolomics datasets has now become routine with the introduction of metabolomic software such as MetaboAnalyst, MathDAMP, MetAlign, MZMine, and XCMS (Xia et al., 2012, 2015; Smith et al., 2006; Baran et al., 2006; Lommen et al., 2009; Katajamaa et al., 2005 & 2006; Tautenhahn et al., 2008).

Some commercial software packages, such as the Chenomx NMR Suite (http://www.chenomx.com/), KnowItAll (BioRad, Philadelphia, PA), AMIX (Bruker, Billerica, MA), provide standard spectral libraries and proprietary databases. Recently, a software package called BAYESIL was developed by Dr. Wishart's group at The Metabolomics Innovation Centre, University of Alberta. BAYESIL can quickly, accurately, and autonomously produce a metabolic profile given a 1D ^{1}H NMR spectrum of a complex biofluid (specifically serum or cerebrospinal fluid) (Ravanbakhsh, 2015). BAYESIL can automatically perform both spectral processing

and spectral profiling. GC-AutoFit (http://gcms.wishartlab.com/), also developed by Dr. Wishart's group, is a web application that automatically identifies and quantifies metabolites using GC-MS spectra.

The most widely used metabolomic software tools are MetaboAnalyst and SIMCA-P (www.umetrics.com; Xia et al., 2012, 2015). MetaboAnalyst is freely available over the web and allows users to upload data, perform data processing, and browse results within a web-based interface. MetaboAnalyst is a web-based suite for high-throughput metabolomic data analysis. GC/LC-MS and NMR raw spectra, peak lists, spectral bins (from NMR spectra), and concentration table can be uploaded and analyzed by MetaboAnalyst. After data processing, the raw data must be normalized (e.g., row-wise normalization, column-wise normalization). After normalization, up to seven different modules (i.e., statistical analysis, enrichment analysis, pathway analysis, time series analysis, power analysis, biomarker analysis, and integrated pathway analysis) are available for further data processing via MetaboAnalyst. For example, in the statistical analysis module, MetaboAnalyst supports both univariate [i.e., *t*-test, non-parametric tests (Wilcoxon rank-sum test), one-way analysis of variance (ANOVA)], and multivariate analyses [i.e., principal component analysis (PCA), partial least square - discriminant analysis (PLS-DA), and orthogonal partial least squares - discriminant analysis (OPLS-DA)], significant feature identification [i.e., significance analysis of microarray (and metabolites) (SAM), empirical Bayesian analysis of microarray (and metabolites) (EBAM)], cluster analysis [i.e., hierarchical clustering: dendrogram heatmaps; partitional clustering: K-means, self-organizing

map (SOM)], and classification and feature selection [i.e., random forest and support vector machine (SVM)]. Please refer to Xia et al. (2012, 2015) for more detailed information on the MetaboAnalyst web server.

1.3.3 Application of metabolomics in dairy cow diseases

Metabolomics has been employed as one of the most powerful approaches in identifying diagnostic biomarkers for the progression of various metabolic diseases or the evaluation of the health status in dairy cows. It enables comprehensive identification and quantification of metabolites in almost any easily accessible biofluid like blood, urine, and milk. Metabolomics enables predictive or diagnostic biomarker discovery that allows one to distinguish between diseases and non-diseased status (Nicholson and Lindon, 2008). However, very few studies have used more than one analytical technique in metabolomics research in dairy cows. Most metabolomics investigations conducted on dairy cows usually have utilized only one platform (either NMR or MS). Blood (i.e., serum or plasma), urine, and milk are the most commonly used body-fluids in metabolomics studies by dairy science researchers in biomarker studies and in understanding the etiology of periparturient disease in dairy cows. All of the three body fluids contain hundreds to thousands of detectable metabolites and can be obtained with minimal invasiveness.

1.3.4 Using blood metabolomics to screen and diagnose periparturient diseases in dairy cows

Blood (i.e., plasma or serum) contributes to maintaining a normal homeostasis in the body by constant regulatory mechanisms. As a result, metabolic fingerprinting of serum or plasma provides a global representation of an animal's immediate metabolic status (Gowda et al., 2008). Moreover, blood perfuses essentially all living cells in the body and thus it is anticipated to carry vital information about virtually all cell types. In this regard, a number of potential plasma biomarkers of ketosis including several AAs, carbohydrates, and fatty acids have been identified by GC-MS, LC-MS, and NMR (Li et al., 2014; Sun et al., 2014; Zhang, et al., 2013).

Metabolomics technologies have been applied by several investigators in the area of periparturient diseases of dairy cows by screening blood samples. GC-MS based blood metabolomics was applied to differentiate metabolic alterations in cows with CK, SCK, and healthy cows (Zhang et al., 2013). Several metabolic pathways including amino acid metabolism, fatty acid metabolism, gluconeogenesis, glycolysis, and the pentose phosphate pathway were identified to be associated with the development of CK and SCK. Several new plasma biomarkers were also reported to be of importance for the diagnosis and prognosis of ketosis (Zhang et al., 2013). In another recent study, NMR-based plasma metabolomics was used to distinguish cows with clinical and subclinical ketosis from healthy controls (Sun et al., 2014). In this study, Sun et al. (2014) demonstrated that 25 metabolites, including acetoacetate,

acetone, lactate, glucose, choline, glutamic acid, and glutamine were different among the 3 groups (i.e., CON, CK, and SCK). In another study of ketosis, LC-MS was applied and the authors (Li et al., 2014) reported that concentrations of valine (Val), glycine (Gly), glycocholic acid, tetradecenoic acid, and palmitoleic acid in plasma increased significantly in cows affected by CK. On the other hand, concentrations of arginine (Arg), aminobutyric acid, leucine (Leu) / isoleucine (Ile), tryptophan (Trp), creatinine, lysine (Lys), norcotinine, and undecanoic acid decreased markedly (Li et al., 2014). In another recent study performed by Xu et al. (2015b), different metabolic profiles were determined for type I ketosis and type II ketosis using ^{1}H-NMR and multivariate analyses. When compared with type II ketosis, cows affected by type I ketosis had greater levels of Ac, acetate, BHBA, Ile, Leu, low density lipoproteins (LDL), Val, and VLDL, and lower concentrations of α-glucose, β-glucose, citrate, creatine, formate, glutamine (Gln), glutamate (Glu), Gly, histidine (His), Lys, O-acetyl glycoprotein, phosphocholine (PC), phenylalanine (Phe), and tyrosine (Tyr) (Xu et al., 2015b). Results from this study seem to indicate the existence of a different pathobiology for type I and type II ketosis in dairy cows.

^{1}H-NMR spectroscopy has also been applied to characterize the serum profile of dairy cows with milk fever (Sun et al., 2014). Results from this study showed that 9 metabolites were distinct between cows with milk fever and control cows with alanine (Ala), gamma-aminobutyrate, glucose, glycerol, and phosphocreatine decreased, and acetone, BHBA, Lys, and pyruvate elevated in the serum of cows affected by milk fever. Most of these alterations were related to carbohydrate and amino acid

networks involved in energy metabolism pathways. Başoğlu et al., (2014) used metabolomics in identifying diagnostic plasma biomarkers in dairy cows affected by displaced abomasum. They reported that plasma levels of BHBA, Gln, and Glu discriminated cows with displaced abomasum from the healthy ones.

Hepatic steatosis (or fatty liver) has also been studied by metabolomics using plasma screening. Imhasly et al. (2014) applied metabolomics in serum of cows affected by fatty liver to identify potential plasma biomarkers of the disease. Twenty-nine metabolites such as AAs, phosphatidylcholines, and sphingomyelin were able to discriminate between healthy dairy cows and those showing different phases of fatty liver. Another serum based metabolomics study (De Buck et al., 2014) was conducted on the early diagnosis of Johne's disease in dairy calves experimentally infected with *Mycobacterium avium subsp. paratuberculosis* (MAP). They used ^{1}H NMR spectrometry and their results indicated significantly metabolic differences in the serum between the healthy and sick calves. This information could be used in the future to perform early diagnosis of infected animals (De Buck et al., 2014).

In a recent publication, our group reported a 3-metabolite plasma biomarker profile (carnitine, propionyl carnitine, and lysophosphatidylcholine acyl C14:0) that could be used to screen dairy cows for the risk of developing periparturient disease, up to 4-6 wks before the occurrence of clinical disease (Hailemariam et al., 2014a). Results from the same study also provided evidence of major alterations in amino acid and sphingolipid plasma profiles in sick cows starting at -4 wks before parturition and continuing during the whole transition period up to +4 wks after calving. These kinds

of results are helpful to better understand periparturient disease pathogenesis and progression during the transition period (Hailemariam et al., 2014b).

1.3.5 Using urine metabolomics to screen and diagnose periparturient diseases in dairy cows

In comparison with other biofluids, urine specimens have a distinct advantage because the collection of samples is non-invasive. Furthermore, urine has relatively low levels of large molecules (e.g., proteins or lipids), and high levels of low molecular weight compounds (i.e., metabolites) minimizing the time for sample preparation and resulting in high quality measurements (Gowda et al., 2008). There are very few urine based metabolomics studies that report biomarkers for dairy cow diseases. It has been reported that metabolic fingerprinting of human urine can be used as an effective approach to diagnose various diseases and to monitor an individual's general health status (Emwas et al., 2015). The same can be done for livestock animals and dairy cattle.

1.3.6 Using milk metabolomics to diagnose diseases and evaluate milk quality

Several milk based metabolomics approaches have been used to investigate diseases in dairy cows and to evaluate milk quality. NMR metabolomics analysis of milk in dairy cows revealed that the milk glycerophosphocholine (GPC)/PC ratio could be used as a biomarker for the risk of ketosis (Klein et al., 2012). In another study, associations between milk metabolites and SCC in bovine milk was studied by

NMR based metabolomics (Sundekilde et al., 2013). Results of this study demonstrated that milk concentrations of acetate, BHBA, lactate, butyrate, and Ile were increased, whereas hippurate and fumarate were decreased in milk with high levels of somatic cells. Fluctuations of milk composition during clinical mastitis have been reported as promising diagnostic biomarkers of the disease (Mansour et al., 2012). Data from this study reported that altered milk metabolites are mainly related to carbohydrate, protein, and lipid pathways. With respects to lipid metabolic pathways, there was an increase in milk levels of arachidonic acid metabolites. Among AAs, Arg metabolites were elevated during mastitis, whereas among carbohydrates, concentrations of galactose metabolites were greater in milk of cows with mastitis caused by *E. coli* and *S. aureus*.

^{1}H-NMR analysis of milk samples has been used to monitor metabolic fluctuation over different lactation stages in dairy cows. It was demonstrated that markers (e.g., Ac, BHBA, and citrate) of energy metabolism were increased during early lactation, then diminished over the first 70 days, before stabilizing, suggesting cows experience energy imbalance at initiation of lactation (Klein et al., 2010). Moreover, MS and NMR based metabolomics revealed changes of metabolic variables in the milk during different lactation stages. The authors demonstrated a strong correlation between biomarkers (i.e., acetone and BHBA) and the metabolic status of individual cows during early lactation (Klein et al., 2010).

A GC-MS based metabolomics study was used to distinguish goat milk, cow milk, and ultra-heat-treated (UHT or pasteurized) milk (Scano et al., 2014). Results

from this study showed that Gly and Val were specific to goat milk, malic acid and talose were unique in cow's milk, and hydroxyglutaric acid was identified in pasteurized samples. Two metabolites (i.e., glucose and fructose) were shared by pasteurized samples and cow milk, whereas ribose was shared by UHT-treated samples and goat milk (Scano et al., 2014). By NMR spectroscopy and LC-MS spectrometry, Yang et al. (2016) showed differential metabolites such as choline and succinic acid in milk between Holstein and other studied animals (i.e., Jersey, yak, buffalo, goat, camel, and horse). Alterations in milk metabolomes during the first month of lactation in dairy cows were investigated by Ilves et al. (2012), and by combining MS and multivariate analysis it was revealed that phosphorylated saccharide levels accounted for most of the fluctuated variables in milk during the first months of lactation.

Metabolomics has also been used for evaluating milk quality in dairy cows. The use of NMR-based metabolomics allowed the identification of novel associations between the metabolite profile and rennet-induced coagulation properties of bovine milk (Sundekilde et al., 2014). Metabolites associated with the prediction of protein content included choline, *N*-acetyl hexosamines, creatinine, GPC, Glu, glucose 1-phosphate (Glu-1P), galactose 1-phosphate (Gal-1P), and orotate. These compounds could also be used as indicators for milk quality (Sundekilde et al., 2014).

1.3.7 Using rumen fluid metabolomics to monitor subacute rumen acidosis

The relationship between rumen metabolism and host health status is widely

accepted, and metabolomics studies on the rumen metabolome can help identify biomarkers for a number of diseases such as sub-acute ruminal acidosis (SARA) in dairy cows (Ametaj et al., 2010). A recent study by Zhao et al., (2014) on the rumen fluid metabolome via ^{1}H-NMR spectroscopy and multivariate analysis identified alterations in the ruminal microbial metabolite profile, especially with regard to biogenic amines, amino acids, and organic acids, that correlated with feeding upon different types of roughage. Three articles published by our group indicate that feeding between 30-40% barley grain in the diet results in decreased rumen pH (i.e., < 5.8 for 6-12 h), and elevated concentrations of butyrate, dimethylamine (DMA), ethanol, endotoxin, fumarate, glycerol, methylamine, Leu, Lys, *N*-nitrosodimethylamine, nicotinate, Phe, and Val in the rumen fluid (Ametaj et al., 2010; Saleem et al., 2012, 2013).

1.4 Proposed etiopathology of ketosis in dairy cows

1.4.1 Importance of dairy cow management during the dry-off period

The dry period, an anatomically and physiologically challenging time for the cow, is a vital phase in the lactation cycle of dairy cows (Dingwell et al., 2001). It is a period of metabolic, nutritional, and mammary change and an adjustment that profoundly impacts the health status (i.e., incidence of disease), reproductivity, and productivity in the next lactation. During the transition from dry-off to early lactation, rumen papillae and microflora must adapt to the newly introduced high concentrate diet. Moreover, the mammary gland undergoes drastic gross and cellular changes at

both milk cessation at the beginning of the dry-off and initiation of milking after parturition. Immediately following milk cessation, there is a major engorgement of the cisternal spaces, ducts and alveoli of the gland, which might create conditions for exogenous factors to infect the gland (Dingwell et al., 2001).

Dairy cows are highly susceptible to udder infections during the dry-off period. Todhunter et al. (1991) demonstrated that the incidence of bovine intra-mammary infections (IMI) during the dry-off period is almost 4-fold greater than during lactation. It is essential to rigorously apply all the drying protocols to prevent infections during the dry-off period.

1.4.2 Involvement of systemic inflammatory insults in the development of ketosis

Several epidemiological studies have shown an association between ketosis and increased susceptibility to infectious diseases like mastitis and metritis (Erb and GroÈhn, 1988). Ametaj et al. (2005a) suggested that there might be a role of immune factors that trigger systemic inflammation during transition period in the pathobiology of metabolic disorders. For example, transition dairy cows with fatty liver (a concurrent disease with type II ketosis) were found to have greater concentrations of two APPs (i.e., Hp and SAA) in the plasma before and after calving, which further supports the assumption that metabolic disorders are associated with subclinical inflammation (Ametaj, 2005b).

Three questions need to be answered with regard to potential inflammatory insults in the etiopathology of metabolic disorders, in general and ketosis, in particular:

1) is there an inflammatory response preceding ketosis?; 2) what is the source of systemic inflammation?; and 3) how does the systemic inflammatory insult contribute to development of ketosis? The hypoglycemic hypothesis of ketosis proposes that the major changes in the blood of ketotic cows include increased concentrations of ketone bodies and fatty acids, and decreased levels of glucose, insulin, free and esterified cholesterol, and phospholipids (Drackley, 1989). Moreover, this hypothesis states that alterations of glucose (e.g., gluconeogenesis, glycolysis, and the TCA cycle) and lipid metabolism (e.g., fatty acid β-oxidation) are associated with the development of ketosis. Therefore, it is of importance to specifically investigate changes of glucose precursors like propionate, gluconeogenic AAs, lactate, and glycerol necessary for gluconeogenesis, intermediates and associated enzymes of the TCA cycle and glycolysis, and triggers for mobilization of fatty acids from adipose tissue in cows with ketosis.

Research conducted by our group reported that cows fed typical early lactation diets trigger a large increase in the concentration of endotoxin [also called lipopolysaccharide (LPS)], in the rumen fluid. This increase correlated with alterations of plasma metabolites related to lipid and carbohydrate metabolism (Zebeli et al., 2011). The effects of LPS on lipid metabolism also have been previously reported in lactating rats (López-Soriano and Williamson, 1994). Feingold et al. (1992) showed that LPS induces alterations in lipid metabolism, which subsequently produce hypertriglyceridemia. Elevated concentrations of blood ketones like BHBA might be related to mobilization of lipids and hypertriglyceridemia during a state of endotoxic

insult. In support of our assumption are data from Kremer et al. (1993) that showed greater blood BHBA and strong correlation between the severity of *E. coli* mastitis and circulatory BHBA. Moreover, infection of the uterine epithelium (i.e., endometritis) postpartum was also shown to be associated with greater concentrations of BHBA in the plasma (Yasui et al., 2014).

It has been reported that ketone bodies, in particular BHBA, are able to suppress non-specific immunity by decreasing the efficiency of chemotaxis and phagocytosis in neutrophils of ketotic ruminants (Sartorelli et al., 1999). Recently, Youm et al. (2015) discovered that ketone body BHBA can regulate the innate immune response by blocking NLRP3 inflammasome, which is a newly identified pattern-recognition receptor (PRR) known as the "pyrin domain-containing protein 3" and belongs to the family of nucleotide-binding and oligomerization domain-like receptors (NLRs) (Inoue et al., 2012; Shao et al., 2015). In addition, BHBA can also inhibit processing of IL-1β in response to the toll-like receptor (TLR)-4 pathogen associated molecular pattern (PAMP) agonist LPS, and the TLR-2 agonist lipoteichoic acid (LTA) (Youm et al., 2015). Therefore, elevated ketone bodies during 'ketotic syndrome' might be associated with the activation of the innate immune response and, in particular, BHBA might serve as a metabolite to quell inflammatory response and alleviate the development of disease. The source of activation of the innate immunity response might be related to infections during the dry off period. The current study (described in this thesis) was not intended to identify the source(s) of inflammatory insults or infections. Rather, we aimed to quantify all detectable metabolites and identify

potential alterations, which might be associated with the pathobiology of ketosis.

1.4.3 Protein mobilization, lipid metabolism, and gluconeogenesis

Ketosis is a metabolic disease of dairy cows characterized by increased concentrations of ketone bodies in the blood during the transition period, which is attributed to a state of NEB. Tóthová et al. (2014) demonstrated that metabolic changes associated with NEB and lipid metabolism might be related to perturbations in immune functions and protein metabolism. It was estimated that the production of glucose from fermentation of carbohydrates from the diet may fall short of glucose demands by as much as 500 g/d during the first week after parturition (Drackley et al., 2001). Glucogenic amino acids from muscle protein mobilization and glycerol from body fat mobilization likely contribute to making up this shortfall (Overton et al., 1998; van der Drift et al., 2012; van Knegsel, et al., 2005).

Perturbations in lipid metabolism and fatty acid transport in cows with ketosis have been previously determined by gene expression studies. A microarray and qPCR based transcriptomics study provided evidence for changes in specific lipid catabolism-related genes in the liver of periparturient dairy cows with nutrition-induced ketosis (Loor et al., 2007). Fluctuations of metabolites involved in fatty acid metabolism have been reported by several metabolomics studies (Zhang et al., 2013). For example, Zhang et al. (2013) reported that several metabolites (e.g., palmitic acid, heptadecanoic acid, stearic acid, trans-9-octadecenoic acid, myristic acid, and cis-9-hexadecenoic acid from the family of NEFA were elevated in ketotic

cows, which confirmed that excessive lipolysis during hypoglycemia leads to ketosis.

It has also been reported that gluconeogenesis, glycolysis, the TCA cycle and the pentose phosphate pathway were altered during ketosis (Xu et al., 2008). Given the evidence that cows with ketosis experienced fluctuations of intermediates involved in the TCA cycle, glycolysis, and gluconeogenesis, more studies are warranted to complete metabolic profiling of the full picture of energy metabolism to reveal the etiopathology of the disease.

1.4.4 Understanding ketosis at the level of systems biology

In the past century, most veterinary scientists have been using the reductionist approach to investigate and interpret the etiopathology of periparturient diseases in dairy cows. For example, the blood concentration of BHBA has been suggested and used as the gold standard for diagnosing ketosis. Is BHBA the only altered variable during the state of ketosis? Can metabolism of BHBA explain all that is happening in cows affected by ketosis? Recent published studies have shown that the number of metabolites perturbed in the blood or milk and metabolic pathways involved during ketosis is quite diverse and complex (Klein et al., 2012; Li et al., 2014; Sun et al., 2014; Zhang et al., 2013). The limitations and drawbacks of the reductionist approach are becoming increasingly apparent and a new approach at the level of systems biology is warranted to better understand the pathobiology of ketosis and to develop screening and prognostic biomarkers of this disease

1.5 Research hypotheses and objectives

Overall it is clear that relatively few investigations have been conducted with regard to the pathobiology of ketosis. Most studies have been focused on methodologies of how to diagnose the incident rate of SCK or CK and the impact of ketosis on other diseases and dairy farm economics. It would be of interest to evaluate whether there are triggering factors for ketosis already present during the dry off period and whether the disease process starts earlier than previously reported. Therefore, the overall hypothesis of this study was that by using NMR and MS based metabolomics technologies and ELISA based immuno-assays, we could identify metabolite fingerprints or protein biomarkers that could be used for screening cows at risk of ketosis, as well as biomarkers offering better and more accurate diagnosis of the disease, and prognosis of treatment for ketosis in dairy cows.

Therefore the objectives of this study were to:

1) Identify alterations in innate immunity reactants as well as carbohydrate and lipid metabolites in the blood of periparturient dairy cows with ketosis starting at -8 wks before the expected day of parturition;
2) Identify potential early screening blood metabolite biomarkers for transition dairy cows starting at -8 wks before parturition affected by ketosis through the use of metabolomics technologies;
3) Identify potential urinary metabolite biomarkers in cows affected by ketosis around calving starting at -8 wks before the expected day of parturition by

using metabolomics technologies including NMR, GC-MS, DI/LC-MS/MS, and ICP-MS.

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Chapter 2 Dairy cows affected by ketosis show alterations in innate immunity and lipid and carbohydrate metabolism during the dry off period and postpartum[1]

ABSTRACT

The objective of this investigation was to search for alterations in blood variables related to innate immunity and carbohydrate and lipid metabolism during the transition period in cows affected by ketosis. One hundred multiparous Holstein dairy cows were involved in the study. Blood samples were collected at -8, -4, weeks of disease diagnosis (+1 to +3 weeks), and +4 weeks relative to parturition from 6 healthy cows (CON) and 6 cows with ketosis and were analyzed for serum variables. Results showed that cows with ketosis had greater concentrations of serum β-hydroxybutyric acid (BHBA), interleukin (IL)-6, tumor necrosis factor (TNF), serum amyloid A (SAA), and lactate in comparison with the CON animals. Serum concentrations of BHBA, IL-6, TNF, and lactate were greater starting at -8 and -4 weeks prior to parturition in cows with ketosis vs those of CON group. Cows with ketosis also had lower DMI and milk production vs CON cows. Milk fat also was lower in ketotic cows at diagnosis of disease. Cows affected by ketosis showed an activated innate immunity and altered carbohydrate and lipid metabolism several

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weeks prior to diagnosis of disease. Serum IL-6 and lactate were the strongest discriminators between ketosis cows and CON ones before the occurrence of ketosis, which might be useful as predictive biomarkers of the disease state.

2.1 Introduction

Ketosis is a common metabolic disorder of transition dairy cows during the early lactation period. It is important to note that ketosis first appears in a subclinical form, known as subclinical ketosis (SCK). In a smaller number of cows the disease progresses to a clinical form known as clinical ketosis (CK). Subclinical ketosis (SCK) is defined as an increase of ketone bodies (i.e. β-hydroxybutyric acid (BHBA), acetoacetate, and acetone) in the blood, urine, or milk, in the absence of obvious clinical symptoms. Almost 40% of dairy cows in North America have different degrees of SCK within a few weeks after calving, with the incidence varying widely and reaching as high as 80%, in some dairy herds (Duffield, 2000). On the other hand clinical ketosis (CK) is characterized by excess ketone bodies in blood, urine and milk, lack of appetite, lower milk production, rapid weight loss, and dry manure (Gordon et al., 2013a). The incidence of CK ranges between 2 to 15% in the first month of lactation (Gordon et al., 2013a).

The “gold standard” test for diagnosis of ketosis has been blood BHBA, which is the predominant circulating ketone body in ruminants and more stable in blood than the other two ketones, acetoacetate and acetone (Työppönen and Kauppinen, 1980). The generally used cut-off value for diagnosis of SCK is ≥ 1,200 and up to 1,400

μmol/L of blood BHBA (Suthar et al., 2013). Clinical ketosis is generally characterized by concentrations of BHBA in the blood greater than 3,000 μmol/L (Oetzel, 2007). Most dairy producers and veterinary practitioners focus more on diagnosis of ketosis and treatment of disease during the postpartum period, mostly disregarding the prepartum period.

Although much is known about the pathobiology of ketosis there is still a need to better understand both the etiology and pathogenesis of the disease. Ketosis has been correlated with a state of negative energy balance (NEB), which results from increased energy demand for milk production, especially during peak lactation (3-6 weeks postpartum) and relatively inadequate feed intake during this period of time (Çağdaş, 2013). All cows commonly undergo a period of NEB during peak lactation and excessive body fat mobilization, but not all animals experience hyperketonemia, and even less develop CK (1.5-4.0% of the herd) (Duffiled et al., 1998; Oetzel, 2007). Mobilization of fatty acids from adipose is accompanied by elevated concentrations of non-esterified fatty acids (NEFA) in the blood circulation. During the state of NEB, gluconeogenesis is enhanced and a large portion of blood NEFA is converted into ketone bodies, as a different form of energy, in the liver hepatocytes (Gordon, 2013b). It was reported that it is not NEB itself, but inadequate metabolic adaptation that contributes to the development of ketosis (Herdt, 2000).

Several epidemiological studies have shown an association between ketosis and increased susceptibility to infectious diseases like mastitis and metritis (Erb and GroÈhn, 1988). Pro-inflammatory cytokines play a pivotal role in activating systemic

inflammatory responses and acute phase proteins (APP) have been used as non-specific biomarkers of inflammation (Petersen et al., 2004; Dantzer and Kelley, 2007). In this study, we hypothesized that alterations in innate immunity reactants, as well as carbohydrate and lipid metabolism in the blood precede occurrence of ketosis. Therefore, the objectives of the current research were to evaluate perturbations of blood metabolites related to innate immunity as well as carbohydrate and lipid metabolism starting at -8 and -4 weeks prepartum. Specifically, concentrations of three serum metabolites: BHBA, non-esterified fatty acids (NEFA), and lactate; three major pro-inflammatory cytokines: interleukin-1 (IL-1); interleukin-6 (IL-6), and tumor necrosis factor (TNF); and two APP: haptoglobin (Hp) and serum amyloid A (SAA) were quantified at -8 and -4 weeks before parturition and during the week of disease diagnosis (+1 to +3 weeks) as well as at +4 weeks after calving.

2.2 Materials and methods

2.2.1 Animals and diets

One hundred pregnant Holstein dairy cows at the Dairy Research and Technology Centre, University of Alberta (Edmonton, AB, Canada) were screened and sampled in this study. All cows (i.e., 100 cows) were urine-tested with Ketostix strips (Bayer Corporation, Elkhart, IN) for the presence of ketone bodies (i.e., urine acetoacetate) on weekly basis from -8 weeks prepartum to +8 weeks postpartum. Diagnosis of ketosis was based on the measurement of serum BHBA by Microplate reader kits (Sigma, St. Louis, MO, USA) at -8, -4, +1, +2, +3, and +4 weeks around

calving, in all 100 cows. However, only 12 cows were selected and used in the study. Six pregnant multiparous (parity: ketosis 3.7 ± 0.8 vs healthy cows (CON) 3.0 ± 0.9; P = 0.58) cows were diagnosed postpartum with ketosis by urine Ketostix strips (Bayer Corporation, Elkhart, IN) and confirmed by a colorimetric method (i.e. serum BHBA ≥ 1,400 μmol/L, kit (Ref. No. 2440-058) provided by Sigma, St. Louis, MO, USA) in the lab. All 6 cows with ketosis used in this study showed Ketostix test score greater than moderate (3,920 μmoL of AcAc/L) and serum BHBA greater than 1,400 μmoL/L. All cows from the CON group had Ketostix test score lower than small (1,470 μmol of AcAc/L) and serum BHBA lower than 1,400 μmoL/L. Cows that had two or more diseases simultaneously (ketosis and another disease) were excluded from the ketotic group. Six clinically healthy cows that were homologous in age, parity, and body condition score (BCS at around 3 after calving) with the other 6 cows diagnosed with ketosis were selected as the control (CON) group. All cows were clinically healthy before parturition. 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 (2009).

The experimental period lasted for 17 weeks from -8 weeks prepartum to +8 weeks postpartum (i.e. -8 to +8 weeks, 0 week means the week of parturition) for each cow. Dry matter intake (DMI) was calculated based on data collected from -8 to +8 weeks. Milk production was calculated from +1 to +8 weeks. Serum variables were analyzed from -8 weeks to +4 weeks. Milk compositions were determined on +2, +3,

+5, and +7 weeks relative to parturition. All cows were offered a total mixed ration (TMR) during the experimental period (Table 2-1 and Table 2-2). Feed was provided for ad libitum intake once daily at 0800 to allow approximately 5% orts (feedstuff not consumed). All TMR were formulated to meet or exceed the nutrient requirements of dry and early lactation 680 kg lactating cows as per National Research Council guidelines (2001). Cows were housed in individual tie stalls (48 inches wide and 6 feet long) bedded with sawdust and with free access to water throughout the 17 weeks of the experimental period. Cows were transferred to an adjoining maternity pen one week prior to calving and returned to their stalls on the following day of calving. Individual DMI was recorded daily throughout the sampling period. Since the onset day of lactation, cows were milked in their stalls twice (at 0500 and 1600) per day, and individual milk yield was recorded electronically. Milk components including milk fat, crude protein (CP), somatic cell count (SCC), lactose, milk urea nitrogen (MUN), and total solids (TS) were analyzed by mid-infrared spectroscopy (MilkoScan 605; A/S Foss Electric, Hillerød, Denmark) at the Central Milk Testing Laboratory, Edmonton, Alberta.

2.2.2 Monitoring the health status of the cows

All cows were monitored daily starting at -8 weeks before the expected date of parturition and continuing up to +8 weeks postpartum for potential health disorders based on clinical signs of diseases by the veterinary technician and trained staff members. Clinical symptoms observed included general appearance, appetite,

alertness, rectal temperature, ease of calving, retained placenta, vaginal discharges (color and consistency), udder edema, gait and pain in the legs. All disease information and veterinary treatments were recorded for each cow throughout the experimental period. Pregnancy check was performed routinely at around 60 to 70 days after insemination by a veterinarian. The expected date of parturition was determined by adding 280 days from the day of artificial insemination and it was also supported by the information of pregnancy diagnosis.

In this study, diagnosis of ketosis was established by a veterinary technician based on Ketostix strip detecting urinary acetoacetate (AcAc) with cut-off points of "trace" (490 μmol of AcAc/L), "small" (1,470 μmol of AcAc/L), "moderate" (3,920 μmol of AcAc/L), "large" (7,840 μmol of AcAc/L), and "very large" (15,700 μmol of AcAc/L) as well as clinical signs (i.e. reduced DMI and milk yield, decreased rumen motility, and ketone odor on the cow's breath) and response to treatment according to the farm standard operating procedure. The disease was further confirmed by testing concentrations of serum BHBA in the lab. If the Ketostix test score was between small and moderate, 250 mL of propylene glycol (Sigma-Aldrich, Oakville, ON) was applied orally twice daily for 3 consecutive days, and then 250 mL once daily for another 3 days. Cows that had obviously depressed appetite were treated orally with approximately 20 L of warm water with 4 kg of bubba mix (Champion Feed Services Ltd., Westlock, AB, Canada) and 250 mL propylene glycol (Alberta Veterinary Laboratories Ltd., Calgary, AB, Canada). If the Ketostix score was greater than large, besides the propylene glycol treatment, the cow was treated i.v. with 500 mL of 50%

dextrose (Sigma-Aldrich, Oakville, ON) solution and 10 mL Flucort (Flumethasone, Zoetis Canada, Kirkland, QC) for 2 consecutive days. All cows affected by ketosis recovered after the treatments.

2.2.3 Sample collection

Blood samples were obtained from the coccygeal vein of all cows (i.e., 100 cows) at 0700 shortly before feeding, at -8 and -4 weeks before the expected day of calving and at week of disease diagnosis and +4 weeks after parturition. All blood samples were collected into 10-mL vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to coagulate and kept at 4 °C until separation of serum. Clotted blood was centrifuged at 2,090 x *g* at 4 °C for 20 min (Rotanta 460 R centrifuge, Hettich Zentrifugan, Tuttlingen, Germany). The supernatant (i.e., serum) was aspirated gradually by transfer pipets (Fisher Scientific, Toronto, ON, Canada) and transferred to sterile 10-mL plastic test tubes (Fisher Scientific, Toronto, ON, Canada). Serum samples were stored at -80 °C until analyses to avoid loss of bioactivity and contamination and were thawed on ice for approximately 2 h before use.

Cows were milked twice per day at 0500 and 1600, and those milk samples collected on days 14, 21, 35, and 49 relative to parturition were used for the analyses of milk composition including CP, milk fat, lactose, SCC, MUN, and TS.

2.2.4 Sample analysis

Serum metabolites. Serum lactate, BHBA, and NEFA were assayed by

commercially available kits provided by Stanbio Laboratory (Boerne, TX, USA), Sigma (St. Louis, MO, USA), and Wako Chemicals (Richmond, VA, USA), respectively, using similar methods as described previously by Ametaj et al. (2009). Each sample was measured in duplicate in microtiter plates according to the manufacturers' instructions. The principles of these three kits have been described in detail previously by Ametaj et al. (2009). Sensitivities of the three assays were 0.06 mg/dL, 0.125 μmol/L, and 0.50 μEq/L, respectively. The intra-assay coefficients of variation (CV) were ≤ 10%. The resulting color reactions for lactate, BHBA, and NEFA were read at 492, 505, and 550 nm, respectively, in a microplate reader (Spectramax 190, Molecular Devices Corporation, EI Cajon, CA, USA).

Serum cytokines. Concentrations of serum interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) were determined in duplicate by bovine enzyme-linked immunosorbent assay (ELISA) kits supplied by Cusabio Biotech Co., Ltd. (Wuhan, Hubei, China), Uscnk Life Science Inc. (Wuhan, Hubei, China), and Bethyl Laboratories, Inc. (Montgomery, TX, USA), respectively. The basic principles of these three kits were reported in our previous article (Zhang et al., 2015). Briefly, all the optical density values were read at 450 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, CA, USA) within 10 min after adding the stop solutions. The detection limits of the three kits were 250 pg/mL, 7.8 pg/mL and 78 pg/mL, respectively. The intra-assay variations of all assays were controlled by CV limits of ≤ 10%.

Serum acute phase proteins (APP). Assays used for measurement of

concentrations of serum Hp (Tridelta Development Ltd., Co.Kildare, Ireland) and SAA (Tridelta Development Ltd., Kildare, Ireland) by ELISA kits were described previously in detail (Emmanuel et al., 2008). In brief, samples were not diluted for the measurement of serum Hp. Samples for SAA analyses were initially diluted 1:500. All samples were tested in duplicate and the optical densities were measured at 600 nm for Hp and 450 nm for SAA by a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, CA, USA). The minimum detectable concentrations for the Hp and SAA assays were 2.5 mg/mL and 18.8 ng/mL, respectively. The intra-assay CV were maintained at ≤ 10%, for all the assays.

2.2.5 Statistical analyses

Analysis of univariate data was conducted using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.2) according to the following model:

$$Y_{ijk} = \mu + S_i + W_j + (SW)_{ij} + e_{ijk}$$

where Y_{ijk} is the observations for dependent variables, μ represents the population mean, S_i is the fixed effect of health status i (i = 1-2, sick cows compared with healthy controls separately), W_j is the fixed effect of measurement week j (j = 1-4, 1-8 or 1-17), SW_{ij} = fixed effect of health status by week interaction, and e_{ijk} is the residual error.

Measurements taken at different weeks on the same cow were considered as repeated measures in the ANOVA. The normality and homoscedasticity for residuals of two groups for each variable were tested and residuals followed normal distribution

and equality of variance. The variance–covariance structure of the repeated measures was modeled separately for each response variable according to the lowest values of the fit statistics based on the Bayesian Information Criteria and an appropriate structure was fitted. Degrees of freedom were approximated by the method of Kenward-Roger (ddfm = kr).

In order to identify early indicators of ketosis, average serum concentrations during the week of disease diagnosis, at -8 and -4 weeks prior to the expected day of parturition were compared using the T-test of SAS 9.2 between healthy controls and cows with ketosis. Data were assessed for normality of distributions using the univariate procedure of SAS. Data are shown as least-squares means (LSM) and the respective standard error of the mean (SEM). All statistical tests were two-sided. Significance was declared at $P \leq 0.05$, and tendency was defined at $0.05 < P < 0.10$.

Multivariate analysis including unsupervised principal component analysis (PCA) and supervised projection to partial least squares - discriminant analysis (PLS-DA) were performed using MetaboAnalyst (Xia et al., 2009). Missing values were replaced by the mean of each variable. Recommended statistical procedures for PCA and PLS-DA were followed according to previously published protocols (Xia et al., 2009). To perform a standard cross-sectional 2-group study, we compared the group of CON cows and those of ketosis cows at -8, -4, week of disease diagnosis, and +4 weeks, separately. In the PLS-DA model, a variable importance in projection (VIP) plot was used to rank the variables based on their importance in discriminating ketosis group from the CON group of cows. Variables with the highest VIP values are the most

powerful group discriminators. Typically, VIP values > 1 are significant and VIP values > 2 are highly significant.

Biomarker profiles and the quality of the biomarker sets were determined using receiver-operator characteristic (ROC) curves as calculated by MetaboAnalyst 3.0 (Xia et al., 2015). Permutation test was conducted for each ROC curve at different time points, and an empirical *P*-value < 0.05 for four ROC curves with 1000 permutations, was attained. Paired sensitivity and false-positive ratios (1-specificity) at different classification decision boundaries were calculated. A ROC curve is plotted with sensitivity values on the Y-axis and the corresponding false-positive rates (1-specificity) on the X-axis. ROC curves are often summarized into a single metric known as the area under the curve (AUC), which indicates the accuracy of a test for correctly distinguishing one group such as ketotic cows from CON ones. If all positive samples are ranked before negative ones, the AUC is 1.0, which indicates a perfectly discriminating test. The 95% confidence interval (CI) and *P* values were calculated. A rough guide for assessing the utility of a biomarker set based on its AUC 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.

To analyze correlations between milk SCC tested at disease weeks and serum variables in specimens collected at the same time and -8, -4, and +4 weeks the CORR procedure of SAS 9.2 was conducted, based on a two-tailed test to calculate the Pearson correlation coefficient and corresponding *P*-values.

2.3 Results

2.3.1 Serum metabolites

Cows with ketosis (1,014 ± 140 μmol/L; Table 2-3) had an overall greater concentration of BHBA in their serum when compared with the CON cows (504 ± 140 μmol/L; $P = 0.04$; Table 2-3) during the whole experimental period. Furthermore, there was an effect of the factor sampling week (Wk) on serum BHBA in the present study ($P < 0.01$; Table 2-3). Concentration of BHBA in the serum increased in both ketotic and CON cows after parturition and reached the peak (1,756 ± 178 μmol/L and 690 ± 80 μmol/L, respectively; $P < 0.01$; Table 2-4) during 1-3 weeks postpartum. In addition, a tendency ($P = 0.08$; Table 2-3) of health status (Hs) X Wk interaction was observed for serum BHBA. Interestingly, serum BHBA in cows with ketosis was greater than CON cows at -4 weeks ($P = 0.02$; Table 2-4) before parturition, and this trend lasted until +4 weeks after parturition.

The concentration of NEFA in the serum did not differ between the two experimental groups (Table 2-3). However, the factor sampling week showed an effect on the concentration of NEFA ($P < 0.01$). Both groups had elevated concentrations of NEFA in the serum and reached a peak during the disease diagnosis week (Table 2-4). No interaction between Hs and Wk on serum NEFA was obtained in this study.

The concentration of lactate in the serum was affected by Hs ($P < 0.01$; Table 2-3). Cows with ketosis (4,236 ± 351 μmol/L) had a greater concentration of lactate in

the serum compared with cows in the CON group (2,240 ± 351 µmol/L). There was no effect of sampling Wk or interaction between Hs and Wk on serum concentration of lactate (Table 2-3). Interestingly, the concentration of serum lactate tended to be higher in ketosis affected cows during the diagnosis week (P = 0.08; Table 2-4), and distinctions of serum lactate between ketosis cows and CON cows were even greater at -4 weeks (4,478 ± 822 µmol/L and 2,162 ± 185 µmol/L, respectively; P = 0.01; Table 2-4) and -8 weeks (5,795 ± 369 µmol/L and 2,455 ± 349 µmol/L, respectively; P = 0.01; Table 2-4) prior to parturition.

2.3.2 Cytokines

Cows with ketosis had greater concentrations of IL-6 in the serum throughout the experimental period versus CON cows (around 10-fold; 255 vs. 27 pg/mL; P = 0.03; Table 2-3). The pronounced distinction of IL-6 was not only evident during the disease week (116 vs. 23 pg/mL; P < 0.01; Table 2-4), but also started at -4 weeks (330 vs. 48 pg/mL; P < 0.01; Table 2-4) and even -8 weeks (183 vs. 19 pg/mL; P < 0.01; Table 2-4) prepartum. Serum IL-6 was still greater in cows with ketosis after recovery until +4 weeks after parturition. There was no effect of the factor Wk alone or the Hs x Wk interaction with respect to serum concentration of IL-6.

The group of ketotic cows had greater overall concentrations of TNF (0.47 ± 0.07 ng/mL) in the serum compared with those of CON cows (0.19 ± 0.07 ng/mL; P = 0.03; Table 2-3) at the 4 tested time points. There was an effect of the Wk of sampling (P < 0.01; Table 2-3) on serum concentration of TNF for the two groups, whereas no

such an effect was evident for the interaction of Hs x Wk (P = 0.49; Table 2-3). Concentrations of TNF in the serum postpartum in both groups were lower compared with prepartum concentrations.

The overall concentrations of IL-1 in the serum were similar between the two groups of cows (P = 0.74; Table 2-3). However, an effect of factor Wk (P < 0.01; Table 2-3) and an interaction between Hs x Wk (P = 0.03; Table 2-3) on concentration of IL-1 in the serum, were obtained. Results indicated a decrease in serum IL-1 in both groups of cows during the week of disease diagnosis and this trend continued and reached at nadir at +4 weeks after parturition. There was a tendency for greater IL-1 in ketotic cows (P = 0.10; Table 2-4) compared with the CON ones during the disease week, while concentrations of serum IL-1 were lower at -4 weeks prepartum (P = 0.02; Table 2-4) in cows with ketosis versus the group of CON cows.

2.3.3 Acute phase proteins

The ANOVA revealed that the overall concentrations of Hp in the serum were not affected by Hs (P = 0.66; Table 2-3). However, there were effects of both Wk (P < 0.01; Table 2-3) and Hs x Wk interaction (P < 0.01; Table 2-3) on concentration of Hp in the serum. Specifically, cows with ketosis had greater concentrations of Hp in the serum at the week of disease diagnosis, which was more than 4-fold greater than CON cows (0.56 vs. 0.12 μg/mL; P = 0.04; Table 2-4). In addition, this pronounced difference was obtained also at -4 weeks prepartum (P = 0.05; Table 2-4).

Results indicated that cows with ketosis had a greater concentration of overall

(including all 4 time points) serum SAA (24,107 vs 8,550 ± 3,457 μg/mL, respectively; $P = 0.01$; Table 2-3) compared with CON cows. The largest difference of serum SAA between the two groups occurred at the disease week (35,835 vs. 10,401; $P = 0.03$; Table 2-4). Furthermore, there was an effect of Wk on serum SAA ($P < 0.01$; Table 2-3). Particularly, concentrations of SAA in the serum decreased in both groups and reached nadir at -4 weeks prepartum. In contrast, serum SAA increased in both groups, reaching peak values at the week of disease diagnosis for ketosis cows and at +4 weeks postpartum for CON cows, respectively. However, no effect of Hs x Wk interaction was observed on serum SAA ($P = 0.14$; Table 2-3).

2.3.4 Dry matter intake (DMI), milk production, and composition

Overall cows with ketosis had a tendency of lower DMI during the entire period of the experiment compared with CON cows (16.59 and 18.64 ± 0.82 kg/d, respectively; $P = 0.10$; Table 2-3). Moreover, there was an effect of Wk on DMI ($P < 0.01$; Table 2-3). There was also an effect of health status (Hs) by Wk interaction with respect to DMI between the two groups ($P = 0.05$, Table 2-3). Specifically, cows with ketosis (14.54 ± 0.88 kg/d) had decreased DMI compared with CON cows (20.34 ± 0.56 kg/d) in the week of disease diagnosis. Moreover, cows with ketosis experienced a decline in DMI during the early lactation (from 0 to +8 weeks) when compared with the group of CON cows. No differences in DMI were observed at -4 ($P = 0.75$; Table 2-4) or -8 ($P = 0.42$; Table 2-4) weeks prior to the expected day of calving.

Health status had a tendency to influence milk production ($P = 0.08$; Table 2-3).

The average daily milk production in ketotic cows (35.25 ± 2.53 kg/d) tended to be lower than CON cows (42.16 ± 2.53 kg/d). There were significant effects of sampling week alone ($P < 0.01$; Table 2-3) and Hs x Wk interaction ($P = 0.05$; Table 2-3) on total daily milk production during the whole experimental period. In particular, milk production was lower for the cows with ketosis (27.02 ± 2.02 kg/d) compared with those of CON ones (40.31 ± 1.62 kg/d; $P < 0.01$; Table 2-4) in the week when the disease was diagnosed.

The effects of Hs, Wk, and Hs x Wk interaction on milk compositions are shown in Table 2-3 and 2-4. There was a tendency for an effect of Hs on milk fat ($P = 0.10$) between two groups and milk fat yield tended to be lower in cows with ketosis than those of the CON group. Milk SCC ($P < 0.01$) was greater in cows with ketosis compared with those in the CON group. In addition, there was an effect of Hs x Wk interaction on milk SCC ($P = 0.03$). Cows with ketosis had greater SCC ($P < 0.01$) during the week of disease and lower milk fat yield ($P = 0.04$) than CON cows. There were no effects of Hs and Hs x Wk on the amounts of milk protein, fat:protein ratio, lactose, MUN, and TS in this study ($P > 0.10$). However, milk protein and SCC were affected by the effect of sampling Wk ($P < 0.05$).

2.3.5 Multivariate analysis of serum variables

Figure 2-1a&b, 2-2a&b, 2-3a&b, and 2-4a&b show the two dimensional PCA scores plots and PLS-DA scores plots based on concentration results of serum variables measured from two groups at 4 different time points. Both PCA (an

unsupervised method) and PLS-DA (a supervised method) analyses revealed a marked and consistent separation between the two groups at the disease diagnosis week (Figure 2-3a&b). Interestingly, when CON cows were compared with cows with ketosis, at -8 and -4 weeks prepartum, again two clearly separated clusters could be observed in both analyses (Figure 2-1a&b; Figure 2-2a&b), which illustrates that innate immunity reactants as well as carbohydrate and lipid metabolites in the serum between CON cows and not-yet-ketotic cows were already different during the dry-off period. Moreover, cows with ketosis continued to experience altered innate immunity reactants and metabolite profiles in the serum after the treatment and recovery from the disease at +4 weeks postpartum (Figure 2-4a&b).

A VIP plot in a PLS-DA model (Permutation test: $P < 0.05$) was used to rank variables based on their contribution to the separation of the ketosis cows from CON ones at 4 time points during the transition period (Figure 2-1c, 2-2c, 2-3c, and 2-4c). The VIP plots indicated that IL-6, lactate, and SAA at -8 weeks; IL-6, BHBA, and lactate at -4 weeks; Hp, IL-6, and TNF at the week of diagnosis of ketosis; and IL-6, TNF, and lactate at +4 weeks were the most important variables responsible for the separation of ketosis cases from CON cows. The heat map on the right side of the 4 VIP plots indicated that these variables were enhanced in cows with ketosis relative to CON cows. It is interesting that during the whole experimental period, IL-6 in the serum obtained the highest VIP scores, which suggests that it might be of importance for prediction and diagnosis of ketosis in transition dairy cows. Moreover, serum lactate was ranked as one of the top 3 variables in the VIP plots during -8 and -4

weeks, which also suggests lactate might be used as a screening and predictive biomarker for the disease.

A ROC curve plot indicating the performance of the top 3 variables in predicting which cows will develop ketosis at -8 and -4 weeks using a PLS-DA model are shown in Figure 2-1d and 2-2d. The AUC for two curves are 0.98 (95% CI, 0.75-1) at -8 weeks and 1.00 (95% CI, 1-1) at -4 weeks, respectively, which indicates that these serum variables (i.e., IL-6, lactate, and SAA at -8 weeks; and IL-6, BHBA, and lactate at -4 weeks) have strong predictive abilities. These results demonstrate that biomarker models developed at -8 and -4 weeks could be used to predict which cows are susceptible to develop ketosis postpartum. The ROC curve developed based on three proteins (i.e., Hp, IL-6, and TNF) at the week of diagnosis of ketosis indicates that this three-metabolite set were a highly significant biomarker for diagnosis of ketosis: AUC, 0.96 (95% CI, 0.75-1, Figure 2-3d). Moreover, multivariate models (ROC curves) combining 3 discriminating variables (i.e., IL-6, TNF, and lactate) at +4 weeks produced an area under the receiver-operating curve of 0.90 (95% CI: 0.5-1, Figure 2-4d).

2.3.6 Correlation analysis between milk SCC and serum variables

Results of correlation analyses between serum variables and milk SCC are presented in Table 2-5. Milk SCC was positively correlated with serum lactate, IL-6, TNF, and SAA at all four sampling weeks with correlation coefficients ranging from r = 0.71 to r = 0.95 (Table 2-5). Correlations between milk SCC and serum BHBA at -4

weeks, disease week, and +4 weeks were strongly positive (r = 0.86, r = 0.89, and r = 0.84, respectively; Table 2-5). There were positive correlations between serum IL-1 and milk SCC at disease week and +4 weeks (r = 0.68, and r = 0.85, respectively; Table 2-5) and a negative correlation between two parameters at -4 weeks prepartum (r = -0.78; Table 2-5). In addition, concentrations of Hp in the serum correlated with milk levels of SCC at -4 weeks, and disease week (r = 0.68, and r = 0.95, respectively; Table 2-5). No pronounced correlations between milk SCC and serum NEFA were observed during the whole period of the experiment.

2.4 Discussion

2.4.1 Alteration in serum metabolites

Blood BHBA has been used as a practical diagnostic biomarker for ketosis in dairy cows (Oetzel, 2007). In this study, cows with ketosis had greater concentrations of BHBA in the serum, not only during the state of ketosis postpartum, but also at -4 weeks before parturition, although concentrations (483 μmol/L) were far lower than the defined ketotic levels of 1,200-1,400 μmol/L. Elevated concentrations of BHBA in the serum during the early lactation period could be attributed to NEB, which leads to intensive mobilization of NEFA from adipose tissue to meet the requirements for maintenance and milk production (Ingvartsen, 2006). Based on DMI data (not different between the two groups), the concept of NEB is not satisfactory to explain the greater BHBA levels found at -4 weeks prior to parturition and therefore there must be other contributing factors for elevated serum BHBA in the pre-ketotic cows.

It is speculated that an endotoxin insult might have occurred in those cows prepartum. The reason for this assumption is that in a previous study we showed that infusing cows around calving (-2 weeks to +1 week peripartum) with increasing doses of lipopolysaccharide (LPS) is associated with greater plasma BHBA starting at -10 day before the expected day of parturition (Zebeli et al., 2011).

In this study, concentrations of NEFA in the serum did not show differences between the healthy and ketosis-affected cows prepartum, althougth there was a numerical difference with greater serum NEFA in ketotic cows during the week of ketosis diagnosis. According to Oetzel (2004), concentrations of NEFA, during presence of NEB prepartum, commonly rise 48 h before calving. However, in our study no samples were analyzed for that period of time. Our data showed that both groups of cows (ketosis and CON ones) had no differences in DMI before calving, which suggest that there was no NEB during that period of time. However, there was a significant difference in DMI between the CON and ketotic cows during the week of diagnosis of ketosis. Results of this study also showed that serum NEFA was increased above 700 μmol/L in both groups of cows during the week of diagnosis of disease. Concentrations of NEFA above 0.7 mmol/L suggest development of subclinical ketosis. Ketotic cows had average NEFA levels, during the week of disease diagnosis, at > 1,038 μmol/L (i.e., severe ketosis), whereas CON cows had border ketotic levels of NEFA at 760 μmol/L. This might be related to the fact that one cow in the CON group, had greater concentration of NEFA compared to the other group members.

Concentrations of lactate in the blood have been used to examine certain diseases in dairy cows. For example, blood lactate was used as prognostic marker for bovine respiratory diseases (Coghe et al., 2000). In another study, Wang et al. (2012) demonstrated that concentration of lactate in the rumen fluid was greater in cows with ketosis than in the healthy controls. In the current study, cows affected by ketosis had greater concentration of serum lactate than CON cows. The major source of blood lactate is anaerobic glycolysis, which is characterized as pyruvate converted into lactic acid by lactate dehydrogenase (Lagutchik et al., 1996). Of note, recent research indicates that lactate also is released by immune cells like monocyte/macrophages as well as T and B cells and it lowers their motility, killing capability, and effector functions and eases the inflammatory process as indicated by suppression of the inflammasome and production of pro-inflammatory cytokines (Fischer et al., 2007; Goetze et al., 2011; Haas et al., 2015). In our study we observed greater serum lactate in cows affected by ketosis during the entire experimental period and it is speculated that blood lactate might be one of the factors that contributes to immune suppression commonly reported in transition dairy cows.

2.4.2 Alterations in innate immunity

A clear correlation does exist between inflammation and metabolic stress, and innate immunity reactants might play important roles as prognostic biomarkers of the cows' heath status (Ametaj et al., 2011; Trevisi et al., 2012). Recent research in dairy cows has documented the role of systemic inflammation in development of metabolic

disorders (Ametaj et al., 2005a). In this study, cows with ketosis had greater concentration of IL-6, TNF, and Hp, not only during the week of disease diagnosis but also at -8 and -4 weeks perpartum, which indicates presence of an inflammatory condition during the dry-off period and early lactation. To our best knowledge this is the first time that cows suffering from ketosis are found to have an inflammatory condition that precedes development of the disease at the beginning of far-off dry period. Our data are also consistent with the results of a recently published study demonstrating associations between serum IL-6 measured at -23 and -33 d prepartum and the incidence of ruminal stasis, abomasal displacement, diarrhea, and retained placenta in the postpartum dairy cows (Amadori et al., 2015).

Although much is known about ketosis, the underlying mechanisms of hyperketonemia are not completely understood and it is not known whether the disease is triggered by an inflammatory insult or it is related more to an imbalanced nutrition. Greater IL-6 in the serum of cows with ketosis during the dry off period suggest that IL-6 might contribute to the metabolic alterations commonly observed in ketotic cows. For example, gene network analysis, in a study by Loor et al. (2007), suggested that upregulation of IL-6 in the liver might affect multiple networks related to lipoprotein metabolism, oxidation of fatty acids, oxidative stress, and protein degradation. Dysfunction in any or all the aforementioned networks might contribute to the overall pathobiology of ketosis and fatty liver (Loor et al., 2007). Intriguingly, human subjects with diabetes and hyperketonemia were reported to have greater concentrations of IL-6 in the systemic circulation (Jain et al., 2003). Interestingly,

incubation of U937 monocytes from the same patients with acetoacetate, but not with BHBA, increased production of IL-6 in vitro (Jain et al., 2003).

Besides IL-6, serum concentrations of TNF were greater in cows affected by ketosis starting at -4 weeks before parturition and during diagnosis week. Tumor necrosis factor is a major pro-inflammatory cytokine, which has been implicated in a wide range of systemic disorders (Popa et al., 2007). For example, correlation between serum TNF and total lipid amounts in cows with fatty liver as well as insulin resistance has been reported (Ohtsuka et al., 2001; Ametaj et al., 2005a). In addition, cytokines such as IL-6 and TNF could stimulate the breakdown of body fat stores by depression of feed intake, triggering of insulin resistance, and direct initiation of lipolysis (Kushibiki et al., 2003), all of which are associated with development of ketosis in dairy cows (Ingvartsen, 2006). Enhanced IL-6 and TNF in the serum of cows with ketosis at -8 and -4 weeks prior to parturition suggests that an acute phase response (APR) was present. During the APR hepatic fatty acid metabolism is redirected toward triglyceride (TG) synthesis and lipogenesis that contribute to increased secretion of TG-rich lipoproteins, important for cleaning endotoxins from blood circulation (Grunfeld et al., 1996; Rauchhaus et al., 2000).

Strong positive correlation between acute phase proteins (e.g., Hp and SAA) and indicators of lipomobilization (i.e., BHBA and NEFA) have been reported previously (Tóthová et al., 2014). Haptoglobin and SAA in the serum are important positive APP in dairy cows and are mainly produced by liver hepatocytes in response to pro-inflammatory cytokines (e.g., IL-6 and TNF) and glucocorticoids (Ametaj et al.,

2011; Trevisi et al., 2012). Ametaj (2005b) reported greater concentrations of Hp and SAA in the plasma of transition dairy cows with fatty liver, which further supports the assumption that metabolic disorders are associated with subclinical inflammation. In this study, cows with ketosis had greater concentrations of Hp and SAA in the serum during the disease week and enhanced serum Hp at -4 weeks prepartum, which indicates presence of a systemic inflammation and that alterations of innate immunity reactants precede development of ketosis.

It should be pointed out that the VIP plots at -8 and -4 weeks prepartum indicated that IL-6 and lactate in the serum were the strongest discriminators between ketosis cows and the healthy ones, which suggest that those two variables might be potential predictive biomarkers of disease state. Moreover, biomarker models (i.e., ROC curves) constructed at 4 time points showed good performance with high AUC values ranging from 0.90 to 1.00. However, it should be noted that the identified screening biomarkers are non-specific biomarkers. Since innate immunity is a non-specific immune response the identified biomarkers (i.e., IL-6 and lactate) can be used as general indicators of non-health but not as specific biomarkers of ketosis in transition dairy cows.

2.4.3 Dry matter intake (DMI), milk production, and composition

Cows affected by ketosis experienced depression of the appetite and a tendency of lower milk production during the early lactation period (i.e., +1 to +8 weeks, specifically after diagnosis of the disease). Our data are in agreement with previous

research work that reported a pronounced decrease in DMI and milk yield as early as 4 to 6 days prior to diagnosis of ketosis and the onset of obvious clinical symptoms (Edwards and Tozer, 2004; González et al., 2008). The main reason for depressed appetite and simultaneous lower milk yield in cows with ketosis at early lactation might be related to presence of a systemic inflammation. Previous evidence has shown that cytokines like IL-1, IL-6, and TNF suppress appetite, and conditions such as infection and inflammation are usually associated with enhanced cytokine production in dairy cows (Kasimanickam et al., 2013). Indeed, our data showed greater pro-inflammatory cytokines in ketotic cows versus the CON ones.

Finally, the results from this study showed lower milk fat in cows with ketosis, during the disease week, which is in contrast with previous research. For example, Miettinen (1994) reported that milk fat yield was greater in cows with subclinical ketosis. However, Duffield et al. (1997) showed that neither milk fat nor fat to protein ratio tests are accurate for determining whether ketosis is present or not, given their poor sensitivities and specificities at various cut-off points. In addition, greater milk SCC in cows with ketosis was found in this study, which support the association between ketosis and increased risk of mastitis (Gröhn et al., 1989). Although our findings are interesting and exciting they have to be taken with caution given the low number of replicates in this study. Therefore the findings must be considered preliminary. More research with a larger cohort of cows is warranted to validate the biomarkers identified as well as to determine the potential causal agent that triggered activation of innate immunity during far-off dry period and the precise role of the

immune system and metabolic alterations in the pathobiology of ketosis.

2.5 Conclusions

In summary, results of this study indicate that cows affected by ketosis display elevated variables of innate immunity and altered carbohydrate and lipid metabolism several weeks ahead of diagnosis of the disease. More specifically, concentrations of BHBA, lactate, IL-6, TNF, and SAA in the serum were greater in pre-ketotic cows and during diagnosis of disease compared with the group of healthy cows. Multivariate analysis (i.e., PCA and PLS-DA) demonstrated two clear separated clusters of healthy and ketotic cows. To the best of our knowledge, this study is the first comprehensive report illustrating that alterations of variables related to innate immunity and lipid and carbohydrate metabolism start at the beginning of dry off period and continue during the dry period as well as during the diagnosis week and postpartum. Results also demonstrated that cows with ketosis experienced lower DMI, elevated SCC, and a tendency of lower milk production, and lower milk fat. More research with a larger cohort of animals is warranted to better understand the agent(s) that contribute(s) to pathobiology of ketosis in transition dairy cows and to validate utilization of these blood variables to screen cows for disease state during the transition period.

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Table 2-1. Prepartum diet for the dry off cows

Item	Close-up diet (CUD)
Ingredient	% of DM
Alfalfa hay	10.0
Barley silage	60.0
CUD grain	30.0
Nutrient composition of CUD grain	% in 100 kg of mix
Ruminant TM Pak[1]	0.2775
Selenium 1000 mg/kg (UNscr FineCr)	0.2
Custom TM Complex Premix[2]	0.33
Vitamin A/D_3-1000-200[3]	0.006
Barley grain, rolled	39.5815
Flo-bond mycotoxin binder	0.5
Limestone	3.7
Magnesium chloride	1.645
Mag Ox-56%[4]	0.54
Scale Molasses (60:40)	2.5
Canola meal	17.0
Vitamin E 50% Ads[5]	0.18
Soybean hulls, ground	33.0
Salt	0.54

[1]Ruminant TM Pak: a premix containing cobalt, copper, iodine, manganese, and zinc.

[2]Custom TM complex premix: a custom product supplying organic sources of cobalt, copper, manganese, and zinc.

[3]Vitamin A/D_3-1000-2003: Vitamin A acetate (retinyl acetate) and Vitamin D_3 (cholecalciferol).

[4]Mag Ox 56%: 56% magnesium guarantee.

[5]Vitamin E 50% Ads contains 226,800 IU of Vitamin E per pound.

Table 2-2. Ingredients of TMR fed to cows during early lactation

Item	Early lactation diet
Ingredient % of DM	%, DM
Alfalfa Hay	9.59
Barley Silage	30.24
Alfalfa Silage	9.64
High 16% dairy ration	50.53
Nutrient composition of dairy ration	% amount per kg
ADE Vit Pak-30 Natural E[1]	0.05
Ruminant TM Pak[2]	0.11
Selenium, 1,000 mg/kg (UNscr FineCr)	0.07
Custom TM Complex premix[3]	0.07
AminoShure - L[4]	0.33
Blood meal	3.50
Barley grain, rolled	39.90
Barley grain, ground	27.50
Di-calcium phosphate 21%	1.00
Vit D-10,000 KIU/kg	0.02
Diamond V XPC[5]	0.13
Dairy Xtract	0.02
Energizer RP10	2.75
Limestone	1.70
Mag Ox-56%[6]	0.43
Scale Molasses (60:40)	1.25
Nutri A-Z C Dry	0.10
Amino Plus (High bypass soy)[7]	8.00
Vitamin E 50% Ads[8]	0.01
Soy bean meal-47.5%	1.25
Sodium bicarbonate	0.80
Salt	0.50
Poultry-Tallow	0.50
Biotin 2%-Rovimix H-2[9]	0.01
Wheat distillers grain (50:50)	10.00

[1]ADE Vit Pak-30 Natural E: a premix containing vitamins A, D3, and E.

[2]Ruminant TM Pak: a premix containing cobalt, copper, iodine, manganese, and zinc.

[3]Custom TM complex premix: a custom product supplying organic sources of cobalt, copper, manganese, and zinc.

[4]AminoShure - L: hydrogenated vegetable oil, and L-lysine monohydrochloride (Halchemix, Port Perry, ON, Canada). [5]Diamond V XPC: concentrated yeast (Diamond V Mills, Cedar Rapids, IA).

[6]Mag Ox 56%: 56% magnesium guarantee. [7]Amino Plus: a high by-pass soy meal.

[8]Vitamin E 50% Ads contains 226,800 IU of Vitamin E per pound.

[9]DSM Nutritional Products (Parsippany, NJ).

Table 2-3. Data of DMI, milk production, milk composition as well as metabolites, cytokines and APP in the serum of dairy cows with (n = 6) and without ketosis during the periparturient period

	Group[1]			Effect,[2] *P*-value		
Item	Ketosis	CON	SEM	Hs	Wk	Hs × Wk
DMI[3] (kg/d)	16.59	18.64	0.82	0.10	<0.01	0.05
Milk production[4] (kg/d)	35.25	42.16	2.53	0.08	<0.01	0.05
Milk composition[5] (g/kg, unless otherwise stated)						
Fat	3.39	3.87	0.17	0.10	0.25	0.72
Protein	2.77	2.86	0.06	0.27	<0.01	0.67
Fat:protein ratio	1.24	1.38	0.08	0.33	0.22	0.87
Lactose	4.51	4.56	0.07	0.66	0.07	0.82
SCC (10^3 cells/mL)	80.51	31.11	5.85	<0.01	0.02	0.03
Milk urea N (mg/dL)	16.44	15.56	1.14	0.52	0.19	0.13
TS	11.94	12.20	0.20	0.40	0.11	0.80
Serum parameters[6]						
BHBA (µmol/L)	1,014	504	140	0.04	<0.01	0.08
NEFA (µmol/L)	348	397	81	0.69	<0.01	0.62
Lactate (µmol/L)	4,236	2,240	351	<0.01	0.16	0.33
IL-1 (pg/mL)	294.61	296.68	4.16	0.74	<0.01	0.03
IL-6 (pg/mL)	254.58	26.76	55.38	0.03	0.67	0.97
TNF (ng/mL)	0.47	0.19	0.07	0.03	<0.01	0.49
Haptoglobin (mg/mL)	0.18	0.15	0.05	0.66	<0.01	<0.01
SAA (ug/mL)	24,107	8,550	3,457	0.01	<0.01	0.14

[1]CON = cows without ketosis (health control); Ketosis = cows with ketosis.

[2]Effect of health status (Hs), sampling week (Wk), and health status by sampling week interaction (Hs × Wk).

[3]DMI was calculated from week -8 to +8 relative to parturition.

[4]Milk production was calculated from week +1 to +8 relative to parturition.

[5]Milk compositions were determined on week +2, +3, +5, +7 relative to parturition.

[6]Serum variables were calculated from week -8, -4, disease and +4 relative to parturition.

Table 2-4. Data of DMI, milk production, milk composition and serum variables at the diagnosis week, and concentrations of serum indicators prior to the diagnosis of ketosis

	-8 weeks before parturation			-4 weeks before parturation			Ketosis diagnosis week[1]		
Item	CON	Ketosis	*P*-value	CON	Ketosis	*P*-value	CON	Ketosis	*P*-value
DMI (kg/d)	16.27 ± 0.95	16.82 ± 1.47	0.75	15.93 ± 0.10	14.22 ± 1.83	0.42	20.34 ± 0.56	14.54 ± 0.88	<0.01
Milk production (kg/d)							43.01 ± 1.62	27.02 ± 2.02	<0.01
Milk composition (g/kg, unless otherwise stated)									
Fat							5.08 ± 0.45	3.77 ± 0.25	0.04
Protein							3.00 ± 0.10	2.92 ± 0.07	0.56
Fat:Protein ratio							1.69 ± 0.12	1.44 ± 0.12	0.17
Lactose							4.54 ± 0.05	4.45 ± 0.12	0.46
SCC (10^3 cells/mL)							28.33 ± 5.63	152 ± 20.43	<0.01
Milk urea N (mg/dL)							15.38 ± 0.76	16.88 ± 1.93	0.46
TS							12.21 ± 0.31	12.55 ± 0.52	0.58
Serum parameters									
BHBA (μmol/L)	352 ± 37.71	374 ± 27.02	0.66	312 ± 18.50	483 ± 71.44	0.02	690 ± 80.17	1,756 ± 178	<0.01
NEFA (μmol/L)	141 ± 32.77	125 ± 32.86	0.74	194 ± 47.17	102 ± 22.48	0.17	757 ± 232.01	1038 ± 189	0.39
Lactate (μmol/L)	2,455 ± 349	5,795 ± 369	0.01	2,162 ± 185	4,478 ± 822	0.01	2,228 ± 321	3,361 ± 321	0.08
IL-1 (pg/mL)	317 ± 6.04	312 ± 7.98	0.61	321 ± 1.59	300 ± 6.10	0.02	277 ± 5.42	289 ± 2.66	0.10
IL-6 (pg/mL)	19.23 ± 5.67	183 ± 61.45	<0.01	48.24 ± 17.51	329.87 ± 89.95	<0.01	23.17 ± 5.18	116 ± 15.55	<0.01
TNF (ng/mL)	0.34 ± 0.03	0.48 ± 0.10	0.36	0.27 ± 0.05	0.64 ± 0.06	0.03	0.06 ± 0.03	0.49 ± 0.09	<0.01
Haptoglobin (mg/mL)	0.19 ± 0.03	0.18 ± 0.01	0.95	0.15 ± 0.01	0.18 ± 0.01	0.05	0.12 ± 0.01	0.56 ± 0.12	0.04
SAA (ug/mL)	8,448 ± 3,373	16,342 ± 7,103	0.31	3,461 ± 342	8,389 ± 4,194	0.13	10,401 ± 1,723	35,835 ± 9,697	0.03

[1]Cows were diagnosed with ketosis (n=6) ranging from week +1 to +3. CON = cows without ketosis (health control); ketosis = cows with ketosis

Table 2-5. Pearson's correlations between milk SCC and serum variables

Item		8 weeks before parturition[1]		4 weeks before parturition[2]		ketosis diagnosis week[3]		4 week after parturition[4]	
Milk	Serum	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
SCC[5]	BHBA	0.33	0.39	0.86	<0.01	0.89	<0.01	0.84	<0.01
	NEFA	0.29	0.45	-0.20	0.58	0.48	0.12	-0.26	0.48
	Lactate	0.88	<0.01	0.89	<0.01	0.89	<0.01	0.93	<0.01
	IL-1	-0.13	0.74	-0.78	0.02	0.68	0.04	0.85	<0.01
	IL-6	0.92	<0.01	0.92	<0.01	0.93	<0.01	0.71	0.05
	TNF	0.83	<0.01	0.80	0.02	0.85	0.02	0.76	0.05
	Hp	0.16	0.69	0.68	0.04	0.95	<0.01	0.27	0.52
	SAA	0.79	0.01	0.94	<0.01	0.95	<0.01	0.89	<0.01

[1]Concentration values of serum variables were used from both healthy and ketosis cows at week -8 relative to parturition.

[2]Concentration values of serum variables were used from both healthy and ketosis cows at week -4 relative to parturition.

[3]Concentration values of serum variables were used from ketosis cows at diagnosis week and the similar week from healthy cows.

[4]Concentration values of serum variables were used from both healthy and ketosis cows at week +4 relative to parturition.

[5]Milk SCC values were used from ketosis cows at diagnosis week and the similar week from healthy cows

Figure 2-1.

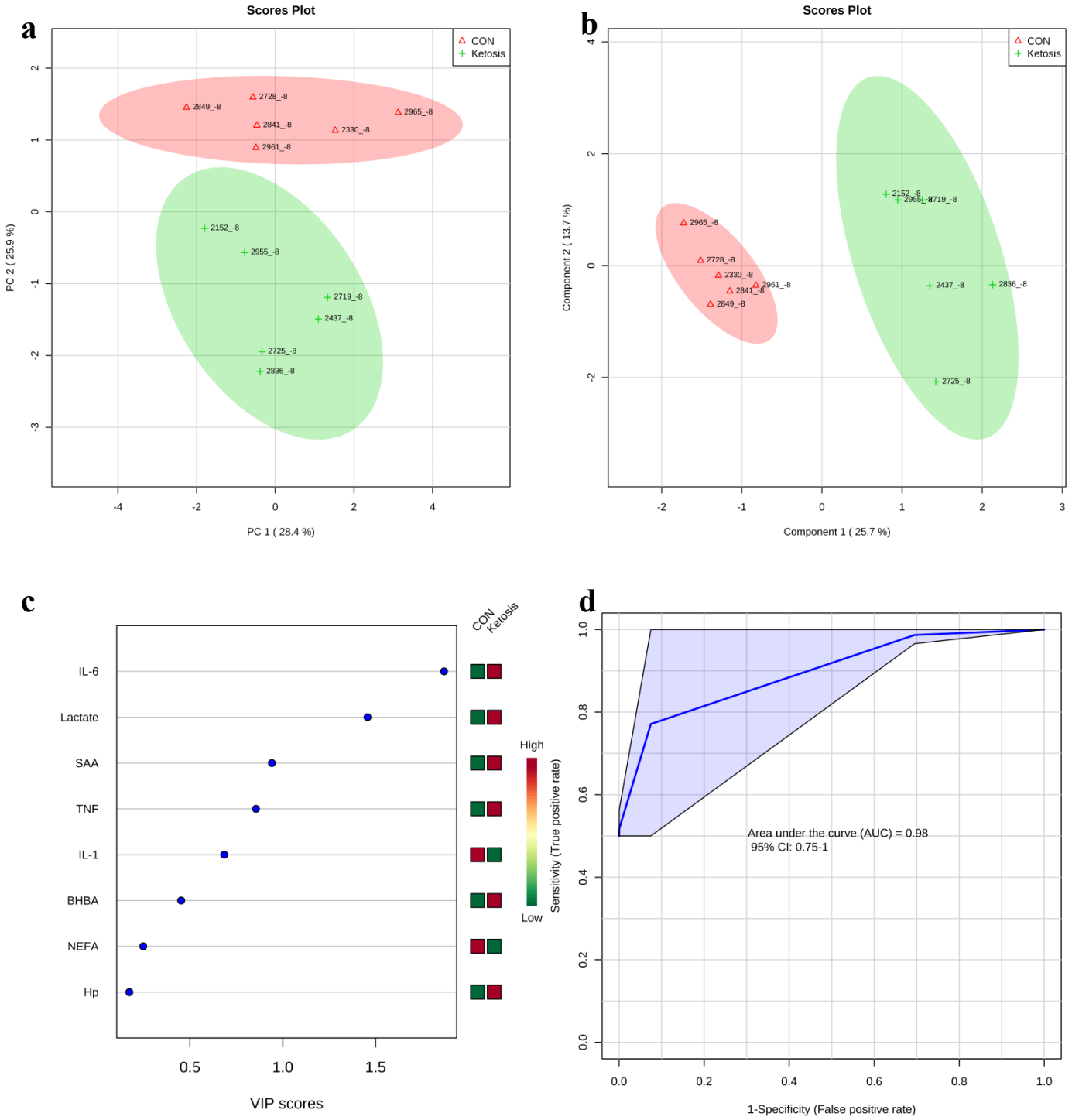

Figure 2-1. a) Principal component analysis (PCA) and b) Partial least squares-discriminant analysis (PLS-DA) of 6 control and 6 ketosis cows at -8 wk before parturition showing 2 separated clusters for 2 groups. c) Variables ranked by variable importance in projection (VIP), and d) Receiver-operator characteristic (ROC) curve of 6 CON and 6 ketosis cows at -8 weeks before parturition for the top 3 serum variables of the VIP plot (i.e., IL-6, lactate, and SAA; empirical $P < 0.05$).

Figure 2-2.

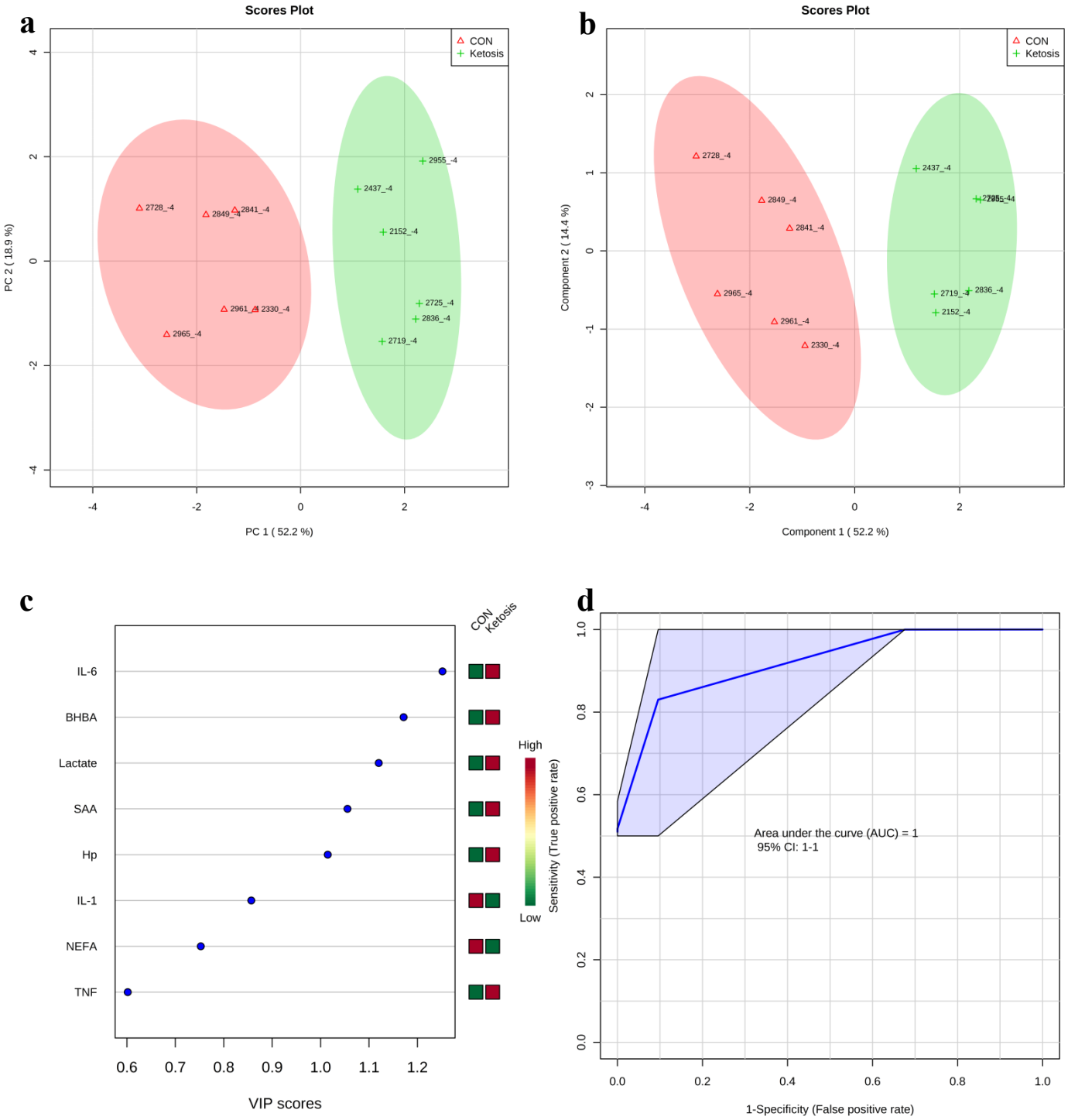

Figure 2-2. a) PCA and b) PLS-DA of 6 control and 6 ketosis cows at -4 wk before parturition showing 2 separated clusters for 2 groups. c) VIP, and d) ROC curve of 6 CON and 6 ketosis cows at -4 weeks before parturition for the top 3 serum variables of the VIP plot (i.e., IL-6, BHBA, and lactate; empirical $P < 0.05$).

Figure 2-3.

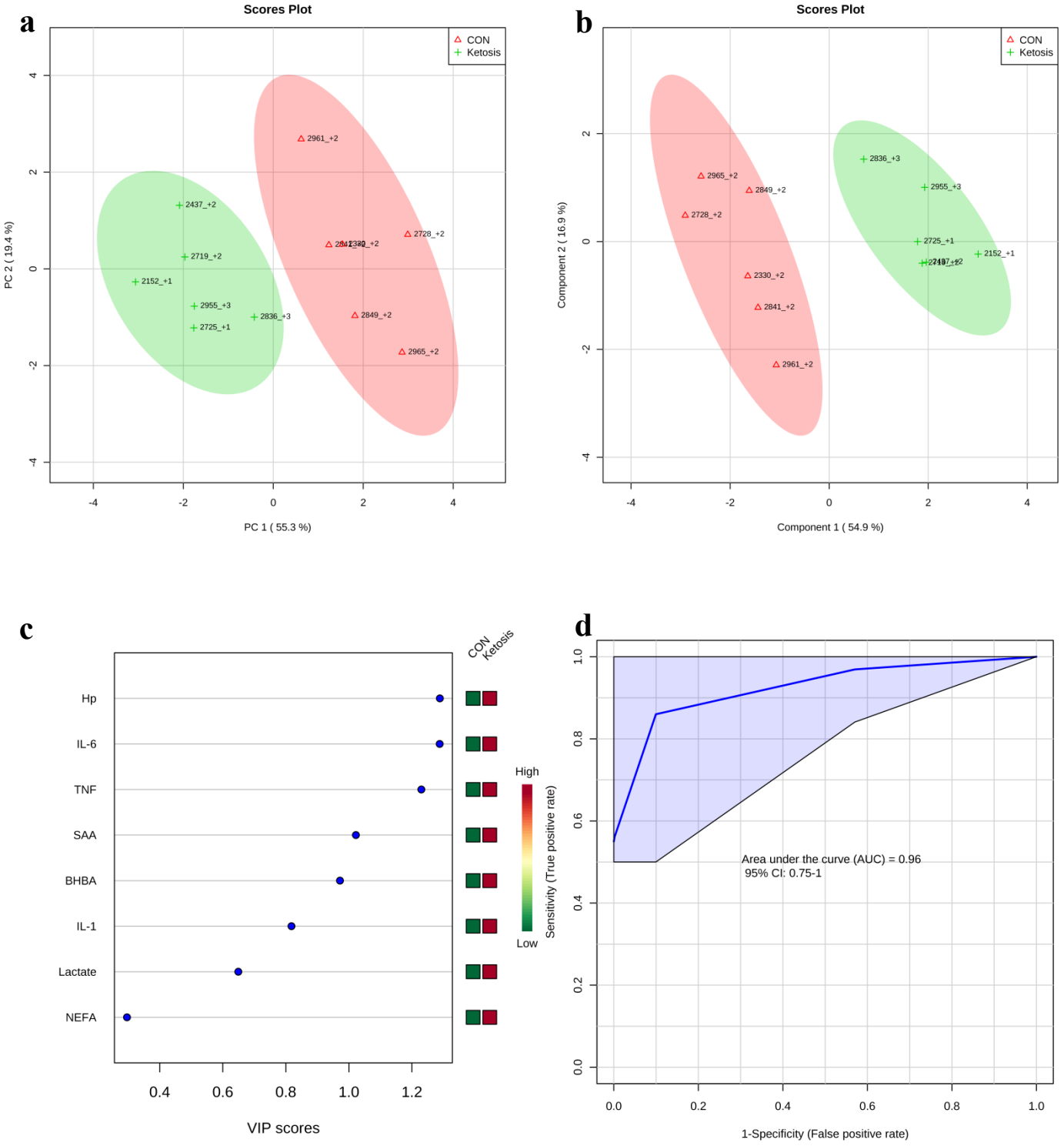

Figure 2-3. a) PCA and b) PLS-DA of 6 control and 6 ketosis cows at disease wk showing 2 separated clusters for 2 groups. c) VIP, and d) ROC curve of 6 CON and 6 ketosis cows at disease wk for the top 3 serum variables of the VIP plot (i.e., Hp, IL-6, and lactate; empirical $P < 0.05$).

Figure 2-4.

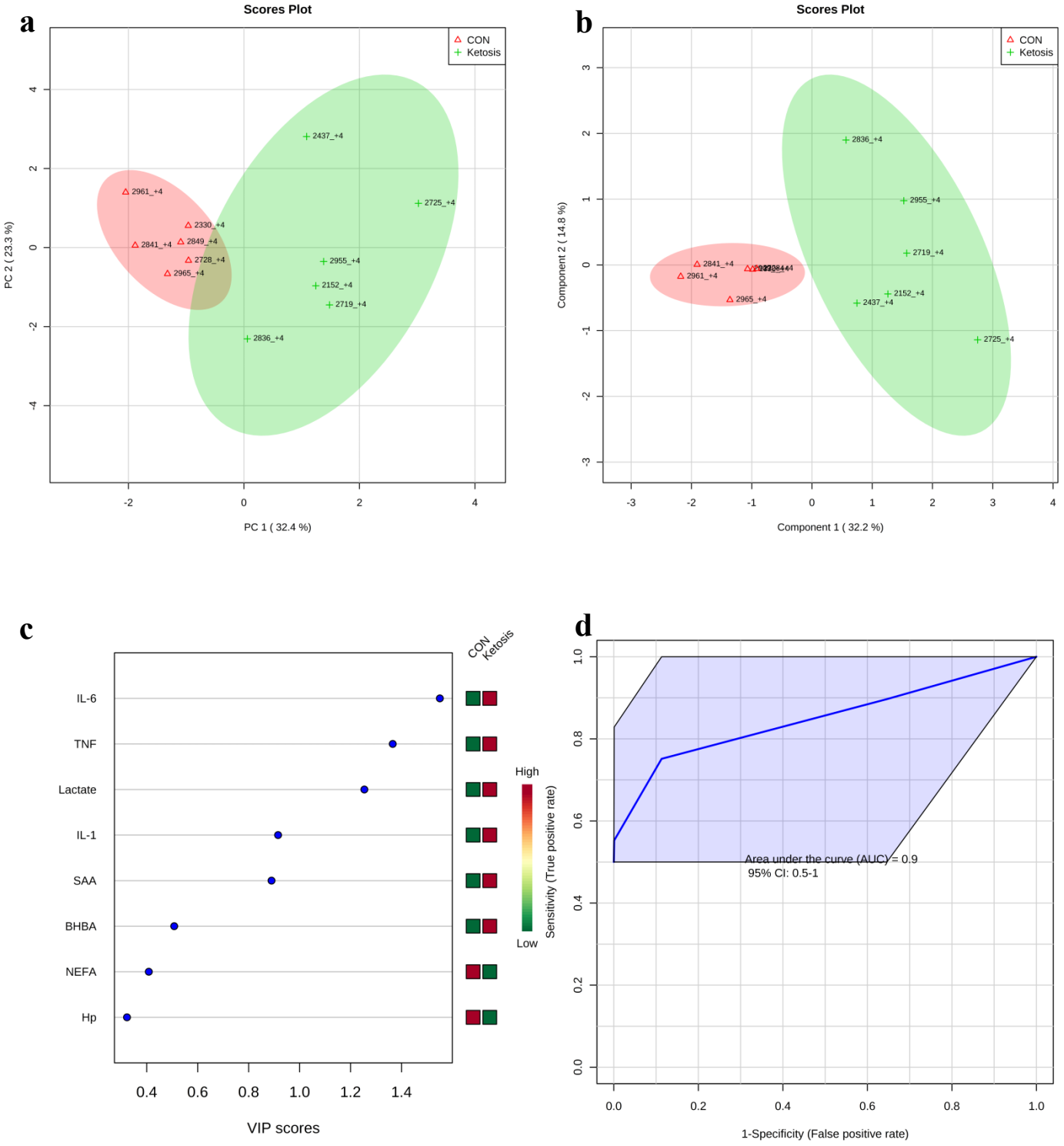

Figure 2-4. a) PCA and b) PLS-DA of 6 control and 6 ketosis at 4 wk after parturition showing 2 separated clusters for 2 groups. c) VIP, and d) ROC curve of 6 CON and 6 ketosis cows at +4 weeks after parturition for the top 3 serum variables of the VIP plot (i.e., IL-6, TNF, and lactate; empirical $P < 0.05$).

Chapter 3 DI/LC-MS/MS-based metabolomics reveals distinct amino acid and lipid profiles in cows with ketosis and identifies predictive serum biomarkers for the disease

ABSTRACT

Ketosis is a prevalent metabolic disease in transition dairy cows and is a risk factor for decreased milk yield as well as the development of other periparturient diseases. The objective of this study was to evaluate alterations of metabolites in the serum of dairy cows with ketosis before, during, and after the diagnosis of disease and identify predictive and diagnostic serum metabolite biomarkers for ketosis. One hundred and twenty-eight serum metabolites were quantitatively profiled in healthy (CON, n = 20) and ketotic (n = 6) cows using a targeted metabolomics approach based on DI/LC-MS/MS at -8 wks, -4 wks, disease at diagnosis, +4 wks, and +8 wks relative to parturition. Significant changes were detected in the levels of several analyte groups of amino acids, glycerophospholipids, sphingolipids, acylcarnitines, biogenic amines, and hexose in the serum of ketotic cows during the entire experimental period. Furthermore, several metabolic pathways were perturbed in cows with ketosis during the onset and progression of disease. Serum biomarkers identified show very accurate predictive, diagnostic, and prognostic abilities for ketosis in transition dairy cows. These new findings give important new insights into our understanding of the pathogenesis of ketosis in dairy cows.

3.1 Introduction

Ketosis is a prevalent metabolic disease in transition dairy cows that commonly occurs during early lactation. Ketosis usually appears in a subclinical form (i.e., subclinical ketosis (SCK)), which is characterized by excess ketone bodies [(i.e., β-hydroxybutyric acid (BHBA), acetoacetate (AcAc), and acetone (Ac)] in the blood, urine, or milk without clinical signs of hyperketonemia. Some cases of severe ketosis can develop into a clinical form (i.e., clinical ketosis (CK)), which is defined as elevated concentrations of ketone bodies in the blood, urine, and milk, and accompanied clinical symptoms including lower milk production, decreased appetite, rapid weight loss, and dry manure (Gordon et al., 2013). The incidence of SCK in dairy cows in North America is relatively high at about 40%, and it might reach as high as 80% in some dairy herds without proper management (Duffield, 2000). About 2 to 15% of the cows are affected by CK (Duffield, 2000). Cows with ketosis have lower milk yield and reproductive performance, greater risk of other periparturient diseases, and a higher culling rate (McArt et al., 2011; 2012).

The ‘gold standard’ for diagnosis of ketosis in dairy cows has been blood BHBA (Työppönen and Kauppinen, 1980). Generally, a cow is diagnosed as having SCK when concentration of BHBA in the blood is between 1,200 to 1,400 μmol/L (Suthar et al., 2013). The clinical form of ketosis is usually recognized with greater levels of blood BHBA at about 3,000 μmol/L or more (Oetzel, 2007). For a long time, ketosis has been attributed to a state of negative energy balance (NEB) during the immediate postpartum period, in which cows experience increased energy demand for milk

production and relatively insufficient feed intake (Kaka, 2013). Although all transition dairy cows commonly experience a period of NEB and excessive body fat mobilization immediately after parturition not all of them undergo hyperketonemia; and even less of them progress to CK. This suggests that something is missing with regards to our understanding of the etiological and susceptibility factors that contribute to development of ketosis in transition dairy cows.

Metabolomics is the newest 'omics' science and a powerful tool for elucidating disease etiologies, developing biomarkers for detection and characterizing biological pathways as well as for monitoring and predicting complex diseases (Martin et al., 2012; Nguyen et al., 2012; Xia et al., 2013). Only a few studies have used metabolomics to investigate metabolic changes and identify biomarkers of ketosis in the plasma and milk of dairy cows. Gas chromatography-mass spectrometry (GC-MS) based metabolomics has suggested several diagnostic biomarkers of ketosis in plasma and associated metabolic perturbations (Zhang et al., 2013). Moreover, Sun et al. (2014) reported that non-targeted nuclear magnetic resonance (NMR) based metabolomics can distinguish cows with SCK and CK from healthy controls. NMR was also used to profile milk metabolites and reveal that the milk glycerophosphocholine to phosphocholine (GPC/PC) ratio can detect cows at risk of ketosis (Klein et al., 2012). However, all previous studies were conducted during the early lactation period when ketosis had already developed in dairy cows.

Recently, our group reported a 3-metabolite predictive biomarker profile (carnitine, propionyl carnitine, and lysophosphatidylcholine acyl C14:0) in the plasma

of transition dairy cows at 4 wks prior to the development of several periparturient diseases (Hailemariam et al., 2014a). In another study using direct injection and liquid chromatography coupled to tandem mass spectrometry (DI/LC-MS/MS) approach we showed that cows with four periparturient diseases (i.e., metritis, mastitis, laminitis, and retained placenta) experienced alterations of amino acid and sphingolipid profiles during the transition period, which gave interesting insights onto the etiopathology of these disease states in dairy cows (Hailemariam et al., 2014b).

In a more recent study we also showed that cows with ketosis experience an activation of innate immunity and altered carbohydrate and lipid metabolism several weeks prior to diagnosis of ketosis (Zhang et al., 2016). We proposed that an inflammatory state during the dry off period and activation of the immune response might contribute to the development of postpartum ketosis. To the best of our knowledge, no metabolic profiling of serum metabolites has been reported in cows before the occurrence of ketosis. Therefore, it would be of great importance to conduct a comprehensive analysis of serum variables in transition dairy cows before, during, and after the diagnosis of ketosis.

In this study, DI/LC-MS/MS in conjunction with multivariate statistical analysis was applied to detect alterations in serum metabolite profiles and related metabolic pathways at two time points prior to parturition, at the time of diagnosis of the disease as well as two other time points during the 2 months after parturition. The objectives of this investigation were: 1) to determine whether there are alterations in serum metabolites related to amino acid, carbohydrate, and lipid metabolism in transition

dairy cows, before, during, and after appearance of clinical signs of ketosis; and 2) to identify predictive metabolite biomarkers of ketosis in the serum of dairy cows that might be useful for screening cows during the dry off period for the potential development of postpartum ketosis.

3.2 Materials and methods

This study was part of a prospective project designed to identify predictive biomarkers of periparturient diseases in dairy cows. 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 (2009). Data related to concentrations of serum cytokines and acute phase proteins, dry matter intake (DMI), milk production and milk composition for this experiment have been reported previously (Zhang et al., 2016). The metabolomics analyses were performed at the Metabolomics Innovation Centre, University of Alberta, Edmonton, AB, Canada.

3.2.1 Animals and diets

Please refer to chapter 2 (section 2.1.1 Animals and diets) of this thesis for detailed information about the animals and diets that were used. In this study, twenty healthy controls and 6 cows that developed ketosis were selected for further metabolomics analyses. Analyses were conducted on samples from 5 time points: at -8 wks (53-59 d) and -4 wks (25-31 d) before parturition, the disease wk (5-21 d, mean: 13 d) and at +4 wks (25-31 d) and +8 wks (53-59 d) after calving from each

cow for serum metabolomics analysis.

3.2.2 Blood sample collection

Blood samples were obtained from the coccygeal vein of all cows (i.e., 100 cows) at 0700, shortly before feeding, at -8 and -4 wks before the expected day of calving, the week of disease diagnosis, +4, and +8 wks after parturition. For more information about detailed procedures of blood sample collection, please refer to chapter 2.

3.2.3 DI/LC-MS/MS compound identification and quantification

To determine the concentration of amino acids (AAs), acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and hexose in the serum samples, a targeted quantitative metabolomics approach was applied using a commercial kit that combined direct injection and tandem mass spectrometry (DI-MS/MS) with a reverse-phase liquid chromatography and tandem mass spectrometry (LC-MS/MS). The kit (Absolute*IDQ* 180) is available from BIOCRATES Life Science AG (Innsbruck, Austria). This kit assay is used in combination with an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex, Foster City, CA) mass spectrometer, and can be used for the targeted identification and quantification of up to 186 different endogenous metabolites from six analyte groups including AAs, acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and hexose. Amino acids and biogenic amines were analyzed by the LC-MS/MS method, whereas all other metabolites were quantified by DI-MS/MS.

The method combines the derivatization and extraction of analytes with the

selective mass spectrometric detection using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards and other internal standards were integrated into a kit plate filter for metabolite quantification. The Absolute*IDQ* 180 kit contains a 96 deep-well plate with a filter plate, along with sealing tape and reagents and solvents used to prepare the plate assay. The first 14 wells (1 blank, 3 zero samples, 7 standards, and 3 quality control samples) in the kit were used for quality control and standardization, with the remaining 82 being available for serum sample analysis. All the serum samples were analyzed with the Absolute*IDQ* 180 kit according to the manufacturer's user manual. Briefly, serum samples were left to thaw on ice and were vortexed and centrifuged at 13,000 × *g* at 4 °C for 3 min. Ten μL of each serum sample were loaded onto the center of the filter on the upper 96-well kit plate and dried under a stream of nitrogen using Zanntek Analytical Evaporator (Glas-Col, Terre Haute, IN, USA). Subsequently, 20 μL of a 5% solution of phenylisothiocyanate was added for derivatization. After incubation, the filter spots were dried again using the same evaporator.

Extraction of the metabolites was accomplished by adding 300 μL methanol containing 5 mM ammonium acetate. The extracts were obtained by centrifugation using Sorvall Evolution RC Superspeed Centrifuge (Fisher Scientific, Toronto, ON, Canada) into the lower 96 deep-well plate, followed by a dilution step with 600 μL of the MS running solvent in the kit. Mass spectrometric analysis was achieved on an ABI 4000 Q-trap tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with a solvent delivery system.

The Biocrates MetIQ software, which was included in the kit was used to control the entire assay workflow, from sample registration to automated calculation of metabolite concentrations to the export of data into other data analysis programs. A targeted profiling scheme was used to do quantification of all detectable metabolites using MRM, neutral loss, and precursor ion scans.

3.2.4 Statistical analysis

Univariate analysis of data was performed using Wilcoxon-Mann-Whitney (rank sum) test provided by R (Version 3.0.3, R Development Core Team, 2008). Statistical significance was declared at $P < 0.05$. All metabolomics data were processed and analyzed using the MetaboAnalyst software (Xia et al., 2009). Recommended statistical procedures for metabolomics analysis were followed according to previously published protocols (Xia et al., 2009). Metabolites that were frequently (>20%) below the limit of detection or with at least 20% missing values were removed from consideration. Otherwise, missing values were replaced by a value of one-half of the minimum positive value in the original data. Data normalization of metabolite concentration was done prior to statistical analysis and pathway analysis to create a Gaussian distribution (Xia et al., 2009). In this study, we used log-transformed and auto scaled metabolite values.

To perform a standard cross-sectional 2-group study, we compared the healthy cows group (control cows, CON) and the group of cows with ketosis at each time point (-8 wks, -4 wks, disease diagnosis, +4 wks, and +8 wks around calving)

separately. Principal component analysis (PCA), partial least squares - discriminant analysis (PLS-DA), quantitative enrichment analysis, and metabolic pathway analysis were performed via MetaboAnalyst. In the PLS-DA model, a VIP (variable importance in projection) plot was used to rank the metabolites based on their importance in discriminating ketosis group from the group of CON cows. Metabolites with the highest VIP values are the most powerful group discriminators. Typically, VIP values > 1 are significant and VIP values > 2 are highly significant. A permutation test with 2,000 random resamplings was implemented to validate the reliability of the model and to determine the probability that the metabolites distinguishing the ketosis and CON groups are a result of chance.

Biomarker profiles and the quality of the biomarker sets were determined using receiver-operator characteristic (ROC) curves as calculated by MetaboAnalyst 3.0 (Xia et al., 2015). Paired sensitivity and false-positive ratios (1-specificity) at different classification decision boundaries were calculated. A ROC curve is plotted with sensitivity values on the Y-axis and the corresponding false-positive rates (1-specificity) on the X-axis. ROC curves are often summarized into a single metric known as the area under the ROC curve (AUC), which indicates the accuracy of a test for correctly distinguishing one group such as ketotic cows from CON ones. If all positive samples are ranked before negative ones, the AUC is 1.0, which indicates a perfect discriminating test. The 95% confidence interval (CI) and *P* values were also calculated. A rough guide for assessing the utility of a biomarker set based on its AUC 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.

Permutation testing was conducted for each ROC curve at different time points with 1,000 random resamplings.

3.3 Results

The analytical characteristics of metabolite profiling based on DI/LC-MS/MS were performed for 6 cases of ketosis and 20 CON cows. In total, one hundred and twenty-eight metabolites were identified and quantified using an in-house mass-spectrometry library. These metabolites can be classified into 6 groups: amino acids (AAs) (21), acylcarnitines (7), biogenic amines (8), glycerophospholipids (77), sphingolipids (14), and hexose (1). By a combination of univariate and multivariate analyses, we compared the ketosis group with the CON group at five time points separately. The results from both univariate and multivariate analyses showed that ketosis exerted a significant effect on the serum metabolome of dairy cows. In particular, a total of 21, 41, 28, 57, and 30 metabolites were found to have significant concentration differences in the serum at -8 wks, -4 wks, disease diagnosis, +4 wks, and +8 wks, respectively, around the expected day of parturition between the two groups. The mean ± SEM concentration values, *P* values along with the fold change, and direction of change (up or down) in cows with ketosis relative to CON cows are provided in Tables 1 and 2.

3.3.1 Metabolic alterations before disease diagnosis

Results of our univariate analysis indicated that there were 21 and 41 distinct metabolites in the serum at -8 wks and -4 wks prepartum between the two groups.

Specifically, 7 AAs, 4 biogenic amines, 4 glycerophospholipids, 1 acylcarnitine, 1 sphingolipid, and hexose were elevated, whereas 1 glycerophospholipid, 1 sphingolipid, and 1 amino acid were decreased in the serum of cows affected by ketosis at -8 wks prepartum (Table 3-1). The same alterations were also observed at -4 wks before occurrence of ketosis (1 acylcarnitine, 22 glycerophospholipids, 2 sphingolipids, 9 AAs, 4 biogenic amines and hexose were increased, and 2 glycerophospholipids were decreased in cows with ketosis compared with CON cows (Table 3-1).

Multivariate analysis showed that when serum metabolites from CON cows were compared with those from not-yet-diseased cows (those that eventually developed ketosis postpartum) at -8 wks and -4 wks, both score plots from the PCA and PLS-DA exhibited two well separated clusters at the two time points (Figure 3-1a&b, Figure 3-2a&b). Permutation testing ($P < 0.05$) revealed that the observed separation was not by chance and the results of cross validation were stable. A VIP plot of the PLS-DA from -8 wks and -4 wks in which the metabolites were ranked based on their importance in discriminating ketosis group from CON ones are shown in Figure 3-1c and Figure 3-2c. The top 15 important metabolites are shown in the VIP plots. The greater the VIP value the greater the contribution of a particular metabolite in distinguishing ketosis from CON cows. The VIP plots indicated that lysine (Lys), lysophosphatidylcholine acyl C17:0 (lysoPC a C17:0), lysophosphatidylcholine acyl C18:0 (lysoPC a C18:0), lysophosphatidylcholine acyl C16:0 (lysoPC a C16:0), isoleucine (Ile), kynurenine, and leucine (Leu) at -8 wks, and Lys, Ile, Leu,

kynurenine, carnosine, arginine (Arg), lysoPC a C17:0, and phosphatidylcholine acyl-alkyl C44:4 (PC ae C44:4) at -4 wks were the strongest discriminating metabolites for separating ketosis 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., lysophosphatidylcholine acyl (lyso PC a C28:0), sphingomyelin C20:2 (SM C20:2), and tryptophan (Trp)) that were decreased in pre-ketotic cows relative to CON ones. A ROC curve plot showing the performance of the top 7 metabolites at -8 wks (empirical $P = 0.001$) and top 8 metabolites at -4 wks (empirical $P = 0.001$) in predicting which cows will develop ketosis using a PLS-DA model is shown in Figure 3-1d & 3-2d. The AUC for two curves are 1 (95% CI, 1-1) at -8 wk and 0.992 (95% CI, 0.921-1) at -4 wks, respectively, which indicates that these serum biomarkers have strong predictive values. Interestingly, none of the 6-ketotic cows exhibited clinical signs of disease (normal concentrations of BHBA in the serum) at -8 and -4 wks prepartum (Table 2-4), which suggests that the subclinical disease state preceded at least -8 wks prior to parturition. The concentration of BHBA was greater in pre-ketotic cows than CON ones at -4 wks prior to parturition but did not reach the subclinical level (Table 2-4). Moreover, these results demonstrate that biomarker models developed at -8 and -4 wks could be used to predict which cows are susceptible to develop ketosis postpartum. In addition the results show that BHBA is not the best metabolite to predict ketosis at -8 or -4 wks prior to calving.

3.3.2 Metabolic alterations during the week of diagnosis of ketosis

Univariate analysis showed that 28 metabolites in serum had significant changes ($P < 0.05$) during the week of diagnosis of disease in ketosis vs CON subjects. Cows with ketosis experienced elevated concentrations of 4 acylcarnitines, 3 glycerophospholipids, 3 sphingolipids, 7 AAs, and 4 biogenic amines in their serum (Table 3-1). While 5 glycerophospholipids, 1 sphingolipid, and 1 amino acid were decreased in ketotic cows (Table 3-1).

When ketotic cows were compared with CON cows at the disease week, unsupervised multivariate analysis (i.e., PCA) and supervised multivariate analysis (i.e., PLS-DA; permutation test: $P < 0.05$) once again revealed a distinctive separation between the two groups of cows (Figure 3-3a, b). In this case, 5 metabolites [i.e., Lys, Leu, Ile, lysoPC a C28:0, sphingomyelin C20:2 (SM C20:2), kynurenine, hexadecanoyl-L-carnitine (C16), and acetyl-L-carnitine (C2)] with greatest VIP scores contributed most significantly to the observed separation (Figure 3-3c). The ROC curve (Figure 3-3d; empirical $P = 0.001$) indicated that this metabolite combination was a highly significant biomarker panel for ketosis with an AUC, 0.998 (95% CI, 1-1).

3.3.3 Metabolic alterations after diagnosis of ketosis

After recovery from ketosis, cows that were affected by ketosis still continued to have alterations in their serum metabolites at +4 and +8 wks postpartum in comparison with CON cows. Interestingly even though cows with ketosis were

healthy in appearance and had normal concentrations of serum BHBA at +4 wks after parturition (Zhang et al., 2016), they still had 57 metabolites altered in the serum compared to CON cows. In particular, all of 57 metabolites (i.e., 3 acylcarnitines, 31 glycerophospholipids, 11 sphingolipids, 7 AAs, 4 biogenic amines, and hexose) were elevated in ketosis cows at +4 wks postpartum. Intriguingly, at the +8 wks after parturition, cows affected with ketosis continued to have alterations of 30 metabolites including 29 (i.e., 2 acylcarnitines, 8 glycerophospholipids, 8 sphingolipids, 7 AAs, and 4 biogenic amines) enhanced, and 1 (i.e., aspartic acid) decreased.

Multivariate analyses [i.e., PCA and PLS-DA (permutation test: $P < 0.05$)] revealed that CON cows and ketosis cows were clearly separated at +4 wks and +8 wks postpartum (Figure 3-4b; Figure 3-5a & 3-5b). The corresponding VIP plots for these two time points are shown in Figure 3-4c & 3-5c, which indicated that Lys, C16, Ile, and Leu were the most discriminating metabolites at +4 wk and Lys, Ile, Leu, and acetylornithine were the top 5 metabolites for the separation of clusters at +8 wk. Multivariate models (ROC curves) combining 4 discriminating metabolites (i.e., Lys, C16, Ile, and Leu) at +4 wks (empirical $P < 0.05$) and 4 metabolites (i.e., Lys, Ile, Leu, and acetylornithine) at +8 wks (empirical $P < 0.05$) produced an area under the receiver-operating curve of 0.98 (95% CI: 0.75-1, Figure 3-4d) and 0.992 (95% CI: 1-1, Figure 3-5d), respectively.

3.3.4 Metabolic pathways associated with the onset and progression of ketosis

Quantitative enrichment analyses and metabolic pathway analyses were

conducted between the two groups at five time-points separately. Metabolic networks that we used in this study included Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2006), The Small Molecule Pathway Database (SMPDB) (Frolkis et al., 2010; Jewison et al., 2014), and Bovine Metabolome Database (BMDB) (http://www.cowmetdb.ca/cgi-bin/browse.cgi). Significant metabolic pathways and related metabolites that were involved in the development of ketosis are presented in Table 3-3. Results showed that the main metabolic alterations in cows with ketosis were related to Lys degradation, biotin metabolism, phospholipid metabolism, Trp metabolism, aspartate (Asp) metabolism, bile acid biosynthesis, and protein biosynthesis at -8 wks before parturition (Table 3-3 and Figure 3-6a). With regards to disturbance of serum metabolites in ketosis cows at -4 wks prepartum, it was observed that the main metabolic pathways affected were those of Lys degradation, biotin metabolism, Trp metabolism, protein biosynthesis, histidine (His) metabolism, Val-Leu-Ile degradation, and beta-alanine (Ala) metabolism (Table 3-3 and Figure 3-6b).

Metabolic pathway analyses also indicated that during the week of diagnosis of ketosis, diseased cows experienced altered concentrations of serum metabolites related to Lys degradation, biotin metabolism, Trp metabolism, Asp metabolism, and Val-Leu-Ile degradation (Table 3-3 and Figure 3-6c). Interestingly, cows with ketosis still encountered alterations of 4 metabolic pathways at +4 wks and 6 metabolic pathways at +8 wks postpartum, respectively, which indicates that metabolic alterations in key pathways in ketosis cows were still present even at +4 and +8 wks

after occurrence of clinical disease (Table 3-3 and Figure 3-6d & e).

It is interesting to note that throughout the 17-wks of the study, 7 serum metabolites (i.e., decanoyl-L-carnitine (C10), His, Ile, Leu, Lys, acetylornithine, and kynurenine) were elevated and appeared to play a consistent role in distinguishing between the CON and ketosis cows before and during the disease state (Table 3-1). Twenty-four metabolites in the serum of ketotic cows including C16, octadecanoylcarnitine (C18), lysoPC a C16:0, PC aa C30:0, PC aa C30:2, PC aa C42:2, PC ae C34:1, PC ae C44:4, SM (OH) C22:1, SM (OH) C22:2, SM (OH) C24:1, SM C16:0, SM C16:1, SM C18:0, SM C24:1, SM C26:0, Ile, Leu, Lys, Phe, Pro, acetylornithine, Kynurenine, and sarcosine were enhanced continuously after the diagnosis of disease (i.e., in both +4 and +8 wks) (Table 3-2). Furthermore, three metabolic pathways (i.e., Lys degradation, biotin metabolism, and Trp metabolism) were perturbed in cows with ketosis during the onset and progression of the disease.

3.4 Discussion

Ketosis is a metabolic disease of dairy cows “officially” characterized by increased concentrations of BHBA in the blood during the transition period. Although much is known about the etiology and pathogenesis of ketosis, the precise pathogenesis and the causal agent(s) is (are) not known. We hypothesized that multiple pathways and metabolites could be altered before, during, and after the diagnosis of ketosis and that these alterations could be used to screen cows before development of the disease as well as to better diagnose the disease and to learn more about the recovery process. Indeed utilization of the DI/LC-MS/MS based metabolic

profiling permitted the identification of numerous altered serum amino acid and phospholipid metabolites and that could be used as potential predictive or diagnostic biomarkers for ketosis. Furthermore, these metabolites may help to understand the biochemical processes associated with the development of this disease. Oscillations in amino acid and lipid metabolism have been previously described in various diseases of dairy cows by our group and others (Hailemariam et al., 2014a&2014b; Lamari et al., 2013).

The central role of amino acids (AAs) in protein synthesis, energy production, and immune functions have been reported and reviewed previously (Li et al., 2007; Moriwaki et al., 2004). It has been reported that catabolism of AAs contributes between 10 to 15% of energy production in humans (Pasquale, 2007). During the degradation processes, compounds related to ketosis also are released. Moreover, based on the types of intermediates generated during degradation of AAs they are classified as ketogenic, glucogenic, or both (D'Mello, 2003). Ketogenic AAs (i.e., Lys and Leu) are degraded into acetoacetate or acetyl-CoA, both of which are precursors of ketone bodies. Glucogenic AAs (i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser, and Val) are metabolized into one of the following five intermediates: α-ketoglutarate, fumarate, oxaloacetate, pyruvate, and succinyl CoA. Five larger AAs including Ile, Phe, Thr, Trp, and Tyr are both ketogenic and glucogenic (D'Mello, 2003). All the intermediates from degradation of AAs can enter the tricarboxylic acid (TCA) cycle, and are either totally oxidized into CO_2 and H_2O or diverted into ketogenesis and gluconeogenesis. More details about the pathways and metabolites

that were involved before, during, and after development of ketosis will be discussed below.

3.4.1 Lysine degradation pathway

Lysine was found to be elevated in the serum of ketotic cows during all time points evaluated in this study. The main question is what is the source of lysine in transition dairy cows? Komaragiri and Erdman (1997), utilizing an isotope dilution technique, estimated body composition in Holstein cows at -2, +5, and +12 wks relative to calving and indicated that transition cows mobilized ~21 kg of body protein along with ~54 kg of body fat between -2 wks and +5 wks around calving. As an essential and exclusively ketogenic AA, Lys is converted into acetoacetyl-CoA, an important component of carbohydrate metabolism and production of energy, and potentially in the generation of ketone bodies (Harvey and Ferrier, 2013). Moreover, lysine helps in the biosynthesis of carnitine, which covers an important role in lipid metabolism, acting as an obligatory cofactor for β-oxidation of fatty acids by facilitating the transport of long-chain fatty acids across the mitochondrial membrane as acylcarnitine esters (Hoppel, 2003). A reduction of the fatty acid transport inside the mitochondria results in the cytosolic accumulation of triglycerides (TG), which is implicated in the pathogenesis of fatty liver disease and insulin resistance.

Our data showed that there was no difference in DMI in both CON and pre-ketotic cows during the dry off period (Zhang et al., 2016), which suggests that there was no NEB in both groups of cows before the clinical appearance of ketosis.

Therefore it is speculated that greater serum Lys in non-yet-ketotic cows prior to parturition could be related to the inflammatory state identified in those cows and activation of immune response during prepartum period (Zhang et al., 2015). Lysine has been reported to strongly stimulate both the inflammatory and immune responses (i.e., humoral and cell mediated), improve healing process in both animal models and human subjects (both acute and chronic), and increase cellular proliferation both *in vitro* and *in vivo* (Datta et al., 2001). Moreover, Lys and other amino acids play an important role in immune system because they are necessary for synthesis of all innate and adaptive immunity proteins including cytokines, antibodies, and acute phase proteins (APP) (Aristoy and Toldrá, 2012). This assumption is based on our previously reported data, in a companion article, that pre-ketotic cows had elevated serum cytokines and APP before parturition (Zhang et al., 2016). Specifically, pre-ketotic cows experienced increased concentrations of serum interleukin (IL)-1 at -8 wks prepartum, and elevated concentrations of serum IL-1, IL-6, tumor necrosis factor (TNF), and haptoglobin (Hp) at -4 wks before parturition, respectively (Zhang et al., 2016).

3.4.2 Tryptophan metabolism pathway

A clear difference in Trp metabolism was detected between ketotic and CON cows before and during the diagnosis of disease. Specifically, cows with ketosis had greater concentrations of an intermediate of Trp metabolism, kynurenine, in their serum. The kynurenine system and its role in immunoregulation have been reported

previously. Conversion of Trp to kynurenine is catalyzed by the enzyme indoleamine 2,3-dioxygenase (IDO) that is induced by interferon (INF)-γ (Taylor and Feng, 1991). Conversion of Trp into kynurenine is an important mechanism to dampen inflammation and prevent growth of pathogenic bacteria that have potentially translocated into the systemic circulation (Mellor et al., 2003). In addition Mándi and Vécsei (2012) demonstrated that after activation of the Trp metabolic pathway by pro-inflammatory stimuli, the anti-inflammatory effect of kynurenic acid provides an effective feedback mechanism in modulating the immune response. It was reported that some of kynurenines, such as 3-hydroxyanthranilic acid and quinolinic acid could effectively suppress T cell proliferation (Fallarino et al., 2003). Furthermore, the production of quinolinic acid is often up-regulated after inflammation and activation of immune responses (Moffett et al., 1997). We believe that elevated levels of kynurenine in the serum of ketotic cows during the tested time points may be associated with its role as an anti-inflammatory compound triggered by activation of innate immunity in cows affected by ketosis (Zhang et al., 2016).

3.4.3 Valine, leucine, and isoleucine degradation pathway

Significantly elevated concentrations of serum metabolites involved in the Val-Leu-Ile degradation pathway were detected in cows with ketosis before, during, and after diagnosis of disease. In contrast with other AAs, the branched-chain AAs (BCAAs) Val, Leu, Ile are metabolized predominantly by the skeletal muscle rather than by the liver (Purpera et al., 2012). BCAAs play roles in regulation of protein

degradation and synthesis in skeletal muscle and other organs (Monirujjaman and Ferdouse, 2014). Cows with ketosis were in a state of NEB during the first two months of lactation as suggested by lower DMI (Zhang et al., 2016). Marked increases of circulating BCAAs might be related to mobilization of muscle proteins for energy yield during early lactation. On the other hand, degradation of BCAAs could also contribute to the development of ketosis. Particularly, Leu is an exclusively ketogenic AA, which is ultimately metabolized to acetoacetate and acetyl CoA and contributes to excessive accumulation of ketone bodies in the blood circulation (Bixel and Hamprecht, 1995). Ile is ketogenic and glucogenic and is metabolized to yield succinyl CoA and acetyl CoA. Valine is glucogenic and ultimately produces succinyl CoA, which enters the TCA cycle for energy production (D'Mello, 2003).

The effects of BCAAs on immune functions have been reviewed by several authors (Calder, 2006; Li et al., 2007). All three BCAAs are required for lymphocyte growth and proliferation (Monirujjaman and Ferdouse, 2014). Konashi et al. (2000) demonstrated that Leu has greater effects on immune function than Ile and Val. It was shown that Leu is a potent activator of mammalian target of rapamycin (mTOR) signaling pathway which accelerates protein synthesis in the liver and other organs (Buse et al., 1979; Li et al., 2007). Elevated concentrations of Leu in ketotic cows are in line with the data reported in our companion article that these cows had greater levels of pro-inflammatory cytokines and APP before and during the state of ketonemia (Zhang et al., 2016).

3.4.4 Bile acid biosynthesis pathway

Another important metabolic pathway affected in pre-ketotic or ketotic dairy cows was the bile acid biosynthesis pathway with glycine and taurine as the main elevated metabolites. Recent studies indicate that bile acids not only regulate their own synthesis and enterohepatic recirculation, they also influence triglyceride, cholesterol, energy, and glucose homeostasis as well as immunity. For example bile acids have been shown to decrease serum TG via decreased production of VLDL and increased VLDL clearance. It is known that transition dairy cows lower production and release of VLDL-TG during the postpartum period (Bobe et al., 2003). This has been suggested to contribute to the development of fatty liver disease in susceptible cows. Another interesting function of bile acids is their effect on cell-mediated immunity. Kawamata et al. (2003) showed that bile acids suppress functions of alveolar macrophages including phagocytosis and lipopolysaccharide-stimulated cytokine productions. Bile acids, including DCA (deoxycholic acid) and CDCA (chenodeoxycholic acid), have been reported to suppress LPS-induced production of cytokines in macrophages, including IL-1, IL-6, and TNF as well as inhibit LPS-induced TNF secretion in human lymphocytes (Greve et al., 1989). Our results suggest that the bile acid synthesis pathway might play a role in controlling the inflammatory state and in the immunosuppression commonly reported in transition dairy cows.

3.4.5 Histidine and glutathione metabolism pathways

Two other important pathways affected in cows with ketosis were the histidine metabolism pathway and the reduced glutathione (GSH) pathway. With regards to the histidine pathway, two metabolites carnosine and histidine were greater in cows affected by ketosis. Carnosine (β-alanil-L-histidine) is an endogenously synthesized peptide found in muscles, brain, and other tissues and is sometimes used as a precursor to histidine (Boldyrev and Severin, 1990). The contribution of carnosine and histidine in health issues has been shown by their ameliorating effects in diabetic animal models where their oral supplementation lowers blood glucose and increases insulin levels (Lee et al., 2005). Carnosine also is a potent antioxidant and has an important role as a pH buffer to protect against damage caused by oxidative stress (Decker et al., 2000). Increased concentrations of those two metabolites in the serum of ketotic cows suggest these compounds are being produced/released to protect against oxidative stress and to help regulate glucose metabolism around parturition.

Another interesting pathway affected in ketotic cows was that of reduced GSH metabolism with glycine as the main amino acid increased in ketotic cows in several time points near the week of disease diagnosis. Glycine availability is lowered during protein malnutrition, sepsis, and inflammatory stimuli (Persaud et al., 1996; Tapiero et al., 2002). Indeed ketotic cows in our experiment exhibited an innate immunity response around calving potentially as a reaction to an inflammatory insult during this period of time (Zhang et al., 2016). Moreover in vivo studies show that glycine availability limits GSH synthesis during states of malnutrition (Persaud et al., 1996).

Provision of sulfur-containing AAs as well as glutamate (glutamine or BCAAs) and glycine (or serine) is critical for maximizing GSH synthesis. Elevated blood glycine and serine in our ketotic cows could be a homeostatic response to regulate GSH synthesis and manage the oxidative stress in those cows.

3.4.6 Protein biosynthesis pathway

Serum levels of 12 AAs from the protein biosynthesis pathway were greater in cows with ketosis during the dry off period. There were no differences in DMI between ketotic and CON cows from -8 wks prepartum until parturition. However, serum BHBA was increased at -4 wks before parturition in cows with ketosis (Zhang et al., 2016). It has been reported that BHBA could suppress AAs (e.g., Leu) oxidation and promote protein synthesis (Nair et al., 1988). Elevated concentrations of serum AAs and increased involvement of protein biosynthesis pathway in ketotic cows before parturition might be correlated with muscle protein breakdown and synthesis of innate immunity reactants (i.e., proteins like pro-inflammatory cytokines, APP, and antibodies), which are initiated by bacterial infection or inflammation.

Beside the anabolic role(s), AAs also serve as important immunoregulators in the following aspects: 1) activation of immune cells such as macrophages, natural killer cells, T lymphocytes, and B lymphocytes; 2) production of cytokines, antibodies, and other cytotoxic substances; and 3) cellular redox state, gene expression, and lymphocyte proliferation (Li et al., 2007). Increased levels of AAs (i.e., Arg, Cit, Glu, His, Ile, Leu, Lys, and Ser) in the serum of both pre-ketotic and ketotic cows suggest

involvement of immune response or presence of systemic inflammation during the onset and progression of ketosis. Activation of systemic inflammation might be associated with bacteria or pathogen-derived insults at the early stages of ketosis. However, it is speculated that elevated concentrations of AAs in the serum of pre-ketotic, ketotic, and post-ketotic cows from -8 wks prepartum until 8 wks postpartum might be attributed to a state of "low-grade" or "chronic" inflammation (i.e., metainflammation or metabolically triggered inflammation).

Elevated concentrations of AAs in the blood and increased protein synthesis lead to impairment of endoplasmic reticulum (ER) functions or ER stress, which are characterized by activation of the unfolded protein response (UPR) and subsequent initiation of major inflammatory and stress-signaling networks via several distinct mechanisms including activation of c-*jun* N-terminal kinase activator protein-1 (JNK-AP-1) and IKB kinase nuclear factor κB (IKK-NFκB) pathways and production of reactive oxygen species (ROS) (Gregor and Hotamisligil, 2007). The ER stress and related signaling networks have been reported as a potential site for the intersection of inflammation and metabolic diseases in both dairy cows (e.g., ketosis and fatty liver) and human subjects (e.g., obesity and type 2 diabetes) (Gessner et al., 2014; Hummasti and Hotamisligil, 2010; Ringseis et al., 2014).

3.4.7 Phospholipid biosynthesis pathway

Ketotic cows were observed to have significant alterations in phospholipid metabolism. In particular, serum levels of lysoPC a C16:0 were greater in ketotic

cows at -8 wks before parturition and +4 wks postpartum. LysoPC derives from the oxidation of low-density lipoproteins (LDL) by ROS, released during inflammatory processes (Matsumoto et al., 2007). LysoPC (e.g., 16:0-, 18:0-, 18:1-, 18:2-, and 20:4-lysoPC species) is a strong proinflammatory mediator and augments inflammation through adhesion molecules, growth factors, monocytes, macrophages, neutrophils, and T lymphocytes (Takatera et al., 2007; Yoder et al., 2014). A growing body of evidence suggests that lysoPC can regulate the function of immune cells to participate in inflammatory processes and immune responses. LysoPC is considered to be an important factor in certain chronic inflammatory diseases, in which local and systemic elevation of lysoPC is characteristic. For example, in a recent study Qin et al. (2014) reported that lysoPC promotes polarization of a pro-inflammatory M1 macrophage that produces proinflammatory cytokines such as IL-1, IL-6, TNF, and IL-12. M1 polarization plays a critical role in the inflammatory process by creating a highly pro-inflammatory milieu to allow differentiation and migration of inflammatory T cells to the inflammation site (Krausgruber et al., 2011). LysoPC a C16:0 has been identified to induce the release of chemokines (i.e., macrophage inflammatory protein 1, monocyte chemoattractant protein 1, and IL-8) (Bath et al., 2010). Moreover, lysoPC a C16:0 can also engage naïve T cells to produce IFN-γ and secrete IL-6 and IL-5 in the blood and support humoral responses through activation of Th2 cells (Bath et al., 2010). Elevated concentrations of LysoPC a C16:0 can be attributed to an inflammatory state in cows with ketosis before and after parturition and mounting of an immune response.

3.4.8 Other metabolites and related metabolic pathways

Alterations of sphingolipids in the serum were also observed in cows with ketosis during all tested time points, which is in agreement with results from our previous study (Hailemariam et al., 2014b). Due to limitations of metabolic pathway databases, most phospholipids are not very well described. However, intermediates of sphingomyelin (SM) like ceramide, ceramide-1-phosphate (C1P), phingosylphosphorylcholine, sphingosine-1-phosphate (S1P), and sphingosine have been documented as being important signaling substances for regulation of several cellular processes that are key to immunity, inflammation, and disease (Brodesser et al., 2003; Kolter and Sandhoff, 2006; Maceyka and Spiegel, 2014). Elevated serum levels of sphingolipids during -8 and -4 wks prepartum, the disease week, and early lactation suggest that inflammation and activation of immunity play important roles in the onset and progression of ketosis.

Several acylcarnitines also were identified to be increased in ketotic cows, especially during the disease week and after parturition. Cows with ketosis were experiencing a state of NEB, due to lower DMI during this period. Rinaldo et al. (2008) reported that acylcarnitines are reliable biomarkers for disorders of fatty acid oxidation. Elevated concentrations of acylcarnitines might be explained by excessive body fat mobilization during the NEB and incomplete β-oxidation of long-chain fatty acids. Furthermore, medium-chain acylcarnitines [i.e., decanoyl-L-carnitine (C10)], can contribute to dysregulated fatty acid oxidation in mitochondria by activation of pro-inflammatory signaling pathways (Adams et al., 2009; Rutkowsky et al., 2014). It

is speculated that accumulation of acylcarnitines might have induced insulin resistance and increased concentration of C10 in the serum of pre-ketotic cows at -8 wks and -4 wks prepartum; also suggesting presence of inflammation (Schooneman et al., 2013).

It is notable that ketotic cows continued to have alterations of serum metabolites and metabolic pathways at +4 and +8 wks postpartum although with no obvious (diagnosed) metabolic or infectious diseases (i.e., healthy in appearance and with normal serum BHBA). Elevated concentrations of acylcarnitines, glycerophospholipids, sphingolipids, AAs, biogenic amines, and hexose in post-ketotic cows indicated a state of sub-acute inflammation lasted until +4 and +8 wks postpartum. The severity of ketosis has only been evaluated with concentrations of ketone bodies (e.g., BHBA) in blood in the past. However, results from the current study demonstrate that the effects of ketosis lasted longer than blood BHBA adjusted to normal levels. we believe it is of great importance for dairy producers to extend their attention to transition dairy cows until +8 wks after parturition.

Due to the low number of cows in the ketotic group, the findings reported here must be considered preliminary. In addition, a limited number of metabolites were quantified from the perturbed metabolic pathways. More research with a larger number of cows and more metabolites or intermediates included in the metabolic analysis is warranted to elucidate their precise role in the pathogenesis of ketosis.

3.5 Conclusions

In conclusion, cows with ketosis experienced altered concentrations of blood

metabolites related to AA, carbohydrate, and lipid metabolism before, during, and after appearance of clinical signs of ketosis. It was notable that 7 serum metabolites (i.e., C10, His, Ile, Leu, Lys, acetylornithine, and kynurenine) were elevated and appeared to play a consistent role in distinguishing between the CON and ketotic cows before and during the disease state. Twenty-four metabolites in the serum of ketotic cows including Lys, C16, Ile, Leu, acetylornithine, lysoPC a C16:0, sarcosine, Phe, and SM (OH) C22:1 were elevated consistently after diagnosis of disease (i.e., at both +4 and +8 wks). Furthermore, several metabolic pathways were perturbed in cows with ketosis during the onset and progression of disease. These new findings give insights into further understanding the pathogenesis of ketosis in dairy cows. Biomarker analysis showed that AUCs for 5 ROC curves were 1 (95% CI, 1-1) at -8 wks, 0.992 (95% CI, 0.921-1) at -4 wks, 0.998 (95% CI, 1-1) at disease wk, 0.98 (95% CI: 0.75-1) at +4 wks and 0.992 (95% CI: 1-1) at +8 wks, respectively, which indicate that serum biomarkers identified have very accurate predictive, diagnostic, and prognostic abilities for ketosis in transition dairy cows.

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Table 3-1. Concentrations of serum metabolites (mean (SEM)) in healthy control (CON) and ketosis cows at 3 time points (-8 wk, -4 wk, and the wk of diagnosis of disease) as determined by DI/LC-MS/MS

	8 week before parturition					4 week before parturition					Ketosis diagnosis week[1]				
Metabolite, μM[2]	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	20	-	-	-	6	20	-	-	-	6	20	-	-	-
C10	0.07 (0.02)	0.02 (0.01)	0.003*	3.5	Up	0.04 (0.002)	0.03 (0.01)	0.003*	1.33	Up	0.06 (0.02)	0.03 (0.01)	0.002*	2	Up
C16	0.02 (0.003)	0.02 (0.003)	1	1	Down	0.01 (0.003)	0.03 (0.003)	0.447	-3	Down	0.05 (0.01)	0.02 (0.00)	0.003*	2.5	Up
C18	0.02 (0.002)	0.03 (0.003)	0.519	-1.5	Down	0.02 (0.004)	0.04 (0.01)	0.192	-2	Down	0.09 (0.02)	0.04 (0.01)	0.014*	2.25	Up
C2	1.48 (0.33)	1.30 (0.10)	0.869	1.14	Up	1.17 (0.08)	1.43 (0.22)	0.717	-1.22	Down	2.59 (0.47)	1.22 (0.11)	0.004*	2.12	Up
C3	0.14 (0.01)	0.14 (0.01)	0.488	1	Up	0.14 (0.02)	0.14 (0.02)	0.621	1	Up	0.13 (0.02)	0.15 (0.05)	0.219	-1.15	Down
C4	0.09 (0.01)	0.08 (0.01)	0.717	1.13	Up	0.08 (0.01)	0.07 (0.01)	0.476	1.14	Up	0.08 (0.01)	0.07 (0.00)	0.242	1.14	Up
C5	0.07 (0.01)	0.08 (0.01)	0.519	-1.14	Down	0.06 (0.004)	0.06 (0.01)	0.974	1	Down	0.05 (0.01)	0.06 (0.00)	0.744	-1.2	Down
lysoPC a C16:0	69.75 (8.39)	27.89 (2.68)	0.002*	2.5	Up	40.52 (8.99)	24.44 (2.64)	0.051	1.66	Up	55.91 (16.13)	23.78 (2.09)	0.157	2.35	Up
lysoPC a C16:1	2.06 (0.22)	1.58 (0.17)	0.071	1.3	Up	1.35 (0.14)	1.26 (0.13)	0.818	1.07	Up	1.51 (0.17)	1.26 (0.11)	0.219	1.2	Up
lysoPC a C17:0	9.43 (0.92)	3.03 (0.36)	0.001*	3.11	Up	7.01 (1.97)	2.00 (0.24)	0.006*	3.51	Up	2.87 (0.94)	1.85 (0.23)	0.533	1.55	Up
lysoPC a C18:0	73.40 (5.18)	25.79 (3.09)	0.001*	2.85	Up	51.51 (11.84)	19.49 (2.33)	0.024*	2.64	Up	41.15 (12.75)	17.67 (1.70)	0.176	2.33	Up
lysoPC a C18:1	20.81 (1.89)	16.09 (1.54)	0.06	1.29	Up	14.35 (1.50)	14.14 (1.58)	0.974	1.01	Up	19.33 (3.49)	13.37 (1.38)	0.196	1.45	Up
lysoPC a C18:2	16.26 (2.55)	18.49 (1.87)	0.767	-1.14	Down	10.44 (1.44)	17.12 (2.09)	0.083	-1.64	Down	12.98 (0.99)	17.43 (2.01)	0.268	-1.34	Down
lysoPC a C20:3	2.67 (0.36)	3.25 (0.33)	0.668	-1.22	Down	1.92 (0.35)	1.67 (0.17)	0.717	1.15	Up	0.78 (0.09)	1.82 (0.22)	0.013*	-2.33	Down
lysoPC a C20:4	2.54 (0.35)	3.61 (0.36)	0.071	-1.42	Down	1.92 (0.30)	2.24 (0.18)	0.447	-1.67	Down	1.18 (0.13)	2.30 (0.26)	0.046*	-1.95	Down
lysoPC a C28:0	0.43 (0.08)	0.83 (0.05)	0.002*	-1.93	Down	0.39 (0.10)	0.61 (0.05)	0.129	-1.56	Down	0.24 (0.05)	0.63 (0.05)	0.001*	-2.63	Down
lysoPC a C28:1	0.48 (0.09)	0.75 (0.10)	0.051	-1.56	Down	0.43 (0.06)	0.51 (0.04)	0.408	-1.19	Down	0.40 (0.06)	0.53 (0.04)	0.176	1.33	Down
PC aa C28:1	2.03 (0.51)	1.86 (0.18)	1	1.09	Up	1.20 (0.11)	1.54 (0.19)	0.447	-1.28	Down	1.99 (0.39)	1.66 (0.15)	0.457	1.2	Up
PC aa C30:0	3.48 (0.84)	2.54 (0.25)	0.336	1.37	Up	2.44 (0.19)	2.17 (0.26)	0.243	1.12	Up	3.74 (0.50)	2.28 (0.21)	0.013*	1.64	Up
PC aa C30:2	0.74 (0.23)	0.33 (0.05)	0.071	2.24	Up	0.59 (0.11)	0.23 (0.04)	0.010*	2.57	Up	0.59 (0.12)	0.28 (0.05)	0.009*	2.11	Up
PC aa C32:0	7.39 (1.51)	8.61 (0.75)	0.371	-1.17	Down	5.23 (0.48)	7.06 (0.78)	0.192	-1.35	Down	6.46 (0.69)	6.68 (0.61)	0.79	-1.03	Down
PC aa C32:1	5.78 (1.49)	7.88 (0.71)	0.169	-1.36	Down	4.01 (0.19)	6.74 (0.81)	0.083	-1.68	Down	7.30 (1.09)	6.70 (0.59)	0.656	1.09	Up
PC aa C32:2	9.32 (3.35)	8.44 (0.89)	0.869	1.1	Up	5.90 (1.16)	5.99 (0.69)	1	-1.02	Down	5.67 (1.06)	6.33 (0.59)	0.656	-1.12	Down
PC aa C32:3	17.29 (6.33)	15.78 (1.97)	1	1.1	Up	9.74 (2.49)	10.64 (1.30)	0.767	-1.09	Down	9.03 (1.43)	11.33 (1.12)	0.421	-1.25	Down
PC aa C34:1	77.36 (13.85)	97.65 (8.90)	0.169	-1.26	Down	56.08 (2.82)	99.96 (14.13)	0.169	-1.78	Down	127.76 (18.00)	94.61 (10.26)	0.072	1.35	Up
PC aa C34:2	101.76 (27.30)	144.82 (13.13)	0.148	-1.42	Down	60.72 (3.33)	154.36 (23.31)	0.035*	-2.54	Down	154.72 (22.48)	147.47 (15.38)	0.533	1.05	Up
PC aa C34:3	17.62 (6.19)	28.61 (2.98)	0.083	-1.62	Down	11.89 (1.26)	23.14 (2.75)	0.035*	-1.95	Down	17.28 (2.49)	24.09 (2.44)	0.123	-1.39	Down

PC aa C34:4	7.61 (2.00)	7.44 (0.91)	0.818	1.02	Up	4.99 (1.28)	3.86 (0.41)	0.575	1.29	Up	2.66 (0.38)	4.39 (0.55)	0.095	-1.65	Down
PC aa C36:0	7.84 (1.10)	6.74 (0.77)	0.488	1.16	Up	8.03 (1.70)	4.40 (0.52)	0.024*	1.83	Up	4.74 (1.03)	4.89 (0.64)	0.976	-1.03	Down
PC aa C36:1	93.96 (8.51)	105.11 (9.08)	0.303	-1.12	Down	80.03 (7.23)	90.50 (10.75)	0.767	-1.13	Down	100.99 (17.81)	87.64 (8.59)	0.656	1.15	Up
PC aa C36:2	136.35 (19.96)	166.65 (14.53)	0.243	-1.22	Down	103.96 (3.55)	157.08 (19.70)	0.071	-1.51	Down	163.40 (25.32)	149.78 (14.23)	0.573	1.09	Up
PC aa C36:3	62.75 (11.39)	79.46 (7.61)	0.303	-1.27	Down	45.79 (2.12)	69.68 (8.48)	0.097	-1.52	Down	70.64 (14.32)	69.02 (6.53)	0.744	1.02	Up
PC aa C36:4	29.93 (4.95)	39.05 (3.45)	0.216	-1.3	Down	21.97 (1.33)	30.96 (3.49)	0.216	-1.41	Down	34.15 (5.49)	31.91 (2.98)	0.929	1.07	Up
PC aa C36:5	8.19 (1.75)	10.24 (0.94)	0.112	-1.25	Down	5.80 (0.76)	7.96 (0.79)	0.243	-1.37	Down	8.17 (1.04)	8.14 (0.82)	0.976	1	Up
PC aa C36:6	3.55 (1.00)	3.30 (0.33)	0.974	1.08	Up	2.63 (0.61)	2.13 (0.20)	0.621	1.23	Up	1.85 (0.42)	2.49 (0.24)	0.219	-1.35	Down
PC aa C38:0	1.89 (0.40)	1.87 (0.20)	0.974	1.01	Up	2.23 (0.48)	1.28 (0.16)	0.083	1.74	Up	0.91 (0.17)	1.34 (0.17)	0.219	-1.47	Down
PC aa C38:1	4.15 (1.25)	4.24 (0.62)	0.621	-1.02	Down	5.51 (1.23)	2.84 (0.39)	0.015*	1.94	Up	2.28 (0.69)	3.22 (0.44)	0.355	-1.41	Down
PC aa C38:3	53.14 (5.72)	55.71 (5.47)	0.974	-1.05	Down	41.21 (3.83)	30.27 (3.09)	0.129	1.39	Up	23.53 (4.82)	34.70 (4.31)	0.268	-1.47	Down
PC aa C38:4	49.87 (4.65)	56.15 (4.79)	0.668	-1.13	Down	40.97 (2.40)	36.75 (3.51)	0.767	1.11	Up	34.61 (4.88)	39.73 (4.36)	0.573	-1.15	Down
PC aa C38:5	24.38 (3.63)	25.35 (2.17)	0.575	-1.04	Down	18.51 (1.00)	21.00 (2.22)	0.519	-1.13	Down	25.72 (3.75)	21.56 (2.14)	0.656	1.19	Up
PC aa C38:6	4.10 (0.78)	4.27 (0.35)	0.53	-1.04	Down	2.90 (0.21)	3.97 (0.52)	0.447	-1.37	Down	5.18 (0.86)	4.18 (0.45)	0.457	1.24	Up
PC aa C40:2	0.43 (0.07)	0.35 (0.05)	0.243	1.23	Up	0.38 (0.07)	0.20 (0.02)	0.019*	1.9	Up	0.17 (0.02)	0.23 (0.03)	0.387	-1.35	Down
PC aa C40:3	4.75 (0.95)	4.41 (0.64)	0.53	1.08	Up	4.90 (0.81)	1.97 (0.35)	0.001*	2.49	Up	0.88 (0.21)	2.17 (0.46)	0.046*	-2.47	Down
PC aa C40:4	14.70 (1.83)	11.72 (1.16)	0.243	1.25	Up	13.93 (1.89)	6.47 (0.84)	0.004*	2.15	Up	4.47 (0.77)	7.40 (1.13)	0.219	-1.66	Down
PC aa C40:5	22.95 (2.59)	18.80 (1.69)	0.303	1.22	Up	18.30 (1.69)	14.34 (1.55)	0.243	1.28	Up	15.40 (2.37)	15.78 (1.65)	0.836	-1.02	Down
PC aa C40:6	3.91 (0.43)	3.49 (0.27)	0.53	1.12	Up	3.26 (0.17)	3.17 (0.35)	0.717	1.03	Up	4.31 (0.67)	3.67 (0.40)	0.421	1.17	Up
PC aa C42:1	0.09 (0.01)	0.07 (0.01)	0.148	1.29	Up	0.09 (0.02)	0.05 (0.00)	0.019*	1.8	Up	0.05 (0.01)	0.05 (0.00)	0.744	1	Down
PC aa C42:2	0.15 (0.01)	0.12 (0.01)	0.169	1.25	Up	0.14 (0.01)	0.08 (0.01)	0.003*	1.75	Up	0.08 (0.01)	0.08 (0.01)	0.692	1	Down
PC aa C42:4	0.25 (0.02)	0.25 (0.03)	0.717	1	Up	0.27 (0.03)	0.13 (0.02)	0.002*	2.08	Up	0.06 (0.01)	0.14 (0.03)	0.139	-2.33	Down
PC aa C42:5	0.90 (0.16)	0.81 (0.09)	0.621	1.11	Up	1.11 (0.27)	0.54 (0.08)	0.035*	2.06	Up	0.25 (0.06)	0.50 (0.09)	0.196	-2	Down
PC aa C42:6	0.40 (0.03)	0.36 (0.02)	0.359	1.11	Up	0.44 (0.06)	0.28 (0.03)	0.007*	1.57	Up	0.26 (0.03)	0.27 (0.02)	0.882	-1.04	Down
PC ae C30:0	0.76 (0.11)	0.72 (0.06)	0.818	1.06	Up	0.57 (0.05)	0.56 (0.05)	0.869	1.02	Up	0.65 (0.08)	0.60 (0.06)	0.614	1.08	Up
PC ae C30:1	1.92 (0.57)	1.32 (0.18)	0.272	1.45	Up	1.59 (0.30)	0.83 (0.10)	0.006*	1.92	Up	1.56 (0.29)	0.90 (0.11)	0.033*	1.73	Up
PC ae C32:1	2.83 (0.77)	3.82 (0.37)	0.169	-1.35	Down	2.05 (0.32)	3.10 (0.33)	0.112	-1.51	Down	2.75 (0.27)	3.07 (0.27)	0.295	-1.12	Down
PC ae C32:2	5.70 (1.60)	7.13 (0.80)	0.408	-1.25	Down	3.61 (0.57)	5.26 (0.68)	0.272	-1.46	Down	4.07 (0.80)	5.48 (0.55)	0.196	-1.35	Down
PC ae C34:0	3.44 (0.50)	3.66 (0.36)	0.974	-1.06	Down	2.85 (0.25)	2.35 (0.24)	0.303	1.21	Up	1.67 (0.20)	2.49 (0.34)	0.324	-1.49	Down
PC ae C34:1	12.60 (2.11)	15.63 (1.42)	0.192	-1.24	Down	10.37 (0.41)	11.80 (1.12)	0.408	-1.14	Down	11.26 (1.68)	11.87 (1.15)	0.614	-1.05	Down
PC ae C34:2	17.06 (4.78)	18.55 (1.95)	0.488	-1.09	Down	11.70 (1.01)	14.92 (1.74)	0.371	-1.28	Down	15.49 (2.69)	14.90 (1.48)	0.882	1.04	Up
PC ae C34:3	19.37 (7.28)	19.30 (2.50)	0.818	1	Up	10.25 (1.53)	15.06 (2.07)	0.243	-1.47	Down	15.20 (2.29)	15.57 (1.71)	0.976	-1.02	Down
PC ae C36:0	1.90 (0.17)	2.45 (0.24)	0.336	-1.29	Down	1.82 (0.15)	1.62 (0.18)	0.575	1.12	Up	0.92 (0.15)	1.75 (0.20)	0.046*	-1.9	Down

PC ae C36:1	17.34 (0.90)	17.77 (1.47)	0.818	-1.02	Down	15.50 (1.45)	12.83 (1.18)	0.53	1.21	Up	12.04 (2.09)	12.63 (1.23)	0.7	-1.05	Down
PC ae C36:2	20.98 (3.73)	23.00 (2.23)	0.575	-1.1	Down	15.18 (1.39)	17.17 (1.59)	0.621	-1.13	Down	14.82 (2.63)	16.98 (1.62)	0.494	-1.15	Down
PC ae C36:3	8.65 (1.78)	10.60 (1.17)	0.53	-1.23	Down	6.46 (0.71)	7.58 (0.74)	0.447	-1.17	Down	6.20 (0.97)	7.83 (0.75)	0.242	-1.26	Down
PC ae C36:4	9.14 (2.36)	9.19 (1.09)	0.869	-1.01	Down	5.72 (0.70)	6.13 (0.68)	0.921	-1.07	Down	5.78 (0.92)	6.37 (0.59)	0.533	-1.1	Down
PC ae C36:5	7.10 (1.59)	7.47 (0.81)	1	-1.05	Down	4.70 (0.58)	5.25 (0.49)	0.488	-1.12	Down	5.09 (0.50)	5.56 (0.52)	0.494	-1.09	Down
PC ae C38:0	1.71 (0.35)	1.59 (0.14)	0.974	1.08	Up	1.57 (0.21)	1.14 (0.09)	0.129	1.38	Up	1.15 (0.17)	1.27 (0.11)	0.573	-1.1	Down
PC ae C38:1	3.60 (0.33)	3.11 (0.31)	0.303	1.16	Up	3.31 (0.30)	1.76 (0.23)	0.006*	1.88	Up	1.60 (0.30)	1.85 (0.28)	0.79	-1.16	Down
PC ae C38:2	3.26 (0.35)	3.66 (0.35)	0.818	-1.12	Down	2.97 (0.07)	2.31 (0.22)	0.216	1.29	Up	1.97 (0.53)	2.37 (0.27)	0.421	-1.2	Down
PC ae C38:3	6.67 (0.60)	6.06 (0.63)	0.575	1.1	Up	5.45 (0.57)	3.21 (0.33)	0.010*	1.7	Up	2.49 (0.44)	3.50 (0.49)	0.421	-1.41	Down
PC ae C38:4	7.07 (0.64)	7.48 (0.71)	0.974	-1.06	Down	5.73 (0.63)	4.71 (0.47)	0.303	1.22	Up	3.35 (0.43)	5.06 (0.68)	0.196	-1.51	Down
PC ae C38:5	4.93 (0.72)	5.31 (0.49)	0.869	-1.08	Down	3.71 (0.30)	3.70 (0.34)	0.575	1	Up	3.20 (0.43)	4.01 (0.40)	0.242	-1.25	Down
PC ae C38:6	4.16 (1.15)	4.02 (0.39)	0.818	1.03	Up	2.89 (0.33)	3.00 (0.32)	0.919	-1.04	Down	3.46 (0.40)	3.47 (0.33)	0.836	1	Down
PC ae C40:1	0.43 (0.07)	0.38 (0.04)	0.717	1.13	Up	0.37 (0.06)	0.28 (0.03)	0.216	1.32	Up	0.17 (0.02)	0.29 (0.04)	0.095	-1.71	Down
PC ae C40:2	1.15 (0.11)	0.94 (0.10)	0.192	1.22	Up	0.93 (0.09)	0.65 (0.06)	0.083	1.43	Up	0.67 (0.10)	0.71 (0.08)	0.656	-1.06	Down
PC ae C40:3	1.63 (0.18)	1.47 (0.16)	0.447	1.11	Up	1.25 (0.14)	0.78 (0.08)	0.015*	1.6	Up	0.56 (0.14)	0.94 (0.15)	0.268	-1.96	Down
PC ae C40:4	1.95 (0.16)	2.08 (0.20)	0.974	-1.07	Down	1.63 (0.14)	1.18 (0.14)	0.129	1.38	Up	0.71 (0.13)	1.39 (0.21)	0.139	-1.33	Down
PC ae C40:5	3.09 (0.35)	2.71 (0.24)	0.53	1.14	Up	2.50 (0.32)	1.94 (0.21)	0.336	1.29	Up	1.60 (0.24)	2.12 (0.24)	0.268	-1.18	Down
PC ae C40:6	1.17 (0.22)	1.05 (0.08)	0.818	1.11	Up	0.92 (0.11)	0.84 (0.07)	0.974	1.1	Up	0.83 (0.12)	0.98 (0.09)	0.355	-1.56	Down
PC ae C42:1	0.15 (0.03)	0.20 (0.02)	0.243	-1.33	Down	0.11 (0.01)	0.13 (0.01)	0.169	-1.18	Down	0.09 (0.01)	0.14 (0.02)	0.107	-1.4	Down
PC ae C42:2	0.24 (0.02)	0.22 (0.02)	0.717	1.09	Up	0.23 (0.03)	0.14 (0.01)	0.010*	1.64	Up	0.10 (0.02)	0.14 (0.02)	0.242	-1.5	Down
PC ae C42:3	0.26 (0.03)	0.23 (0.02)	0.408	1.13	Up	0.22 (0.03)	0.13 (0.01)	0.019*	1.69	Up	0.10 (0.03)	0.15 (0.02)	0.268	1	Down
PC ae C44:3	0.06 (0.01)	0.05 (0.00)	0.336	1.2	Up	0.05 (0.01)	0.03 (0.003)	0.035*	1.67	Up	0.03 (0.01)	0.03 (0.00)	0.744	1	Down
PC ae C44:4	0.10 (0.004)	0.08 (0.004)	0.019*	1.25	Up	0.09 (0.01)	0.06 (0.003)	0.007*	1.5	Up	0.05 (0.01)	0.05 (0.01)	0.882	1	Down
PC ae C44:5	0.08 (0.01)	0.07 (0.01)	0.668	1.14	Up	0.08 (0.01)	0.05 (0.004)	0.029*	1.6	Up	0.05 (0.01)	0.05 (0.00)	0.79	1	Down
SM (OH) C14:1	13.89 (1.41)	10.95 (0.87)	0.097	1.27	Up	8.98 (0.71)	8.94 (0.90)	0.767	1	Up	10.91 (1.80)	9.73 (0.83)	0.614	1.12	Up
SM (OH) C16:1	11.49 (0.97)	8.48 (0.81)	0.071	1.35	Up	8.65 (0.83)	6.26 (0.56)	0.097	1.38	Up	7.03 (1.13)	6.84 (0.65)	1	1.03	Up
SM (OH) C22:1	20.88 (3.81)	12.57 (1.34)	0.051	1.66	Up	12.64 (1.39)	11.97 (1.76)	0.371	1.06	Up	24.28 (5.19)	12.23 (1.26)	0.016*	1.99	Up
SM (OH) C22:2	7.80 (0.96)	5.55 (0.55)	0.097	1.41	Up	5.57 (0.52)	4.36 (0.46)	0.192	1.28	Up	7.29 (1.30)	4.45 (0.41)	0.046*	1.64	Up
SM (OH) C24:1	2.13 (0.25)	1.24 (0.12)	0.015*	1.72	Up	1.43 (0.13)	1.00 (0.10)	0.051	1.43	Up	1.50 (0.27)	1.03 (0.09)	0.083	1.46	Up
SM C16:0	100.15 (13.66)	83.03 (7.45)	0.303	1.21	Up	69.02 (4.20)	70.51 (8.21)	0.717	-1.02	Down	103.53 (15.86)	74.72 (7.00)	0.139	1.39	Up
SM C16:1	11.72 (1.52)	9.75 (0.86)	0.303	1.2	Up	8.13 (0.42)	6.85 (0.70)	0.371	1.19	Up	8.66 (1.29)	7.54 (0.69)	0.614	1.15	Up
SM C18:0	12.17 (1.66)	9.37 (0.85)	0.234	1.3	Up	9.03 (0.65)	8.17 (0.87)	0.371	1.11	Up	13.05 (2.01)	8.48 (0.81)	0.046*	1.54	Up
SM C18:1	4.31 (0.54)	3.97 (0.37)	0.488	1.09	Up	3.17 (0.20)	3.27 (0.34)	0.575	-1.03	Down	4.36 (0.62)	3.43 (0.34)	0.295	1.27	Up

SM C20:2	0.07 (0.04)	0.28 (0.05)	0.013*	-4	Down	0.06 (0.03)	0.17 (0.02)	0.053	-2.83	Down	0.07 (0.03)	0.16 (0.02)	0.041*	-2.29	Down
SM C24:0	25.35 (4.57)	17.37 (2.01)	0.112	1.46	Up	21.52 (2.45)	12.94 (1.35)	0.012*	1.66	Up	19.10 (3.18)	13.85 (1.53)	0.108	1.38	Up
SM C24:1	10.12 (1.82)	8.08 (0.79)	0.336	1.25	Up	7.04 (1.20)	7.65 (0.78)	0.668	-1.09	Down	11.29 (2.07)	7.45 (0.80)	0.095	1.52	Up
SM C26:0	0.39 (0.07)	0.24 (0.02)	0.071	1.63	Up	0.38 (0.05)	0.19 (0.02)	0.002*	2	Up	0.26 (0.05)	0.19 (0.02)	0.108	1.37	Up
SM C26:1	0.25 (0.13)	0.10 (0.02)	0.475	2.5	Up	0.14 (0.10)	0.13 (0.02)	0.262	1.08	Up	0.16 (0.05)	0.12 (0.02)	0.543	1.33	Up
Hexose	4,102 (213)	3,093 (232)	0.019*	1.33	Up	3,268 (219)	2,458 (202)	0.035*	1.33	Up	2,823 (188)	2,354 (179)	0.176	1.2	Up
Alanine	198.80 (35.25)	195.40 (10.64)	0.76	1.02	Up	218.02 (18.15)	158.36 (10.07)	0.032*	1.38	Up	186.68 (31.03)	135.39 (12.93)	0.235	1.38	Up
Arginine	189.27 (47.79)	108.14 (7.43)	0.045*	1.75	Up	162.04 (10.92)	103.05 (5.91)	0.002*	1.57	Up	133.06 (16.38)	96.03 (7.56)	0.051	1.38	Up
Asparagine	16.35 (3.04)	30.59 (3.78)	0.06	-1.87	Down	23.98 (6.38)	26.45 (2.54)	0.76	-1.1	Down	13.14 (2.88)	26.11 (2.56)	0.012*	-1.99	Down
Aspartic acid	20.11 (3.56)	16.06 (3.49)	0.144	1.25	Up	14.83 (1.81)	14.60 (3.92)	0.164	1.02	Up	17.02 (2.17)	12.33 (2.49)	0.083	1.38	Up
Citrulline	89.45 (19.86)	53.75 (3.47)	0.024*	1.66	Up	86.41 (19.86)	56.30 (4.31)	0.083	1.53	Up	86.06 (14.79)	51.87 (4.50)	0.011*	1.66	Up
Glutamine	304.68 (33.06)	286.15 (21.62)	1	1.06	Up	307.32 (31.07)	247.88 (25.27)	0.262	1.24	Up	219.57 (12.34)	218.18 (21.60)	1	1.01	Up
Glutamate	161.50 (39.42)	90.53 (7.67)	0.174	1.78	Up	133.03 (31.01)	71.90 (7.54)	0.072	1.85	Up	105.73 (17.44)	68.42 (6.18)	0.045*	1.54	Up
Glycine	323.63 (34.36)	208.55 (16.17)	0.007*	1.55	Up	260.76 (22.69)	269.50 (26.26)	0.786	-1.03	Down	391.03 (90.27)	252.83 (34.64)	0.123	1.55	Up
Histidine	69.36 (5.79)	51.23 (3.37)	0.019*	1.35	Up	62.13 (4.20)	39.51 (3.03)	0.003*	1.57	Up	54.21 (6.43)	38.33 (2.69)	0.033*	1.41	Up
Isoleucine	276.75 (20.94)	113.87 (10.02)	0.001*	2.43	Up	278.24 (23.07)	99.88 (9.23)	0.001*	2.79	Up	297.57 (14.11)	100.92 (8.94)	0.001*	2.95	Up
Leucine	380.86 (34.80)	150.69 (15.62)	0.001*	2.53	Up	359.29 (65.09)	134.57 (12.29)	0.001*	2.67	Up	434.28 (42.90)	129.56 (11.81)	0.001*	3.35	Up
Lysine	557.84 (80.64)	90.61 (5.80)	0.001*	6.16	Up	450.59 (73.72)	74.14 (4.78)	0.001*	6.08	Up	575.69 (65.56)	69.24 (6.51)	0.001*	8.31	Up
Methionine	32.52 (12.94)	26.94 (3.19)	0.621	1.21	Up	42.43 (15.67)	21.86 (1.91)	0.659	1.94	Up	25.64 (6.43)	19.19 (2.08)	0.171	1.34	Up
Ornithine	29.65 (11.12)	40.48 (2.60)	0.336	-1.37	Down	39.95 (16.22)	30.55 (2.94)	0.659	1.31	Up	27.19 (8.59)	29.23 (3.42)	0.7	-1.08	Down
Phenylalanine	64.55 (7.05)	50.22 (3.84)	0.148	1.29	Up	70.51 (5.21)	43.73 (3.87)	0.003*	1.61	Up	43.91 (7.72)	41.71 (3.33)	0.927	1.05	Up
Proline	71.86 (17.73)	72.70 (5.72)	0.668	-1.01	Down	78.25 (3.43)	63.36 (4.22)	0.062	1.24	Up	59.95 (13.67)	57.62 (4.77)	0.7	1.04	Up
Serine	108.46 (28.38)	66.84 (6.58)	0.083	1.62	Up	92.43 (8.45)	66.76 (6.96)	0.042*	1.38	Up	85.54 (11.85)	59.38 (5.25)	0.039*	1.44	Up
Threonine	82.00 (9.09)	78.58 (8.16)	0.408	1.04	Up	82.07 (6.01)	70.66 (7.01)	0.072	1.16	Up	74.03 (6.44)	64.22 (6.47)	0.157	1.15	Up
Tryptophan	21.18 (2.34)	41.67 (3.33)	0.004*	-1.97	Down	21.20 (1.55)	29.48 (3.29)	0.371	-1.39	Down	19.14 (1.66)	29.90 (3.47)	0.095	-1.56	Down
Tyrosine	44.13 (6.51)	44.12 (3.12)	0.974	1	Up	47.71 (5.98)	35.55 (3.30)	0.097	1.34	Up	37.51 (3.32)	35.00 (3.41)	0.79	1.07	Up
Valine	242.78 (35.72)	218.05 (17.60)	0.61	1.11	Up	262.34 (32.90)	185.31 (14.62)	0.032*	1.42	Up	206.36 (18.27)	183.46 (16.27)	0.494	1.12	Up
Acetylornithine	13.13 (4.40)	3.73 (0.47)	0.042*	3.52	Up	11.92 (3.91)	3.72 (0.59)	0.010*	3.2	Up	9.30 (2.16)	3.32 (0.47)	0.006*	2.8	Up
Asymmetric dimethylarginine	0.71 (0.11)	0.79 (0.06)	0.415	-1.11	Down	0.74 (0.11)	0.60 (0.06)	0.272	1.23	Up	0.52 (0.06)	0.55 (0.06)	0.465	-1.06	Down
total Dimethylarginine	0.96 (0.05)	0.77 (0.06)	0.129	1.25	Up	1.02 (0.09)	0.68 (0.06)	0.024*	1.5	Up	0.88 (0.05)	0.64 (0.05)	0.023*	1.38	Up
Carnosine	8.56 (1.32)	5.60 (0.42)	0.032*	1.53	Up	8.76 (1.13)	4.96 (0.33)	0.004*	1.77	Up	6.17 (0.95)	4.25 (0.37)	0.107	1.45	Up
Creatinine	78.36 (4.89)	77.12 (6.00)	0.53	1.02	Up	80.77 (6.67)	68.88 (3.98)	0.185	1.17	Up	75.48 (6.12)	57.76 (4.36)	0.063	1.31	Up
Kynurenine	16.43 (1.99)	8.34 (0.69)	0.006*	1.97	Up	16.00 (1.73)	6.54 (0.60)	0.001*	2.45	Up	14.31 (0.70)	6.58 (0.77)	0.001*	2.17	Up

Sarcosine	4.62 (0.62)	2.74 (0.44)	0.019*	1.69	Up	3.81 (0.43)	3.51 (0.65)	0.336	1.09	Up	4.58 (0.72)	2.62 (0.64)	0.016*	1.75	Up
Taurine	53.35 (7.98)	37.70 (2.49)	0.071	1.42	Up	48.38 (6.97)	36.44 (3.11)	0.072	1.33	Up	47.68 (5.44)	33.93 (4.02)	0.095	1.41	Up

[1]Cows were diagnosed with ketosis (n=6) ranging from wk +1 to +3 (i.e., 5 - 21 d, mean 13 d).

[2]C10: decanoyl-L-carnitine; C16: hexadecanoyl-L-carnitine; C18: octadecenoyl-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.

Table 3-2. Concentrations of serum metabolites (mean (SEM)) in healthy control (CON) and ketosis cows at +4, and +8 wks after parturition as determined by DI/LC-MS/MS

	4 week after parturition					8 week after parturition				
Metabolite[1], μM	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
C10	0.06 (0.02)	0.07 (0.03)	0.574	-1.17	Down	0.05 (0.01)	0.06 (0.02)	0.378	-1.2	Down
C16	0.04 (0.002)	0.01 (0.002)	0.002*	4	Up	0.03 (0.01)	0.02 (0.01)	0.041*	1.5	Up
C18	0.07 (0.01)	0.03 (0.01)	0.004*	2.33	Up	0.07 (0.01)	0.04 (0.02)	0.045*	1.75	Up
C2	1.89 (0.28)	1.01 (0.17)	0.015*	1.87	Up	1.34 (0.02)	1.06 (0.29)	0.394	1.26	Up
C3	0.18 (0.04)	0.17 (0.04)	0.818	1.06	Up	0.21 (0.04)	0.21 (0.07)	0.818	1	Up
C4	0.07 (0.01)	0.06 (0.01)	0.297	1.17	Up	0.07 (0.01)	0.06 (0.01)	0.485	1.17	Up
C5	0.05 (0.01)	0.04 (0.01)	0.31	1.25	Up	0.05 (0.01)	0.05 (0.01)	0.818	1	Up
lysoPC a C16:0	109.48 (19.29)	20.80 (5.86)	0.004*	5.26	Up	36.73 (2.15)	24.87 (6.72)	0.485	1.48	Up
lysoPC a C16:1	3.10 (0.65)	1.08 (0.35)	0.009*	2.87	Up	2.38 (0.18)	1.26 (0.33)	0.015*	1.89	Up
lysoPC a C17:0	5.54 (1.15)	1.31 (0.43)	0.009*	4.23	Up	2.25 (0.14)	2.07 (0.56)	0.818	1.09	Up
lysoPC a C18:0	75.47 (13.17)	18.63 (6.33)	0.009*	4.05	Up	27.33 (2.05)	26.18 (7.37)	0.937	1.04	Up
lysoPC a C18:1	33.92 (5.24)	13.72 (4.40)	0.015*	2.47	Up	26.28 (2.23)	20.72 (5.54)	0.699	1.27	Up
lysoPC a C18:2	28.92 (5.74)	20.19 (6.89)	0.24	1.43	Up	40.66 (3.22)	32.40 (9.30)	0.31	1.25	Up
lysoPC a C20:3	1.44 (0.33)	1.48 (0.46)	0.699	-1.03	Down	2.30 (0.25)	2.50 (0.65)	0.818	-1.09	Down
lysoPC a C20:4	1.81 (0.27)	1.37 (0.34)	0.31	1.32	Up	2.32 (0.18)	2.02 (0.49)	0.589	1.15	Up
lysoPC a C28:0	0.39 (0.09)	0.43 (0.06)	0.31	-1.1	Down	0.45 (0.05)	0.65 (0.13)	0.18	-1.44	Down
lysoPC a C28:1	0.61 (0.10)	0.50 (0.16)	0.31	1.22	Up	0.98 (0.06)	0.86 (0.24)	0.818	1.14	Up
PC aa C28:1	3.17 (0.46)	1.61 (0.45)	0.041*	1.97	Up	4.44 (0.25)	2.99 (0.75)	0.394	1.48	Up
PC aa C30:0	5.13 (0.58)	1.96 (0.55)	0.009*	2.62	Up	7.20 (0.48)	3.44 (0.85)	0.004*	2.09	Up
PC aa C30:2	1.09 (0.22)	0.24 (0.09)	0.004*	4.54	Up	1.46 (0.16)	0.52 (0.18)	0.009*	2.81	Up
PC aa C32:0	8.57 (0.96)	4.64 (1.35)	0.041*	1.85	Up	11.53 (0.47)	8.12 (2.15)	0.699	1.42	Up
PC aa C32:1	9.95 (1.33)	5.49 (1.86)	0.093	1.81	Up	15.73 (0.46)	9.82 (2.78)	0.24	1.6	Up
PC aa C32:2	10.35 (2.87)	4.88 (1.73)	0.132	2.12	Up	16.62 (2.23)	11.70 (3.31)	0.394	1.42	Up
PC aa C32:3	20.23 (5.93)	12.97 (4.61)	0.31	1.56	Up	36.90 (4.69)	30.98 (9.31)	0.589	1.19	Up
PC aa C34:1	159.62 (16.54)	73.67 (20.57)	0.026*	2.17	Up	192.74 (11.79)	100.50 (31.88)	0.065	1.92	Up
PC aa C34:2	218.86 (22.08)	148.13 (41.69)	0.18	1.48	Up	299.73 (15.05)	240.57 (57.45)	0.818	1.25	Up
PC aa C34:3	24.87 (4.53)	15.42 (4.81)	0.18	1.61	Up	50.23 (3.73)	29.18 (8.14)	0.173	1.72	Up
PC aa C34:4	5.17 (1.52)	2.59 (0.89)	0.132	2	Up	9.72 (1.34)	5.78 (1.65)	0.128	1.68	Up
PC aa C36:0	9.31 (2.46)	4.05 (1.44)	0.132	2.3	Up	17.62 (2.36)	8.89 (2.80)	0.093	1.98	Up

PC aa C36:1	127.19 (17.09)	67.97 (22.34)	0.065	1.87	Up	172.41 (10.53)	117.92 (31.75)	0.485	1.46	Up
PC aa C36:2	213.03 (22.52)	150.60 (44.63)	0.31	1.41	Up	296.92 (14.41)	269.20 (66.38)	1	1.1	Up
PC aa C36:3	98.44 (14.87)	60.78 (19.68)	0.24	1.62	Up	162.33 (12.30)	118.78 (32.30)	0.575	1.37	Up
PC aa C36:4	47.89 (5.39)	23.00 (6.74)	0.026*	2.08	Up	72.33 (4.35)	44.92 (12.16)	0.18	1.61	Up
PC aa C36:5	10.94 (1.42)	4.96 (1.39)	0.026*	2.21	Up	16.06 (1.45)	10.49 (3.34)	0.485	1.53	Up
PC aa C36:6	3.00 (0.71)	1.25 (0.31)	0.026*	2.4	Up	4.46 (0.59)	3.18 (1.09)	0.485	1.4	Up
PC aa C38:0	1.81 (0.55)	1.21 (0.45)	0.31	1.5	Up	3.31 (0.47)	2.62 (0.81)	0.485	1.26	Up
PC aa C38:1	3.88 (1.29)	3.41 (1.77)	0.394	1.14	Up	7.78 (1.39)	5.63 (1.68)	0.394	1.38	Up
PC aa C38:3	36.23 (5.76)	23.40 (7.64)	0.18	1.55	Up	60.16 (4.86)	54.03 (16.28)	0.937	1.11	Up
PC aa C38:4	44.11 (4.16)	24.78 (7.10)	0.093	1.78	Up	61.26 (3.82)	48.63 (14.52)	0.818	1.26	Up
PC aa C38:5	32.15 (2.84)	13.40 (3.63)	0.009*	2.4	Up	41.67 (4.07)	26.05 (8.37)	0.24	1.6	Up
PC aa C38:6	6.56 (0.76)	2.68 (0.75)	0.009*	2.45	Up	8.61 (0.85)	5.00 (1.54)	0.18	1.72	Up
PC aa C40:2	0.27 (0.05)	0.12 (0.04)	0.041*	2.25	Up	0.53 (0.06)	0.37 (0.12)	0.132	1.43	Up
PC aa C40:3	2.29 (0.88)	2.06 (1.02)	0.699	1.11	Up	6.90 (1.07)	6.06 (2.06)	0.699	1.14	Up
PC aa C40:4	9.50 (2.02)	5.21 (2.14)	0.041*	1.82	Up	18.41 (1.55)	12.38 (3.69)	0.31	1.49	Up
PC aa C40:5	22.07 (2.13)	9.40 (2.93)	0.015*	2.35	Up	29.51 (3.39)	17.57 (5.91)	0.24	1.68	Up
PC aa C40:6	5.52 (0.49)	2.52 (0.68)	0.015*	2.19	Up	6.92 (1.00)	3.67 (1.14)	0.093	1.89	Up
PC aa C42:1	0.06 (0.01)	0.03 (0.01)	0.015*	2	Up	0.10 (0.01)	0.06 (0.01)	0.092	1.67	Up
PC aa C42:2	0.12 (0.02)	0.04 (0.01)	0.004*	3	Up	0.18 (0.02)	0.08 (0.02)	0.015*	2.25	Up
PC aa C42:4	0.07 (0.01)	0.05 (0.02)	0.394	1.4	Up	0.19 (0.02)	0.15 (0.05)	0.394	1.27	Up
PC aa C42:5	0.66 (0.19)	0.43 (0.21)	0.24	1.53	Up	1.46 (0.28)	1.02 (0.33)	0.24	1.43	Up
PC aa C42:6	0.36 (0.04)	0.31 (0.05)	0.394	1.16	Up	0.65 (0.08)	0.46 (0.10)	0.18	1.41	Up
PC ae C30:0	0.91 (0.14)	0.48 (0.15)	0.041*	1.9	Up	1.35 (0.09)	0.87 (0.21)	0.31	1.55	Up
PC ae C30:1	2.87 (0.71)	1.07 (0.35)	0.093	2.68	Up	4.23 (0.51)	2.27 (0.75)	0.065	1.86	Up
PC ae C32:1	4.04 (0.58)	2.01 (0.67)	0.065	2.01	Up	5.85 (0.34)	3.85 (0.99)	0.18	1.52	Up
PC ae C32:2	7.12 (1.60)	4.14 (1.39)	0.24	1.72	Up	11.88 (1.09)	8.59 (2.48)	0.485	1.38	Up
PC ae C34:0	2.63 (0.44)	1.35 (0.46)	0.093	1.95	Up	4.07 (0.28)	2.63 (0.71)	0.229	1.55	Up
PC ae C34:1	17.18 (2.38)	8.07 (2.61)	0.041*	2.13	Up	25.01 (1.21)	13.62 (3.75)	0.015*	1.84	Up
PC ae C34:2	29.11 (4.73)	13.70 (4.37)	0.041*	2.12	Up	43.34 (2.75)	25.57 (7.02)	0.065	1.69	Up
PC ae C34:3	29.37 (6.14)	15.82 (5.46)	0.18	1.86	Up	49.76 (4.24)	33.61 (9.76)	0.24	1.48	Up
PC ae C36:0	1.52 (0.28)	0.85 (0.25)	0.093	1.79	Up	2.47 (0.30)	1.88 (0.53)	0.589	1.31	Up
PC ae C36:1	16.51 (2.30)	7.94 (2.37)	0.026*	2.08	Up	24.22 (1.65)	17.48 (4.98)	0.589	1.39	Up
PC ae C36:2	24.46 (3.87)	16.00 (5.22)	0.24	1.53	Up	41.92 (3.19)	33.49 (9.83)	0.818	1.25	Up

PC ae C36:3	10.17 (1.72)	6.55 (2.21)	0.31	1.55	Up	16.93 (0.83)	12.71 (3.63)	0.485	1.33	Up
PC ae C36:4	10.17 (1.63)	5.89 (2.03)	0.31	1.73	Up	16.69 (1.18)	9.87 (2.62)	0.041*	1.69	Up
PC ae C36:5	7.74 (0.84)	3.92 (1.17)	0.041*	1.97	Up	11.06 (0.40)	6.72 (2.13)	0.065	1.65	Up
PC ae C38:0	1.84 (0.40)	0.90 (0.28)	0.093	2.04	Up	2.76 (0.36)	1.58 (0.46)	0.31	1.75	Up
PC ae C38:1	2.04 (0.42)	0.93 (0.38)	0.093	2.19	Up	3.10 (0.42)	1.85 (0.533)	0.041*	1.68	Up
PC ae C38:2	2.74 (0.42)	2.03 (0.77)	0.394	1.35	Up	5.32 (0.57)	3.93 (1.16)	0.589	1.35	Up
PC ae C38:3	3.76 (0.56)	2.13 (0.71)	0.093	1.77	Up	6.78 (0.50)	4.75 (1.36)	0.589	1.43	Up
PC ae C38:4	4.52 (0.54)	2.40 (0.74)	0.093	1.88	Up	7.01 (0.30)	4.66 (1.31)	0.24	1.5	Up
PC ae C38:5	4.47 (0.56)	2.17 (0.63)	0.041*	2.06	Up	6.54 (0.26)	4.14 (1.15)	0.065	1.58	Up
PC ae C38:6	4.89 (0.68)	2.05 (0.57)	0.015*	2.39	Up	6.80 (0.53)	4.11 (1.32)	0.132	1.65	Up
PC ae C40:1	0.31 (0.05)	0.22 (0.08)	0.18	1.41	Up	0.51 (0.06)	0.28 (0.08)	0.093	1.82	Up
PC ae C40:2	0.88 (0.11)	0.36 (0.11)	0.015*	2.44	Up	1.32 (0.12)	0.75 (0.20)	0.132	1.76	Up
PC ae C40:3	0.75 (0.10)	0.47 (0.17)	0.18	1.6	Up	1.31 (0.10)	1.00 (0.30)	0.589	1.31	Up
PC ae C40:4	0.95 (0.11)	0.63 (0.21)	0.24	1.51	Up	1.52 (0.12)	1.28 (0.38)	0.589	1.19	Up
PC ae C40:5	2.19 (0.32)	1.17 (0.36)	0.065	1.87	Up	3.42 (0.32)	2.23 (0.69)	0.31	1.53	Up
PC ae C40:6	1.23 (0.19)	0.63 (0.19)	0.093	1.95	Up	1.76 (0.12)	1.10 (0.31)	0.132	1.6	Up
PC ae C42:1	0.10 (0.01)	0.07 (0.02)	0.093	1.43	Up	0.17 (0.01)	0.13 (0.03)	0.699	1.31	Up
PC ae C42:2	0.15 (0.02)	0.09 (0.03)	0.132	1.67	Up	0.25 (0.02)	0.20 (0.07)	0.394	1.25	Up
PC ae C42:3	0.14 (0.01)	0.09 (0.03)	0.18	1.56	Up	0.20 (0.02)	0.16 (0.06)	0.394	1.25	Up
PC ae C44:3	0.03 (0.003)	0.03 (0.01)	0.378	1	Up	0.06 (0.01)	0.05 (0.01)	0.394	1.2	Up
PC ae C44:4	0.08 (0.01)	0.03 (0.01)	0.004*	2.67	Up	0.08 (0.01)	0.05 (0.01)	0.004*	1.6	Up
PC ae C44:5	0.05 (0.01)	0.02 (0.004)	0.013*	2.5	Up	0.07 (0.01)	0.05 (0.01)	0.699	1.4	Up
SM (OH) C14:1	15.13 (1.91)	7.86 (2.38)	0.065	1.92	Up	20.56 (1.25)	11.94 (3.05)	0.065	1.72	Up
SM (OH) C16:1	10.10 (1.13)	4.68 (1.38)	0.015*	2.16	Up	14.90 (1.21)	8.97 (2.80)	0.18	1.66	Up
SM (OH) C22:1	35.95 (3.34)	11.36 (2.83)	0.002*	3.16	Up	45.87 (4.71)	15.82 (3.78)	0.002*	2.9	Up
SM (OH) C22:2	10.39 (1.09)	3.49 (1.10)	0.004*	2.98	Up	13.88 (1.14)	5.98 (1.59)	0.002*	2.32	Up
SM (OH) C24:1	2.05 (0.24)	0.76 (0.23)	0.009*	2.7	Up	2.78 (0.26)	1.28 (0.29)	0.004*	2.17	Up
SM C16:0	148.65 (14.13)	61.80 (16.84)	0.009*	2.4	Up	193.82 (11.31)	98.38 (23.09)	0.004*	1.97	Up
SM C16:1	13.04 (1.63)	6.33 (2.04)	0.041*	2.06	Up	19.57 (1.11)	11.35 (2.97)	0.041*	1.72	Up
SM C18:0	19.32 (1.88)	6.49 (1.90)	0.004*	2.98	Up	24.75 (1.39)	12.45 (2.91)	0.002*	1.99	Up
SM C18:1	6.01 (0.58)	2.33 (0.86)	0.015*	2.58	Up	7.86 (0.54)	5.23 (1.93)	0.31	1.5	Up
SM C20:2	0.17 (0.10)	0.10 (0.05)	0.807	1.7	Up	0.22 (0.06)	0.43 (0.10)	0.18	-1.95	Down
SM C24:0	33.59 (6.09)	14.80 (4.38)	0.041*	2.27	Up	58.18 (4.25)	33.38 (9.40)	0.065	1.74	Up

SM C24:1	14.18 (1.77)	5.15 (1.51)	0.009*	2.75	Up	19.50 (2.01)	10.06 (2.61)	0.026*	1.94	Up
SM C26:0	0.36 (0.05)	0.15 (0.04)	0.015*	2.4	Up	0.50 (0.03)	0.23 (0.05)	0.004*	2.17	Up
SM C26:1	0.19 (0.03)	0.07 (0.03)	0.065	2.71	Up	0.07 (0.04)	0.10 (0.04)	0.55	-1.43	Down
Hexose	2,658 (274)	1,489 (380)	0.026*	1.79	Up	2,682 (532)	2,228 (633)	0.589	1.2	Up
Alanine	226.67 (30.41)	120.78 (26.97)	0.041*	1.88	Up	186.22 (21.25)	142.52 (31.82)	0.394	1.31	Up
Arginine	132.98 (10.87)	83.48 (18.62)	0.078	1.59	Up	130.97 (15.50)	84.35 (14.63)	0.093	1.55	Up
Asparagine	17.95 (2.20)	33.66 (6.81)	0.24	-1.88	Down	25.13 (6.02)	37.05 (8.47)	0.24	-1.47	Down
Aspartic acid	22.89 (3.35)	38.23 (7.08)	0.132	-1.67	Down	17.16 (2.18)	44.98 (8.71)	0.015*	-2.62	Down
Citrulline	86.53 (9.13)	55.08 (14.62)	0.132	1.57	Up	89.97 (5.31)	66.27 (13.63)	0.589	1.36	Up
Glutamine	199.17 (40.64)	154.43 (45.07)	0.485	1.29	Up	260.78 (45.83)	163.42 (42.98)	0.093	1.6	Up
Glutamate	179.22 (31.98)	113.15 (32.27)	0.31	1.58	Up	122.24 (23.94)	109.50 (27.84)	0.699	1.12	Up
Glycine	477.39 (81.52)	298.00 (62.05)	0.18	1.6	Up	485.72 (38.48)	230.00 (43.46)	0.009*	2.11	Up
Histidine	57.65 (6.19)	34.99 (8.43)	0.132	1.65	Up	52.32 (7.26)	34.70 (6.15)	0.132	1.51	Up
Isoleucine	276.43 (22.01)	52.28 (12.76)	0.002*	5.29	Up	342.63 (27.75)	55.50 (14.77)	0.002*	6.17	Up
Leucine	383.90 (41.42)	65.75 (19.78)	0.005*	5.84	Up	465.04 (85.06)	83.95 (20.12)	0.002*	5.54	Up
Lysine	548.04 (53.10)	46.43 (10.19)	0.005*	11.8	Up	487.72 (71.25)	54.80 (12.93)	0.002*	8.9	Up
Methionine	28.47 (10.26)	23.76 (5.84)	0.937	1.2	Up	28.26 (7.97)	32.75 (8.75)	0.937	-1.16	Down
Ornithine	29.46 (10.58)	17.31 (4.34)	0.589	1.7	Up	25.69 (7.47)	23.01 (4.61)	0.937	1.12	Up
Phenylalanine	57.96 (3.92)	16.61 (5.13)	0.005*	3.49	Up	60.01 (7.09)	24.70 (4.64)	0.002*	2.43	Up
Proline	78.23 (5.95)	44.75 (11.12)	0.041*	1.75	Up	76.55 (7.62)	43.62 (10.22)	0.041*	1.75	Up
Serine	100.19 (7.75)	54.03 (20.71)	0.065	1.85	Up	105.57 (8.81)	49.07 (9.53)	0.004*	2.15	Up
Threonine	81.38 (7.33)	71.38 (18.82)	0.485	1.14	Up	76.58 (6.13)	76.85 (19.07)	0.485	1	Down
Tryptophan	21.02 (1.90)	14.78 (4.54)	0.31	1.42	Up	19.80 (1.58)	21.41 (5.33)	1	-1.08	Down
Tyrosine	38.39 (3.54)	33.26 (11.58)	0.699	1.15	Up	33.50 (4.55)	28.79 (7.49)	0.699	1.16	Up
Valine	211.14 (19.45)	105.88 (21.45)	0.015*	2	Up	184.27 (25.02)	122.80 (31.22)	0.18	1.5	Up
Acetylornithine	11.83 (3.58)	1.36 (0.40)	0.008*	8.7	Up	10.95 (2.52)	1.76 (0.49)	0.004*	6.22	Up
Asymmetric dimethylarginine	0.57 (0.09)	0.52 (0.06)	0.688	1.1	Up	0.46 (0.06)	0.53 (0.02)	0.128	-1.15	Down
total Dimethylarginine	0.97 (0.11)	0.72 (0.06)	0.065	1.35	Up	0.80 (0.04)	0.72 (0.04)	0.24	1.11	Up
Carnosine	9.93 (1.84)	5.08 (1.05)	0.041*	1.95	Up	11.10 (1.75)	7.43 (2.35)	0.173	1.49	Up
Creatinine	82.11 (9.72)	53.18 (11.38)	0.18	1.54	Up	68.19 (3.46)	54.05 (10.98)	0.589	1.26	Up
Kynurenine	15.61 (1.45)	8.67 (1.92)	0.026*	1.8	Up	13.27 (1.21)	8.19 (1.39)	0.041*	1.62	Up
Sarcosine	4.91 (0.56)	1.46 (0.41)	0.002*	3.36	Up	5.28 (0.72)	1.20 (0.39)	0.002*	4.4	Up
Taurine	46.72 (6.77)	26.91 (7.43)	0.093	1.74	Up	56.79 (7.51)	24.77 (3.27)	0.002*	2.29	Up

[1]C10: decanoyl-L-carnitine; C16: hexadecanoyl-L-carnitine; C18: octadecenoyl-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.

Table 3-3. Significant metabolic pathways involved in the onset and progression of ketosis in dairy cows from quantitative enrichment analysis

Metabolite Set	Total Cmpd	Hits	Significant Cmp
Lysine degradation[1,2,3,4,5]	13	1	L-Lysine
Biotin metabolism[1,2,3,4,5]	4	1	L-Lysine
Phospholipid biosynthesis[1,4]	19	1	LysoPC a C16:0
Tryptophan metabolism[1,2,3]	34	1	L-Kynurenine
Aspartate metabolism[1,3]	12	3	L-Asparagine; Citrulline; D-Aspartic acid
Bile acid biosynthesis[1,5]	49	2	Glycine; Taurine
Protein biosynthesis[1,2]	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
Histidine metabolism[2]	11	2	Carnosine; L-Histidine
Valine, leucine and isoleucine degradation[2,3,4,5]	36	3	L-Leucine; L-Valine, L-Isoleucine
Beta-alanine metabolism[2]	13	1	Carnosine
Taurine and hypotaurine metabolism[5]	7	1	Taurine
Glutathione metabolism[5]	10	1	Glycine
Porphyrin metabolism[5]	22	1	Glycine

[1]Significant metabolic pathways at -8 wks before parturition; Holm $P < 0.05$.

[2]Significant metabolic pathways at -4 wks before parturition; Holm $P < 0.05$.

[3]Significant metabolic pathways at the wk of diagnosis of disease; Holm $P < 0.05$.

[4]Significant metabolic pathways at +4 wks after parturition; Holm $P < 0.05$.

[5]Significant metabolic pathways at +8 wks after parturition; Holm $P < 0.05$.

Figure 3-1.

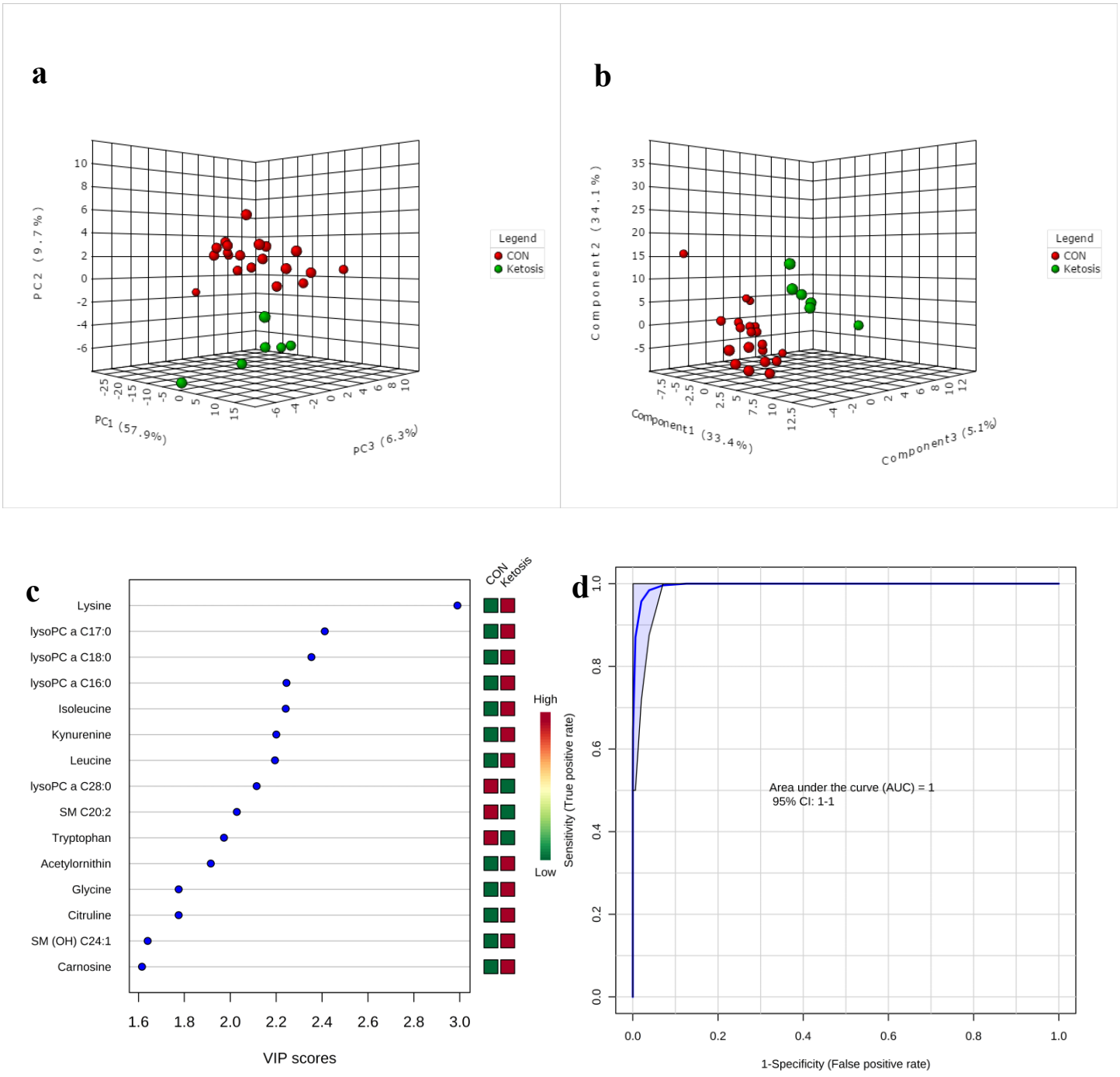

Figure 3-1. (a) Principal component analysis (PCA) and (b) Partial least squares-discriminant analysis (PLS-DA, Permutation test: $P < 0.05$) of 20 control (CON) and 6 ketosis cows at -8 wks before parturition showing 2 separated clusters for 2 groups. (c) Variables ranked by variable importance in projection (VIP), and (d) Receiver-operator characteristic (ROC) curve of 20 CON and 6 ketosis cows at -8 wks before parturition for the top 7 serum variables (i.e., Lys, lysoPC a C17:0, lysoPC a C18:0, lysoPC a C16:0, Ile, kynurenine, Leu; empirical $P = 0.001$).

Figure 3-2.

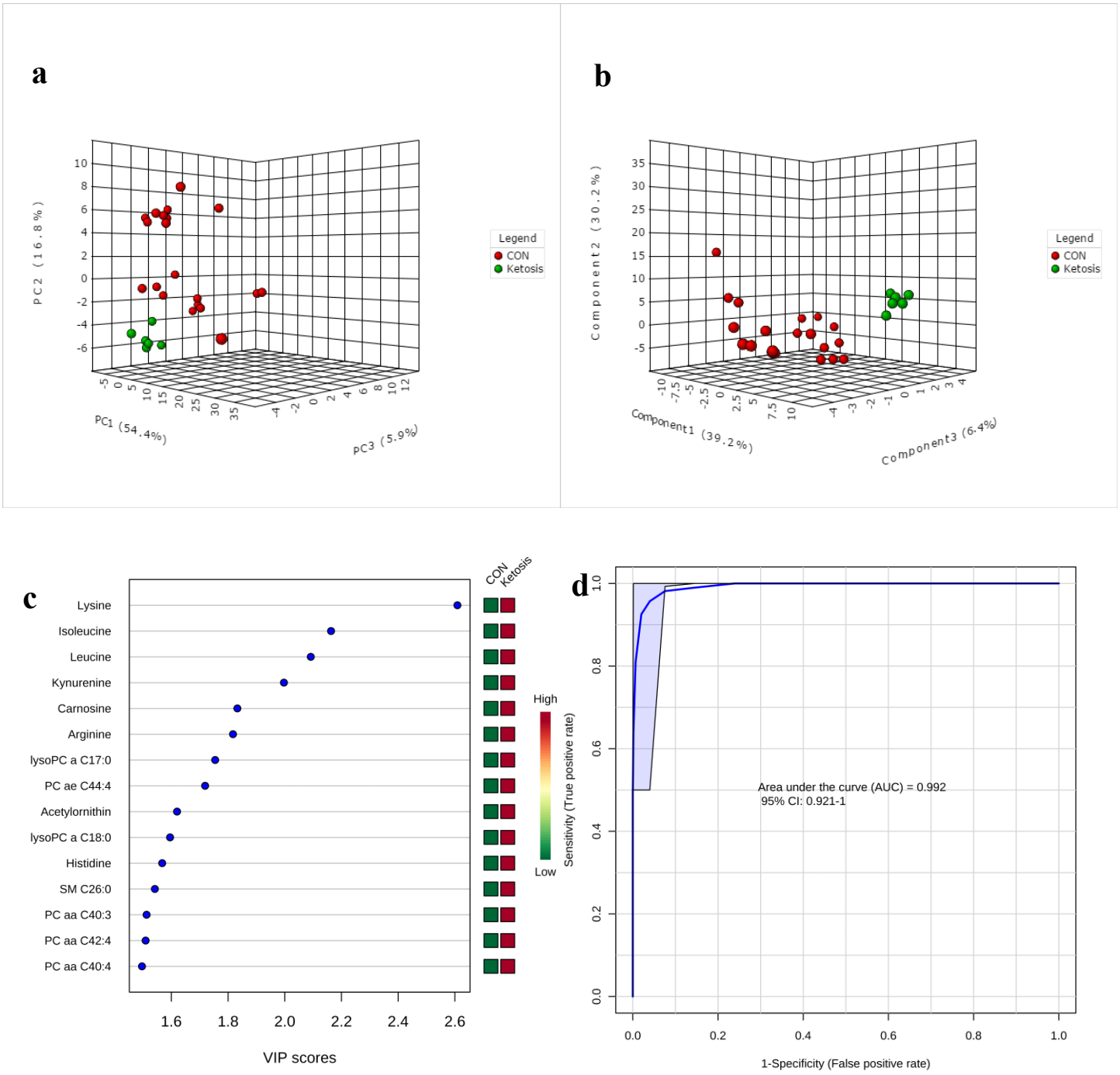

Figure 3-2. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 20 CON and 6 ketosis cows at -4 wks before parturition showing 2 separated clusters for 2 groups. (c) VIP, and (d) ROC curve of 20 CON and 6 ketosis cows at -4 wks before parturition for the top 8 serum variables (i.e., Lys, Ile, Leu, kynurenine, carnosine, Arg, lysoPC a C17:0, and PC ae C44:4; empirical $P = 0.001$).

Figure 3-3.

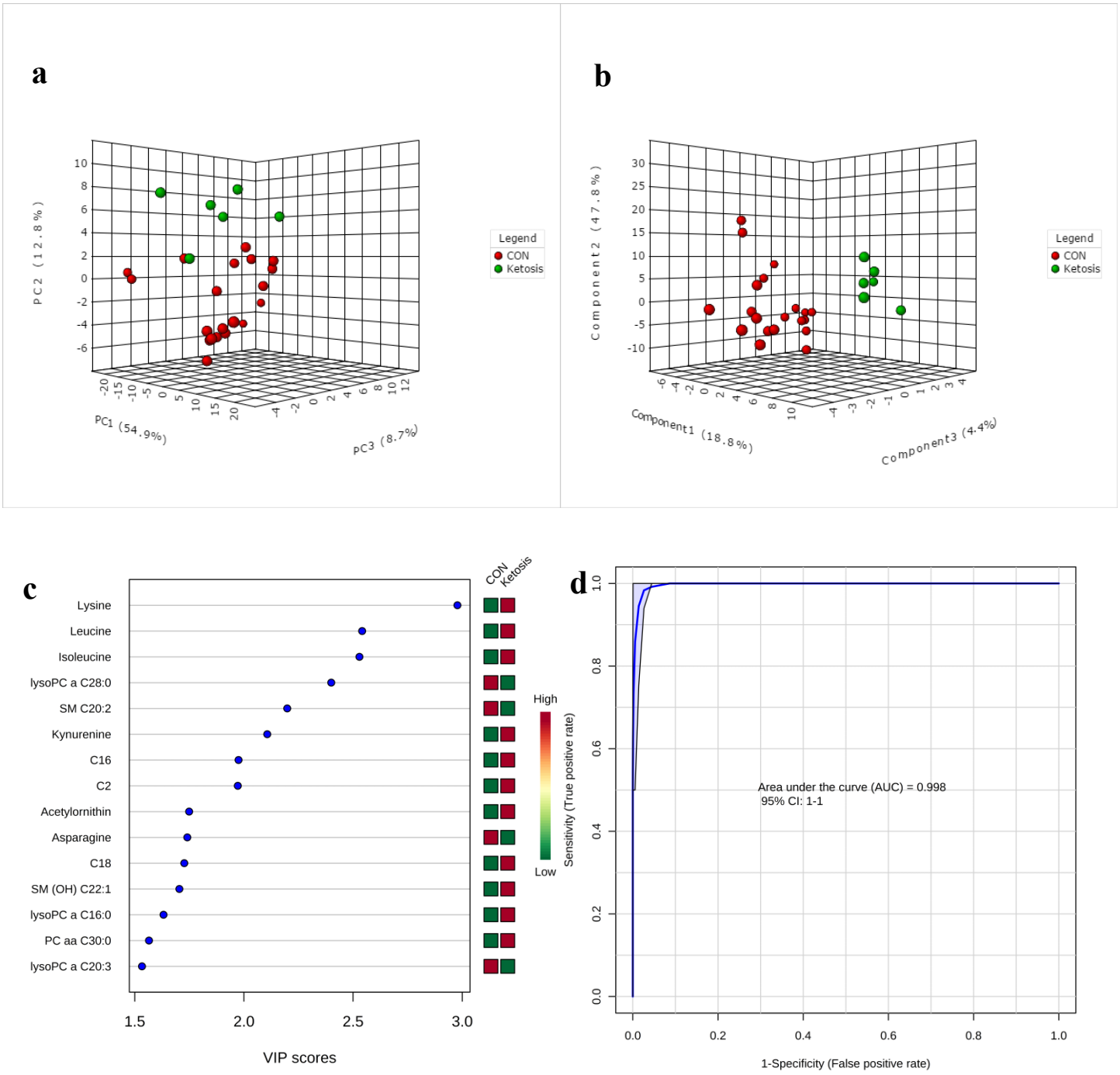

Figure 3-3. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 20 CON and 6 ketosis cows at disease wk showing 2 separated clusters for 2 groups. (c) VIP, and (d) ROC curve of 20 CON and 6 ketosis cows at disease wk for the top 8 serum variables (i.e., Lys, Leu, Ile, lysoPC a C28:0, SM C20:2, kynurenine, C16, and C2; empirical $P = 0.001$).

Figure 3-4.

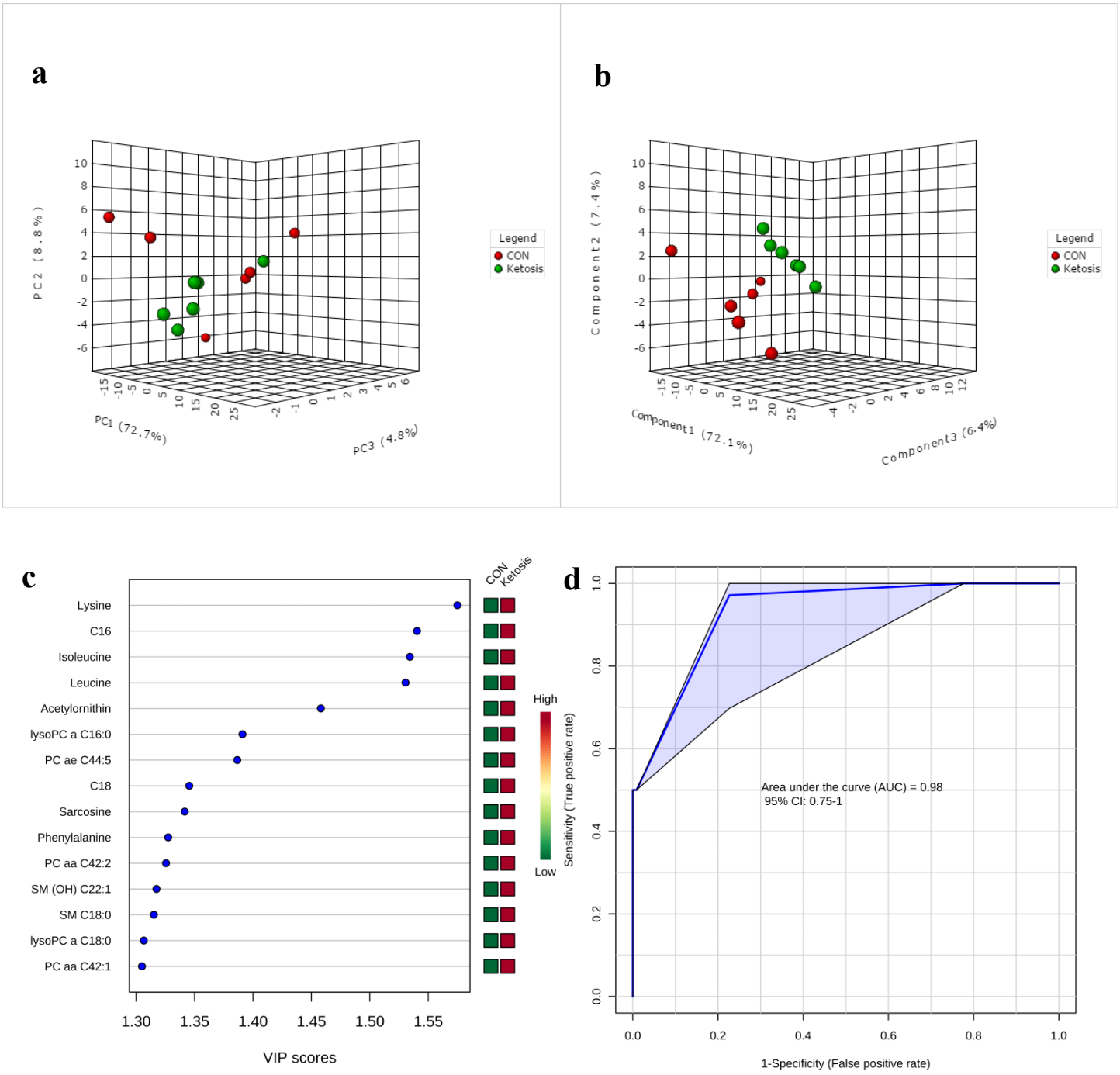

Figure 3-4. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 6 CON and 6 ketosis cows at +4 wks after parturition showing 2 separated clusters for 2 groups. (c) VIP, and (d) ROC curve of 6 CON and 6 ketosis cows at +4 wks after parturition for the top 4 serum variables (i.e., Lys, C16, Ile, and Leu; empirical $P < 0.05$).

Figure 3-5.

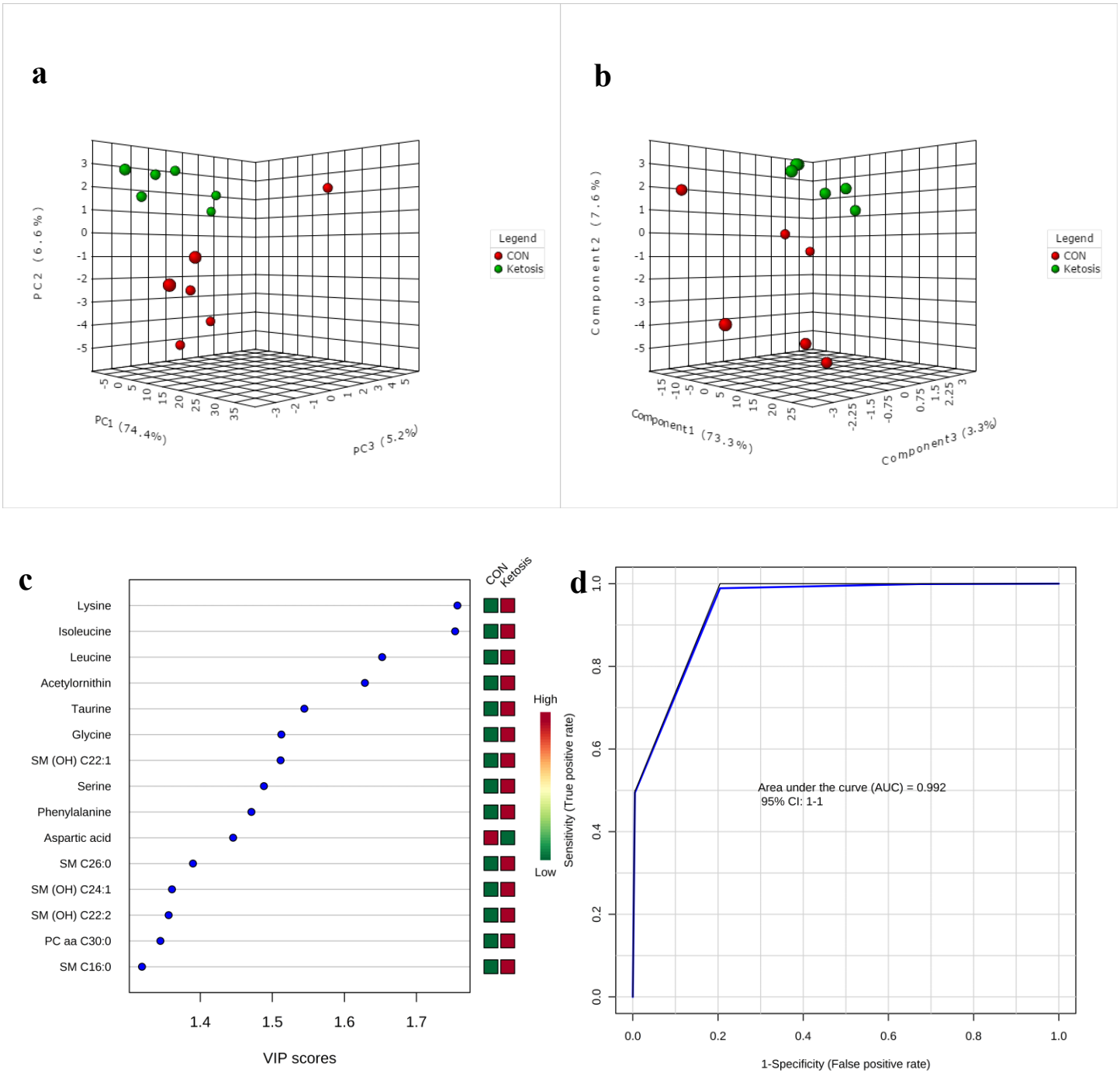

Figure 3-5. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 6 CON and 6 ketosis cows at +8 wks after parturition showing 2 separated clusters for 2 groups. (c) VIP, and (d) ROC curve of 6 CON and 6 ketosis cows at +8 wks after parturition for the top 4 serum variables (i.e., Lys, Ile, Leu, and acetylornithine; empirical $P < 0.05$).

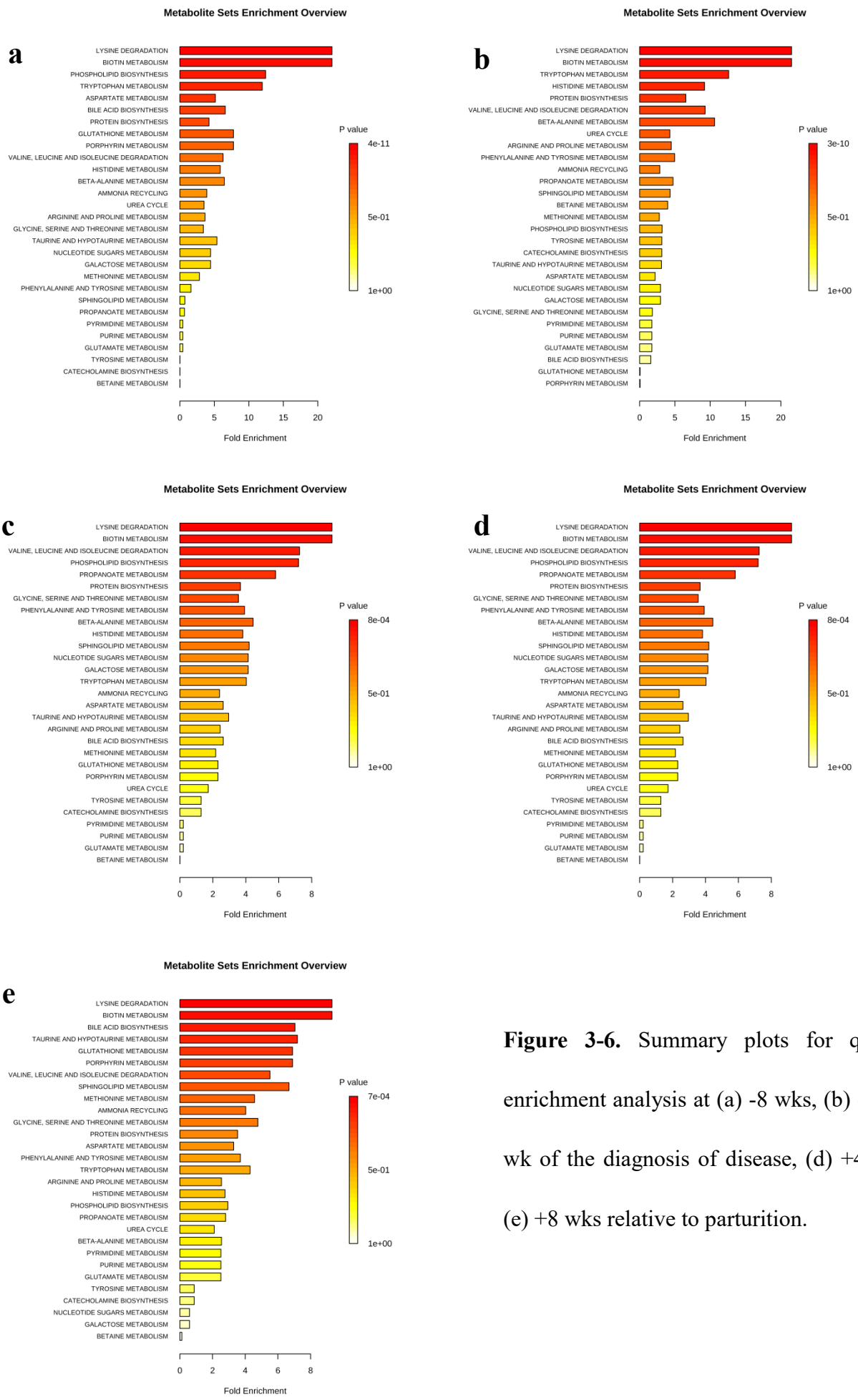

Figure 3-6. Summary plots for quantitative enrichment analysis at (a) -8 wks, (b) -4 wks, (c) wk of the diagnosis of disease, (d) +4 wks, and (e) +8 wks relative to parturition.

Chapter 4 NMR and MS based metabolomics identifies new predictive urinary biomarkers and highlights the pathobiology of ketosis in periparturient dairy cows

ABSTRACT

The aim of this study was to investigate metabolic fingerprints in the urine of ketotic cows before, during, and after the occurrence of disease and to identify newly predictive and diagnostic urine biomarkers that can be used to distinguish cows with ketosis from healthy controls (CON). In this study, NMR, DI/LC-MS/MS, GC-MS, and ICP-MS based metabolomics were performed to analyze urine samples from 6 cows with ketosis and 20 CON cows, at -8 wks, -4 wks, disease diagnosis, +4 wks and +8 wks relative to parturition. Univariate and multivariate analyses and biomarker analysis were used to select metabolite-sets for the noninvasive prediction and diagnosis of ketosis. A total of 72, 66, 49, 25, and 53 differential metabolites between the two groups were identified at -8 wks, -4 wks, disease diagnosis, +4 wks, and +8 wks, respectively. VIP plots ranked the most significant differential metabolites, which contributed to the onset and progression of ketosis. These involve the alteration of several key metabolic pathways. Moreover, two promising biomarker models including a 7-metabolite biomarker set [i.e., methylglutaryl-L-carnitine (C5-M-DC), octadecadienyl-L-carnitine (C18:2), hydroxypropionyl-L-carnitine (C3-OH), hexadecanoyl-L-carnitine (C16), Al, hydroxytetradecenoyl-L-carnitine (C14:1-OH), and hexadecadienyl-L-carnitine (C16:2)] at -8 wks and a 9-metabolite biomarker set [i.e., C3-OH, methanol, C16, phosphatidylcholine acyl-alkyl C30:2 (PC ae C30:2),

C18:2, Al, *N*-acetylglutamine, C5-M-DC, and tetradecenoyl-L-carnitine (C14:1)] at -4 wks, were identified for prediction of ketosis with excellent sensitivity and specificity. Overall, multiple urine metabolite alterations were identified in pre-ketotic, ketotic, and post-ketotic cows which could be used as potential screening biomarker, as well as to better understand the pathobiology of disease, and to develop new preventive treatments.

4.1 Introduction

Several recent metabolomics articles on ketosis in dairy cows have been focused mainly on blood or milk specimens at the time of disease diagnosis. These represent an important contribution to the understanding of the pathobiology of the disease and should help to further evolve the diagnostic tools. However, it would be of interest to develop screening biomarkers of ketosis before the clinical disease develops. Moreover, using urine as an analytical biofluid would be more advantageous because it is non-invasively acquired and easily accessible in large volumes. It also has a low content of proteins or lipids that would otherwise interfere (as in plasma or serum samples) in the analytical process. Metabolomic analysis of urine samples has been used for biomarker studies in human research for a very long time (Emwas et al., 2015, 2016). To the best of our knowledge, no urine metabolic profiling for predictive biomarker identification has been previously reported in dairy science.

The traditional urinary biomarker of ketosis, acetoacetate (AcAc), has been widely used by the dairy industry for diagnosing ketosis in the form of a semi-quantitative dipstick (i.e., Ketostix); however, this test is qualitative and has low

sensitivity. Furthermore it is used mainly for diagnostic purposes (Oetzel, 2004). Therefore, more sensitive and more specific urine biomarkers for the prediction and diagnosis of ketosis are warranted. The ideal predictive biomarkers should help identify pre-ketotic cows at early stages of disease, resulting in timely prophylactic strategies. Previous metabolomics studies have demonstrated that cows affected by ketosis have numerous alterations of metabolites, besides ketone bodies in the blood and milk. This suggests that ketosis is a very complex disease (Klein et al., 2011; Li et al., 2014). Similar fluctuations of metabolites might be present in the urine and might indicate subclinical disease in the earliest stages of ketosis.

In this study, a combination of four metabolomics analytical platforms including high resolution nuclear magnetic resonance (NMR) spectroscopy, direct injection/liquid chromatography tandem mass spectrometry (DI/LC-MS/MS), inductively coupled plasma mass spectrometry (ICP-MS), and gas chromatography mass spectrometry (GC-MS) were applied to improve metabolite coverage of urine metabolic profiles. Alterations in urine metabolites and associated metabolic pathways in cows with ketosis at different time periods around calving [i.e., -8/-4 wks prepartum, diagnosis of disease (+1 to +3 wks postpartum), and +4/+8 wks postpartum] when the disease is prevalent were comprehensively investigated. Therefore the objectives of this investigation were to evaluate alterations of urinary metabolites that can be used to distinguish pre-ketotic cows from healthy controls (CON) during the dry off period and help in better understanding the pathobiology of ketosis.

4.2 Materials and methods

4.2.1 Animals and diets

In this study, twenty healthy controls and 6 cows that developed ketosis were selected for further metabolomics analyses. Urine metabolomics analyses were conducted on samples from 5 time points: at -8 wks (53-59 d) and -4 wks (25-31 d) prior to parturition, the disease wk (5-21 d, mean: 13 d), at +4 wks (25-31 d) and +8 wks (53-59 d) after calving from each cow. Please refer to chapter 2 (section 2.1.1 Animals and diets) of this thesis for detailed information about the animals and diets used.

4.2.2 Urine sample collection

Urine samples were obtained from 100 transition Holstein dairy cows once per week at 0700 before feeding from -8 wks before parturition to +8 wks postpartum. Twenty healthy controls and 6 cows that developed ketosis (the same cows used in Chapter 3) were selected for further metabolomics analyses. Analyses were conducted on samples from 5 time points: at -8 wks (53-59 d) and -4 wks (25-31 d) before parturition, the disease (5-21 d, mean: 13 d) wk and at +4 wks (25-31 d) and +8 wks (53-59 d) after calving from each cow for urine metabolomics analysis. Urine samples were stored at -80 °C until analysis to avoid loss of bioactivity and contamination. All samples were thawed on ice for approximately 2 h before use.

4.2.3 DI/LC-MS/MS compound identification and quantification

To determine concentrations of amino acids (AAs), acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and hexose in the urine samples, a targeted quantitative metabolomics approach was applied using a commercial kit (Absolute*IDQ* 180) that combined direct injection and tandem mass spectrometry (DI-MS/MS) with a reverse-phase liquid chromatography and tandem mass spectrometry (LC-MS/MS). The kit is available from BIOCRATES Life Science AG (Innsbruck, Austria). This kit assay is used in combination with an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex, Foster City, CA) mass spectrometer and can be used for the targeted identification and quantification of up to 186 different endogenous metabolites from six analyte groups. Please refer to chapter 3 (section 3.2.3) for detailed procedures of DI/LC-MS/MS analysis.

4.2.4 NMR compound identification and quantification

After thawing, urine samples were centrifuged for 5 min at 10,000 rpm (Eppendorf Centrifuge 5424, Eppendorf AG, Hamburg, Germany) under +4 °C. A 600 µL aliquot of sample supernatant was added in an Eppendorf tube (1.5 mL) followed by the addition of 70 µL of D_2O (Deuterium oxide, Sigma-Aldrich Co., St. Louis, MO, USA) and 30 µL of a standard buffer solution [11.667 mM DSS (disodium-2,2-dimethyl-2-silapentane-5-sulphonate), 730 mM imidazole, and 0.47% NaN_3 in H_2O]. Urine samples were vortexed and mixed completedly and then centrifuged (Heraeus Instruments GmbH, Germany) under 10,000 rmp × 5 min × 4 °C.

The urine samples (700 μL) were then transferred to a standard glass NMR tube (5 mm thin wall, Wilmad LabGlass, Vineland, NJ, USA) for subsequent NMR spectral analysis. The pH of urine samples ranged from 7.3 to 7.7. A total of 102 urine samples were prepared under this protocol.

All proton NMR (^{1}H-NMR) spectra were obtained on a 500 MHz Inova spectrometer (Varian Inc., Palo Alto, CA) equipped with a 5 mm hydrogen, carbon, and nitrogen (HCN) Z-gradient pulsed-field gradient (PFG) Varian cold-probe. ^{1}H-NMR spectra were acquired at 25 °C using the first transient of the Varian tnnoesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy (Saude et al., 2006). Spectra were collected with 128 transients and 8 steady-state scans using a 4 s acquisition time and a 1 s recycle delay (Bouatra et al., 2013).

The ^{1}H-NMR spectra were processed and analyzed with the Chenomx NMR Suite Professional software package (version 7.6, Chenomx Inc., Edmonton, AB, Canada) as previously described (Weljie et al., 2006; Wishart, 2008a). Prior to spectral analysis, all FIDs were zero-filled to 64,000 data points and line broadened 0.5 Hz. The singlet produced by DSS was used as an internal standard for both chemical shift referencing (set to 0 ppm) and for metabolite quantification. Each spectrum was processed and analyzed independently by at least two experienced NMR spectroscopists to minimize errors of compound identification and quantification.

4.2.5 GC-MS compound identification and quantification

The extraction and derivatization protocol for organic acids was adapted from a previously reported method (Tanaka et al., 1980). Briefly, an aliquot of 200 µL of urine sample, quality control (QC), and blank (100 µL HPLC water), were mixed with 40 µL (75 g/L in H_2O) methoxylamine hydrochloride solution in a 2 mL glass vial and incubated at 60 °C for 30 min, to convert ketoacids to their methoxime derivatives. The mixture was transferred in to a 1.5 mL Eppendorf tube and extracted two times with ethyl acetate. Add 20 µL (3.64 mM) of cholesterol as internal standard into the mixture before the first extraction. For each extraction, add 600 µL of ethyl acetate in the mixture, vortex thoroughly for 5 min and spin samples at 10,000 rpm for 3 min. The supernatant from the first and second extraction was combined in a glass vial with screw cap (Agilent, Santa Clara, CA, USA). The supernatant was evaporated in a 35 °C heating block under nitrogen. After evaporation, the residue was dissolved in 160 µL of hexane and 40 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added. The resulting mixture was incubated in a heating block at 70-90 °C for 15 min after with the BSTFA-supplemented extract was transferred into an insert (250 µL, Agilent, Santa Clara, CA, USA) of a vial using a transfer pipet and placed into a GC-MS auto-sampler vial.

Derivatized extracts were injected by Agilent 7683 Series autosampler (Agilent Technologies, Palo Alto, CA, USA) followed by the analysis employing Agilent 6890N GC system coupled with electron impact (EI) ionization mode 5973N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). A 2 µL aliquot was

injected with a 5:1 split ratio onto a 30 m × 0.25 mm × 0.25 µm DB-5 column (Agilent Technologies). The injector port temperature was held at 250 °C and the helium carrier gas flow rate was set to 1 mL/min at an initial oven temperature of 70 °C. The oven temperature was increased at 6 °C/min to 310 °C for a final run time of 54 min. Full scan spectra (50-500 m/z; 1.7 scans/sec) were acquired after a 5 min solvent delay, with an MS ion source temperature of 200 °C. The QC was treated and analyzed in the same way as serum samples to investigate the reproducibility and repeatability of the methods. A QC was run every 10 samples to monitor the stability and reproducibility of the method. In addition, hexane and a blank sample was run as well for the elution of residual impurities and analytes from the glass liner and the capillary column at the beginning of the sequence. All the derivatized samples were run within 24 h after preparation. After running all the samples, a mixture of alkane standard solution C8-C20 and C21-C40 (1:1 vol/vol, Sigma-Aldrich, Oakvile, ON, Canada) was injected to get the retention times of n-alkanes for the calculation of the Kovat's retention index of metabolites instantly.

Raw MS data (".D" file format) were first transformed into CDF format by the ChemStation Data Analysis software (Agilent Technologies, Palo Alto, CA, USA) prior to data pretreatment. Identification and quantification of metabolites was firstly processed and analyzed automatically by GC-AutoFit (http://gcms.wishartlab.com/) and results were confirmed manually following the method as previously described (Wishart et al., 2008b). Briefly, the Automated Mass Spectral Deconvolution and Identification System (AMDIS) spectral deconvolution software (Version 2.70) from

NIST (National Institute of Standards and Technology) was used to process the total ion chromatogram and the EI-MS spectra of each GC peak. After deconvolution, the purified mass spectrum of each of the trimethylsilylated metabolites was identified using the NIST MS Search program (version 2.0d) linked to the NIST mass spectral library (2008). Retention Indices (RIs) were calculated using a C8-C20 and C21-C40 alkane mixture solution (Fluka, Sigma-Aldrich) which served as an external alkane standard. Metabolites were identified by matching the EI-MS spectra with those of reference compounds from the NIST library. In AMDIS, each search produces a list of library spectra ("hits"), which is ranked by the similarity to the target spectrum according to a mathematically computed "match factor". The match factor indicates the likelihood that our spectrum and the reference NIST spectrum arose from the same compound. In the current case, we considered hits with a match factor of >60% and a probability >20%. In addition, authenticity checks were performed by using additional published retention index libraries. RIs and EI spectra were subsequently used for producing external seven-point calibration curves (for absolute quantification).

4.2.6 ICP-MS compound identification and quantification

Before trace element analysis by ICP-MS was performed, all urine samples were processed based on a protocol adapted from previously described (Cava-Montesinos et al., 2005). In particular, 1 mL urine sample was aliquoted and sonicated in an ultrasound water bath for 10 min in order to obtain a homogeneous dispersion. 0.1 mL

HNO_3, 0.1 mL internal standards (i.e., In, Bi, and Sc) and 8.8 mL DI H_2O was added and the samples shaken for 20 s prior to analysis. The sample was subjected to acid digestion by adding 2 mL of 8 M HNO_3, boiling for 10 min and then centrifuging the sample in a Beckman GPKR centrifuge (Beckman Coulter, Inc., Brea, CA, USA) at 3,000 rpm for 10 min. Prior to conducting the ICP-MS analysis, the supernatant was taken and filtered through Whatman™ (grade 2) filter paper (Fisher Scientific, Toronto, ON, Canada). The remaining filtrate was dried and used for ICP-MS based elemental analysis. The ICP-MS analysis was carried out using a Perkin-Elmer Sciex® Elan™ 6000 quadrupole ICP-MS (PerkinElmer, Inc., Waltham, MA, USA) operating in a dual detector mode with an ICP RF power of 1,300 W. Blank subtraction was applied after internal standard correction with Bi, Sc and In being used as internal standards. Quantification of all elements was done by four point calibration curves (0, 0.25, 0.50, and 1.00 ppm for Na, Ca, Mg, Fe, K, P, and 0, 0.005, 0.010, and 0.020 ppm for other remaining elements). The flow rate was approximately 1 mL/min with 35 sweeps per reading using one reading per replicate with three replicates being measured. Dwell times were 150 ms for Se, 10 ms for Na, Al, K, Cu, Zn, Sr, and 20 ms for all other elements. The integration times were 5,250 ms for Se, 350 ms for Na, Al, K, Cu, Zn, Sr and 700 ms for all other elements. The relative standard deviation (2σ level) for As, Ni, Pb, and Zn was between 5 and 10%. The accuracy of the ICP-MS analytical protocol was periodically evaluated via the analysis of certified reference standard materials (whole rock powders) BE-N and DR-N available from the SARM laboratory at the CRPG (Centre de Recherches Pétrographiques et

Géologiques, Nancy, France) (Bouatra et al., 2013).

4.2.7 Statistical analysis

All metabolite concentrations measured from different analytical methods were normalized to each urine sample's corresponding creatinine value (assuming a constant rate creatinine excretion for each urine sample) to compensate for variations in urine volume. The concentration of each metabolite is expressed as μM/mM creatinine. All concentration data from the different methods (i.e., DI/LC-MS/MS, NMR, GC-MS, and ICP-MS) was pooled together for multivariate analysis, pathway analysis, and biomarker analysis.

Univariate analysis of data was performed using Wilcoxon-Mann-Whitney (rank sum) test provided by R (Version 3.0.3, R Development Core Team, 2008). Statistical significance was declared at $P < 0.05$. All metabolomics data were processed and analyzed using the MetaboAnalyst software (Xia et al., 2009). Recommended statistical procedures for metabolomics analysis were followed according to previously published protocols (Xia et al., 2009). Biomarker profiles and the quality of the biomarker sets were determined using receiver-operator characteristic (ROC) curves as calculated by MetaboAnalyst 3.0 (Xia et al., 2015). For information regarding the details of the univariate and multivariate data analyses, metabolic pathway analysis, and biomarker analysis, please refer to 'Statistical analysis' section (Section 3.2.4) in chapter 3.

4.3 Results

The DI/LC-MS/MS targeted metabolomics analysis using the Biocrates Absolute*IDQ* kit quantified 140 metabolites [40 acylcarnitines, 9 lysophosphatidylcholine (lysoPC), 67 phosphatidylcholine (PC), 9 hydroxysphingomyelin [SM (OH)] or sphingomyelin (SM), hexose, 11 amino acids, and 3 biogenic amines] in urine of both ketotic and CON groups of cows (Table 4-1 and Table 4-2). Eighty-eight metabolites (26 organic acids, 36 amino acids and derivatives, 6 saccharides, 3 ketones, 3 alcohols, and 14 misc) were identified and quantified in each urine sample by ^{1}H NMR analysis (Table 4-3 and Table 4-4). A total of 47 metabolites were identified by GC-MS, however, only 13 metabolites (organic acids) were both identified and quantified in most urine samples (Table 4-5 and Table 4-6). Targeted multi-elemental analysis of urinary minerals by ICP-MS provided quantitative results for a total of 18 metals or trace minerals in both CON and cows with ketosis (Table 4-5 and Table 4-6). The complete list of metabolite concentrations [means ± standard deviations (SD)], *p*-value, fold change, and direction of change (up or down) in pre-ketotic cows and cows diagnosed with ketosis relative to CON cows are shown in Table 4-1, 4-2, 4-3, 4-4, 4-5, and 4-6. Metabolites detected but with more than 30% missing values are not reported in this paper.

4.3.1 Metabolic urine alterations before disease diagnosis and newly predictive biomarkers

Univariate and multivariate analyses were performed to compare urine metabolite

fingerprints of ketotic cows versus CON cows at two time points during the dry-off period (i.e., -8 wks and -4 wks prepartum) to identify predictive biomarkers for ketosis. Univariate analysis (i.e., *t*-test or Wilcoxon Mann Whitney test) indicated 72 and 66 urinary metabolites were altered ($P < 0.05$) in cows with ketosis at -8 wks and -4 wks, respectively (Table 4-1, 4-3, and 4-5). Specifically, the majority of metabolites were elevated in pre-ketotic cows including 20 acylcarnitines [e.g., methylglutaryl-L-carnitine (C5-M-DC), octadecadienyl-L-carnitine (C18:2), and hydroxypropionyl-L-carnitine (C3-OH)], 1 lysoPC [i.e., lysophosphatidylcholine acyl C28:1 (lysoPC a C28:1)], 10 PC [e.g., phosphatidylcholine diacyl C28:1 (PC aa C28:1), PC aa C42:6, and phosphatidylcholine acyl-alkyl C38:0 (PC ae C38:0)] hexose, 12 amino acids or derivatives [e.g., L-lysine (L-Lys), L-tryptophan (L-Trp), and pantothenic acid], 3 biogenic amines [i.e., symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA), and carnosine], 3 organic acids (i.e., ascorbic acid, gluconic acid, and isocitric acid), 1 alcohol (i.e., methanol), 5 minerals [i.e., aluminum (Al), boron (B), chromium (Cr), potassium (K), and rubidium (Rb)], 3 from the misc group (i.e., hypoxanthine, imidazole, and myo-inositol) at -8 wks, and 13 acylcarnitines [e.g., C3-OH, Hexadecanoyl-L-carnitine (C16), and C18:2], 3 PC (i.e., PC aa C42:5, PC ae C38:6, and PC ae C44:3), 19 amino acids or derivatives [e.g., L-phenylalanine (L-Phe), L-Lys, and L-arginine (L-Arg)], 3 biogenic amines (i.e., SDMA, ADMA, and carnosine), 1 alcohol (methonol), 8 minerals [e.g., Al, B, and calcium (Ca)], 3 from the misc group (e.g., *myo*-inositol, hypoxanthine, and trimethylamine) at -4 wks (Table 4-1, 4-3, and 4-5). Additionally, a few urinary

metabolites, most of which belong to phosphatidylcholines, were lower in pre-ketotic cows. Examples of this class of metabolites include PC aa C32:2 and PC ae C30:2, which were decreased in the urine of pre-ketotic cows compared with CON cows at both -8 and -4 wks (Table 4-1). Another two interesting metabolites were SM C16:0 and urea, both of which decreased in pre-ketotc cows at both time points during the dry off period.

In multivariate analysis, both 2-dimensional and 3-dimensional principal component analysis (PCA) and partial least squares - discriminant analysis (PLS-DA) score plots revealed clearly distinguished clusters between the two groups of cows based on a combination of all urinary metabolites measured from four metabolic profiling instruments at -8 wks and -4 wks (Figure 4-1a&b; Figure 4-2a&b). The data from -8 wk showed that metabolite fingerprints of pre-ketotic were separate from those of CON cows with principle component (PC) 1 at 28.1 % and PC2 at 15.1 % for PCA, and PC1 at 27.5 % and PC2 at 9.9 % for PLS-DA, respectively (Figure 4-1a&b). To understand the contribution of individual urine metabolites for the variation of the first two PC's (i.e., PC1 and PC2), we used the corresponding loading plot for both PCA and PLS-DA models. Examples of a loading plot for the PLS-DA models at -8 and -4 wks are presented in Figure 4-1c and Figure 4-2c. The loading plot shows similar or distinct behaviors between variables. Metabolites with the same distance and similar directions from 0 are positively correlated, whereas those in opposite directions are negatively correlated. For instance, C5-M-DC, C18:2, C3-OH, C16, and hydroxytetradecenoyl-L-carnitine (C14:1-OH) were positively correlated with each

other in ketotic cows. These metabolites, however, are negatively correlated with PC aa C38:5 and urea. Moreover, those five urinary metabolites that are positively associated with each other in ketotic cows appear to contribute significantly in the separation along the PC1 axis. The *P*-value for a permutation test with 2,000 resampling steps for the PLS-DA model was lower than 0.05.

The variable importance in projection (VIP) plot from PLS-DA ranked the top 15 significant metabolites that contributed mostly to the separation of pre-ketotic cows from CON cows at -8 wks and -4 wks (Figure 4-1d; Figure 4-2d). The VIP plots indicated that C5-M-DC, C18:2, C3-OH, C16, Al, C14:1-OH, and hexadecadienyl-L-carnitine (C16:2) at -8 wks, and C3-OH, methanol, C16, PC ae C30:2, C18:2, Al, *N*-acetylglutamine, C5-M-DC, and tetradecenoyl-L-carnitine (C14:1) at -4 wks were the strongest differential metabolites in urine for separating pre-ketotic cows from CON ones. To visualize the relationship and distinctions in the levels of urine metabolites among each sample at -8 wks and -4 wks, a heat map was constructed based on the PLS-DA VIP scores and top 25 metabolites are shown in Figure 4-1e and Figure 4-2e. For example, Figure 4-1e shows that among the top 25 metabolites, concentrations of 19 urine metabolites (e.g. Lys, Al, and myo-inositol) were greater in 6 pre-ketotic cows, whereas the other 6 metabolites, all of which were lysoPC's (e.g., lysoPC a C17:0) and PC's (e.g., PC aa C38:5) groups were lower in pre-ketotic cows at -8 wks. A 7-metabolite biomarker set (i.e., C5-M-DC, C18:2, C3-OH, C16, Al, C14:1-OH, and C16:2) at -8 wks, and a 9-metabolite biomarker set (i.e., C3-OH, methanol, C16, PC ae C30:2, C18:2, Al, *N*-acetylglutamine, C5-M-DC,

and C14:1) at -4 wks prepartum were built as predictive urine biomarkers for ketosis based on VIP scores. The performance of these biomarkers was evaluated by a receiver operating characteristic (ROC) curve at -8 wks and -4 wks using a PLS-DA model (Figure 4-1f; Figure 4-2f). The area under the ROC curve (AUC) for two ROC curves are 0.99 (95% CI, 0.938-1) with empirical *P*-value = 0.001 (under 1,000 permutations) at -8 wks and 0.993 (95% CI, 0.906-1) with empirical *P*-value = 0.003 (under 1,000 permutations) at -4 wks, respectively, which suggests that these urine biomarkers identified are very strongly predictive for ketosis in dairy cows.

4.3.2 Metabolic urine alterations during the week of diagnosis of ketosis and newly diagnostic biomarkers

Results of the univariate analysis during the wk of ketosis diagnosis showed that 49 urine metabolites were different ($P < 0.05$) between the two groups of cows (Table 4-1, 4-3, and 4-5). The majority of elevated metabolites in the urine of ketotic cows at the disease wk were from acylcarnitines [e.g., C5-M-DC, dodecanoyl-L-carnitine (C12), and C18:2], and organic acids (i.e., ascorbic acid, glycolic acid, and L-lactic acid) (Table 4-1 and 4-3). Moreover, concentrations of two urinary ketones [i.e., 3-hydroxybutyric acid (3HBA or BHBA), acetoacetic acid (AcAc)] were significantly greater whereas those of acetone (Ac)] had a tendency ($P = 0.07$) to be greater in ketotic cows versus the CON cows (Table 4-3). On the contrary, concentrations of most PC metabolites (e.g., PC ae C30:2, PC aa C40:4, and PC aa C38:3) decreased in cows that developed ketosis during the disease wk.

Both PCA and PLS-DA (*P*-value < 0.05 in a 2,000 permutation test) analyses

indicated consistently separated clusters between ketotic and CON cows (Figure 4-3a&b). Figure 4-3c&e shows the PLS-DA loading plot and the heat map with the top 25 metabolites based on PLS-DA VIP. Five metabolites including PC ae C30:2, C5-M-DC, PC aa C40:4, SDMA, and PC aa C38:3 (Figure 4-3d) were selected as new diagnostic biomarkers for ketosis. The 5-metabolite biomarker set was assessed by ROC curve analysis and exhibited very high sensitivity and specificity with an AUC at 0.994 (95% CI: 0.969-1) (Figure 4-3f). The empirical *P*-value was 0.001 for the 1,000 permutation test of the ROC curve.

4.3.3 Metabolic urine alterations in post-ketotic cows

Comparisons of urinary profiles between the two groups of cows by a *t*-test or Wilcoxon Mann Whitney test revealed that post-ketotic cows experienced alterations in 25 and 53 urine metabolites at +4 wks and +8 wks, respectively (Table 4-2, 4-4, and 4-6). No differences were observed regarding concentrations of three ketone bodies (BHBA, AcAc, and Ac) in the urine between the two groups, although all of them were numerically greater in post-ketotic cows at +4 and +8 wks after parturition (Table 4-4). In the absence of ketosis, post-ketotic cows still had 8 and 20 acylcarnitines elevated at +4 and +8 wks after parturition (Table 4-2). Interestingly, numerous phosphotidylcholines were elevated in post-ketotic cows, especially, during the +8 wks (11 PC's increased in cows with ketosis) (Table 4-2). Moreover, metals or trace minerals were remarkably increased at both postpartum time points. Specifically, nine [e.g. Al, phosphorus (P), and magnesium (Mg)], and 13 other [e.g., B and Cr]

urinary minerals were increased in post-ketotic cows compared with CON ones at +4 and +8 wks, respectively.

Multivariate analyses, including PCA and PLS-DA, showed clearly separated clusters between the two groups of cows at +4 wks and +8 wks postpartum (Figure 4-4a&b; Figure 4-5a&b). The PLS-DA loading plots and heat map, showing the top 25 metabolites for each sample at two postpartum time points, are shown in Figure 4-4c&e and Figure 4-5c&e. The VIP plots indicate that the top 6 urine metabolites (i.e., PC ae C36:4, Al, C16:2, *N*-acetylglutamic acid, C5-M-DC, and P) at +4 wks, and the top 6 urine metabolites [i.e., decenoyl-L-carnitine (C10:1), methylmalonyl-L-carnitine/hydroxyvaleryl-L-carnitine: C5-OH (C3-DC-M), PC aa C38:1, C16:2, P, and C3-OH] at +8 wks were significantly different between post-ketotic cows and CON cows (Figure 4-4d and Figure 4-5d). ROC curves for the two sets of differential metabolites revealed that the AUC for the 6-metabolite set at +4 wks and the 6-metabolite set at +8 wks were both 1 (95% CI, 1-1) (Figure 4-4f and Figure 4-5f).

4.3.4 Metabolic pathways associated with the onset and progression of ketosis

Both univariate and multivariate analyses revealed significant alterations of urinary metabolites in the pre-ketotic cows starting at -8 wks prepartum, ketotic (at diagnosis wk) as well as in post-ketotic cows at +8 wks parturition. Biomarker analysis also identified highly specific predictive and diagnostic biomarkers for ketosis or discriminators between the two groups of cows at different time points.

Although some biomarker overlap appeared consistently before and at the wk of diagnosis, most biomarkers of the perspective of disease are restricted to a certain sampling time. In order to have a better understanding of ketosis from the disease initiation to progression, and until after recovery, a longitudinal view of metabolic alterations and screening consistently perturbed metabolites at different stage of ketosis is warranted.

Three metabolites, all acylcarnitines (i.e., C5-M-DC, C16, and C16:2), were elevated at all five tested time points (Table 4-1 and 4-2). Moreover, another 9 metabolites including 3 acylcarnitines [i.e., C3-OH, C5-OH (C3-DC-M), and pimelyl-L-carnitine (C7-DC)], 3 PC (i.e., PC aa C32:2, PC ae C30:2, and PC ae C44:3), SDMA, pantothenic acid, and myo-inositol before and during the wk of diagnosis (i.e., three time points at -8, -4 wks, and disease wk) (Table 4-1 and 4-3). Interestingly, after the 3 ketone bodies in the urine of ketotic cows returned to similar levels with those of CON cows, remarkable increases of seven urinary minerals such as Al, B, P, K, Ca, Mg, and Cr were seen in post-ketotic cows at both +4 and +8 wks after parturition.

Metabolic pathway analysis such as metabolite sets enrichment analysis was done by MetaboAnalyst 3.0 (Xia et al., 2009; 2015) at five different time points. A few databases including 'The Small Molecule Pathway Database' (SMPDB) (Frolkis et al., 2010; Jewison et al., 2014), 'The Urine Metabolome Database' (UMD) (Bouatra et al., 2013), 'The Bovine Metabolome Database' (BMDB) (http://www.cowmetdb.ca/cgi-bin/browse.cgi), and 'The Kyoto Encyclopedia of

Genes and Genomes' (KEGG) (Kanehisa and Goto, 2000) were referenced in the summary of altered metabolic pathways during ketosis. Significantly altered urine metabolites and corresponding metabolic pathways (the main 51 pathways are listed) at the five tested time points are shown in Table 4-7. In particular, fatty acid transport metabolism (FFT) and lipid catabolism (LC) were significantly perturbed in cows with ketosis at all 5 time points from -8 wks to +8 wks. Besides, glycerophospholipid metabolism (GPL), free fatty acid metabolism (FFAM), methane metabolism (MM), beta-alanine metabolism (BAM), pantothenate and CoA biosynthesis (PCB), galactose metabolism (GAM), and inositol metabolism (IM) were altered before and during diagnosis of ketosis.

4.4 Discussion

In this study, four types of quantitative metabolic fingerprinting methodologies (i.e., DI/LC-MS/MS, NMR, GC-MS, and ICP-MS) were performed on 102 urine samples collected from 20 CON and 6 cows affected by ketosis at five time points (-8 wks, -4 wks, at diagnosis of disease, +4 wks, and +8 wks relative to parturition). Urinary metabolic profiles were not only altered at the time when ketosis was diagnosed at early lactation, but also were already significantly changed at the earlier time points during the dry off period (i.e., -8 wks and -4 wks before calving), when no hyperketonemia was present. Therefore, the current hypothesis of negative energy balance (NEB) and hyperketonemia being a single phenotype is insufficient in interpreting the mechanism of ketosis, especially during the early stages of disease initiation. By using quantitative metabolomics, we identified several predictive and

diagnostic urinary biomarkers of ketosis. The major metabolites and associated metabolic pathways that might be involved in the onset and progress of ketosis are discussed below.

4.4.1 Acylcarnitines and fatty acid metabolism

Acylcarnitines (ACs) are present in cells and tissues of all mammalian species. They play an important role as transporters of long-chain fatty acids (LCFA) into mitochondria for β-oxidation (Jones et al., 2010). The main source of ACs is predominantly the diet; however, endogenous biosynthesis also plays a role. ACs can be synthesized *in vivo* from L-methionine (L-Met), and L-lysine (L-Lys), mostly in the liver and kidney (Borum, 1983). Transport of acyl groups from acyl-Coenzyme A (acyl-CoA) in the cytosol to carnitine to generate ACs occurs through the action of the enzyme carnitine palmitoyltransferase (CPT)-1 in mitochondria. Then, ACs are catabolized by the enzyme CPT-2 to produce free acyl-CoA, which is further metabolized into fatty acids through β-oxidation or ketogenic pathways (Ramsay and Zammit, 2004). Moreover, ACs are transported out of the mitochondria into the cytosol across the inner mitochondrial membrane by carnitine-acylcarnitine translocase, and then transported throughout the body by systemic circulation to deliver various acyl groups for a wide range of functions (Jones et al., 2010). Therefore, ACs are critical substrates for both β-oxidation and ketogenesis, and metabolic perturbations of ACs might be closely associated with the development of ketosis.

Acylcarnitine changes detected in various disorders of fatty acid β-oxidation and organic acid metabolism have been reported in several AC profile analyses (Jones et al., 2010; Rinaldo et al., 2008). For example, both carnitine-acylcarnitine translocase (CACT) deficiency and CPT-2 deficiency are associated with increased levels of C16, C18:2, octadecenoyl-L-carnitine (C18:1), and octadecanoyl-L-carnitine (C18). Deficiencies of very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and short-chain acyl-CoA dehydrogenase (SCAD) all contribute to elevated ACs with a chain length of different carbons, indicating incomplete long-chain, medium-chain, and short chain fatty acid β-oxidation (Adams et al., 2009, Jones et al., 2010). In humans, a rapid increase of both short- and long-chain ACs in the plasma/serum and urine have been observed during fasting or diabetic ketosis (Frohlich, et al., 1978; Genuth and Hoppel, 1979; Hoppel and Genuth, 1980). It was also reported that hepatic levels of CPT-2, LCAD, CPT-1, 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS), and acetyl-CoA carboxylase (ACC) were decreased in ketotic cows (Li et al., 2012). We found that cows with ketosis had increased concentrations of numerous ACs including C16, C16:2, C18:2, hydroxyhexadecadienyl-L-carnitine (C16-OH), C5-M-DC, C5-OH (C3-DC-M), and C7-DC in their urine when they were diagnosed with ketosis.. Interestingly, concentrations of these urinary ACs were already elevated in pre-ketotic cows at -8 wks or -4 wks prepartum, suggesting that disorders of fatty acid β-oxidation were present in those cows during the dry-off period. This suggests that concentrations of ACs in the urine can serve as important predictive biomarkers

for ketosis in dairy cows. Increased urinary excretion of acylcarnitines might be a protective mechanism to alleviate systemic inflammation because they contribute to the activation of pro-inflammatory signaling pathways (Rutkowsky et al., 2014).

4.4.2 lysoPC/PC and glycerophospholipid metabolism

Phosphatidylcholine (PC) is an essential and the most abundant phospholipid (varying from 60% to 80% in different species) in all mammalian cells and tissues (Li and Vance, 2008; Pison et al., 1994). As the main phospholipid in ruminants, PC is critical for cell membrane structure, free fatty acid metabolism (FFAM), lipid absorption, cell signaling, and synthesis of lipoproteins (Zeisel and Holmes-McNary, 2001). In animals, PC is biosynthesized from choline, which is obtained from the diet through the choline pathway or via the *de novo* biosynthesis through the methylation of phosphatidylethanolamine (PE), and then catabolized by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT) (Bremer and Greenberg., 1961). Both exogenous and endogenous choline are converted into PC, which constitutes approximately 95% of the total choline pool in most animal tissues (Li and Vance, 2008). In a rodent study with a choline-deficient diet it was shown that limiting PC precursors lowered PC synthesis (i.e., a 50% decrease in hepatic PC content) and mice treated with the choline-deficient diet developed severe steatohepatitis, steatosis, and liver failure after 3 days (Walkey et al., 1998). Normal concentrations of PCs are required for secretion of very low density lipoprotein (VLDL) from hepatocytes to remove triglycerides (TG) (Yao and Vance, 1988).

Results from the current study showed that ketotic cows had decreased levels of PCs (e.g., PC aa C32:2, PC ae C30:2, PC aa C32:0, PC aa C36:4, and PC ae C42:1) in urine during the week of ketosis diagnosis and even during the dry-off period. Especially, a variety of metabolites from the group of PCs such as PC ae C30:2, PC aa C36:4, PC aa C38:3, PC aa C40:4, and PC aa C38:3 were decreased during the disease wk and are included as diagnostic biomarkers of ketosis. We believe that cows with ketosis might have had a mild case of fatty liver disease before parturition and subclinical symptoms of ketosis will need to be addressed.

Indeed, a metabolomics study in dairy cows identified several PC biomarkers of hepatic lipidosis (also known as fatty liver disease, which is a complication of type II ketosis) including PC aa C36:4, PC aa C38:3, PC aa C40:4, and PC aa C38:3, which were lower in the serum of cows with hepatic lipidosis (Imhasly et al., 2014). Our results of urine PC profiles in cows with ketosis are in agreement with serum PC profiles of cows with hepatic lipidosis in a recent study by Imhasly et al. (2014). Lowered concentrations of PCs in the urine of pre-ketotic cows in our study at -8 wks and -4 wks prepartum suggests that those cows might be in their early stages of mild hepatic lipidosis. Since pre-ketotic cows and CON cows had no difference in their dry matter intake (DMI) at -8 wks and -4 wks prepartum, perturbations to the choline pathway might not be the main reason for shortage of PC biosynthesis. It was reported that the PEMT pathway for PC biosynthesis, catalyzed by the transferase enzyme PEMT, is uniquely significant in the liver (Vance and Ridgway, 1988). Therefore, the second pathway (i.e., the endogenous pathway for PC biosynthesis in animals) by

PEMT might be suppressed in cows with ketosis, which reduces secretion of VLDL and further contributes to the accumulation of TG in the liver affecting liver functions.

4.4.3 Amino acid metabolism

Besides their role as building blocks of polypeptides and proteins, amino acids also are important precursors for gluconeogenesis and ketogenesis (D'Mello, 2003; Pasquale, 2007), and critical regulators or intermediates in various metabolic pathways that are associated with maintenance, growth, reproduction, immunity, cell signaling, and oxidative stress (Galli, 2007; Li et al., 2007; Moriwaki et al., 2004; Wu, 2009). Therefore, homeostasis of amino acid metabolism is vital for maintaining health and preventing metabolic or infectious diseases. In this study, we found that several amino acids [e.g., L-arginine (L-Arg), L-aspartic acid (L-Asp), L-glutamine (L-Gln), glycine (Gly), β-alanine (β-Ala), L-cysteine (L-Cys), L-isoleucine (L-Ile), L-lysine (L-Lys), L-phenylalanine (L-Phe), and L-tyrosine (L-Tyr)] and their metabolic products such as ADMA, SDMA, carnosine, urea, 3-aminoisobutyric acid, N-acetylglutamic acid, pantothenic acid, 1-methylhistidine, and 3-methylhistidine, were increased in the urine of cows with ketosis, especially at -8 wks and -4 wks before parturition.

A disturbance of amino acid metabolism in ketotic cows has been previously noted (Zhang et al., 2013). A comparative proteomic study of liver tissue showed that arginase-1, a key enzyme that catalyzes the first step of Arg degradation through the urea cycle, was decreased in cows with ketosis (Xu and Wang, 2008). In the current

study, the concentration of L-Arg in the urine was greater, whereas, that of urea was lower in ketotic cows. Our data are consistent with the reported findings of Xu and Wang (2008) that the degradation of Arg is suppressed due to the downregulation of hepatic argnase-1 in ketotic cows. Interestingly, both ADMA and SDMA, two analogues of L-Arg were increased in the urine of pre-ketotic cows. Increased levels of ADMA have been shown to be strong risk predictors for various diseases such as diabetes mellitus, chronic renal failure, atherosclerosis, hypercholesterolemia, and cardiovascular diseases in humans (Sibal et al., 2010).

It is important to understand why concentrations of numerous amino acids were increased in pre-ketotic cows at -8 wks and -4 wks prepartum, long before ketosis was diagnosed after parturition? As important precursors of gluconeogenesis (e.g., L-Arg, L-Asp, L-Cys, L-Gln, Gly) and ketogenesis (e.g., L-Lys), amino acids are degraded into multiple intermediates including α-ketoglutarate, fumarate, oxaloacetate, pyruvate, succinyl CoA, acetoacetate and acetyl-CoA, which enter the tricarboxylic acid (TCA) cycle to be used for energy and catabolized into end-products like CO_2 and H_2O, or they are diverted into the process of ketogenesis (D'Mello, 2003; Wu, 2009).

Besides their roles in gluconeogenesis or ketogenesis, amino acids also play significant roles in immune functions (Li et al., 2007; Wu, 2009). Indeed, in a companion article we reported that pre-ketotic cow had greater concentrations of interleukin-6 (IL-6), tumor necrosis factor (TNF), and haptoglobin (Hp) in the serum at -8 wks and -4 wks before parturition versus CON cows (Zhang et al., 2016).

Elevated concentrations of these innate immunity reactants in the serum of pre-ketotic cows during the dry-off suggest that an acute phase response or systemic immune response was activated. An unidentified factor during the dry-off period might be involved in activation of innate immune responses during this time period and promote excessive breakdown of proteins from muscles to release sufficient amino acids, necessary for biosynthesis of antimicrobials like antibodies, cytokines, and APPs. Moreover, it was reported that six functional amino acids including Arg, Cys, Gln, Leu, Pro, and Trp play important roles in enhancing immune status of the body and regulating lymphocytes and macrophages' immune response to stimuli (Li et al., 2007; Wu, 2009).

Six metabolites including β-Ala, carnosine, L-Asp, pantothenic acid, 3-methylhistidine which are involved in beta-alanine metabolism were elevated in the urine of pre-ketotic cows at -8 wks and -4 wks prepartum. Carnosine, a dipeptide made of the precursors β-Ala and L-histidine (L-His), has been reported to be an important antioxidant for oxidative stress reduction (Boldyrev and Severin, 1990; Prokopieva et al., 2016). Several *in vivo* studies demonstrated that carnosine and His can lower hepatic TG and cholesterol in diabetic condition and alleviate hepatic steatosis (Lee et al., 2005; Mong et al., 2011). Pantothenic acid, or vitamin B5, has considerable metabolic importance in production of energy from carbohydrates, fatty acids, and amino acids as it is an integral part of CoA, phosphopantetheine, and acyl-carrier-protein (ACP), all of which are involved in fatty acid metabolism (Ragaller et al., 2011). Ruminants have two sources of pantothenic acid including

feedstuff and biosynthesis by microorganisms in the rumen (Bechdel et al., 1928). Increased levels of carnitine, and β-Ala, and a tendency towards increased L-His in pre-ketotic cows suggest that the animal's antioxidant protection machinery was activated. The reason for elevated levels of pantothenic acid in the urine of cows with ketosis is not known; however, this should raise concerns regarding dairy cow nutrition and emphasized the need to balance the diet so that the amount of the pantotheic acid excreted is equal to intake. Increased urinary pantothenic acid in pre-ketotic and ketotic cows suggests that more pantothenic acid is present in free form rather than conjugated to CoA, fatty acid synthetase, phosphopantetheine, or ACP, which might affect amino acid or fatty acid metabolism.

4.4.4 Ketone body metabolism, glycolysis/gluconeogenesis, and energy balance

Ketosis or hyperketonemia has been traditionally defined as a condition of abnormally increased concentrations of ketone bodies in the blood, urine, and milk of cows. Disturbed energy balance in ketotic cows enhances gluconeogenesis, lipolysis, proteolysis, and glycogenolysis, all of which are aimed at providing sufficient energy substrates. Three ketone bodies (i.e., BHBA, AcAc, and Ac) are mostly formed in the liver from fatty acid β-oxidation and also partly from two ketogenic amino acids (i.e., Lys and Leu) and five both ketogenic and glucogenic amino acids (i.e., Ile, Phe, Thr, Trp, and Tyr). Urine levels of BABA and AcAc were greater in ketotic cows compared with CON cows during the wk of diagnosis. The data regarding urine ketone bodies are consistent with the serum data from the same cows (Zhang et al.,

2016). No differences among the three ketone bodies were observed at other time points in the study between the two groups of cows. Our results indicate that urine ketones are reliable for the diagnosis of ketosis. However, when it comes to their performance in predicting the disease, they are clearly not good predictive biomarkers for ketosis.

There were no distinct differences in terms of intermediates or their precursors involved in glycolysis, gluconeogenesis, the TCA cycle, or other energy metabolism in the urine of cows with ketosis versus controls. In particular, concentrations of glucogenic amino acids in the urine were not different between the two groups of cows; On the other hand, very different results were obtained from the serum samples (chapter 3) showing elevated levels of both glucogenic and ketogenic amino acids in ketotic cows. This suggests that urine glucogenic amino acids cannot be used as indicators of energy status in cows with ketosis.

Urine concentrations of hexose (e.g., aldohexoses such as D-glucose and D-mannose; and ketohexoses such as D-fructose), 1,3-dihydroxyacetone (DHA), and L-lactic acid were greater, whereas D-galactose was lower in cows with ketosis during the disease wk. Greater levels of L-lactic acid and hexose in the urine of ketotic cows agrees with our serum findings (chapter 3). In ruminants, the major precursors for gluconeogenesis are glucogenic volatile fatty acids including propionate, isobutyrate, and valerate as well as L-lactic acid, glycerol, and glucogenic amino acids (Larsen and Kristensen, 2013). The energy demand for milk production increases immediately after parturition when the mammary gland requires excessive glucose and amino acids

for the synthesis of milk lactose and protein . As a result the nutritional needs for milk production increases from virtually zero to around 1 kg/day within a few days after parturition (Larsen and Kristensen, 2013; Reynolds et al., 2003). A review paper on precursors for liver gluconeogenesis in periparturient dairy cows pointed out that the most important adaptation of metabolism to meet the increased requirements for glucose in the immediate postpartum period is endogenous recycling of glucogenic carbon originating from the lactic acid (Cori) cycle (Larsen and Kristensen, 2013). Increased concentrations of monosaccharides such as hexose and DHA, and precursors of gluconeogenesis (e.g., L-lactic acid) in the urine suggest a blockade of tubular reabsorption of these metabolites in the kidney, producing marked glucosuria.

4.4.5 Other metabolites and related metabolic pathways

It is interesting that concentrations of *myo*-inositol in urine were greater in both pre-ketotic (i.e., at -8 wks and -4 wks prepartum) and ketotic (i.e., at the disease wk) cows compared with CON cows. *Myo*-inositol is the most abundant form of 9 stereoisomers of inositol. *Myo*-inositol is an important lipotropic factor and its role in signal transmission for various growth factors, neurotransmitters, and hormones has been well documented (Holub, 1986). Additionally, it was reported that *myo*-inositol is involved in fat lipolysis and in lowering blood cholesterol levels (Rapiejko et al., 1986). *Myo*-inositol deficiency has been recognized as an important factor that might contribute to the accumulation of TG in the liver or intestines. Recently it was reported that an increased excretion of *myo*-inositol in the urine of human subjects

with hyperglycemia suggesting *myo*-inositol in the urine as is a good marker of glucose intolerance (Ikezaki et al., 2013). In animals, the initial committed step in the metabolism of *myo*-inositol occurs exclusively in the kidney for the production of D-glucuronic acid, yielding D-xylulose 5-phosphate that enters the pentose phosphate pathway for energy production (Howard and Anderson, 1967). Additionally, Donà et al. (2012) reported that *myo*-inositol administration can lower oxidative abnormalities in patients with polycystic ovary syndrome, suggesting that *myo*-inositol could act as an antioxidant. Increased levels of *myo*-inositol in urine of pre-ketotic and ketotic cows suggests that an increase in fat mobilization may occur before and during the occurrence of ketosis, which requires more *myo*-inositol to maintain fatty acid metabolism.

Another interesting finding from this study was that several urinary minerals were found to fluctuate significantly in pre-ketotic and post-ketotic cows. For example B, Na, Mg, Al, P, K, Ca, Cr, Mn, Co, Se, Rb, and strontium (Sr) were greater in the urine of pre-ketotic or post-ketotic cows versus CON ones. The importance of trace metal elements in health and disease conditions of humans and animals has been reviewed previously (Soetan et al., 2010; Yasothai, 2014). In particular, Ca plays a pivotal role in activation of various enzymes such as lipase, adenosine triphosphatase (ATPase), and succinic dehydrogenase. Functions of P as a vital constituent of energy compounds [e.g., adenosine triphosphate (ATP)], phosphorylated metabolic intermediates, and its involvement in the synthesis of phospholipids and phosphoproteins are well known (Soetan et al., 2010). Sodium (Na) and K are the

principle cations in extracellular fluids and involved in the maintenance of osmotic pressure of body fluids, muscle function, and Na^+/K^+-ATPase. Specifically, K is involved in glycogenesis, helping transfer phosphate from ATP to pyruvic acid. Trace elements [e.g., Cu, cobalt (Co), Mg, manganese (Mn), Zinc (Zn), Cr, and selenium (Se)] are essential components of enzyme systems, which are important in glucose utilization, lipid metabolism, iron transport, and free radical metabolism (Ceylan et al., 2008; Hostetler et al., 2003). For instance, Mg is an active component of several enzyme systems such as creatine kinase, myokinase, and several important enzymes (e.g., pyruvic acid carboxylase, pyruvic acid oxidase) involved in the TCA cycle. Trivalent Cr is a constituent of glucose tolerance factor, which potentiates insulin action, and the role of Cr in amino acid and lipid metabolism is now well known (Soetan et al., 2010). Cu is a constituent of various enzyme systems and plays an important role in immune function (Chandra, 1990).

Urine levels of B, Al, and Rb were increased in pre-ketotic cows at both -8 wks and -4 wks prepartum. Moreover, B and Al were increased in the urine of ketotic cows at four tested time points (i.e., -8 wks, -4 wks, +4 wks, and +8 wks). Boron has been reported as an important mineral for various nutritional, hormonal, metabolic, and physiological processes that is vital for humans and animals (Hunt, 1998; Kabu and Akosman, 2013; Nielsen, 1997). It was reported that the administration of sodium borate (B as the functional component) reduced concentrations of TG, VLDL, NEFA, high density lipoprotein (HDL), low density lipoprotein (LDL), and cholesterol in the blood of dairy cows (Kabu et al., 2013). Therefore, normal levels of B in the blood

can help improve the cow's metabolic status during the periparturient period (Kabu and Civelek, 2012). Excessive excretion of B in the urine might result in elevated levels of NEFA and an accumulation of TG in the liver, which subsequently contributes to progression of ketosis. Additionally, elevated urinary secretion of Al has been associated with various diseases such as multiple sclerosis (Exley et al., 2006).

Elevated concentrations of all the aforementioned mineral elements in the urine suggest that pre-ketotic cows and post-ketotic cows experienced excessive excretion of minerals by the kidney. Since there were no differences of DMI between the two groups of cows before parturition, and even lower DMI in post-ketotic cows at +4 wks and +8 wks postpartum (Zhang et al., 2016), greater levels of urinary trace metals suggests excessive loss of minerals before and after the occurrence of ketosis. In a human diabetes study, Feng et al. (2015) reported that multiple metals (e.g., Al, Co, Se, and Sr) in urine are associated with fasting plasma glucose, impaired fasting glucose, and diabetes risk. Severe diabetes might be accompanied by renal impairment, which may cause abnormal urine excretion of trace mineral elements (Feng et al., 2015). Since two types of ketosis including type I ketosis (hypoinsulinemia) and type II ketosis (insulin resistance) are classified based on their metabolic counterparts (i.e., type I and type II diabetes mellitus) (Oetzel, 2007), similar renal impairment and excessive urinary excretion of metals might also exist in cows with ketosis. In acid base abnormalities such as severe cases of metabolic acidosis, bone demineralization is initiated (Van der Aa et al., 2011). The ketotic cows

used in this study were reported had greater levels of lactate in their serum before and during the occurrence the disease, suggesting a blood acidosis state (Zhang et al., 2016). Enhanced urinary excretion of minerals might be associated with neutralization of excessive acids in the blood. Elevated urinary excretion of various mineral elements might be associated with development of ketosis and this trend might last until +8 wks postpartum. There were no differences of urine metals at the disease wk between cows with ketosis and CON ones. This might be attributed to numerically greater levels of creatinine in urine of ketotic cows since all urinary metabolites are normalized by urinary creatinine. Creatinine production is related to muscle mass and renal elimination of creatinine has been used as an important parameter to estimate renal function (Smith, 1988). During the week that ketosis was diagnosed, renal function such as glomerular filtration rate in ketotic cows were affected based on numerically increased levels of urinary creatinine. Though relative concentrations of minerals in urine of ketotic cows seem to be normal. The urinary extraction of minerals might be altered in cows with ketosis.

4.5 Conclusions

Results of this study show that both NMR and MS based metabolomics provides a powerful approach to discover predictive and diagnostic biomarkers of ketosis in dairy cows. A comprehensive analysis of metabolic patterns in urine before, during, and after the occurrence of ketosis in dairy cows is presented. We identified two sets of early candidate biomarkers of ketosis that can predict ketosis much earlier than the measurement of ketone bodies during the early lactation. More specifically, a

seven-metabolite biomarker set (i.e., C5-M-DC, C18:2, C3-OH, C16, Al, C14:1-OH, and C16:2) at -8 wks, and a 9-metabolite biomarker set (i.e., C3-OH, methanol, C16, PC ae C30:2, C18:2, Al, *N*-acetylglutamine, C5-M-DC, and C14:1) at -4 wks, were identified for the prediction of ketosis with excellent sensitivity and specificity. Moreover, five metabolites including PC ae C30:2, C5-M-DC, PC aa C40:4, SDMA, and PC aa C38:3 were selected as an improved set of diagnostic biomarkers for ketosis with an AUC at 0.994 (95% CI: 0.969-1). Since urine samples require non-invasive collection, the newly identified early predictive and diagnostic urinary biomarkers show remarkable advantages compared to the current 'golden standard' of blood biomarker (e.g. serum BHBA) or urine ketostix test (measurement of acetoacetate). More research is warranted to validate these results in a larger cohort of transition dairy cows.

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Figure captions

Figure 4-1. (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 ketosis cows at -8 wks before parturition showing 2 separated clusters for 2 groups; (c) Loading plot for PLS-DA model; (d)Variables ranked by variable importance in projection (VIP); (e) Heat map based on PLS-DA VIP scores and top 25 metabolites; and (f) Receiver-operator characteristic (ROC) curve of 20 CON and 6 ketosis cows at -8 wks before parturition for the top 7 urine variables (i.e., C5-M-DC, C18:2, C3-OH, C16, Al, C14:1-OH, and C16:2; empirical $P = 0.001$).

Figure 4-2. (a) PCA and (b) PLS-DA (Permutation test: $P <0.05$) of 20 CON and 6 ketosis cows at -4 wks before parturition showing 2 separated clusters for 2 groups; (c) Loading plot for PLS-DA model; (d) VIP; (e) Heat map based on PLS-DA VIP scores and top 25 metabolites; and (f) ROC curve of 20 CON and 6 ketosis cows at -4 wks before parturition for the top 9 urine variables (i.e., C3-OH, methanol, C16, PC ae C30:2, C18:2, Al, *N*-acetylglutamine, C5-M-DC, and C14:1; empirical $P = 0.003$).

Figure 4-3. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 20 CON and 6 ketosis cows at disease wk showing 2 separated clusters for 2 groups; (c) Loading plot for PLS-DA model; (d) VIP; (e) Heat map based on PLS-DA VIP scores and top 25 metabolites; and (f) ROC curve of 20 CON and 6 ketosis cows at disease wk for the top 5 urine variables (i.e., PC ae C30:2, C5-M-DC, PC aa C40:4, SDMA, and PC aa

C38:3; empirical $P = 0.001$).

Figure 4-4. (a) PCA and (b) PLS-DA (Permutation test: $P < 0.05$) of 6 CON and 6 ketosis cows at +4 wks after parturition showing 2 separated clusters for 2 groups; (c) Loading plot for PLS-DA model; (d) VIP; (e) Heat map based on PLS-DA VIP scores and top 25 metabolites; and (f) ROC curve of 6 CON and 6 ketosis cows at +4 wks after parturition for the top 6 urine variables (i.e., PC ae C36:4, Al, C16:2, *N*-acetylglutamine, C5-M-DC, and P; empirical $P < 0.05$).

Figure 4-5. (a) PCA and (b) PLS-DA (Permutation test: $P <0.05$) of 6 CON and 6 ketosis cows at +8 wks after parturition showing 2 separated clusters for 2 groups; (c) Loading plot for PLS-DA model; (d) VIP; (e) Heat map based on PLS-DA VIP scores and top 25 metabolites; and (f) ROC curve of 6 CON and 6 ketosis cows at +8 wks after parturition for the top 6 urine variables [i.e., C10:1, C5-OH (C3-DC-M), PC aa C38:1, C16:2, P, and C3-OH, empirical $P < 0.05$].

Table 4-1. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at 3 time points (-8 wk, -4 wk, and the wk of diagnosis of disease) as determined by DI/LC-MS/MS

	8 week before parturition					4 week before parturition					Ketosis diagnosis week[1]				
Metabolite, μM/mM creatinine[2]	Ketosis	CON	*P*-value[3]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	20	-	-	-	6	20	-	-	-	6	20	-	-	
Acylcarnitines															
C0	0.914 (0.357)	0.843 (0.267)	0.6002	1.08	Up	1.241 (0.991)	0.863 (0.517)	0.3244 (W)	1.44	Up	0.550 (0.151)	0.848 (0.499)	0.1082 (W)	-1.54	Down
C10	0.053 (0.040)	0.024 (0.009)	0.1365	2.24	Up	0.045 (0.036)	0.027 (0.017)	0.1565 (W)	1.63	Up	0.040 (0.017)	0.030 (0.020)	0.1082 (W)	1.34	Up
C10:1	0.055 (0.037)	0.027 (0.009)	0.0029 (W)	2.05	Up	0.077 (0.075)	0.031 (0.012)	0.0029 (W)	2.48	Up	0.038 (0.013)	0.025 (0.012)	0.0668 (W)	1.52	Up
C10:2	0.019 (0.008)	0.010 (0.004)	0.0043 (W)	1.82	Up	0.022 (0.016)	0.010 (0.007)	0.0107 (W)	2.2	Up	0.010 (0.003)	0.012 (0.007)	0.9764 (W)	-1.13	Down
C12	0.138 (0.222)	0.028 (0.017)	0.0949 (W)	4.95	Up	0.146 (0.286)	0.024 (0.020)	0.2185 (W)	6.05	Up	0.158 (0.115)	0.053 (0.036)	0.0011 (W)	2.99	Up
C12-DC	0.027 (0.010)	0.018 (0.006)	0.0093 (W)	1.52	Up	0.032 (0.026)	0.017 (0.004)	0.2464 (W)	1.88	Up	0.026 (0.014)	0.016 (0.005)	0.0765 (W)	1.63	Up
C12:1	0.088 (0.110)	0.063 (0.038)	0.6999 (W)	1.4	Up	0.091 (0.125)	0.069 (0.068)	1.0000 (W)	1.31	Up	0.077 (0.039)	0.086 (0.072)	0.8823 (W)	-1.12	Down
C14	0.009 (0.005)	0.007 (0.002)	0.3244 (W)	1.31	Up	0.015 (0.017)	0.007 (0.005)	0.1229 (W)	2.18	Up	0.008 (0.003)	0.007 (0.005)	0.6999 (W)	1.04	Up
C14:1	0.004 (0.003)	0.003 (0.001)	0.0391 (W)	1.75	Up	0.011 (0.013)	0.003 (0.002)	0.0008 (W)	4.09	Up	0.005 (0.002)	0.004 (0.002)	0.0720 (W)	1.3	Up
C14:1-OH	0.007 (0.002)	0.003 (0.001)	0.0002 (W)	2.27	Up	0.007 (0.009)	0.003 (0.002)	0.3875 (W)	2.11	Up	0.007 (0.003)	0.003 (0.002)	0.0034 (W)	2.14	Up
C14:2	0.003 (0.001)	0.002 (0.001)	0.6565 (W)	1.09	Up	0.005 (0.004)	0.003 (0.002)	0.2681 (W)	1.81	Up	0.002 (0.001)	0.003 (0.002)	0.7445 (W)	-1.04	Down
C14:2-OH	0.005 (0.001)	0.003 (0.001)	0.0077	1.56	Up	0.025 (0.043)	0.004 (0.005)	0.0331 (W)	5.64	Up	0.010 (0.008)	0.006 (0.006)	0.3244 (W)	1.56	Up
C16	0.013 (0.007)	0.004 (0.002)	0.0001 (W)	3.35	Up	0.012 (0.008)	0.004 (0.002)	0.0055 (W)	3.31	Up	0.008 (0.004)	0.004 (0.002)	0.0069 (W)	2.2	Up
C16-OH	0.006 (0.004)	0.002 (0.001)	0.0004 (W)	2.62	Up	0.006 (0.006)	0.003 (0.002)	0.1963 (W)	2.25	Up	0.005 (0.001)	0.003 (0.002)	0.0062	1.73	Up
C16:1	0.013 (0.004)	0.007 (0.003)	0.0006 (W)	2.06	Up	0.023 (0.030)	0.007 (0.005)	0.0279 (W)	3.28	Up	0.011 (0.007)	0.008 (0.004)	0.6999 (W)	1.27	Up
C16:1-OH	0.005 (0.003)	0.002 (0.001)	0.0849 (W)	1.9	Up	0.007 (0.007)	0.003 (0.001)	0.4335 (W)	2.2	Up	0.003 (0.001)	0.002 (0.001)	0.3354 (W)	1.21	Up
C16:2	0.005 (0.002)	0.002 (0.001)	0.018	2.63	Up	0.006 (0.006)	0.002 (0.001)	0.0055 (W)	2.94	Up	0.004 (0.002)	0.002 (0.001)	0.0043 (W)	1.94	Up
C16:2-OH	0.007 (0.005)	0.004 (0.001)	0.1409 (W)	1.74	Up	0.012 (0.012)	0.005 (0.002)	0.1101 (W)	2.52	Up	0.005 (0.001)	0.004 (0.001)	0.2027 (W)	1.24	Up
C18	0.006 (0.005)	0.003 (0.001)	0.0417 (W)	2.4	Up	0.006 (0.004)	0.003 (0.001)	0.0133 (W)	2.48	Up	0.004 (0.002)	0.002 (0.001)	0.0580 (W)	1.81	Up
C18:1	0.008 (0.006)	0.004 (0.002)	0.0261 (W)	1.76	Up	0.006 (0.004)	0.004 (0.001)	0.7304 (W)	1.66	Up	0.005 (0.002)	0.004 (0.002)	0.0996 (W)	1.28	Up
C18:1-OH	0.010 (0.011)	0.005 (0.002)	0.1101 (W)	2.12	Up	0.008 (0.006)	0.004 (0.001)	0.0358 (W)	2.03	Up	0.008 (0.006)	0.005 (0.003)	0.4373 (W)	1.65	Up
C18:2	0.011 (0.009)	0.002 (0.001)	0.0619	5.84	Up	0.008 (0.008)	0.002 (0.001)	0.0020 (W)	4.51	Up	0.007 (0.003)	0.002 (0.002)	0.0279 (W)	3.2	Up
C2	0.121 (0.046)	0.092 (0.038)	0.1301	1.31	Up	0.198 (0.292)	0.111 (0.073)	0.9764 (W)	1.79	Up	0.142 (0.057)	0.124 (0.099)	0.3244 (W)	1.14	Up
C3	0.018 (0.006)	0.013 (0.006)	0.1196	1.33	Up	0.016 (0.006)	0.013 (0.008)	0.1565 (W)	1.26	Up	0.009 (0.006)	0.014 (0.007)	0.2073	-1.46	Down
C3-DC (C4-OH)	0.022 (0.004)	0.020 (0.010)	0.4215 (W)	1.06	Up	0.036 (0.046)	0.021 (0.013)	0.6999 (W)	1.74	Up	0.018 (0.004)	0.025 (0.015)	0.4570 (W)	-1.4	Down
C3-OH	0.048 (0.026)	0.010 (0.006)	0.0152	4.59	Up	0.049 (0.031)	0.010 (0.005)	0.0257	4.92	Up	0.025 (0.021)	0.013 (0.009)	0.0279 (W)	1.89	Up
C3:1	0.010 (0.005)	0.021 (0.009)	0.0069 (W)	-1.99	Down	0.021 (0.026)	0.022 (0.012)	0.0622 (W)	-1.02	Down	0.011 (0.005)	0.027 (0.018)	0.0015 (W)	-2.51	Down

C4	0.107 (0.033)	0.080 (0.046)	0.2129	1.32	Up	0.081 (0.037)	0.080 (0.052)	0.7445 (W)	1.01	Up	0.031 (0.029)	0.030 (0.021)	0.9292 (W)	1.04	Up
C4:1	0.033 (0.024)	0.029 (0.012)	0.6814	1.15	Up	0.041 (0.049)	0.033 (0.015)	0.2681 (W)	1.24	Up	0.023 (0.019)	0.037 (0.025)	0.1756 (W)	-1.55	Down
C6 (C4:1-DC)	0.015 (0.005)	0.012 (0.002)	0.0913 (W)	1.31	Up	0.020 (0.019)	0.017 (0.021)	0.4942 (W)	1.16	Up	0.010 (0.002)	0.010 (0.003)	0.1966 (W)	1.01	Up
C5	0.027 (0.012)	0.019 (0.008)	0.0536 (W)	1.47	Up	0.029 (0.023)	0.022 (0.016)	0.2681 (W)	1.36	Up	0.014 (0.007)	0.017 (0.011)	0.2954 (W)	-1.27	Down
C5-M-DC	0.054 (0.033)	0.012 (0.005)	< 0.0001 (W)	4.38	Up	0.054 (0.053)	0.014 (0.009)	0.0008 (W)	3.95	Up	0.053 (0.018)	0.017 (0.011)	< 0.0001 (W)	3.15	Up
C5-OH (C3-DC-M)	0.056 (0.026)	0.024 (0.007)	0.0046 (W)	2.33	Up	0.069 (0.065)	0.027 (0.015)	0.0160 (W)	2.52	Up	0.058 (0.011)	0.036 (0.025)	0.0017 (W)	1.63	Up
C5:1	0.052 (0.019)	0.042 (0.013)	0.1628	1.23	Up	0.055 (0.057)	0.044 (0.022)	0.5327 (W)	1.24	Up	0.039 (0.011)	0.042 (0.025)	0.8823 (W)	-1.07	Down
C5:1-DC	0.010 (0.010)	0.007 (0.002)	0.9764 (W)	1.47	Up	0.013 (0.016)	0.008 (0.007)	1.0000 (W)	1.56	Up	0.011 (0.002)	0.010 (0.006)	0.2681 (W)	1.09	Up
C5-DC (C6-OH)	0.016 (0.006)	0.010 (0.004)	0.0033	1.61	Up	0.028 (0.037)	0.011 (0.006)	0.0949 (W)	2.56	Up	0.021 (0.005)	0.015 (0.011)	0.0069 (W)	1.47	Up
C6:1	0.020 (0.010)	0.012 (0.005)	0.0026 (W)	1.75	Up	0.027 (0.031)	0.012 (0.006)	0.1082 (W)	2.2	Up	0.017 (0.007)	0.012 (0.007)	0.1082 (W)	1.37	Up
C7-DC	0.015 (0.005)	0.007 (0.003)	< 0.0001	2.07	Up	0.017 (0.017)	0.007 (0.004)	0.0131 (W)	2.42	Up	0.014 (0.003)	0.007 (0.005)	0.0008 (W)	1.99	Up
C8	0.036 (0.022)	0.020 (0.006)	0.0307 (W)	1.74	Up	0.035 (0.028)	0.019 (0.005)	0.2729 (W)	1.84	Up	0.020 (0.007)	0.017 (0.005)	0.2256 (W)	1.17	Up
C9	0.047 (0.017)	0.038 (0.025)	0.1390 (W)	1.24	Up	0.088 (0.096)	0.041 (0.021)	0.2425 (W)	2.13	Up	0.040 (0.017)	0.030 (0.018)	0.2373	1.34	Up
Lysophosphatidylcholines															
lysoPC a C16:0	0.019 (0.029)	0.016 (0.011)	0.4335 (W)	1.22	Up	0.022 (0.023)	0.086 (0.203)	0.2218 (W)	-3.86	Down	0.013 (0.008)	0.018 (0.025)	1.0000 (W)	-1.35	Down
lysoPC a C16:1	0.010 (0.007)	0.006 (0.003)	0.1586 (W)	1.55	Up	0.010 (0.019)	0.007 (0.003)	0.0741 (W)	1.53	Up	0.008 (0.012)	0.007 (0.004)	0.1620 (W)	1.17	Up
lysoPC a C17:0	0.003 (0.003)	0.005 (0.002)	0.1583 (W)	-1.75	Down	0.030 (0.019)	0.035 (0.047)	0.4215 (W)	-1.17	Down	0.003 (0.004)	0.004 (0.002)	0.2502 (W)	-1.42	Down
lysoPC a C18:0	0.041 (0.041)	0.032 (0.039)	0.5727 (W)	1.29	Up	0.030 (0.019)	0.035 (0.047)	0.4215 (W)	-1.17	Down	0.015 (0.012)	0.031 (0.031)	0.0949 (W)	-2.02	Down
lysoPC a C18:1	0.003 (0.003)	0.004 (0.003)	0.4332 (W)	-1.46	Down	0.007 (0.010)	0.016 (0.029)	0.1247 (W)	-2.3	Down	0.005 (0.004)	0.007 (0.005)	0.1814 (W)	-1.44	Down
lysoPC a C18:2	0.007 (0.011)	0.009 (0.005)	0.2215 (W)	-1.27	Down	0.007 (0.007)	0.023 (0.041)	0.0261 (W)	-3.12	Down	0.008 (0.005)	0.013 (0.015)	0.5549 (W)	-1.63	Down
lysoPC a C20:3	0.026 (0.018)	0.017 (0.006)	0.1586 (W)	1.54	Up	0.029 (0.024)	0.019 (0.006)	0.6384 (W)	1.57	Up	0.014 (0.013)	0.019 (0.010)	0.0996 (W)	-1.32	Down
lysoPC a C20:4	0.005 (0.005)	0.003 (0.002)	0.2456 (W)	1.93	Up	0.004 (0.005)	0.006 (0.020)	0.9512 (W)	-1.46	Down	0.004 (0.009)	0.003 (0.004)	0.0571 (W)	1.19	Up
lysoPC a C28:1	0.004 (0.009)	0.003 (0.002)	0.0470 (W)	1.26	Up	0.002 (0.004)	0.003 (0.006)	0.2822 (W)	-1.51	Down	0.003 (0.002)	0.003 (0.002)	0.4748 (W)	-1.28	Down
Phosphatidylcholines															
PC aa C24:0	0.005 (0.002)	0.005 (0.002)	0.3315 (W)	1.13	Up	0.010 (0.007)	0.005 (0.003)	0.1990 (W)	1.78	Up	0.004 (0.003)	0.005 (0.002)	0.3354 (W)	-1.16	Down
PC aa C28:1	0.011 (0.006)	0.009 (0.003)	0.0358 (W)	1.32	Up	0.019 (0.019)	0.013 (0.010)	0.8755 (W)	1.49	Up	0.011 (0.003)	0.008 (0.002)	0.1442 (W)	1.28	Up
PC aa C30:0	0.027 (0.036)	0.013 (0.005)	0.6838 (W)	2.1	Up	0.013 (0.004)	0.013 (0.008)	0.9750 (W)	-1.05	Down	0.010 (0.006)	0.010 (0.003)	0.4373 (W)	1	Up
PC aa C30:2						0.002 (0.003)	0.001 (0.002)	0.6449 (W)	1.54	Up	0.003 (0.003)	0.001 (0.001)	0.4745 (W)	2.14	Up
PC aa C32:0	0.004 (0.004)	0.004 (0.003)	0.6384 (W)	1.09	Up	0.002 (0.002)	0.024 (0.055)	0.0188 (W)	-10.07	Down	0.002 (0.005)	0.003 (0.005)	0.0399 (W)	-1.39	Down
PC aa C32:1	0.001 (0.002)	0.002 (0.002)	0.1777 (W)	-1.39	Down						0.002 (0.002)	0.003 (0.003)	0.7797 (W)	-1.49	Down
PC aa C32:2	0.003 (0.003)	0.007 (0.006)	0.0261 (W)	-2.33	Down	0.002 (0.002)	0.010 (0.012)	0.0013 (W)	-4.88	Down	0.002 (0.002)	0.006 (0.003)	0.0047 (W)	-2.39	Down
PC aa C34:1	0.009 (0.008)	0.036 (0.071)	0.2185 (W)	-4.17	Down	0.005 (0.009)	0.194 (0.762)	0.0279 (W)	-36.47	Down	0.014 (0.019)	0.042 (0.085)	0.6565 (W)	-3.04	Down
PC aa C34:2	0.013 (0.025)	0.090 (0.203)	0.0131 (W)	-7.1	Down	0.012 (0.011)	0.432 (1.700)	0.0622 (W)	-37.38	Down	0.012 (0.014)	0.094 (0.188)	0.1565 (W)	-7.87	Down

PC aa C34:3	0.001 (0.001)	0.006 (0.015)	0.3767 (W)	-5.18	Down	0.001 (0.002)	0.016 (0.060)	0.4424 (W)	-13.41	Down	0.001 (0.001)	0.006 (0.013)	0.0297 (W)	-9.73	Down
PC aa C34:4	0.001 (0.002)	0.003 (0.002)	0.0413 (W)	-2.73	Down	0.002 (0.002)	0.004 (0.007)	0.8312 (W)	-1.78	Down	0.001 (0.001)	0.004 (0.004)	0.0720 (W)	-3.07	Down
PC aa C36:0	0.052 (0.030)	0.030 (0.012)	0.0417 (W)	1.73	Up	0.049 (0.028)	0.032 (0.019)	0.0742 (W)	1.52	Up	0.023 (0.011)	0.021 (0.005)	0.7325 (W)	1.1	Up
PC aa C36:1	0.003 (0.002)	0.018 (0.044)	0.9515 (W)	-5.54	Down	0.003 (0.004)	0.035 (0.110)	0.0940 (W)	-10.78	Down	0.004 (0.004)	0.017 (0.045)	0.6049 (W)	-4.19	Down
PC aa C36:2	0.012 (0.020)	0.058 (0.139)	0.1390 (W)	-4.76	Down	0.006 (0.008)	0.175 (0.633)	0.0634 (W)	-28.4	Down	0.007 (0.005)	0.059 (0.129)	0.2185 (W)	-8.72	Down
PC aa C36:3	0.004 (0.008)	0.022 (0.048)	0.0161 (W)	-5.88	Down	0.004 (0.004)	0.103 (0.395)	0.1756 (W)	-24.22	Down	0.003 (0.004)	0.028 (0.055)	0.0622 (W)	-8.56	Down
PC aa C36:4	0.005 (0.004)	0.031 (0.064)	0.1229 (W)	-6.37	Down	0.005 (0.007)	0.293 (1.238)	0.0459 (W)	-56.84	Down	0.004 (0.005)	0.031 (0.057)	0.0551 (W)	-8.6	Down
PC aa C36:5	0.001 (0.002)	0.003 (0.007)	0.0159 (W)	-5.01	Down	0.001 (0.001)	0.025 (0.102)	0.1185 (W)	-28.39	Down	0.002 (0.003)	0.003 (0.006)	0.3294 (W)	-2	Down
PC aa C38:0	0.005 (0.002)	0.003 (0.001)	0.0484 (W)	1.42	Up	0.005 (0.006)	0.009 (0.016)	0.1586 (W)	-1.8	Down	0.003 (0.002)	0.003 (0.002)	0.4015 (W)	-1	Down
PC aa C38:1	0.004 (0.005)	0.003 (0.001)	0.8264 (W)	1.71	Up	0.010 (0.014)	0.004 (0.010)	0.8787 (W)	2.49	Up	0.004 (0.003)	0.002 (0.002)	0.0658 (W)	2.2	Up
PC aa C38:3	0.005 (0.005)	0.005 (0.004)	0.3977 (W)	-1.03	Down	0.004 (0.006)	0.050 (0.125)	0.0188 (W)	-14.16	Down	0.001 (0.001)	0.008 (0.015)	0.0069 (W)	-7.61	Down
PC aa C38:4	0.008 (0.008)	0.022 (0.043)	0.8358 (W)	-2.63	Down	0.024 (0.024)	0.110 (0.433)	0.6565 (W)	-4.63	Down	0.014 (0.009)	0.027 (0.043)	0.7445 (W)	-1.89	Down
PC aa C38:5	0.001 (0.001)	0.010 (0.020)	0.0026 (W)	-17.47	Down	0.001 (0.002)	0.041 (0.164)	0.0931 (W)	-40.06	Down	0.002 (0.003)	0.010 (0.020)	0.1130 (W)	-4.92	Down
PC aa C38:6	0.002 (0.002)	0.017 (0.042)	0.1706 (W)	-8.03	Down	0.003 (0.004)	0.118 (0.490)	0.0552 (W)	-42.28	Down	0.002 (0.002)	0.016 (0.037)	0.1524 (W)	-9.07	Down
PC aa C40:1	0.035 (0.013)	0.033 (0.011)	0.6838 (W)	1.07	Up	0.050 (0.041)	0.034 (0.010)	0.8755 (W)	1.48	Up	0.019 (0.009)	0.031 (0.012)	0.0274 (W)	-1.63	Down
PC aa C40:2	0.001 (0.001)	0.002 (0.002)	0.1609 (W)	-2.11	Down	0.003 (0.004)	0.002 (0.005)	0.7840 (W)	1.26	Up	0.001 (0.001)	0.002 (0.002)	0.1204 (W)	-2.07	Down
PC aa C40:3	0.001 (0.002)	0.001 (0.002)	0.7354 (W)	1.04	Up	0.001 (0.002)	0.002 (0.006)	0.4825 (W)	-1.87	Down	0.001 (0.002)	0.001 (0.001)	0.2308 (W)	-1.15	Down
PC aa C40:4						0.002 (0.005)	0.004 (0.012)	0.1346 (W)	-1.8	Down	0.001 (0.001)	0.003 (0.004)	0.0551 (W)	-4.23	Down
PC aa C40:6	0.041 (0.029)	0.026 (0.009)	0.3013 (W)	1.6	Up	0.047 (0.037)	0.060 (0.106)	0.8264 (W)	-1.28	Down	0.019 (0.008)	0.019 (0.012)	0.6411 (W)	-1.02	Down
PC aa C42:0	0.006 (0.011)	0.005 (0.002)	0.0417 (W)	1.21	Up	0.005 (0.002)	0.004 (0.003)	0.4712 (W)	1.2	Up	0.002 (0.001)	0.003 (0.002)	0.1442 (W)	-1.34	Down
PC aa C42:1	0.001 (0.001)	0.001 (0.001)	0.8041	1.15	Up	0.002 (0.002)	0.002 (0.002)	0.9292 (W)	-1.1	Down	0.002 (0.003)	0.002 (0.003)	0.8310 (W)	1.26	Up
PC aa C42:2	0.009 (0.005)	0.007 (0.004)	0.2512	1.35	Up	0.008 (0.007)	0.007 (0.006)	0.9292 (W)	1.09	Up	0.007 (0.006)	0.007 (0.005)	0.5327 (W)	-1.02	Down
PC aa C42:4	0.002 (0.003)	0.001 (0.000)	0.5099 (W)	2.52	Up	0.001 (0.001)	0.001 (0.002)	0.0357 (W)	-2.42	Down	0.001 (0.001)	0.001 (0.001)	0.9752 (W)	1.19	Up
PC aa C42:5	0.001 (0.001)	0.001 (0.001)	0.5722 (W)	-1.4	Down	0.009 (0.006)	0.004 (0.003)	0.0283 (W)	2.06	Up	0.008 (0.005)	0.003 (0.002)	0.0433 (W)	2.58	Up
PC aa C42:6	0.057 (0.035)	0.027 (0.010)	0.0307 (W)	2.16	Up	0.058 (0.056)	0.024 (0.009)	0.1409 (W)	2.39	Up	0.026 (0.014)	0.019 (0.005)	0.4373 (W)	1.4	Up
PC ae C30:0	0.021 (0.020)	0.014 (0.004)	0.7780 (W)	1.49	Up	0.021 (0.017)	0.012 (0.004)	0.5517 (W)	1.69	Up	0.012 (0.010)	0.011 (0.004)	0.4373 (W)	1.09	Up
PC ae C30:2	0.004 (0.003)	0.011 (0.012)	0.0560 (W)	-3.02	Down	0.002 (0.002)	0.012 (0.011)	0.0036 (W)	-7.71	Down	0.000 (0.001)	0.016 (0.016)	0.0017 (W)	-35.06	Down
PC ae C32:2	0.000 (0.001)	0.001 (0.003)	0.0911 (W)	-4.16	Down	0.001 (0.002)	0.002 (0.005)	0.6350 (W)	-1.35	Down	0.002 (0.003)	0.001 (0.003)	0.8297 (W)	1.41	Up
PC ae C34:0	0.001 (0.001)	0.002 (0.002)	0.3452 (W)	-2.23	Down	0.003 (0.002)	0.003 (0.005)	0.6701 (W)	-1.23	Down	0.001 (0.001)	0.002 (0.002)	0.9272 (W)	-1.25	Down
PC ae C34:1	0.001 (0.002)	0.002 (0.001)	0.3012 (W)	-1.21	Down	0.004 (0.004)	0.011 (0.025)	0.2464 (W)	-3.22	Down	0.001 (0.001)	0.002 (0.003)	0.0869 (W)	-2.17	Down
PC ae C34:2											0.002 (0.002)	0.003 (0.006)	0.6914 (W)	-1.57	Down
PC ae C34:3	0.002 (0.003)	0.001 (0.001)	0.7758 (W)	2.54	Up	0.001 (0.002)	0.008 (0.029)	0.5421 (W)	-6.47	Down	0.001 (0.002)	0.001 (0.002)	0.1684 (W)	-1.6	Down
PC ae C36:0	0.011 (0.010)	0.009 (0.004)	0.9251 (W)	1.2	Up	0.011 (0.011)	0.010 (0.005)	0.4712 (W)	1.11	Up	0.004 (0.002)	0.009 (0.004)	0.0057 (W)	-2.04	Down

PC ae C36:1	0.009 (0.007)	0.004 (0.004)	0.0391 (W)	2.2	Up	0.005 (0.003)	0.012 (0.041)	0.2681 (W)	-2.75	Down	0.003 (0.002)	0.006 (0.006)	0.6999 (W)	-1.66	Down
PC ae C36:2	0.001 (0.001)	0.006 (0.008)	0.0056 (W)	-4.39	Down	0.004 (0.006)	0.017 (0.051)	0.4839 (W)	-3.93	Down	0.002 (0.002)	0.007 (0.010)	0.1756 (W)	-3.29	Down
PC ae C36:3	0.004 (0.003)	0.002 (0.004)	0.0282 (W)	2.25	Up	0.001 (0.003)	0.006 (0.022)	0.2005 (W)	-4.64	Down	0.001 (0.001)	0.003 (0.005)	0.7377 (W)	-2.8	Down
PC ae C36:4	0.008 (0.002)	0.004 (0.004)	0.0020 (W)	2.01	Up	0.015 (0.013)	0.020 (0.073)	0.0087 (W)	-1.33	Down	0.009 (0.004)	0.004 (0.005)	0.0194 (W)	2.03	Up
PC ae C36:5	0.001 (0.001)	0.001 (0.001)	0.2331 (W)	-1.35	Down						0.003 (0.006)	0.002 (0.002)	0.1055 (W)	1.61	Up
PC ae C38:0	0.048 (0.035)	0.023 (0.008)	0.0484 (W)	2.12	Up	0.037 (0.030)	0.021 (0.012)	0.5944 (W)	1.73	Up	0.022 (0.011)	0.017 (0.008)	0.1130 (W)	1.3	Up
PC ae C38:1	0.001 (0.001)	0.001 (0.003)	0.4833 (W)	-1.07	Down	0.002 (0.003)	0.003 (0.011)	0.2964 (W)	-1.33	Down	0.002 (0.003)	0.001 (0.002)	0.4424 (W)	1.19	Up
PC ae C38:2	0.001 (0.001)	0.001 (0.001)	0.4711 (W)	1.11	Up	0.001 (0.001)	0.004 (0.012)	0.8789 (W)	-4.7	Down					
PC ae C38:3	0.001 (0.001)	0.001 (0.001)	0.2995 (W)	1.72	Up	0.004 (0.005)	0.007 (0.027)	0.9025 (W)	-2.02	Down	0.002 (0.002)	0.002 (0.002)	0.4745 (W)	-1.06	Down
PC ae C38:4	0.002 (0.001)	0.003 (0.004)	1.0000 (W)	-1.81	Down	0.002 (0.003)	0.016 (0.058)	0.6999 (W)	-6.5	Down	0.001 (0.001)	0.004 (0.005)	0.5427 (W)	-2.81	Down
PC ae C38:5	0.004 (0.006)	0.002 (0.004)	0.9757 (W)	1.75	Up	0.001 (0.001)	0.014 (0.059)	0.4110 (W)	-13.98	Down	0.001 (0.002)	0.003 (0.004)	0.4651 (W)	-1.81	Down
PC ae C38:6	0.008 (0.005)	0.005 (0.003)	0.1229 (W)	1.56	Up	0.014 (0.010)	0.013 (0.035)	0.0160 (W)	1.1	Up	0.010 (0.012)	0.005 (0.005)	0.4942 (W)	2.05	Up
PC ae C40:1	0.002 (0.002)	0.002 (0.001)	0.9251 (W)	1.17	Up	0.003 (0.004)	0.003 (0.010)	0.4105 (W)	1.04	Up	0.001 (0.001)	0.002 (0.002)	0.0666 (W)	-2.42	Down
PC ae C40:2	0.001 (0.001)	0.001 (0.001)	0.4124 (W)	-1.07	Down	0.003 (0.004)	0.006 (0.012)	0.6162 (W)	-1.84	Down					
PC ae C40:3	0.002 (0.004)	0.001 (0.001)	0.5476 (W)	2.85	Up						0.001 (0.002)	0.001 (0.001)	0.3602 (W)	1.32	Up
PC ae C40:4	0.007 (0.004)	0.006 (0.002)	0.0969 (W)	1.09	Up	0.010 (0.011)	0.010 (0.012)	0.5517 (W)	-1.05	Down	0.003 (0.004)	0.006 (0.004)	0.0668 (W)	-1.82	Down
PC ae C40:6	0.003 (0.003)	0.002 (0.001)	0.8753 (W)	1.73	Up	0.002 (0.004)	0.006 (0.022)	0.1201 (W)	-3.35	Down	0.002 (0.002)	0.001 (0.001)	0.6190 (W)	1.29	Up
PC ae C42:0	0.054 (0.043)	0.090 (0.043)	0.0858	-1.66	Down	0.074 (0.046)	0.097 (0.097)	0.5727 (W)	-1.32	Down	0.031 (0.015)	0.105 (0.088)	0.0043 (W)	-3.37	Down
PC ae C42:1	0.003 (0.003)	0.008 (0.004)	0.0053	-2.89	Down	0.004 (0.006)	0.008 (0.007)	0.1906 (W)	-1.96	Down	0.002 (0.002)	0.008 (0.008)	0.0055 (W)	-4.06	Down
PC ae C42:2						0.003 (0.003)	0.002 (0.003)	0.3447 (W)	1.65	Up	0.001 (0.001)	0.002 (0.002)	0.0878 (W)	-3.07	Down
PC ae C42:5	0.080 (0.040)	0.077 (0.033)	0.8755 (W)	1.04	Up	0.067 (0.059)	0.070 (0.022)	0.2218 (W)	-1.04	Down	0.038 (0.015)	0.072 (0.037)	0.0069 (W)	-1.87	Down
PC ae C44:3	0.005 (0.003)	0.002 (0.002)	0.0107 (W)	2.16	Up	0.010 (0.009)	0.003 (0.003)	0.0026 (W)	4.01	Up	0.007 (0.005)	0.002 (0.002)	0.0279 (W)	3.16	Up
PC ae C44:4	0.009 (0.007)	0.006 (0.004)	0.2425 (W)	1.41	Up	0.015 (0.015)	0.008 (0.006)	0.6565 (W)	1.94	Up	0.008 (0.007)	0.008 (0.005)	0.9764 (W)	1.05	Up
PC ae C44:5	0.007 (0.006)	0.007 (0.003)	0.5517 (W)	1.04	Up	0.007 (0.005)	0.008 (0.006)	0.6838 (W)	-1.07	Down	0.004 (0.002)	0.006 (0.002)	0.0874 (W)	-1.64	Down
PC ae C44:6	0.002 (0.001)	0.003 (0.002)	0.0969 (W)	-1.53	Down	0.001 (0.002)	0.003 (0.004)	0.1513 (W)	-2.42	Down	0.000 (0.001)	0.002 (0.002)	0.0023 (W)	-6.81	Down
Sphingomyelins															
SM (OH) C16:1	0.001 (0.001)	0.001 (0.000)	1.0000 (W)	1.33	Up	0.004 (0.006)	0.004 (0.015)	0.8021 (W)	-1.02	Down	0.002 (0.004)	0.001 (0.001)	0.6857 (W)	1.93	Up
SM (OH) C22:1	0.001 (0.001)	0.001 (0.002)	0.0470 (W)	-2.68	Down						0.003 (0.006)	0.003 (0.004)	0.5140 (W)	1.2	Up
SM (OH) C24:1	0.002 (0.003)	0.001 (0.001)	0.9512 (W)	3.22	Up	0.001 (0.002)	0.001 (0.003)	0.8247 (W)	1.04	Up	0.000 (0.001)	0.001 (0.001)	0.1881 (W)	-1.56	Down
SM C16:0	0.005 (0.008)	0.020 (0.037)	0.0081 (W)	-4.1	Down	0.004 (0.007)	0.134 (0.544)	0.0552 (W)	-31.09	Down	0.004 (0.005)	0.021 (0.042)	0.3152 (W)	-4.77	Down
SM C16:1	0.003 (0.002)	0.003 (0.004)	0.7445 (W)	-1.15	Down	0.002 (0.002)	0.020 (0.080)	0.4825 (W)	-11.03	Down	0.001 (0.001)	0.003 (0.006)	0.7840 (W)	-2.31	Down
SM C18:0	0.003 (0.004)	0.002 (0.001)	0.6827 (W)	1.67	Up	0.005 (0.005)	0.032 (0.104)	0.5025 (W)	-6.8	Down	0.002 (0.003)	0.002 (0.004)	0.7309 (W)	-1.12	Down
SM C22:3						0.001 (0.001)	0.003 (0.007)	0.1487 (W)	-4.28	Down	0.001 (0.000)	0.001 (0.001)	0.6630 (W)	1.02	Up

SM C24:0	0.019 (0.018)	0.009 (0.006)	0.0969 (W)	2.1	Up	0.024 (0.022)	0.033 (0.066)	0.5944 (W)	-1.41	Down	0.008 (0.003)	0.008 (0.005)	0.3675 (W)	-1	Down
SM C24:1	0.003 (0.005)	0.005 (0.006)	0.0731 (W)	-2.02	Down						0.001 (0.002)	0.006 (0.010)	0.0755 (W)	-3.76	Down
Hexose	586.956 (892.842)	167.809 (136.369)	0.0536 (W)	3.5	Up	595.632 (1122.887)	127.923 (107.469)	0.1963 (W)	4.66	Up	676.828 (440.753)	317.042 (251.094)	0.0107 (W)	2.13	Up
Amino acids															
L-Alanine	19.211 (4.128)	20.807 (18.063)	0.3244 (W)	-1.08	Down	17.192 (14.016)	17.334 (13.315)	1.0000 (W)	-1.01	Down	28.385 (19.726)	39.377 (39.020)	0.4942 (W)	-1.39	Down
L-Arginine	4.236 (2.082)	1.660 (0.929)	0.0004 (W)	2.55	Up	3.661 (1.977)	1.625 (1.033)	0.0526	2.25	Up	2.830 (0.548)	2.232 (1.677)	0.1082 (W)	1.27	Up
L-Asparagine	5.079 (0.706)	4.528 (1.933)	0.3008	1.12	Up	4.970 (2.480)	4.861 (2.700)	0.7897 (W)	1.02	Up	5.929 (2.269)	7.453 (6.640)	1.0000 (W)	-1.26	Down
L-Aspartic acid	12.116 (1.923)	5.403 (3.316)	< 0.0001	2.24	Up	13.100 (8.473)	5.547 (4.454)	0.0043 (W)	2.36	Up	10.777 (7.565)	9.257 (6.956)	0.5727 (W)	1.16	Up
L-Glutamine	72.655 (53.188)	34.293 (18.939)	0.0194 (W)	2.12	Up	68.891 (40.520)	40.401 (24.753)	0.1229 (W)	1.71	Up	49.503 (14.022)	42.708 (33.225)	0.2425 (W)	1.16	Up
L-Glutamate	14.801 (7.770)	15.436 (14.160)	0.6140 (W)	-1.04	Down	15.008 (11.611)	13.192 (7.081)	0.9292 (W)	1.14	Up	12.623 (9.047)	15.732 (12.394)	0.4215 (W)	-1.25	Down
Glycine	77.556 (86.769)	20.064 (11.634)	0.0087 (W)	3.87	Up	44.987 (31.625)	32.120 (40.947)	0.1229 (W)	1.4	Up	142.226 (151.547)	109.707 (137.548)	0.4215 (W)	1.3	Up
L-Histidine	11.366 (5.978)	6.285 (2.877)	0.0931	1.81	Up	12.140 (8.216)	6.871 (4.543)	0.1390 (W)	1.77	Up	11.617 (7.370)	10.474 (8.571)	0.4942 (W)	1.11	Up
L-Serine	18.766 (7.817)	13.916 (7.957)	0.1229 (W)	1.35	Up	17.212 (9.782)	15.162 (9.308)	0.5727 (W)	1.14	Up	24.178 (8.033)	23.788 (15.278)	0.7445 (W)	1.02	Up
L-Threonine	19.195 (5.669)	10.845 (4.950)	0.0018	1.77	Up	22.528 (18.132)	11.791 (7.553)	0.0949 (W)	1.91	Up	10.600 (4.422)	11.141 (7.550)	0.7897 (W)	-1.05	Down
L-Tryptophan	7.153 (2.704)	2.864 (1.516)	0.0002 (W)	2.5	Up	8.536 (6.344)	3.023 (2.310)	0.0055 (W)	2.82	Up	4.205 (2.730)	2.948 (2.211)	0.1963 (W)	1.43	Up
Biogenic amines															
ADMA	6.074 (1.405)	3.576 (1.985)	0.0087	1.7	Up	5.519 (4.123)	3.139 (2.356)	0.0459 (W)	1.76	Up	1.458 (0.779)	1.528 (1.488)	0.4942 (W)	-1.05	Down
SDMA	13.196 (10.632)	4.535 (1.556)	< 0.0001 (W)	2.91	Up	25.891 (35.428)	5.072 (2.504)	0.0536 (W)	5.1	Up	19.125 (18.576)	4.899 (3.686)	0.0011 (W)	3.9	Up
Carnosine	2.434 (0.637)	1.226 (0.467)	< 0.0001	1.99	Up	2.700 (1.879)	1.431 (0.943)	0.0194 (W)	1.89	Up	1.718 (1.137)	1.424 (1.318)	0.3244 (W)	1.21	Up

[1]Cows were diagnosed with ketosis (n=6) ranging from wk +1 to +3.

[2] C0: DL-Carnitine; C10: Decanoyl-L-carnitine; C10:1: Decenoyl-L-carnitine; C10:2: Decadienyl-L-carnitine; C12: Dodecanoyl-L-carnitine; C12-DC: Dodecanedioyl-L-carnitine; C12:1: Dodecenoyl-L-carnitine; C14: Tetradecanoyl-L-carnitine; C14:1 Tetradecenoyl-L-carnitine; C14:1-OH: Hydroxytetradecenoyl-L-carnitine; C14:2: Tetradecadienyl-L-carnitine; C14:2-OH: Hydroxytetradecadienyl-L-carnitine; C16: Hexadecanoyl-L-carnitine; C16-OH: Hydroxyhexadecanoyl-L-carnitine; C16:1: Hexadecenoyl-L-carnitine; C16:1-OH: Hydroxyhexadecenoyl-L-carnitine; C16:2: Hexadecadienyl-L-carnitine; C16:2-OH: Hydroxyhexadecadienyl-L-carnitine; C18: Octadecanoyl-L-carnitine; C18:1: Octadecenoyl-L-carnitine; C18:1-OH: Hydroxyoctadecenoyl-L-carnitine; C18:2: Octadecadienyl-L-carnitine; C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C3-DC (C4-OH): Malonyl-L-carnitine / Hydroxybutyryl-L-carnitine; C3-OH: Hydroxypropionyl-L-carnitine; C3:1: Propenyl-L-carnitine; C4: Butyryl-L-carnitine; C4:1: Butenyl-L-carnitine; C6 (C4:1-DC): Fumaryl-L-carnitine / Hexanoyl-L-carnitine; C5: Valeryl-L-carnitine; C5-M-DC: Methylglutaryl-L-carnitine; C5-OH (C3-DC-M): Methylmalonyl-L-carnitine / Hydroxyvaleryl-L-carnitine; C5:1: Tiglyl-L-carnitine; C5:1-DC: Glutaconyl-L-carnitine; C5-DC (C6-OH): Glutaryl-L-carnitine / Hydroxyhexanoyl-L-carnitine; C6:1: Hexenoyl-L-carnitine; C7-DC: Pimelyl-L-carnitine; C8: Octanoyl-L-carnitine; C9: Nonayl-L-carnitine; lysoPC a: lysophosphatidylcholine acyl; PC aa: phosphatidylcholine diacyl; PC ae: phosphatidylcholine acyl-alkyl; SM (OH): hydroxy sphingomyelin; SM: sphingomyelin; ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine.

[3]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

Table 4-2. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at +4, and +8 wks after parturition as determined by DI/LC-MS/MS

	4 week after parturition					8 week after parturition				
Metabolite[1], μM/mM creatinine	Ketosis	CON	*P*-value[2]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Acylcarnitines										
C0	1.254 (0.820)	1.200 (0.400)	0.5887 (W)	1.05	Up	1.026 (0.354)	0.803 (0.183)	0.2007	1.28	Up
C10	0.052 (0.029)	0.043 (0.020)	0.5462	1.21	Up	0.043 (0.019)	0.024 (0.006)	0.0578	1.8	Up
C10:1	0.093 (0.077)	0.034 (0.019)	0.0087 (W)	2.76	Up	0.062 (0.029)	0.018 (0.002)	0.0022 (W)	3.49	Up
C10:2	0.024 (0.010)	0.016 (0.006)	0.1146	1.5	Up	0.023 (0.010)	0.011 (0.003)	0.0319	2.05	Up
C12	0.101 (0.028)	0.063 (0.026)	0.034	1.6	Up	0.072 (0.041)	0.039 (0.021)	0.1027	1.87	Up
C12-DC	0.039 (0.017)	0.027 (0.012)	0.1796	1.45	Up	0.035 (0.026)	0.018 (0.005)	0.0411 (W)	1.93	Up
C12:1	0.103 (0.038)	0.066 (0.058)	0.2195	1.56	Up	0.064 (0.028)	0.025 (0.008)	0.0164	2.61	Up
C14	0.014 (0.009)	0.008 (0.003)	0.1691	1.75	Up	0.010 (0.006)	0.006 (0.001)	0.1207	1.85	Up
C14:1	0.011 (0.011)	0.006 (0.003)	0.3197	1.8	Up	0.011 (0.011)	0.003 (0.001)	0.1301	3.56	Up
C14:1-OH	0.010 (0.005)	0.006 (0.002)	0.1542	1.68	Up	0.012 (0.007)	0.003 (0.001)	0.043	3.35	Up
C14:2	0.011 (0.015)	0.003 (0.001)	0.6991 (W)	3.28	Up	0.005 (0.004)	0.003 (0.001)	0.2373	1.84	Up
C14:2-OH	0.031 (0.033)	0.012 (0.011)	0.3095 (W)	2.53	Up	0.053 (0.077)	0.005 (0.001)	0.2403 (W)	10.99	Up
C16	0.014 (0.010)	0.005 (0.002)	0.0087 (W)	2.59	Up	0.013 (0.007)	0.003 (0.001)	0.0176	3.74	Up
C16-OH	0.010 (0.005)	0.005 (0.002)	0.0260 (W)	2.08	Up	0.008 (0.005)	0.003 (0.001)	0.0998	2.31	Up
C16:1	0.027 (0.021)	0.017 (0.010)	0.2957	1.62	Up	0.033 (0.032)	0.013 (0.005)	0.1771	2.62	Up
C16:1-OH	0.005 (0.002)	0.003 (0.001)	0.0805	1.52	Up	0.008 (0.005)	0.002 (0.001)	0.0329	3.61	Up
C16:2	0.007 (0.005)	0.003 (0.001)	0.0022 (W)	2.68	Up	0.006 (0.003)	0.002 (0.001)	0.0043 (W)	3.07	Up
C16:2-OH	0.010 (0.006)	0.006 (0.003)	0.1894	1.67	Up	0.014 (0.010)	0.004 (0.002)	0.0260 (W)	3.25	Up
C18	0.005 (0.002)	0.003 (0.001)	0.0338	1.69	Up	0.006 (0.002)	0.002 (0.001)	0.0118	2.98	Up
C18:1	0.007 (0.003)	0.004 (0.002)	0.1128	1.53	Up	0.006 (0.002)	0.003 (0.001)	0.0254	1.9	Up
C18:1-OH	0.011 (0.007)	0.007 (0.004)	0.5887 (W)	1.51	Up	0.013 (0.008)	0.005 (0.002)	0.042	2.84	Up
C18:2	0.009 (0.005)	0.003 (0.002)	0.0329	2.56	Up	0.008 (0.004)	0.002 (0.001)	0.024	3.21	Up
C2	0.213 (0.160)	0.247 (0.086)	0.3095 (W)	-1.16	Down	0.176 (0.087)	0.144 (0.057)	0.4621	1.22	Up
C3	0.030 (0.029)	0.031 (0.012)	0.4848 (W)	-1.05	Down	0.021 (0.010)	0.022 (0.009)	0.7747	-1.08	Down
C3-DC (C4-OH)	0.037 (0.021)	0.042 (0.016)	0.6385	-1.14	Down	0.032 (0.016)	0.030 (0.014)	0.8308	1.06	Up
C3-OH	0.068 (0.055)	0.023 (0.006)	0.099	3.02	Up	0.064 (0.026)	0.019 (0.007)	0.0066	3.43	Up
C3:1	0.027 (0.022)	0.046 (0.018)	0.1444	-1.67	Down	0.018 (0.012)	0.033 (0.014)	0.0649 (W)	-1.83	Down
C4	0.061 (0.077)	0.063 (0.035)	0.3095 (W)	-1.03	Down	0.056 (0.045)	0.049 (0.033)	0.6991 (W)	1.14	Up
C4:1	0.048 (0.027)	0.054 (0.008)	0.6638	-1.11	Down	0.062 (0.040)	0.032 (0.009)	0.1166	1.98	Up

C6 (C4:1-DC)	0.022 (0.018)	0.020 (0.006)	0.5887 (W)	1.12	Up	0.013 (0.004)	0.016 (0.004)	0.327	-1.19	Down
C5	0.032 (0.029)	0.030 (0.012)	0.4848 (W)	1.07	Up	0.029 (0.009)	0.019 (0.010)	0.0411 (W)	1.5	Up
C5-M-DC	0.066 (0.036)	0.023 (0.007)	0.0327	2.87	Up	0.047 (0.020)	0.017 (0.005)	0.014	2.69	Up
C5-OH (C3-DC-M)	0.065 (0.034)	0.045 (0.012)	0.2398	1.43	Up	0.057 (0.014)	0.028 (0.005)	0.003	2.04	Up
C5:1	0.058 (0.038)	0.070 (0.020)	0.2403 (W)	-1.2	Down	0.050 (0.024)	0.038 (0.007)	0.2631	1.33	Up
C5:1-DC	0.019 (0.009)	0.016 (0.005)	0.4821	1.2	Up	0.013 (0.004)	0.011 (0.003)	0.3248	1.2	Up
C5-DC (C6-OH)	0.025 (0.016)	0.022 (0.006)	0.7264	1.12	Up	0.021 (0.005)	0.014 (0.005)	0.0594	1.44	Up
C6:1	0.026 (0.014)	0.020 (0.004)	0.6991 (W)	1.29	Up	0.023 (0.010)	0.015 (0.003)	0.1259	1.51	Up
C7-DC	0.016 (0.010)	0.011 (0.005)	0.2953	1.44	Up	0.013 (0.006)	0.007 (0.002)	0.0829	1.76	Up
C8	0.037 (0.023)	0.024 (0.005)	0.3095 (W)	1.52	Up	0.031 (0.010)	0.016 (0.006)	0.0089	1.93	Up
C9	0.047 (0.023)	0.064 (0.033)	0.3343	-1.35	Down	0.053 (0.030)	0.041 (0.017)	0.374	1.32	Up
Lysophosphatidylcholines										
lysoPC a C16:0	0.033 (0.033)	0.011 (0.007)	0.1641	3.04	Up	0.028 (0.029)	0.005 (0.002)	0.1797 (W)	5.62	Up
lysoPC a C16:1	0.018 (0.036)	0.008 (0.008)	1.0000 (W)	2.26	Up	0.009 (0.004)	0.005 (0.005)	0.3095 (W)	1.68	Up
lysoPC a C17:0	0.012 (0.017)	0.006 (0.008)	0.6304 (W)	1.87	Up	0.006 (0.005)	0.003 (0.002)	0.1744	2.22	Up
lysoPC a C18:0	0.044 (0.047)	0.026 (0.008)	0.6991 (W)	1.71	Up	0.023 (0.025)	0.014 (0.004)	0.9372 (W)	1.64	Up
lysoPC a C18:1	0.006 (0.013)	0.008 (0.008)	0.2130 (W)	-1.36	Down	0.007 (0.007)	0.008 (0.006)	1.0000 (W)	-1.18	Down
lysoPC a C18:2	0.013 (0.011)	0.022 (0.017)	0.3087	-1.67	Down	0.009 (0.004)	0.010 (0.011)	0.8182 (W)	-1.2	Down
lysoPC a C20:3	0.034 (0.050)	0.014 (0.012)	0.5887 (W)	2.37	Up	0.027 (0.010)	0.011 (0.004)	0.0043 (W)	2.42	Up
lysoPC a C20:4	0.013 (0.015)	0.005 (0.005)	0.3048	2.32	Up	0.004 (0.006)	0.002 (0.001)	0.8705 (W)	1.57	Up
lysoPC a C28:1	0.012 (0.017)	0.002 (0.002)	0.2215 (W)	5.4	Up					
Phosphatidylcholines										
PC aa C24:0	0.010 (0.011)	0.005 (0.003)	0.3095 (W)	2.02	Up	0.011 (0.010)	0.005 (0.002)	0.0649 (W)	2.21	Up
PC aa C28:1	0.019 (0.019)	0.008 (0.004)	0.2403 (W)	2.41	Up	0.016 (0.006)	0.010 (0.004)	0.0723	1.67	Up
PC aa C30:0	0.021 (0.008)	0.013 (0.006)	0.0693	1.67	Up	0.020 (0.009)	0.009 (0.004)	0.0312	2.06	Up
PC aa C30:2	0.002 (0.002)	0.002 (0.001)	0.5206	1.49	Up	0.003 (0.005)	0.000 (0.000)	0.7976 (W)	10.09	Up
PC aa C32:0	0.012 (0.021)	0.001 (0.001)	0.0904 (W)	12.84	Up	0.004 (0.004)	0.001 (0.001)	0.1432	3.09	Up
PC aa C32:1	0.007 (0.012)	0.001 (0.001)	0.6781 (W)	12.71	Up	0.003 (0.002)	0.001 (0.001)	0.0904 (W)	4.62	Up
PC aa C32:2	0.009 (0.018)	0.004 (0.005)	0.9338 (W)	2.21	Up	0.004 (0.002)	0.003 (0.002)	0.279	1.49	Up
PC aa C34:1	0.009 (0.008)	0.005 (0.002)	0.3662	1.65	Up	0.012 (0.019)	0.005 (0.004)	0.6991 (W)	2.46	Up
PC aa C34:2	0.011 (0.011)	0.009 (0.004)	0.5887 (W)	1.2	Up	0.018 (0.024)	0.011 (0.014)	0.9372 (W)	1.62	Up
PC aa C34:3	0.001 (0.002)	0.000 (0.000)	0.6775 (W)	4.42	Up	0.003 (0.005)	0.001 (0.001)	0.8033 (W)	3.92	Up
PC aa C34:4	0.009 (0.008)	0.013 (0.015)	0.5494	-1.5	Down	0.012 (0.023)	0.003 (0.004)	0.8068 (W)	4.18	Up

PC aa C36:0	0.047 (0.039)	0.032 (0.011)	0.8182 (W)	1.48	Up	0.049 (0.017)	0.028 (0.007)	0.0132	1.8	Up
PC aa C36:1	0.002 (0.002)	0.001 (0.001)	0.2818	1.9	Up	0.004 (0.003)	0.001 (0.001)	0.4848 (W)	3.18	Up
PC aa C36:2	0.006 (0.006)	0.004 (0.004)	0.7483 (W)	1.76	Up	0.007 (0.006)	0.004 (0.002)	0.2116	1.95	Up
PC aa C36:3	0.006 (0.004)	0.003 (0.001)	0.0807	2.23	Up	0.004 (0.004)	0.001 (0.001)	0.2678	2.48	Up
PC aa C36:4	0.004 (0.004)	0.004 (0.003)	0.9081	1.07	Up	0.005 (0.004)	0.002 (0.002)	0.0931 (W)	2.46	Up
PC aa C36:5						0.001 (0.001)	0.001 (0.001)	0.1240 (W)	-1.63	Down
PC aa C38:0	0.015 (0.020)	0.006 (0.003)	0.4848 (W)	2.67	Up	0.007 (0.002)	0.002 (0.002)	0.0037	2.99	Up
PC aa C38:1	0.005 (0.007)	0.003 (0.004)	0.5721	1.57	Up	0.007 (0.002)	0.003 (0.001)	0.0002	2.78	Up
PC aa C38:3	0.004 (0.004)	0.003 (0.002)	0.443	1.65	Up	0.005 (0.005)	0.002 (0.001)	0.2524	2.04	Up
PC aa C38:4	0.009 (0.004)	0.005 (0.002)	0.0931 (W)	1.62	Up	0.013 (0.008)	0.005 (0.002)	0.0696	2.72	Up
PC aa C38:5	0.001 (0.001)	0.001 (0.002)	0.6049	-1.44	Down	0.004 (0.004)	0.002 (0.001)	0.3507	1.86	Up
PC aa C38:6	0.004 (0.004)	0.002 (0.001)	0.2631	1.89	Up	0.002 (0.003)	0.005 (0.007)	0.4704 (W)	-2.1	Down
PC aa C40:1	0.047 (0.043)	0.032 (0.013)	0.9372 (W)	1.48	Up	0.040 (0.014)	0.023 (0.009)	0.0315	1.77	Up
PC aa C40:2	0.004 (0.004)	0.002 (0.002)	0.2994	1.84	Up	0.001 (0.002)	0.001 (0.002)	0.6248 (W)	-1.09	Down
PC aa C40:3	0.003 (0.005)	0.002 (0.002)	0.6085	1.53	Up	0.002 (0.002)	0.000 (0.000)	0.1436	7.19	Up
PC aa C40:4	0.002 (0.001)	0.000 (0.000)	0.1044 (W)	7.91	Up	0.001 (0.001)	0.001 (0.001)	0.5497 (W)	-1.2	Down
PC aa C40:6	0.054 (0.048)	0.023 (0.013)	0.17	2.39	Up	0.040 (0.014)	0.018 (0.010)	0.0106	2.22	Up
PC aa C42:0	0.014 (0.017)	0.003 (0.003)	0.1655	4.27	Up	0.005 (0.005)	0.004 (0.005)	0.8555	1.12	Up
PC aa C42:1	0.002 (0.003)	0.001 (0.001)	0.5611 (W)	2.06	Up	0.001 (0.002)	0.000 (0.001)	0.4460 (W)	1.81	Up
PC aa C42:2	0.008 (0.008)	0.008 (0.004)	0.9865	-1.01	Down	0.011 (0.009)	0.007 (0.006)	0.6991 (W)	1.57	Up
PC aa C42:4	0.004 (0.006)	0.000 (0.001)	0.4217 (W)	12.77	Up	0.003 (0.004)	0.001 (0.001)	0.9357 (W)	3	Up
PC aa C42:5	0.001 (0.003)	0.000 (0.001)	0.5497 (W)	3.1	Up	0.006 (0.006)	0.002 (0.001)	0.117	3.78	Up
PC aa C42:6	0.059 (0.048)	0.024 (0.014)	0.1401	2.49	Up	0.035 (0.020)	0.016 (0.007)	0.0611	2.24	Up
PC ae C30:0	0.012 (0.004)	0.012 (0.006)	0.7992	-1.06	Down	0.012 (0.005)	0.010 (0.003)	0.4027	1.22	Up
PC ae C30:2	0.003 (0.007)	0.002 (0.001)	0.0870 (W)	1.4	Up	0.002 (0.003)	0.002 (0.002)	0.1659 (W)	-1.28	Down
PC ae C32:2	0.001 (0.001)	0.001 (0.001)	0.7976 (W)	1.14	Up	0.001 (0.001)	0.000 (0.001)	0.7526 (W)	1.96	Up
PC ae C34:0	0.001 (0.001)	0.001 (0.001)	0.2215 (W)	-1.49	Down	0.002 (0.003)	0.000 (0.001)	0.5320 (W)	4.6	Up
PC ae C34:1	0.001 (0.002)	0.002 (0.002)	0.0874 (W)	-2.58	Down	0.003 (0.006)	0.001 (0.001)	0.8705 (W)	4.69	Up
PC ae C34:2	0.002 (0.004)	0.001 (0.001)	0.9289 (W)	3.02	Up	0.006 (0.013)	0.001 (0.000)	0.5611 (W)	10.44	Up
PC ae C34:3	0.005 (0.006)	0.000 (0.000)	0.1044 (W)	17.57	Up	0.002 (0.002)	0.000 (0.000)	0.1811	6.14	Up
PC ae C36:0	0.010 (0.012)	0.011 (0.006)	0.3939 (W)	-1.19	Down	0.008 (0.004)	0.007 (0.005)	0.4848 (W)	1.1	Up
PC ae C36:1	0.011 (0.006)	0.004 (0.004)	0.0442	2.57	Up	0.009 (0.007)	0.002 (0.001)	0.0698	4.25	Up
PC ae C36:2	0.002 (0.002)	0.002 (0.002)	0.4151 (W)	-1.3	Down	0.004 (0.003)	0.003 (0.001)	0.4263	1.32	Up

PC ae C36:3						0.003 (0.003)	0.002 (0.001)	0.44	1.72	Up
PC ae C36:4	0.014 (0.011)	0.003 (0.002)	0.0022 (W)	4.51	Up	0.018 (0.011)	0.004 (0.004)	0.0256	4.09	Up
PC ae C36:5	0.003 (0.003)	0.001 (0.001)	0.1498	3.23	Up	0.005 (0.011)	0.000 (0.000)	0.6781 (W)	13.33	Up
PC ae C38:0	0.052 (0.057)	0.014 (0.005)	0.0260 (W)	3.61	Up	0.033 (0.011)	0.016 (0.006)	0.0095	2.07	Up
PC ae C38:1	0.003 (0.005)	0.000 (0.001)	0.7750 (W)	9.85	Up	0.002 (0.002)	0.000 (0.001)	0.2805 (W)	4.53	Up
PC ae C38:2	0.001 (0.002)	0.001 (0.001)	0.7973 (W)	1.84	Up	0.001 (0.002)	0.000 (0.000)	0.2458 (W)	4.71	Up
PC ae C38:3	0.003 (0.004)	0.001 (0.001)	0.1999 (W)	5.32	Up	0.002 (0.003)	0.001 (0.001)	0.2807	2.67	Up
PC ae C38:4	0.002 (0.002)	0.001 (0.001)	0.1196	3.39	Up	0.002 (0.002)	0.001 (0.001)	0.1643	2.32	Up
PC ae C38:5	0.001 (0.003)	0.001 (0.000)	0.4550 (W)	2.61	Up	0.001 (0.001)	0.001 (0.001)	1.0000 (W)	1.41	Up
PC ae C38:6	0.011 (0.007)	0.005 (0.003)	0.0764	2.3	Up	0.016 (0.013)	0.006 (0.002)	0.0022 (W)	2.64	Up
PC ae C40:1	0.002 (0.003)	0.002 (0.001)	0.9767	1.02	Up	0.002 (0.001)	0.001 (0.001)	0.1901	2.15	Up
PC ae C40:2	0.007 (0.009)	0.000 (0.001)	0.1147 (W)	14.91	Up	0.001 (0.002)	0.002 (0.004)	0.0874 (W)	-2.27	Down
PC ae C40:3	0.004 (0.006)	0.000 (0.000)	0.0730 (W)	12.74	Up	0.007 (0.005)	0.001 (0.001)	0.0289	9.13	Up
PC ae C40:4	0.006 (0.003)	0.004 (0.004)	0.361	1.43	Up	0.004 (0.004)	0.004 (0.002)	0.7592	1.14	Up
PC ae C40:6	0.002 (0.002)	0.001 (0.002)	0.8033 (W)	1.71	Up	0.002 (0.002)	0.001 (0.001)	0.4069	2.08	Up
PC ae C42:0	0.069 (0.030)	0.059 (0.029)	0.5367	1.18	Up	0.062 (0.029)	0.054 (0.022)	0.6076	1.15	Up
PC ae C42:1	0.005 (0.009)	0.005 (0.003)	0.3751 (W)	-1.01	Down	0.005 (0.004)	0.005 (0.003)	0.7573	-1.13	Down
PC ae C42:2	0.001 (0.001)	0.000 (0.001)	0.7745 (W)	1.66	Up	0.001 (0.002)	0.001 (0.001)	0.5611 (W)	1.14	Up
PC ae C42:5	0.075 (0.063)	0.072 (0.037)	0.6991 (W)	1.04	Up	0.073 (0.037)	0.048 (0.018)	0.1602	1.53	Up
PC ae C44:3	0.008 (0.002)	0.002 (0.002)	0.0050 (W)	4.34	Up	0.006 (0.008)	0.003 (0.002)	0.3213	2.36	Up
PC ae C44:4	0.014 (0.010)	0.008 (0.005)	0.2742	1.68	Up	0.011 (0.005)	0.004 (0.001)	0.0142	2.69	Up
PC ae C44:5	0.006 (0.003)	0.007 (0.006)	0.9372 (W)	-1.22	Down	0.007 (0.006)	0.007 (0.003)	0.8954	-1.05	Down
PC ae C44:6	0.002 (0.002)	0.001 (0.001)	0.6884 (W)	1.31	Up	0.002 (0.002)	0.001 (0.001)	0.2958	1.88	Up
Sphingomyelins										
SM (OH) C16:1	0.002 (0.002)	0.001 (0.002)	1.0000 (W)	1.14	Up	0.002 (0.003)	0.000 (0.001)	0.6081 (W)	4.8	Up
SM (OH) C22:1	0.005 (0.009)	0.001 (0.001)	1.0000 (W)	4.25	Up	0.003 (0.004)	0.000 (0.000)	0.2215 (W)	6.33	Up
SM (OH) C24:1	0.000 (0.000)	0.000 (0.001)	0.7745 (W)	-1.37	Down	0.002 (0.002)	0.001 (0.001)	0.1581 (W)	3.58	Up
SM C16:0	0.004 (0.004)	0.003 (0.003)	0.6137	1.41	Up	0.007 (0.009)	0.002 (0.001)	0.2469	3.07	Up
SM C16:1	0.003 (0.004)	0.001 (0.002)	0.3124	2.86	Up	0.002 (0.002)	0.001 (0.001)	0.3221	2.32	Up
SM C18:0	0.000 (0.001)	0.002 (0.001)	0.1140 (W)	-3.48	Down	0.002 (0.001)	0.000 (0.001)	0.0562 (W)	5.52	Up
SM C22:3	0.001 (0.001)	0.000 (0.001)	1.0000 (W)	1.43	Up	0.002 (0.002)	0.000 (0.001)	0.8033 (W)	3.61	Up
SM C24:0	0.024 (0.033)	0.009 (0.005)	0.4848 (W)	2.61	Up	0.009 (0.008)	0.007 (0.003)	0.4481	1.4	Up
SM C24:1	0.001 (0.001)	0.002 (0.002)	0.3403	-1.81	Down	0.001 (0.001)	0.000 (0.000)	0.1224	3.8	Up

Hexose	346.719 (149.641)	340.847 (107.336)	0.9393	1.02	Up	331.868 (139.113)	218.833 (102.644)	0.1403	1.52	Up
Amino acids										
L-Alanine	133.817 (218.586)	95.228 (49.189)	0.1320 (W)	1.41	Up	64.069 (36.797)	59.306 (42.245)	0.8392	1.08	Up
L-Arginine	3.947 (1.976)	4.301 (1.924)	0.7596	-1.09	Down	4.720 (1.168)	2.908 (1.206)	0.0260 (W)	1.62	Up
L-Asparagine	8.836 (3.756)	11.126 (3.717)	0.1320 (W)	-1.26	Down	8.725 (2.339)	5.631 (1.976)	0.0328	1.55	Up
L-Aspartic acid	37.396 (53.900)	24.671 (22.303)	0.8182 (W)	1.52	Up	19.896 (3.152)	14.594 (9.936)	0.0649 (W)	1.36	Up
L-Glutamine	48.628 (25.678)	96.033 (48.197)	0.0594	-1.97	Down	64.920 (33.811)	44.943 (29.456)	0.3008	1.44	Up
L-Glutamate	74.605 (109.210)	40.438 (24.117)	0.8182 (W)	1.84	Up	21.532 (12.910)	20.500 (11.842)	0.9372 (W)	1.05	Up
Glycine	156.463 (102.252)	317.795 (141.928)	0.0260 (W)	-2.03	Down	149.078 (140.345)	230.661 (185.570)	0.4105	-1.55	Down
L-Histidine	15.214 (3.045)	23.086 (9.007)	0.0879	-1.52	Down	17.682 (5.664)	12.725 (4.527)	0.125	1.39	Up
L-Serine	27.040 (5.955)	44.854 (18.200)	0.0931 (W)	-1.66	Down	29.967 (9.560)	23.070 (5.752)	0.161	1.3	Up
L-Threonine	16.793 (6.087)	25.282 (8.958)	0.0838	-1.51	Down	20.129 (6.719)	14.938 (10.030)	0.3175	1.35	Up
L-Tryptophan	6.530 (3.163)	5.093 (1.891)	0.3621	1.28	Up	6.873 (2.003)	3.071 (1.542)	0.0042	2.24	Up
Biogenic amines										
ADMA	1.386 (0.553)	2.385 (1.596)	0.1964	-1.72	Down	4.570 (3.487)	2.568 (1.644)	0.2323	1.78	Up
SDMA	20.009 (16.649)	8.368 (4.097)	0.1509	2.39	Up	23.187 (22.503)	5.808 (1.339)	0.1172	3.99	Up
Carnosine	3.343 (1.111)	2.866 (1.102)	0.4728	1.17	Up	2.789 (0.814)	1.435 (0.396)	0.0044	1.94	Up

[1]C0: DL-Carnitine; C10: Decanoyl-L-carnitine; C10:1: Decenoyl-L-carnitine; C10:2: Decadienyl-L-carnitine; C12: Dodecanoyl-L-carnitine; C12-DC: Dodecanedioyl-L-carnitine; C12:1: Dodecenoyl-L-carnitine; C14: Tetradecanoyl-L-carnitine; C14:1: Tetradecenoyl-L-carnitine; C14:1-OH: Hydroxytetradecenoyl-L-carnitine; C14:2: Tetradecadienyl-L-carnitine; C14:2-OH: Hydroxytetradecadienyl-L-carnitine; C16: Hexadecanoyl-L-carnitine; C16-OH: Hydroxyhexadecanoyl-L-carnitine; C16:1: Hexadecenoyl-L-carnitine; C16:1-OH: Hydroxyhexadecenoyl-L-carnitine; C16:2: Hexadecadienyl-L-carnitine; C16:2-OH: Hydroxyhexadecadienyl-L-carnitine; C18: Octadecanoyl-L-carnitine; C18:1: Octadecenoyl-L-carnitine; C18:1-OH: Hydroxyoctadecenoyl-L-carnitine; C18:2: Octadecadienyl-L-carnitine; C2: Acetyl-L-carnitine; C3: Propionyl-L-carnitine; C3-DC (C4-OH): Malonyl-L-carnitine / Hydroxybutyryl-L-carnitine; C3-OH: Hydroxypropionyl-L-carnitine; C3:1: Propenyl-L-carnitine; C4: Butyryl-L-carnitine; C4:1: Butenyl-L-carnitine; C6 (C4:1-DC): Fumaryl-L-carnitine / Hexanoyl-L-carnitine; C5: Valeryl-L-carnitine; C5-M-DC: Methylglutaryl-L-carnitine; C5-OH (C3-DC-M): Methylmalonyl-L-carnitine / Hydroxyvaleryl-L-carnitine; C5:1: Tiglyl-L-carnitine; C5:1-DC: Glutaconyl-L-carnitine; C5-DC (C6-OH): Glutaryl-L-carnitine / Hydroxyhexanoyl-L-carnitine; C6:1: Hexenoyl-L-carnitine; C7-DC: Pimelyl-L-carnitine; C8: Octanoyl-L-carnitine; C9: Nonayl-L-carnitine; lysoPC a: lysophosphatidylcholine acyl; PC aa: phosphatidylcholine diacyl; PC ae: phosphatidylcholine acyl-alkyl; SM (OH): hydroxy sphingomyelin; SM: sphingomyelin; ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine.

[2]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

Table 4-3. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at 3 time points (-8 wk, -4 wk, and the wk of diagnosis of disease) as determined by NMR

	8 week before parturition					4 week before parturition					Ketosis diagnosis week[1]				
Metabolite[2], μM/mM creatinine	Ketosis	CON	*P*-value[3]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	20	-	-	-	6	20	-	-	-	6	20	-	-	
Ketones															
3-Hydroxybutyric acid	25.246 (11.676)	28.375 (15.580)	0.9764 (W)	-1.12	Down	25.217 (18.953)	19.812 (10.528)	0.6140 (W)	1.27	Up	69.267 (40.517)	38.512 (22.349)	0.0297 (W)	1.8	Up
Acetoacetic acid	10.753 (4.476)	17.634 (11.278)	0.0828 (W)	-1.64	Down	15.925 (12.055)	12.791 (6.194)	0.9764 (W)	1.25	Up	50.235 (27.826)	24.091 (12.914)	0.0067 (W)	2.09	Up
Acetone	8.219 (3.917)	17.460 (21.282)	0.1390 (W)	-2.12	Down	12.164 (8.906)	9.895 (9.997)	0.9292 (W)	1.23	Up	65.841 (41.547)	35.852 (39.485)	0.0726 (W)	1.84	Up
Saccharides															
1,3-Dihydroxyacetone (DHA)	9.582 (9.879)	7.568 (8.954)	0.5327 (W)	1.27	Up	3.214 (2.478)	2.728 (3.441)	0.2185 (W)	1.18	Up	23.284 (21.716)	9.103 (9.578)	0.0087 (W)	2.56	Up
Arabinose	48.045 (57.689)	21.320 (13.284)	0.2185 (W)	2.25	Up	46.787 (44.443)	18.660 (15.938)	0.0331 (W)	2.51	Up	26.045 (10.066)	23.691 (17.741)	0.3244 (W)	1.1	Up
D-Galactose	35.230 (16.184)	49.648 (29.686)	0.2681 (W)	-1.41	Down	35.394 (22.728)	42.679 (23.832)	0.2954 (W)	-1.21	Down	16.774 (14.061)	59.357 (105.344)	0.0279 (W)	-3.54	Down
D-Glucose	50.126 (15.552)	42.442 (15.788)	0.3047	1.18	Up	51.996 (26.694)	37.067 (12.683)	0.2356	1.4	Up	55.409 (26.056)	56.344 (33.883)	0.6999 (W)	-1.02	Down
D-Xylose	9.889 (5.206)	11.721 (3.774)	0.1082 (W)	-1.19	Down	11.407 (6.393)	8.092 (2.427)	0.3244 (W)	1.41	Up	9.134 (4.077)	11.860 (5.747)	0.2924	-1.3	Down
Lactose	57.151 (62.335)	30.101 (38.035)	1.0000 (W)	1.9	Up	73.734 (124.581)	36.706 (80.928)	0.1963 (W)	2.01	Up	33.153 (22.719)	24.388 (19.635)	0.2185 (W)	1.36	Up
Amino acids and derivatives															
2-Aminobutyric acid	17.163 (11.088)	21.276 (8.442)	0.0828 (W)	-1.24	Down	21.104 (15.266)	15.070 (4.575)	0.9764 (W)	1.4	Up	14.251 (5.555)	19.962 (13.991)	0.4570 (W)	-1.4	Down
3-Aminoisobutyric acid	21.187 (7.816)	16.440 (6.346)	0.1398	1.29	Up	20.879 (7.638)	13.072 (4.522)	0.0043	1.6	Up	20.562 (5.313)	20.129 (11.004)	0.5327 (W)	1.02	Up
beta-Alanine	15.173 (8.445)	7.915 (7.296)	0.0131 (W)	1.92	Up	19.299 (9.514)	7.701 (4.566)	0.0294	2.51	Up	12.743 (10.833)	10.220 (7.205)	0.5327 (W)	1.25	Up
Betaine	43.073 (29.269)	41.129 (39.013)	0.4942 (W)	1.05	Up	33.677 (25.259)	20.089 (13.470)	0.2954 (W)	1.68	Up	47.751 (41.546)	44.205 (47.712)	0.8823 (W)	1.08	Up
Creatine	404.335 (231.779)	537.239 (230.096)	0.2273	-1.33	Down	637.932 (241.996)	487.787 (198.752)	0.1349	1.31	Up	963.918 (258.242)	839.356 (327.776)	0.4033	1.15	Up
Guanidoacetic acid	155.972 (252.183)	70.801 (46.911)	0.9764 (W)	2.2	Up	28.911 (11.698)	34.922 (20.700)	0.6999 (W)	-1.21	Down	130.941 (93.853)	86.237 (58.843)	0.3244 (W)	1.52	Up
L-Alloisoleucine	14.585 (5.215)	10.751 (8.089)	0.1390 (W)	1.36	Up	18.740 (9.165)	7.821 (5.209)	0.0107 (W)	2.4	Up	16.154 (8.808)	13.769 (11.500)	0.3551 (W)	1.17	Up
L-Cysteine	18.436 (16.806)	9.092 (4.899)	0.1082 (W)	2.03	Up	22.031 (16.731)	10.311 (9.118)	0.0391 (W)	2.14	Up	23.630 (24.107)	11.181 (6.476)	0.1082 (W)	2.11	Up
L-Isoleucine	62.101 (27.536)	39.322 (13.743)	0.1004	1.58	Up	64.746 (19.874)	35.163 (14.131)	0.0011 (W)	1.84	Up	79.777 (77.860)	55.395 (21.891)	0.7897 (W)	1.44	Up
L-Leucine	335.272 (528.955)	316.259 (310.570)	0.9764 (W)	1.06	Up	90.713 (56.062)	101.260 (131.036)	0.5327 (W)	-1.12	Down	480.603 (183.480)	394.396 (271.802)	0.4763	1.22	Up
L-Lysine	13.778 (5.475)	5.495 (2.685)	0.0126	2.51	Up	12.861 (5.305)	4.388 (2.224)	0.0103	2.93	Up	14.509 (10.120)	9.930 (11.685)	0.1756 (W)	1.46	Up
L-Methionine	40.199 (66.466)	13.063 (10.452)	0.4215 (W)	3.08	Up	30.351 (48.198)	6.801 (3.568)	0.0949 (W)	4.46	Up	11.069 (9.697)	15.312 (18.697)	0.6565 (W)	-1.38	Down
L-Phenylalanine	14.885 (8.412)	9.585 (3.470)	0.0720 (W)	1.55	Up	16.583 (8.415)	6.817 (3.047)	< 0.0001 (W)	2.43	Up	13.353 (4.837)	12.943 (9.023)	0.4215 (W)	1.03	Up
L-Tyrosine	27.640 (11.445)	15.146 (8.427)	0.0072	1.82	Up	27.903 (8.230)	15.564 (8.026)	0.0031	1.79	Up	22.204 (17.498)	19.355 (14.687)	0.8823 (W)	1.15	Up
L-Valine	1820.749 (670.094)	6181.864 (5171.103)	0.1565 (W)	-3.4	Down	1923.557 (1183.203)	6035.988 (4370.962)	0.0720 (W)	-3.14	Down	1529.750 (696.721)	7893.428 (6576.605)	0.0004	-5.16	Down
N,N-Dimethylglycine	16.031 (7.261)	17.210 (5.189)	0.2954 (W)	-1.07	Down	15.690 (5.539)	12.610 (3.410)	0.1069	1.24	Up	18.429 (2.361)	20.130 (9.981)	1.0000 (W)	-1.09	Down
N-Acetylaspartic acid	2.984 (0.746)	4.079 (1.924)	0.0498	-1.37	Down	3.165 (3.944)	4.019 (3.176)	0.0720 (W)	-1.27	Down	2.357 (3.343)	4.575 (3.250)	0.0160 (W)	-1.94	Down

N-Acetylglutamic acid	17.363 (4.966)	10.066 (4.351)	0.0019	1.72	Up	22.644 (7.688)	8.507 (4.424)	< 0.0001	2.66	Up	10.760 (7.199)	12.654 (7.851)	0.6565 (W)	-1.18	Down
N-Acetylglutamine	14.970 (7.581)	13.584 (4.464)	0.7897 (W)	1.1	Up	13.544 (5.642)	10.846 (2.501)	0.3015	1.25	Up	25.605 (14.652)	19.852 (8.225)	0.2245	1.29	Up
N-Phenylacetylglycine	26.981 (21.215)	37.794 (22.208)	0.1756 (W)	-1.4	Down	26.629 (11.238)	22.915 (11.154)	0.482	1.16	Up	50.862 (15.949)	57.733 (29.221)	0.5896	-1.14	Down
Pantothenic acid	3.415 (2.679)	1.440 (1.410)	0.0391 (W)	2.37	Up	2.900 (1.791)	1.256 (2.306)	0.0055 (W)	2.31	Up	3.036 (1.743)	1.544 (1.993)	0.0279 (W)	1.97	Up
Sarcosine	22.780 (8.574)	35.469 (24.395)	0.1229 (W)	-1.56	Down	19.658 (4.871)	22.229 (9.144)	0.8358 (W)	-1.13	Down	14.866 (7.702)	41.404 (48.702)	0.0720 (W)	-2.79	Down
Tiglylglycine	9.940 (5.280)	10.494 (5.263)	0.8358 (W)	-1.06	Down	9.656 (3.332)	6.576 (2.041)	0.0101	1.47	Up	8.721 (4.145)	8.895 (5.407)	0.8823 (W)	-1.02	Down
1-Methylhistidine	9.932 (5.811)	7.158 (5.364)	0.1229 (W)	1.39	Up	9.925 (4.300)	4.813 (2.641)	0.0015 (W)	2.06	Up	9.006 (5.350)	9.724 (6.695)	0.8125	-1.08	Down
3-Methylhistidine	19.731 (8.209)	13.075 (8.092)	0.0536 (W)	1.51	Up	17.892 (3.015)	12.466 (6.255)	0.0194 (W)	1.44	Up	20.058 (17.692)	17.129 (9.769)	0.7897 (W)	1.17	Up
Organic acids															
1,3-Dimethyluric acid	3.171 (1.505)	4.793 (2.528)	0.1565 (W)	-1.51	Down	6.333 (3.785)	3.667 (2.226)	0.1229 (W)	1.73	Up	8.009 (4.550)	8.458 (9.571)	0.2681 (W)	-1.06	Down
2-Hydroxy-3-methylpentanoic acid	49.724 (20.709)	56.393 (39.061)	0.6999 (W)	-1.13	Down	48.550 (13.666)	37.890 (18.706)	0.0720 (W)	1.28	Up	112.934 (56.590)	102.016 (55.232)	0.6764	1.11	Up
2-Hydroxybutyric acid	34.249 (6.275)	41.201 (15.927)	0.6140 (W)	-1.2	Down	42.609 (15.504)	29.577 (9.454)	0.0177	1.44	Up	48.803 (14.901)	49.176 (28.044)	0.6140 (W)	-1.01	Down
2-Hydroxyisobutyric acid	5.858 (1.329)	8.599 (9.199)	0.8358 (W)	-1.47	Down	6.735 (3.393)	4.744 (1.213)	0.1229 (W)	1.42	Up	5.678 (2.543)	5.739 (2.083)	0.9526	-1.01	Down
2-Hydroxyvaleric acid	83.308 (25.381)	87.432 (39.658)	0.6999 (W)	-1.05	Down	73.902 (16.933)	67.675 (19.982)	0.4968	1.09	Up	128.826 (80.920)	104.574 (34.264)	0.5035	1.23	Up
2-Methylglutaric acid	19.479 (11.159)	16.175 (6.067)	0.7445 (W)	1.2	Up	26.165 (13.338)	15.143 (6.664)	0.0391 (W)	1.73	Up	23.969 (13.911)	21.621 (9.483)	0.6373	1.11	Up
2-Oxobutyric acid	13.944 (1.472)	14.934 (4.802)	0.9764 (W)	-1.07	Down	15.226 (4.800)	12.593 (4.406)	0.2199	1.21	Up	13.528 (7.248)	15.780 (10.288)	0.5727 (W)	-1.17	Down
2-Oxoglutaric acid	10.299 (7.373)	9.197 (3.881)	0.8823 (W)	1.12	Up	13.336 (9.044)	7.428 (3.712)	0.0622 (W)	1.8	Up	15.567 (6.967)	11.244 (7.556)	0.1229 (W)	1.38	Up
2-Oxoisocaproate	5.979 (3.422)	8.349 (4.568)	0.1963 (W)	-1.4	Down	8.040 (3.492)	5.980 (3.052)	0.0828 (W)	1.34	Up	6.142 (2.508)	9.033 (4.068)	0.1390 (W)	-1.47	Down
3-Hydroxy-3-methylglutaric acid	6.909 (4.338)	9.751 (3.711)	0.1259	-1.41	Down	9.653 (6.380)	7.386 (3.253)	0.435	1.31	Up	11.857 (5.416)	12.522 (6.308)	0.9764 (W)	-1.06	Down
4-Hydroxyphenylacetic acid	7.797 (2.829)	12.036 (9.997)	0.5327 (W)	-1.54	Down	7.968 (5.145)	9.339 (5.077)	0.5683	-1.17	Down	11.873 (12.535)	18.979 (13.147)	0.1756 (W)	-1.6	Down
Acetic acid	165.813 (123.232)	175.555 (173.722)	0.6999 (W)	-1.06	Down	183.630 (203.515)	126.380 (127.162)	0.5327 (W)	1.45	Up	261.225 (261.481)	255.389 (216.192)	0.6140 (W)	1.02	Up
Adipic acid	66.451 (15.532)	87.581 (24.096)	0.0558	-1.32	Down	71.075 (23.153)	69.233 (15.465)	0.8823 (W)	1.03	Up	78.942 (40.203)	96.982 (33.539)	0.2425 (W)	-1.23	Down
Ascorbic acid	25.449 (22.066)	11.883 (6.338)	0.0194 (W)	2.14	Up	16.923 (9.777)	11.613 (6.159)	0.1565 (W)	1.46	Up	20.464 (8.747)	11.719 (5.971)	0.0093	1.75	Up
Citric acid	68.193 (62.556)	26.363 (24.936)	0.0622 (W)	2.59	Up	67.220 (56.622)	25.087 (19.635)	0.0536 (W)	2.68	Up	50.335 (38.797)	71.807 (83.222)	0.7897 (W)	-1.43	Down
Ethylmalonic acid	20.354 (3.893)	36.103 (25.669)	0.1963 (W)	-1.77	Down	21.239 (5.568)	20.790 (8.421)	0.6565 (W)	1.02	Up	32.907 (17.621)	35.047 (19.209)	0.6999 (W)	-1.07	Down
Formic acid	19.926 (10.166)	16.258 (5.712)	0.2635	1.23	Up	17.218 (6.420)	14.360 (5.011)	0.2612	1.2	Up	13.901 (3.257)	15.382 (7.135)	0.8823 (W)	-1.11	Down
Gluconic acid	18.197 (20.052)	6.641 (4.856)	0.0331 (W)	2.74	Up	13.263 (10.419)	7.594 (7.516)	0.1082 (W)	1.75	Up	11.107 (5.246)	7.161 (5.194)	0.1565 (W)	1.55	Up
Glyceric acid	34.234 (22.286)	19.182 (9.062)	0.1619	1.78	Up	26.865 (16.858)	15.428 (6.457)	0.16	1.74	Up	21.870 (5.382)	17.479 (12.440)	0.1082 (W)	1.25	Up
Glycolic acid	107.723 (122.265)	146.271 (457.951)	0.3875 (W)	-1.36	Down	26.898 (11.762)	30.571 (17.231)	0.6999 (W)	-1.14	Down	133.673 (55.879)	96.566 (136.661)	0.0536 (W)	1.38	Up
Hippuric acid	137.806 (150.713)	88.799 (47.744)	0.6999 (W)	1.55	Up	66.544 (19.403)	55.034 (18.857)	0.2048	1.21	Up	139.884 (85.142)	107.234 (44.397)	0.4021	1.3	Up
Isocitric acid	37.433 (15.832)	24.020 (9.660)	0.017	1.56	Up	33.832 (9.635)	31.295 (14.421)	0.6914	1.08	Up	21.064 (8.441)	31.523 (14.082)	0.0994	-1.5	Down
L-Lactic acid	10.297 (4.924)	10.025 (3.577)	0.3875 (W)	1.03	Up	11.110 (6.566)	7.514 (3.153)	0.1565 (W)	1.48	Up	11.526 (1.713)	9.184 (2.243)	0.0275	1.26	Up
Methylmalonic acid	23.203 (10.512)	21.961 (6.321)	0.7213	1.06	Up	22.241 (6.287)	16.399 (5.510)	0.0233 (W)	1.36	Up	22.101 (8.635)	26.179 (12.136)	0.8358 (W)	-1.18	Down

Succinic acid	101.099 (60.231)	115.391 (43.448)	0.2954 (W)	-1.14	Down	84.647 (24.985)	81.426 (27.992)	0.8027	1.04	Up	137.242 (49.185)	118.860 (62.853)	0.3551 (W)	1.15	Up
trans-Aconitic acid	24.306 (12.585)	18.539 (9.914)	0.2508	1.31	Up	27.093 (19.098)	15.268 (8.239)	0.1941	1.77	Up	19.446 (13.102)	23.955 (21.524)	0.5727 (W)	-1.23	Down
Alcohols															
Ethanol	51.493 (23.780)	61.878 (32.309)	0.6140 (W)	-1.2	Down	39.357 (13.041)	42.796 (12.548)	0.2185 (W)	-1.09	Down	40.636 (7.542)	56.190 (22.208)	0.1565 (W)	-1.38	Down
Methanol	34.202 (23.902)	18.444 (7.778)	0.0459 (W)	1.85	Up	44.579 (21.449)	15.843 (6.796)	< 0.0001 (W)	2.81	Up	30.583 (14.662)	24.976 (14.654)	0.3551 (W)	1.22	Up
Propylene glycol	33.331 (6.270)	42.388 (22.863)	0.1263	-1.27	Down	34.353 (12.083)	34.344 (14.779)	0.9989	1	Up	31.752 (12.974)	47.599 (29.134)	0.1565 (W)	-1.5	Down
Misc															
1,7-Dimethylxanthine	20.044 (35.659)	10.120 (6.809)	0.4942 (W)	1.98	Up	3.296 (1.841)	4.937 (3.452)	0.6999 (W)	-1.5	Down	22.719 (20.191)	14.650 (10.813)	0.6140 (W)	1.55	Up
3-Indoxyl sulfate	40.896 (17.259)	77.506 (48.890)	0.0331 (W)	-1.9	Down	73.067 (38.848)	51.167 (23.789)	0.1013	1.43	Up	49.885 (28.945)	94.579 (68.913)	0.1963 (W)	-1.9	Down
Creatinine[4]	4039.620 (1860.974)	4723.310 (1621.231)	0.389	-1.17	Down	4060.483 (2160.656)	5024.240 (1519.214)	0.2279	-1.24	Down	7045.717 (3357.020)	4984.760 (2670.551)	0.1963 (W)	1.41	Up
Dimethyl sulfone	103.975 (69.952)	129.998 (60.236)	0.2681 (W)	-1.25	Down	113.746 (83.981)	135.866 (130.289)	0.4215 (W)	-1.19	Down	56.398 (27.406)	150.634 (123.789)	0.0087 (W)	-2.67	Down
Dimethylamine	43.943 (24.738)	57.231 (70.490)	0.4570 (W)	-1.3	Down	83.897 (111.182)	29.543 (35.629)	0.7445 (W)	2.84	Up	11.240 (6.002)	42.322 (103.940)	0.0536 (W)	-3.77	Down
Hypoxanthine	33.484 (12.170)	19.818 (9.909)	0.0095	1.69	Up	26.445 (6.114)	15.358 (5.047)	0.0001	1.72	Up	21.763 (7.517)	22.664 (10.565)	0.8482	-1.04	Down
Imidazole	5.496 (3.620)	1.775 (1.127)	0.053	3.1	Up	4.192 (2.976)	1.730 (1.378)	0.0008 (W)	2.42	Up	2.637 (1.633)	1.770 (2.208)	0.1082 (W)	1.49	Up
myo-Inositol	31.890 (23.131)	10.727 (7.825)	0.0020 (W)	2.97	Up	26.347 (10.136)	9.169 (5.178)	0.0001 (W)	2.87	Up	21.854 (9.242)	13.256 (8.300)	0.04	1.65	Up
N-Carbamoyl-beta-alanine	11.668 (4.412)	16.601 (9.103)	0.1565 (W)	-1.42	Down	11.440 (3.626)	12.230 (3.164)	0.6082	-1.07	Down	19.377 (5.612)	26.633 (16.675)	0.6140 (W)	-1.37	Down
O-Phosphocholine	103.082 (34.477)	297.378 (583.481)	0.1756 (W)	-2.88	Down	112.245 (40.298)	157.265 (122.158)	0.4942 (W)	-1.4	Down	90.645 (56.855)	330.677 (537.163)	0.0459 (W)	-3.65	Down
Trimethylamine	22.359 (8.931)	22.730 (20.018)	0.3875 (W)	-1.02	Down	22.528 (6.068)	15.265 (7.728)	0.046	1.48	Up	51.183 (28.844)	46.590 (36.616)	0.4570 (W)	1.1	Up
Trimethylamine N-oxide	1.966 (1.217)	1.999 (0.888)	0.6565 (W)	-1.02	Down	2.107 (1.012)	1.914 (2.252)	0.1963 (W)	1.1	Up	2.623 (3.072)	1.970 (1.754)	0.7897 (W)	1.33	Up
Uracil	15.186 (7.734)	15.405 (5.471)	0.4942 (W)	-1.01	Down	14.526 (14.033)	13.066 (5.051)	0.5727 (W)	1.11	Up	13.780 (5.571)	24.442 (10.664)	0.0284	-1.77	Down
Urea	41.789 (30.263)	146.549 (99.053)	0.0004	-3.51	Down	44.508 (21.820)	144.302 (96.802)	0.0391 (W)	-3.24	Down	43.771 (18.384)	194.566 (153.121)	0.0003	-4.45	Down

[1]Cows were diagnosed with ketosis (n=6) ranging from wk +1 to +3.

[2]Only metabolites unique to NMR are shown. More metabolites were measured but because their concentrations were also measured by DI/LC-MS/MS and GC-MS and were not found to be statistically different, these data are not given here.

[3]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

[4]Concentration of metabolite (Mean ± SD) is expressed by μM.

Table 4-4. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at +4, and +8 wks after parturition as determined by NMR

	4 week after parturition					8 week after parturition				
Metabolite[1], μM/mM creatinine	Ketosis	CON	*P*-value[2]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	6	-	-	-	6	6	-	-	-
Ketones										
3-Hydroxybutyric acid	458.375 (580.927)	169.260 (325.217)	0.4848 (W)	2.71	Up	307.163 (554.574)	34.096 (22.741)	0.6991 (W)	9.01	Up
Acetoacetic acid	94.240 (100.092)	41.965 (65.668)	0.1320 (W)	2.25	Up	137.534 (213.312)	27.261 (23.897)	0.3095 (W)	5.05	Up
Acetone	327.638 (467.313)	81.026 (174.335)	0.0931 (W)	4.04	Up	250.120 (539.194)	12.392 (6.634)	0.2403 (W)	20.18	Up
Saccharides										
1,3-Dihydroxyacetone (DHA)	16.200 (10.741)	6.069 (2.767)	0.0692	2.67	Up	17.963 (9.112)	6.169 (4.140)	0.0162	2.91	Up
Arabinose	38.842 (17.822)	53.044 (26.873)	0.306	-1.37	Down	53.004 (38.465)	32.678 (20.232)	0.2786	1.62	Up
D-Galactose	64.158 (106.494)	37.044 (27.499)	0.6991 (W)	1.73	Up	33.153 (13.122)	246.373 (510.047)	0.3095 (W)	-7.43	Down
D-Glucose	127.762 (148.017)	55.679 (36.293)	0.3095 (W)	2.29	Up	66.666 (32.022)	62.177 (22.973)	0.9372 (W)	1.07	Up
D-Xylose	16.240 (8.192)	14.854 (7.864)	1.0000 (W)	1.09	Up	13.770 (10.470)	12.049 (4.633)	0.5887 (W)	1.14	Up
Lactose	473.724 (1011.324)	30.325 (21.761)	0.2403 (W)	15.62	Up	307.925 (647.753)	107.515 (158.526)	1.0000 (W)	2.86	Up
Amino acids and derivatives										
2-Aminobutyric acid	24.534 (11.156)	26.386 (13.520)	0.8011	-1.08	Down	23.357 (18.873)	22.306 (13.585)	0.6991 (W)	1.05	Up
3-Aminoisobutyric acid	49.654 (24.905)	31.239 (16.369)	0.1611	1.59	Up	36.201 (32.624)	31.653 (18.139)	0.6991 (W)	1.14	Up
beta-Alanine	34.495 (21.164)	17.193 (4.290)	0.1026	2.01	Up	19.504 (14.064)	22.150 (22.758)	0.8182 (W)	-1.14	Down
Betaine	140.345 (212.829)	57.317 (63.348)	0.8182 (W)	2.45	Up	60.447 (54.063)	33.263 (28.154)	0.3003	1.82	Up
Creatine	916.915 (574.519)	687.648 (411.988)	0.4454	1.33	Up	806.056 (441.587)	752.553 (353.573)	0.8215	1.07	Up
Guanidoacetic acid	76.454 (25.837)	83.202 (29.908)	0.3939 (W)	-1.09	Down	75.614 (35.921)	76.209 (38.452)	0.9785	-1.01	Down
L-Alloisoleucine	29.857 (18.764)	19.294 (19.544)	0.3095 (W)	1.55	Up	17.382 (10.060)	16.943 (6.302)	0.9296	1.03	Up
L-Cysteine	35.138 (27.772)	20.105 (13.032)	0.3939 (W)	1.75	Up	23.863 (20.497)	23.391 (21.096)	0.6991 (W)	1.02	Up
L-Isoleucine	138.086 (158.591)	61.353 (40.151)	0.1320 (W)	2.25	Up	104.115 (65.755)	57.848 (13.017)	0.0931 (W)	1.8	Up
L-Leucine	258.605 (128.993)	244.645 (122.971)	0.8517	1.06	Up	342.210 (109.620)	294.526 (132.560)	0.5125	1.16	Up
L-Lysine	20.653 (7.755)	11.039 (8.507)	0.068	1.87	Up	13.044 (9.380)	5.792 (3.975)	0.1320 (W)	2.25	Up
L-Methionine	32.400 (24.035)	23.740 (27.313)	0.5728	1.36	Up	39.564 (53.393)	24.299 (30.510)	0.4848 (W)	1.63	Up
L-Phenylalanine	23.680 (12.933)	16.650 (10.146)	0.1797 (W)	1.42	Up	17.653 (5.000)	12.330 (8.927)	0.1320 (W)	1.43	Up
L-Tyrosine	28.370 (21.923)	36.057 (28.075)	0.6991 (W)	-1.27	Down	28.033 (11.419)	32.253 (13.445)	0.5708	-1.15	Down
L-Valine	2318.793 (949.790)	1260.075 (805.155)	0.0639	1.84	Up	2630.603 (1580.904)	1473.110 (1077.430)	0.0931 (W)	1.79	Up
N,N-Dimethylglycine	23.612 (7.598)	26.213 (13.616)	0.6914	-1.11	Down	24.660 (13.375)	21.196 (7.577)	0.5932	1.16	Up
N-Acetylaspartic acid	13.630 (25.738)	6.529 (7.572)	0.3939 (W)	2.09	Up	5.152 (6.583)	9.800 (9.043)	0.5887 (W)	-1.9	Down

N-Acetylglutamic acid	31.575 (19.729)	11.275 (3.833)	0.0022 (W)	2.8	Up	15.179 (2.822)	17.305 (9.756)	0.6269	-1.14	Down
N-Acetylglutamine	56.817 (18.253)	43.822 (27.950)	0.3628	1.3	Up	38.877 (21.317)	26.176 (11.702)	0.2296	1.49	Up
N-Phenylacetylglycine	66.421 (31.827)	74.650 (35.840)	0.683	-1.12	Down	56.665 (9.821)	49.978 (17.265)	0.4288	1.13	Up
Pantothenic acid	4.420 (4.109)	2.488 (1.307)	0.3144	1.78	Up	3.662 (2.422)	3.942 (3.671)	0.879	-1.08	Down
Sarcosine	52.575 (37.514)	25.714 (14.516)	0.1329	2.04	Up	42.940 (32.915)	36.055 (35.106)	0.9372 (W)	1.19	Up
Tiglylglycine	18.367 (9.831)	10.873 (6.744)	0.1547	1.69	Up	13.714 (7.195)	12.457 (12.827)	0.3095 (W)	1.1	Up
1-Methylhistidine	17.345 (18.874)	11.297 (8.905)	0.4848 (W)	1.54	Up	10.697 (3.862)	6.655 (4.902)	0.1437	1.61	Up
3-Methylhistidine	34.672 (25.549)	24.467 (9.084)	0.3908	1.42	Up	34.131 (20.375)	24.419 (18.383)	0.3095 (W)	1.4	Up
Organic acids										
1,3-Dimethyluric acid	13.885 (5.446)	14.066 (5.616)	0.956	-1.01	Down	13.092 (8.036)	10.157 (8.345)	0.2403 (W)	1.29	Up
2-Hydroxy-3-methylpentanoic acid	146.632 (30.268)	129.480 (68.289)	0.5862	1.13	Up	122.132 (57.797)	114.619 (54.392)	0.8213	1.07	Up
2-Hydroxybutyric acid	85.432 (59.207)	67.727 (33.528)	0.4848 (W)	1.26	Up	70.360 (71.978)	53.367 (22.752)	0.6991 (W)	1.32	Up
2-Hydroxyisobutyric acid	9.887 (4.070)	9.255 (4.176)	0.7959	1.07	Up	7.432 (3.823)	10.290 (8.970)	0.5887 (W)	-1.38	Down
2-Hydroxyvaleric acid	140.021 (73.492)	97.868 (41.720)	0.2498	1.43	Up	118.579 (68.679)	90.468 (34.577)	0.3916	1.31	Up
2-Methylglutaric acid	54.192 (44.309)	38.498 (35.735)	0.3939 (W)	1.41	Up	45.759 (38.856)	34.453 (38.741)	0.4848 (W)	1.33	Up
2-Oxobutyric acid	31.987 (26.416)	22.689 (10.312)	1.0000 (W)	1.41	Up	28.204 (29.676)	17.707 (9.350)	0.9372 (W)	1.59	Up
2-Oxoglutaric acid	29.671 (29.784)	23.626 (12.049)	0.8182 (W)	1.26	Up	15.904 (11.569)	18.175 (12.432)	0.4848 (W)	-1.14	Down
2-Oxoisocaproate	12.674 (4.802)	9.475 (6.682)	0.3634	1.34	Up	8.936 (2.909)	8.804 (6.111)	0.3939 (W)	1.02	Up
3-Hydroxy-3-methylglutaric acid	19.834 (11.183)	15.980 (8.255)	0.5125	1.24	Up	10.358 (4.161)	10.073 (4.879)	0.9154	1.03	Up
4-Hydroxyphenylacetic acid	49.293 (36.078)	28.858 (51.413)	0.0931 (W)	1.71	Up	34.317 (33.565)	11.142 (18.002)	0.0931 (W)	3.08	Up
Acetic acid	1411.596 (1649.546)	772.016 (1283.069)	0.3939 (W)	1.83	Up	713.494 (593.061)	136.268 (97.716)	0.0627	5.24	Up
Adipic acid	120.652 (37.307)	111.790 (38.864)	0.6955	1.08	Up	102.706 (45.099)	95.081 (16.690)	0.8182 (W)	1.08	Up
Ascorbic acid	20.527 (15.308)	18.237 (8.166)	0.8182 (W)	1.13	Up	13.545 (3.598)	19.285 (9.603)	0.0931 (W)	-1.42	Down
Citric acid	163.426 (236.465)	157.503 (141.950)	0.9372 (W)	1.04	Up	38.976 (35.855)	39.861 (22.074)	0.6991 (W)	-1.02	Down
Ethylmalonic acid	47.775 (20.340)	43.790 (25.927)	0.7731	1.09	Up	37.771 (29.530)	92.704 (154.726)	0.3939 (W)	-2.45	Down
Formic acid	26.622 (9.592)	22.530 (8.359)	0.4491	1.18	Up	24.820 (9.690)	24.472 (15.950)	0.8182 (W)	1.01	Up
Gluconic acid	14.882 (11.856)	17.467 (12.953)	0.7258	-1.17	Down	18.921 (9.173)	10.635 (9.230)	0.1499	1.78	Up
Glyceric acid	52.085 (44.930)	25.973 (11.272)	0.1797 (W)	2.01	Up	36.208 (13.792)	23.340 (12.075)	0.1163	1.55	Up
Glycolic acid	1572.371 (3430.254)	62.492 (25.274)	0.3095 (W)	25.16	Up	561.988 (809.047)	77.907 (29.028)	0.0411 (W)	7.21	Up
Hippuric acid	101.948 (20.830)	99.843 (24.451)	0.6991 (W)	1.02	Up	99.687 (36.872)	93.433 (32.135)	0.7606	1.07	Up
Isocitric acid	44.605 (41.802)	30.728 (5.871)	0.9372 (W)	1.45	Up	26.702 (8.706)	27.659 (9.939)	0.8627	-1.04	Down
L-Lactic acid	21.743 (17.645)	14.643 (11.379)	0.4268	1.48	Up	13.674 (7.863)	7.813 (4.528)	0.1447	1.75	Up
Methylmalonic acid	20.526 (6.506)	31.021 (8.295)	0.0349	-1.51	Down	26.946 (11.666)	28.593 (13.972)	0.8291	-1.06	Down

Succinic acid	133.553 (59.044)	117.216 (58.755)	0.6413	1.14	Up	130.391 (66.373)	149.608 (22.961)	0.0649 (W)	-1.15	Down
trans-Aconitic acid	46.754 (52.995)	26.538 (15.544)	0.5887 (W)	1.76	Up	24.384 (12.492)	24.387 (10.671)	0.9997	-1	Down
Alcohols										
Ethanol	138.203 (196.290)	86.663 (80.363)	0.4848 (W)	1.59	Up	100.213 (54.155)	74.614 (30.616)	0.3372	1.34	Up
Methanol	49.606 (28.921)	24.941 (15.264)	0.0944	1.99	Up	38.791 (25.085)	28.947 (16.013)	0.3095 (W)	1.34	Up
Propylene glycol	69.048 (60.574)	43.005 (30.200)	0.3682	1.61	Up	61.499 (61.995)	32.298 (22.223)	0.3095 (W)	1.9	Up
Misc										
1,7-Dimethylxanthine	10.119 (5.534)	16.429 (7.387)	0.125	-1.62	Down	7.765 (6.763)	12.470 (6.280)	0.2402	-1.61	Down
3-Indoxyl sulfate	147.618 (87.275)	100.128 (71.689)	0.3273	1.47	Up	109.685 (88.476)	79.611 (55.363)	0.9372 (W)	1.38	Up
Creatinine[3]	3440.867 (1251.254)	3914.033 (1707.166)	0.596	-1.14	Down	3560.017 (1146.611)	4645.567 (1458.799)	0.1824	-1.3	Down
Dimethyl sulfone	112.708 (65.829)	97.621 (42.540)	0.8182 (W)	1.15	Up	99.685 (31.659)	76.055 (24.768)	0.1804	1.31	Up
Dimethylamine	29.117 (16.432)	29.795 (27.579)	0.6991 (W)	-1.02	Down	29.861 (22.231)	23.005 (13.868)	0.536	1.3	Up
Hypoxanthine	53.963 (35.430)	39.390 (20.109)	0.6991 (W)	1.37	Up	49.565 (53.054)	32.644 (22.343)	0.8182 (W)	1.52	Up
Imidazole	5.324 (4.902)	3.119 (1.232)	0.5887 (W)	1.71	Up	3.875 (1.365)	5.024 (3.633)	0.4849	-1.3	Down
myo-Inositol	48.302 (37.558)	26.803 (15.344)	0.1320 (W)	1.8	Up	33.364 (16.568)	29.693 (17.934)	0.7204	1.12	Up
N-Carbamoyl-beta-alanine	23.298 (7.163)	19.985 (6.976)	0.4359	1.17	Up	19.794 (5.525)	16.087 (4.201)	0.22	1.23	Up
O-Phosphocholine	159.304 (67.607)	128.349 (92.295)	0.3095 (W)	1.24	Up	133.480 (87.832)	101.820 (39.175)	0.4388	1.31	Up
Trimethylamine	66.894 (17.338)	69.675 (72.077)	0.3095 (W)	-1.04	Down	49.085 (15.591)	45.954 (27.989)	0.8156	1.07	Up
Trimethylamine N-oxide	77.585 (185.058)	1.578 (0.745)	0.2403 (W)	49.16	Up	2.197 (1.528)	6.823 (13.192)	1.0000 (W)	-3.11	Down
Uracil	55.867 (64.521)	26.955 (13.021)	0.3939 (W)	2.07	Up	39.823 (28.331)	21.478 (12.106)	0.1320 (W)	1.85	Up
Urea	62.431 (51.310)	39.379 (18.851)	0.3395	1.59	Up	61.897 (31.577)	51.388 (28.931)	0.8182 (W)	1.2	Up

[1]Only metabolites unique to NMR are shown. More metabolites were measured but because their concentrations were also measured by DI/LC-MS/MS and GC-MS and were not found to be statistically different, these data are not given here.

[2]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

[3]Concentration of metabolite (Mean ± SD) is expressed by μM.

Table 4-5. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at 3 time points (-8 wk, -4 wk, and the wk of diagnosis of disease) as determined by ICP-MS or GC-MS

	8 week before parturition					4 week before parturition					Ketosis diagnosis week[1]				
Metabolite[2], μM/mM creatinine	Ketosis	CON	*P*-value[3]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	20	-	-	-	6	20	-	-	-	6	20	-	-	
Minerals															
Boron (B)	241.386 (277.807)	71.488 (47.350)	0.0043 (W)	3.38	Up	163.051 (120.821)	63.945 (37.644)	0.0069 (W)	2.55	Up	141.096 (85.357)	157.880 (150.511)	0.6999 (W)	-1.12	Down
Sodium (Na)	7542.996 (6142.867)	3595.124 (4045.119)	0.1229 (W)	2.1	Up	13301.638 (20685.023)	1896.448 (1707.628)	0.0034 (W)	7.01	Up	5107.843 (4378.642)	15801.274 (17509.082)	0.1082 (W)	-3.09	Down
Magnesium (Mg)	3327.665 (3895.454)	1038.638 (581.844)	0.0622 (W)	3.2	Up	3593.074 (2925.971)	1212.490 (1642.240)	0.0008 (W)	2.96	Up	2570.440 (1946.073)	2270.255 (2210.912)	0.5727 (W)	1.13	Up
Aluminum (Al)	0.392 (0.524)	0.050 (0.021)	0.0003 (W)	7.82	Up	0.169 (0.114)	0.058 (0.050)	0.0003 (W)	2.92	Up	0.071 (0.075)	0.072 (0.053)	0.4570 (W)	-1.01	Down
Phosphorus (P)	144.920 (146.652)	68.755 (54.274)	0.0720 (W)	2.11	Up	168.091 (117.214)	58.645 (41.546)	0.0020 (W)	2.87	Up	147.095 (141.060)	532.880 (1477.910)	0.9292 (W)	-3.62	Down
Potassium (K)	96272.735 (73291.223)	51389.717 (38369.249)	0.0459 (W)	1.87	Up	100746.353 (84842.449)	44502.516 (25185.200)	0.0720 (W)	2.26	Up	39793.269 (32405.918)	42485.253 (27658.019)	0.6565 (W)	-1.07	Down
Calcium (Ca)	115.377 (122.935)	61.528 (49.656)	0.1963 (W)	1.88	Up	175.442 (155.421)	60.046 (67.977)	0.0006 (W)	2.92	Up	65.239 (29.463)	81.580 (68.959)	0.9764 (W)	-1.25	Down
Chromium (Cr)	2.659 (1.894)	1.335 (1.308)	0.0233 (W)	1.99	Up	2.757 (2.476)	1.091 (0.495)	0.0622 (W)	2.53	Up	2.315 (1.545)	2.590 (2.486)	0.9764 (W)	-1.12	Down
Manganese (Mn)	0.064 (0.057)	0.065 (0.038)	0.7445 (W)	-1.01	Down	0.077 (0.080)	0.053 (0.029)	0.7897 (W)	1.46	Up	0.042 (0.027)	0.083 (0.058)	0.1963 (W)	-1.98	Down
Cobalt (Co)	0.008 (0.009)	0.008 (0.009)	1.0000 (W)	-1.05	Down	0.046 (0.057)	0.010 (0.012)	0.1229 (W)	4.61	Up	0.012 (0.010)	0.026 (0.025)	0.1082 (W)	-2.19	Down
Copper (Cu)	0.091 (0.042)	0.064 (0.035)	0.1082 (W)	1.43	Up	0.128 (0.085)	0.060 (0.071)	0.0160 (W)	2.12	Up	0.069 (0.041)	0.160 (0.099)	0.0415	-2.3	Down
Zinc (Zn)	0.033 (0.027)	0.032 (0.013)	0.8727 (W)	1.04	Up	0.047 (0.094)	0.012 (0.012)	0.9292 (W)	3.91	Up	0.095 (0.051)	0.100 (0.092)	0.5404 (W)	-1.05	Down
Arsenic (As)	0.077 (0.067)	0.114 (0.102)	0.3551 (W)	-1.48	Down	0.131 (0.111)	0.103 (0.061)	0.4152	1.28	Up	0.176 (0.096)	0.205 (0.230)	0.6565 (W)	-1.17	Down
Selenium (Se)	0.352 (0.250)	0.453 (0.562)	0.8823 (W)	-1.29	Down	0.990 (1.192)	0.521 (0.354)	0.6999 (W)	1.9	Up	0.402 (0.162)	0.703 (0.619)	0.4215 (W)	-1.75	Down
Rubidium (Rb)	32.936 (22.801)	18.492 (14.125)	0.0131 (W)	1.78	Up	42.704 (39.843)	17.689 (8.469)	0.0331 (W)	2.41edd	Up	15.482 (11.727)	16.234 (11.104)	0.8358 (W)	-1.05	Down
Strontium (Sr)	1.942 (1.723)	1.235 (1.212)	0.2681 (W)	1.57	Up	1.798 (1.374)	1.099 (1.144)	0.1082 (W)	1.64	Up	0.962 (0.574)	1.145 (1.077)	1.0000 (W)	-1.19	Down
Molybdenum (Mo)	0.318 (0.411)	0.187 (0.163)	0.4215 (W)	1.7	Up	0.179 (0.134)	0.191 (0.174)	0.8823 (W)	-1.07	Down	0.191 (0.157)	0.221 (0.152)	0.6140 (W)	-1.16	Down
Lead (Pb)	0.002 (0.003)	0.007 (0.025)	1.0000 (W)	-3.18	Down	0.000 (0.000)	0.000 (0.000)	0.5629 (W)	-1.02	Down					
Organic acids															
Pyruvic acid	0.062 (0.029)	0.050 (0.028)	0.1130 (W)	1.24	Up						0.046 (0.000)	0.046 (0.022)	0.1868 (W)	1	Up
Malonic acid	0.107 (0.043)	0.147 (0.069)	0.0737 (W)	-1.38	Down						0.135 (0.081)	0.202 (0.156)	0.6308 (W)	-1.49	Down
3-Hydroxyisovaleric acid	0.272 (0.169)	0.472 (0.544)	0.8049 (W)	-1.73	Down						0.917 (0.452)	1.224 (1.116)	0.7325 (W)	-1.34	Down
Adipic acid	0.007 (0.001)	0.007 (0.008)	0.5078 (W)	-1	Down	0.003 (0.002)	0.003 (0.004)	0.5227 (W)	-1	Down	0.008 (0.005)	0.010 (0.006)	0.7780 (W)	-1.22	Down
Pimelic acid	0.021 (0.013)	0.026 (0.020)	0.6194 (W)	-1.21	Down	0.010 (0.009)	0.013 (0.015)	0.8791 (W)	-1.29	Down	0.013 (0.005)	0.012 (0.010)	0.6411 (W)	1.02	Up
m-Hydroxyphenylacetic acid	0.009 (0.004)	0.006 (0.004)	0.0713 (W)	1.47	Up	0.005 (0.001)	0.005 (0.003)	0.9500 (W)	-1.1	Down	0.007 (0.006)	0.014 (0.017)	0.2005 (W)	-2.17	Down
p-Hydroxyphenylacetic acid	0.050 (0.022)	0.040 (0.030)	0.4162 (W)	1.24	Up	0.018 (0.018)	0.032 (0.032)	0.4112 (W)	-1.8	Down	0.051 (0.002)	0.052 (0.031)	1.0000 (W)	-1.02	Down
Suberic acid	0.023 (0.016)	0.015 (0.015)	0.0709 (W)	1.55	Up	0.010 (0.006)	0.017 (0.017)	0.4587 (W)	-1.69	Down	0.011 (0.005)	0.019 (0.022)	0.7609 (W)	-1.74	Down

Azelaic acid	0.052 (0.056)	0.045 (0.050)	0.6922 (W)	1.16	Up	0.009 (0.014)	0.044 (0.043)	0.0101 (W)	-5.07	Down	0.032 (0.038)	0.038 (0.034)	0.4467 (W)	-1.2	Down
Sebacic acid	0.034 (0.038)	0.020 (0.009)	0.3473 (W)	1.71	Up	0.007 (0.004)	0.026 (0.033)	0.0726 (W)	-3.95	Down	0.026 (0.011)	0.037 (0.030)	0.3308 (W)	-1.43	Down
Phenaceturic acid	0.138 (0.072)	0.197 (0.085)	0.0957 (W)	-1.43	Down	0.120 (0.179)	0.167 (0.328)	0.9273 (W)	-1.39	Down	0.343 (0.104)	0.398 (0.213)	0.5642 (W)	-1.16	Down
Hydroxyphenyllactic acid	0.004 (0.003)	0.008 (0.008)	0.1743 (W)	-1.85	Down	0.005 (0.003)	0.004 (0.005)	0.2868 (W)	1.1	Up	0.011 (0.006)	0.015 (0.013)	0.5106 (W)	-1.44	Down
m-Hydroxyhippuric acid	0.016 (0.004)	0.016 (0.006)	0.9741 (W)	1.05	Up	0.009 (0.007)	0.017 (0.031)	0.9273 (W)	-1.84	Down	0.012 (0.003)	0.014 (0.009)	0.9755 (W)	-1.18	Down
Stearic acid	0.027 (0.020)	0.030 (0.042)	0.4570 (W)	-1.12	Down	0.020 (0.013)	0.021 (0.016)	0.9515 (W)	-1.01	Down	0.036 (0.039)	0.029 (0.029)	0.9764 (W)	1.24	Up

[1]Cows were diagnosed with ketosis (n=6) ranging from wk +1 to +3.

[2]Only metabolites unique to ICP-MS and GC-MS are shown. More metabolites were measured but because their concentrations were also measured by NMR and DI/LC-MS/MS and were not found to be statistically different, these data are not given here.

[3]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

Table 4-6. Concentrations of urine metabolites (mean (SD)) in healthy control (CON) and ketotic cows at +4, and +8 wks after parturition as determined by ICP-MS or GC-MS

	4 week after parturition					8 week after parturition				
Metabolite[1], μM/mM creatinine	Ketosis	CON	*P*-value[2]	Fold change	Ketosis / CON	Ketosis	CON	*P*-value	Fold change	Ketosis / CON
Number of cases	6	6	-	-	-	6	6	-	-	-
Minerals										
Boron (B)	269.454 (169.427)	126.805 (67.988)	0.0411 (W)	2.12	Up	231.740 (81.865)	85.297 (43.800)	0.0031	2.72	Up
Sodium (Na)	28551.413 (23414.219)	12532.133 (10691.671)	0.1584	2.28	Up	14603.583 (6851.069)	6832.816 (4444.682)	0.042	2.14	Up
Magnesium (Mg)	3881.233 (3032.941)	1714.747 (1007.241)	0.0411 (W)	2.26	Up	4268.175 (1440.452)	1512.591 (870.295)	0.0025	2.82	Up
Aluminum (Al)	0.214 (0.137)	0.075 (0.030)	0.0043 (W)	2.86	Up	0.173 (0.044)	0.087 (0.061)	0.0260 (W)	1.99	Up
Phosphorus (P)	255.461 (169.605)	94.192 (44.788)	0.0087 (W)	2.71	Up	179.035 (33.009)	66.201 (39.282)	0.0043 (W)	2.7	Up
Potassium (K)	82279.300 (47093.348)	36862.445 (19452.690)	0.0152 (W)	2.23	Up	73041.470 (23466.122)	38707.068 (15855.184)	0.0141	1.89	Up
Calcium (Ca)	147.776 (138.687)	60.356 (57.030)	0.0411 (W)	2.45	Up	112.764 (45.805)	53.788 (34.183)	0.03	2.1	Up
Chromium (Cr)	3.527 (3.036)	1.469 (0.498)	0.0043 (W)	2.4	Up	2.370 (0.649)	1.137 (0.483)	0.0039	2.08	Up
Manganese (Mn)	0.476 (0.616)	0.058 (0.043)	0.0043 (W)	8.25	Up	0.325 (0.397)	0.119 (0.200)	0.0931 (W)	2.73	Up
Cobalt (Co)	0.069 (0.060)	0.032 (0.014)	0.3939 (W)	2.17	Up	0.057 (0.021)	0.030 (0.014)	0.0265	1.89	Up
Copper (Cu)	0.341 (0.265)	0.136 (0.048)	0.0931 (W)	2.52	Up	0.165 (0.040)	0.094 (0.040)	0.0122	1.75	Up
Zinc (Zn)	0.307 (0.260)	0.109 (0.109)	0.2539 (W)	2.81	Up	0.141 (0.114)	0.121 (0.108)	0.7584	1.17	Up
Arsenic (As)	0.271 (0.201)	0.212 (0.075)	0.8182 (W)	1.28	Up	0.201 (0.064)	0.162 (0.074)	0.3457	1.24	Up
Selenium (Se)	1.828 (1.643)	0.943 (0.340)	0.6991 (W)	1.94	Up	1.699 (1.039)	0.805 (0.371)	0.0411 (W)	2.11	Up
Rubidium (Rb)	37.720 (27.344)	14.747 (8.575)	0.0260 (W)	2.56	Up	29.477 (12.421)	13.872 (5.594)	0.0186	2.12	Up
Strontium (Sr)	1.756 (1.352)	1.088 (0.813)	0.3939 (W)	1.61	Up	1.480 (0.412)	0.881 (0.514)	0.0501	1.68	Up
Molybdenum (Mo)	0.219 (0.114)	0.225 (0.067)	0.9117	-1.03	Down	0.387 (0.313)	0.248 (0.229)	0.4001	1.56	Up
Lead (Pb)	0.001 (0.000)	0.001 (0.000)	0.5324	-1.2	Down	0.001 (0.000)	0.000 (0.000)	0.221	1.36	Up
Organic acids										
Pyruvic acid										
Malonic acid						0.074 (0.030)	0.099 (0.070)	1.0000 (W)	-1.33	Down
3-Hydroxyisovaleric acid										
Adipic acid	0.036 (0.024)	0.066 (0.077)	0.6553 (W)	-1.84	Down	0.010 (0.006)	0.017 (0.013)	0.3700 (W)	-1.77	Down
Pimelic acid	0.049 (0.029)	0.087 (0.086)	0.6553 (W)	-1.76	Down	0.022 (0.013)	0.038 (0.035)	0.8068 (W)	-1.75	Down
m-Hydroxyphenylacetic acid	0.041 (0.031)	0.098 (0.118)	0.3611 (W)	-2.36	Down	0.020 (0.012)	0.040 (0.045)	0.3261	-2.01	Down
p-Hydroxyphenylacetic acid	0.159 (0.029)	0.175 (0.044)	0.9241 (W)	-1.11	Down	0.078 (0.046)	0.066 (0.032)	0.615	1.18	Up
Suberic acid	0.015 (0.014)	0.055 (0.070)	0.1255 (W)	-3.75	Down	0.006 (0.007)	0.033 (0.024)	0.0087 (W)	-5.54	Down
Azelaic acid	0.024 (0.028)	0.079 (0.072)	0.1139	-3.27	Down	0.023 (0.027)	0.120 (0.093)	0.0508	-5.21	Down

Sebacic acid	0.033 (0.001)	0.034 (0.018)	0.7526 (W)	-1.03	Down					
Phenaceturic acid	0.142 (0.019)	0.126 (0.019)	0.2184 (W)	1.12	Up					
Hydroxyphenyllactic acid	0.018 (0.005)	0.023 (0.025)	0.8489 (W)	-1.24	Down					
m-Hydroxyhippuric acid	0.010 (0.002)	0.009 (0.002)	0.2184 (W)	1.18	Up					
Stearic acid	0.024 (0.013)	0.025 (0.015)	0.9264	-1.03	Down	0.020 (0.009)	0.068 (0.091)	0.1320 (W)	-3.3	Down

[1]Only metabolites unique to ICP-MS and GC-MS are shown. More metabolites were measured but because their concentrations were also measured by NMR and DI/LC-MS/MS and were not found to be statistically different, these data are not given here.

[2]*p*-value is calculated with t-test as a default, *p*-value with (W) is calculated by the Wilcoxon Mann Whitney test.

Table 4-7. Significant metabolic pathways involved in the onset and progression of ketosis in dairy cows

Metabolite	Metabolic pathway[6]
Fatty acid metabolism	
C0[5]	FFT, LC
C10:1[1,2,4,5]	FFT, LC
C10:2[1,2,5]	FFT, LC
C12[3,4]	FFT, LC
C12-DC[1,5]	FFT, LC
C12:1[5]	FFT, LC
C14:1[1,2]	FFT, LC
C14:1-OH[1,3,5]	FFT, LC
C14:2-OH[1]	FFT, LC
C16[1,2,3,4,5]	FFT, LC
C16-OH[1,3,4]	FFT, LC
C16:1-OH[5]	FFT, LC
C16:1[1,2]	FFT, LC
C16:2[1,2,3,4,5]	FFT, LC
C16:2-OH[5]	FFT, LC
C18[1,2,4,5]	FFT, LC
C18:1[1,5]	FFT, LC
C18:1-OH[2,5]	FFT, LC
C18:2[2,3,4,5]	FFT, LC
C3-OH[1,2,3,5]	FFT, LC
C3:1[1,3]	FFT, LC
C5[1,5]	FFT, LC
C5-M-DC[1,2,3,4,5]	FFT, LC
C5-OH (C3-DC-M) [1,2,3,5]	FFT, LC
C5-DC (C6-OH) [1,3,5]	FFT, LC
C6:1[1]	FFT, LC
C7-DC[1,2,3]	FFT, LC
C8[1,5]	FFT, LC
Glycerophospholipid metabolism	
lysoPC a C18:0[3]	GPL, FFAM, MC
lysoPC a C18:2[2]	GPL, FFAM, MC
lysoPC a C20:3[5]	GPL, FFAM, MC
lysoPC a C20:4[3]	GPL, FFAM, MC
lysoPC a C28:1[1]	GPL, FFAM, MC
PC aa C28:1[1]	GPL, FFAM, MC
PC aa C30:0[5]	GPL, FFAM, MC
PC aa C32:0[2,3]	GPL, FFAM, MC
PC aa C32:2[1,2,3]	GPL, FFAM, MC
PC aa C34:1[2]	GPL, FFAM, MC
PC aa C34:2[1]	GPL, FFAM, MC

PC aa C34:3[3]	GPL, FFAM, MC
PC aa C34:4[1]	GPL, FFAM, MC
PC aa C36:0[1,5]	GPL, FFAM, MC
PC aa C36:3[1]	GPL, FFAM, MC
PC aa C36:4[2,3]	GPL, FFAM, MC
PC aa C36:5[1]	GPL, FFAM, MC
PC aa C38:0[1,5]	GPL, FFAM, MC
PC aa C38:1[5]	GPL, FFAM, MC
PC aa C38:3[2,3]	GPL, FFAM, MC
PC aa C38:5[1]	GPL, FFAM, MC
PC aa C38:6[2]	GPL, FFAM, MC
PC aa C40:1[3,5]	GPL, FFAM, MC
PC aa C40:4[3]	GPL, FFAM, MC
PC aa C40:6[5]	GPL, FFAM, MC
PC aa C42:0[1]	GPL, FFAM, MC
PC aa C42:4[2]	GPL, FFAM, MC
PC aa C42:5[2,3]	GPL, FFAM, MC
PC aa C42:6[1]	GPL, FFAM, MC
PC ae C30:2[1,2,3]	GPL, FFAM, MC
PC ae C36:0[3]	GPL, FFAM, MC
PC ae C36:1[1 ,4]	GPL, FFAM, MC
PC ae C36:2[1]	GPL, FFAM, MC
PC ae C36:3[1]	GPL, FFAM, MC
PC ae C36:4[1,2,3,4,5]	GPL, FFAM, MC
PC aa C36:5[1]	GPL, FFAM, MC
PC ae C38:0[1,4,5]	GPL, FFAM, MC
PC ae C38:6[2,5]	GPL, FFAM, MC
PC ae C40:3[5]	GPL, FFAM, MC
PC ae C42:0[3]	GPL, FFAM, MC
PC ae C42:1[1,3]	GPL, FFAM, MC
PC ae C42:5[3]	GPL, FFAM, MC
PC ae C44:3[1,2,3,4]	GPL, FFAM, MC
PC ae C44:4[5]	GPL, FFAM, MC
PC ae C44:6[3]	GPL, FFAM, MC
O-Phosphocholine[3]	GPL
Sphingolipid metabolism	
SM C16:0[1,2]	MC, CS, SM
Amino acid metabolism	
L-Arginine[1,2,5]	APM, UC, GNG, PB
L-Asparagine[5]	AM, AR, GNG, PB
L-Aspartic acid[1,2]	BAM, APM, UC, AM, GNG, PB, MAS
L-Glutamine[1,4]	UC, AR, GM, PPM, GNG, PB
Glycine[1,4]	GNG, GTM, PPM
L-Threonine[1]	GSTM, GNG, PB, KTG, PDA, MM

L-Tryptophan[1,2,5]	GNG, PB, KTG, FM, PDA
Carnosine[1,2,5]	BAM, HM
3-Aminoisobutyric acid[2]	PPM
beta-Alanine[1]	BAM, GNG, AM, PPM, PNM
L-Alloisoleucine[2]	A stereo-isomer of L-Isoleucine
L-Cysteine[2]	GNG, PB, GTM, GSTM, MEM, PCB
L-Isoleucine[2]	GNG, KTG, PB, VLID
L-Lysine[1,2]	KTG, PB, PNM, LD, BM
L-Phenylalanine[2]	GNG, KTG, PB, PTM
L-Tyrosine[1]	GNG, KTG, PB, PTM, CB
L-Valine[3]	GNG, PB, PNM, VLID
N-Acetylaspartic acid[1,3]	ASGM
N-Acetylglutamic acid[1,2,4]	AB, BAB
Pantothenic acid[1,2,3]	BAM, PCB
Tiglylglycine[2]	An acyl glycine
1-Methylhistidine[2]	HM
3-Methylhistidine[1]	BAM, HM
2-Hydroxybutyric acid[2]	PNM
Ascorbic acid[1,3]	GTM
Methylmalonic acid[2,4]	VLID, PNM, PPM
Urea[1,2,3]	APM, UC, PPM
Ketone body metabolism	
3-Hydroxybutyric acid[3]	KBM
Acetoacetic acid[3]	KBM, PTM, VLID, BUM
Glycolysis / Gluconeogenesis	
Hexose[1,3]	N/A
1,3-Dihydroxyacetone (DHA) [3,5]	GLM, GL, MM
Arabinose[2]	ASNSM
D-Galactose[3]	NSM, GAM, SM
L-Lactic acid[3]	GNG, GL, PNM, PYM
TCA cycle	
2-Methylglutaric acid[2]	A metabolite of succinic acid
Citric acid[2]	TCA
Isocitric acid[1]	TCA
Pentose phosphate pathway	
Gluconic acid[2]	PPP
Others	
Adipic acid[1]	CD
Methanol[1]	PNM, MM
3-Indoxyl sulfate[1]	OS
Dimethyl sulfone[3]	SUM
Dimethylamine[3]	MM
ADMA[1,2]	MM
SDMA[1,2,3]	MM

Hypoxanthine[1,2]	PPM
Imidazole[1,2]	N/A
myo-Inositol[1,2,3]	GAM, IM
Trimethylamine[2]	MM
Uracil[3]	PPM, MM, BAM
Boron (B)[1,2,4,5]	N/A
Sodium (Na)[2,5]	N/A
Magnesium (Mg)[2,4,5]	N/A
Aluminum (Al)[1,2,4,5]	N/A
Phosphorus (P)[4,5]	N/A
Potassium (K)[1,4,5]	N/A
Calcium (Ca)[2,4,5]	N/A
Chromium (Cr)[1,4,5]	N/A
Manganese (Mn)[4]	N/A
Cobalt (Co)[5]	N/A
Copper (Cu)[2,3,5]	N/A
Selenium (Se)[5]	N/A
Rubidium (Rb)[1,2,4]	N/A
Strontium (Sr)[5]	N/A
Suberic acid[5]	FAO
Azelaic acid[2]	PPM

[1]Significant metabolic pathways at -8 wks before parturition;
[2]Significant metabolic pathways at -4 wks before parturition;
[3]Significant metabolic pathways at the wk of diagnosis of disease;
[4]Significant metabolic pathways at +4 wks after parturition;
[5]Significant metabolic pathways at +8 wks after parturition;
[6]Fatty acid transport: **FFT**; Lipid catabolism: **LC**; Glycerophospholipid metabolism: **GPL**; Free fatty acid metabolism: **FFAM**; Membrane component: **MC**; Cell signaling: **CS**; Sphingolipid metabolism: **SM**; Arginine and proline metabolism: **APM**; Urea cycle: **UC**; Gluconeogenesis: **GNG**, Protein biosynthesis: **PB**; Aspartate metabolism: **AM**; Ammonia recycling: **AR**; Beta-alanine metabolism: **BAM**; Malate aspartate shuttle: **MAS**; Glutamate metabolism: **GM**; Purine/pyrimidine metabolism: **PPM**; Glutathione metabolism: **GTM**; Glycine, serine, and threonine metabolism: **GSTM**; Ketogenesis: **KTG**; Protein digestion and absorption: **PDA**; Methane metabolism: **MM**; Histidine metabolism: **HM**; Propanoate metabolism: **PNM**; Valine, leucine and isoleucine degradation: **VLID**; Methionine metabolism: **MEM**; Pantothenate and CoA biosynthesis: **PCB**; Lysine degradation: **LD**; Biotin metabolism: **BM**; Phenylalanine and tyrosine metabolism: **PTM**; Catecholamine biosynthesis: **CB**; Arginine biosynthesis: **AB**; Alanine, aspartate and glutamate metabolism: **ASGM**; Ketone body metabolism: **KBM**; Butyrate metabolism: **BM**; Glycerolipid metabolism: **GLM**; Glycolysis: **GL**; Amino sugar and nucleotide sugar metabolism: **ASNSM**; Nucleotide sugars metabolism: **NSM**; Galactose metabolism: **GAM**; Pyruvate metabolism: **PYM**; Citric acid cycle: **TCA cycle**; Pentose phosphate pathway: **PPP**; Caprolactam degradation: **CD**; Oxidative stress: **OS**; Sulfur metabolism: **SUM**;

Inositol metabolism: **IM**; Mineral absorption: **MA**; Fatty acid oxidation: **FAO**.

Figure 4-1.

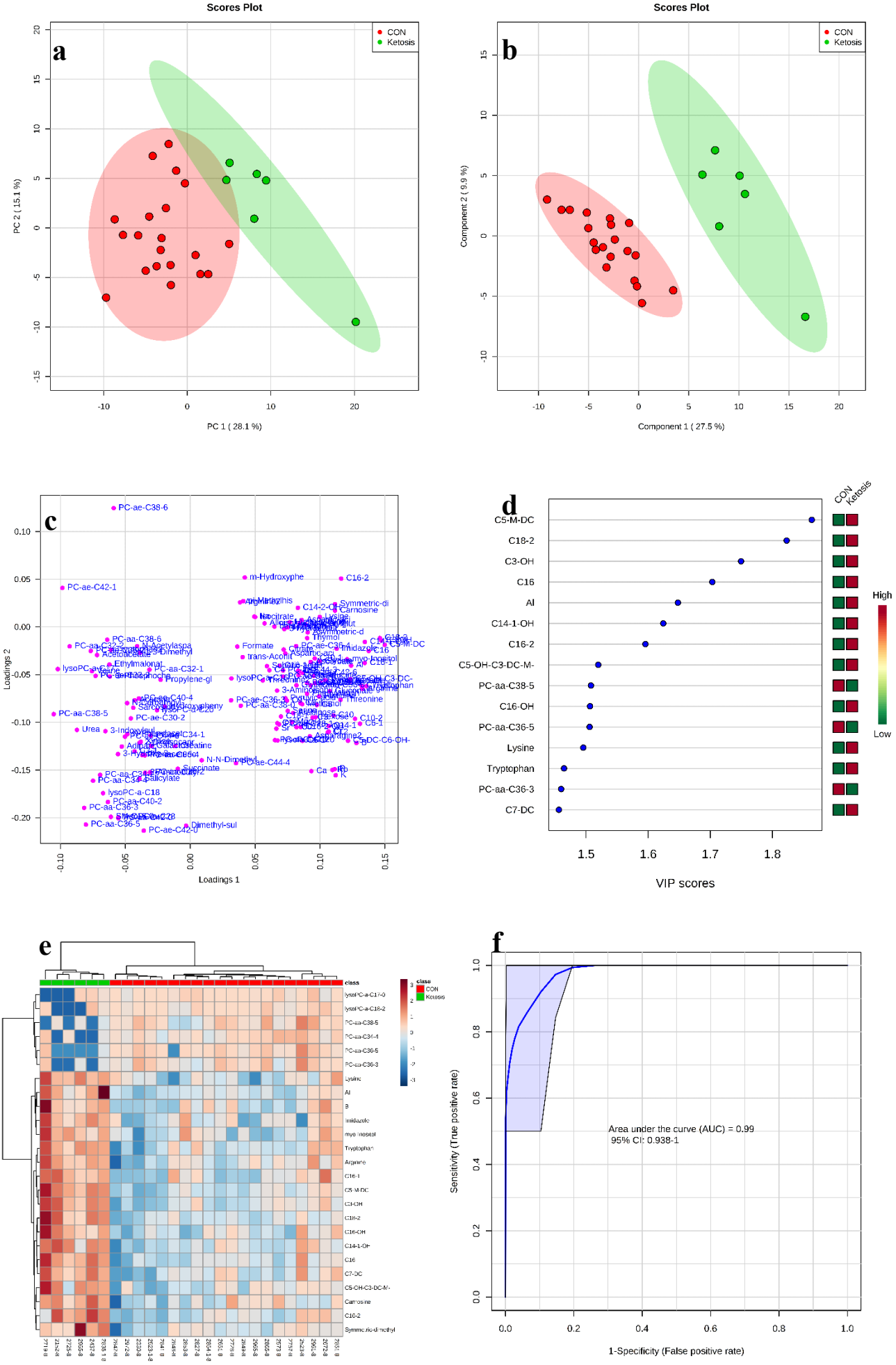
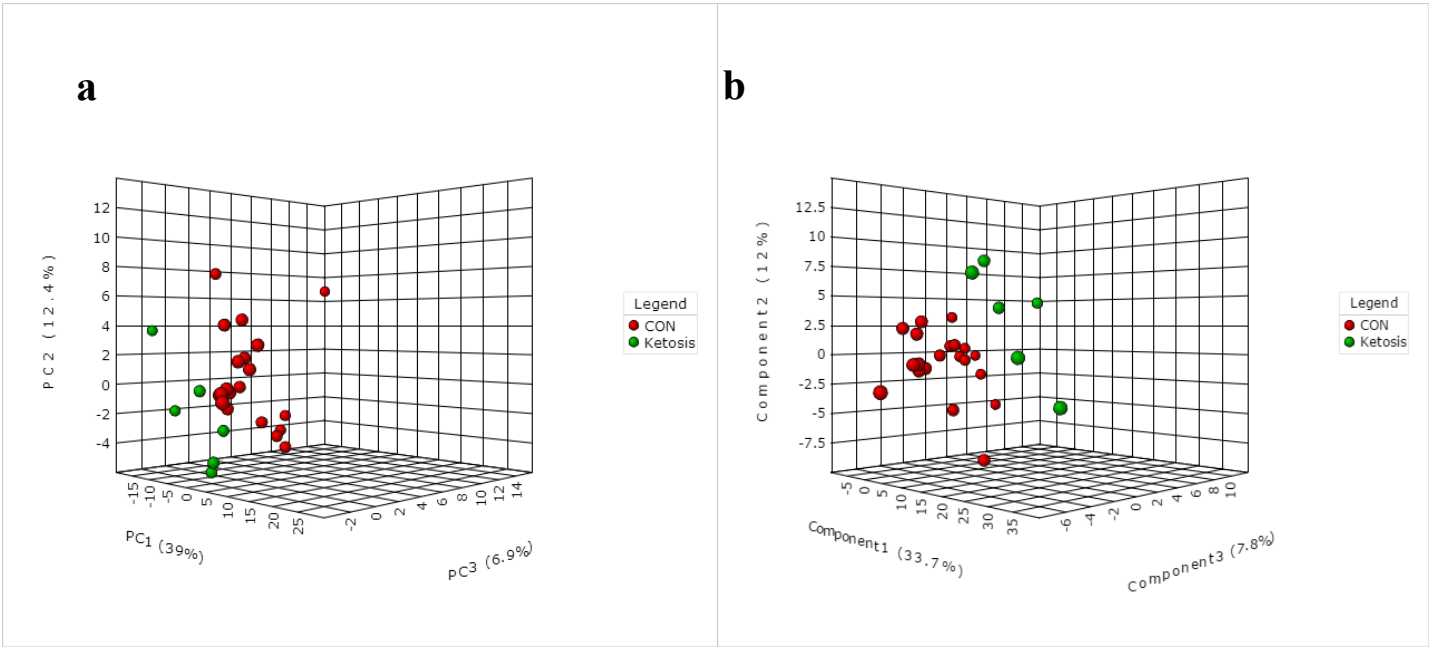
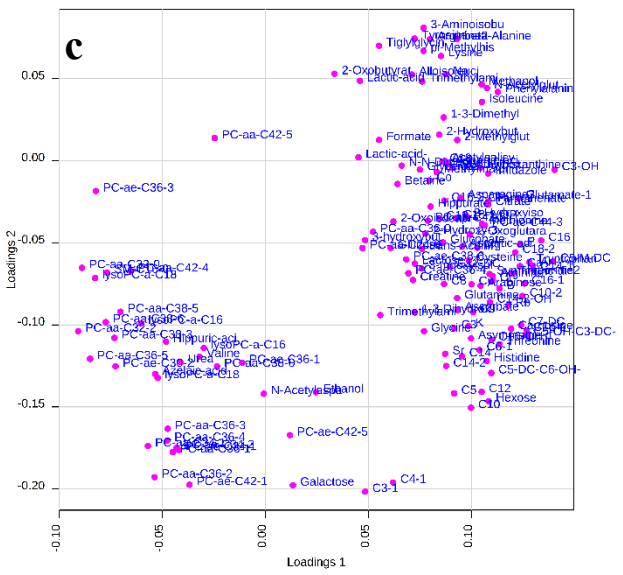
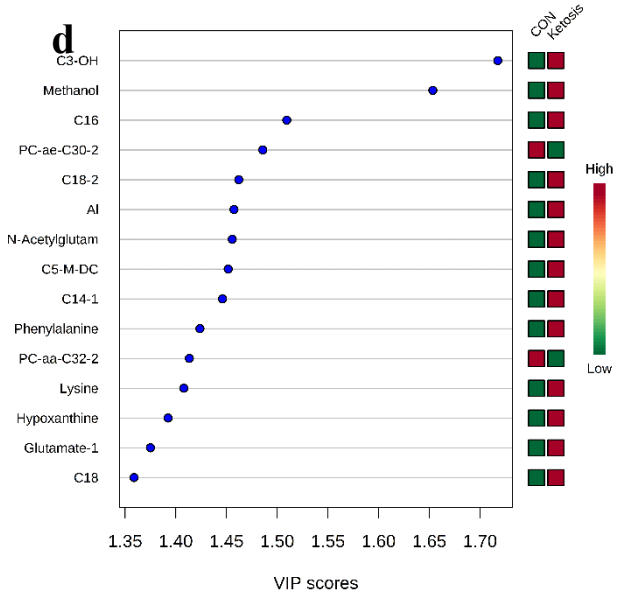
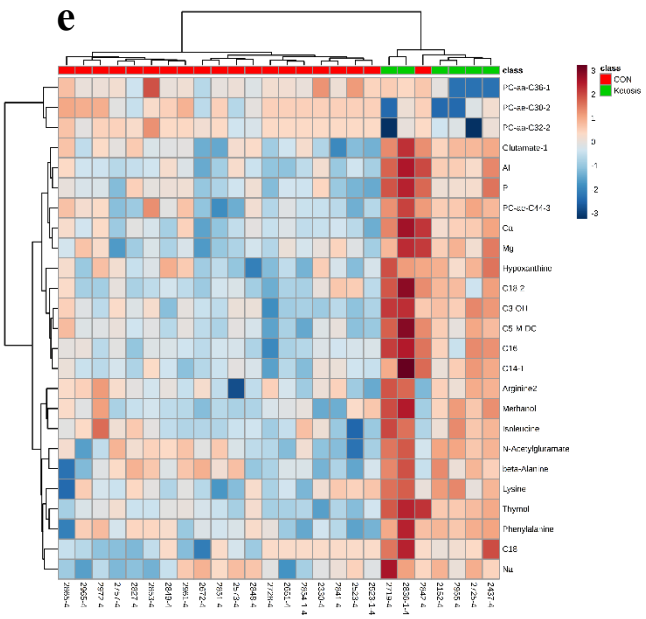

Scores Plot
a
CON
Ketosis
PC 2 (15.1 %)
PC 1 (28.1 %)
Scores Plot
b
CON
Ketosis
Component 2 (9.9 %)
Component 1 (27.5 %)
c
PC-ae-C38-6
PC-ae-C42-1
m-Hydroxyphe
C16-2
Formate
trans-Aconit
Propylene-gl
PC-aa-C38-5
PC-ae-C30-2
Urea
N-N-Dimethyl
PC-ae-C44-4
lysoPC-a-C18
PC-aa-C40-2
PC-aa-C36-3
PC-aa-C36-5
PC-ae-C42-0
Dimethyl-sul
Loadings 1
Loadings 2
d
C5-M-DC
C18-2
C3-OH
C16
AI
C14-1-OH
C16-2
C5-OH-C3-DC-M-
PC-aa-C38-5
C16-OH
PC-aa-C36-5
Lysine
Tryptophan
PC-aa-C36-3
C7-DC
CON
Ketosis
High
Low
VIP scores
e
class
CON
Ketosis
lysoPC-a-C17-0
lysoPC-a-C18-2
PC-aa-C38-5
PC-aa-C36-4
PC-aa-C36-5
PC-aa-C36-3
Lysine
AI
B
Imidazole
myo-Inositol
Tryptophan
Arginine
C16-1
C5-M-DC
C14-OH
C18-2
C16-OH
C14-1-OH
C16
C7-DC
C5-OH-C3-DC-M-
Carnosine
C16-2
Symmetric-dimethyl
f
Area under the curve (AUC) = 0.99
95% CI: 0.938-1
Sensitivity (True positive rate)
1-Specificity (False positive rate)

Figure 4-2.


a
PC 2 (12.4%)
PC1 (39%)
PC3 (6.9%)
Legend
CON
Ketosis
b
Component2 (12%)
Component1 (33.7%)
component3 (7.8%)
Legend
CON
Ketosis


c
Loadings 2
Loadings 1
PC-aa-C42-5
PC-ae-C36-3
PC-aa-C38-5
PC-aa-C36-5
PC-aa-C36-3
PC-aa-C36-4
PC-aa-C36-2
PC-ae-C42-5
PC-ae-C42-1
Galactose
C4-1
C3-1
N-Acetylglutam
Ethanol
Formate
Betaine
Isoleucine
Lysine
Creatine
Glutamine
Histidine
Hexose
C5
C12
C10
C16
C14-1
C5-DC-C6-OH-
Trimethylamine


d
C3-OH
Methanol
C16
PC-ae-C30-2
C18-2
Al
N-Acetylglutam
C5-M-DC
C14-1
Phenylalanine
PC-aa-C32-2
Lysine
Hypoxanthine
Glutamate-1
C18
CON
Ketosis
High
Low
1.35 1.40 1.45 1.50 1.55 1.60 1.65 1.70
VIP scores


e
class
CON
Ketosis
PC-aa-C36-1
PC-aa-C30-2
PC-aa-C32-2
Glutamate-1
Al
P
PC-aa-C44-3
Cu
Mg
Hypoxanthine
C18:2
C3-OH
C5-M-DC
C16
C14-1
Arginine2
Methanol
Isoleucine
N-Acetylglutamate
beta-Alanine
Lysine
Thymol
Phenylalanine
C18
Na

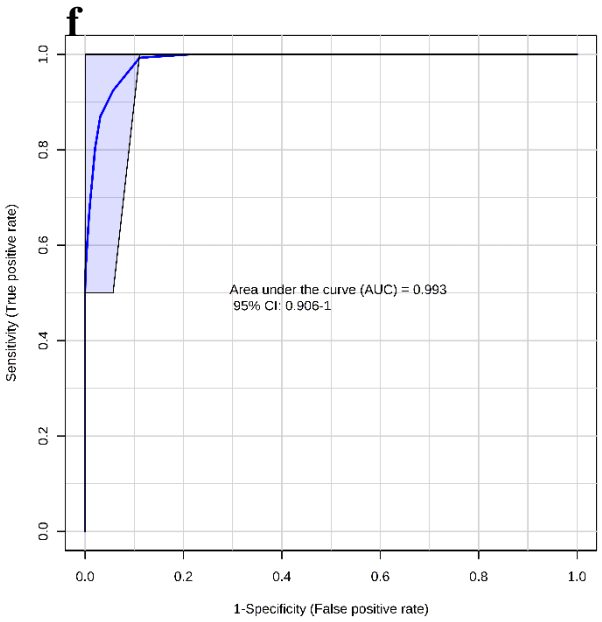

f
Sensitivity (True positive rate)
1-Specificity (False positive rate)
Area under the curve (AUC) = 0.993
95% CI: 0.906-1
0.0 0.2 0.4 0.6 0.8 1.0

Figure 4-3.

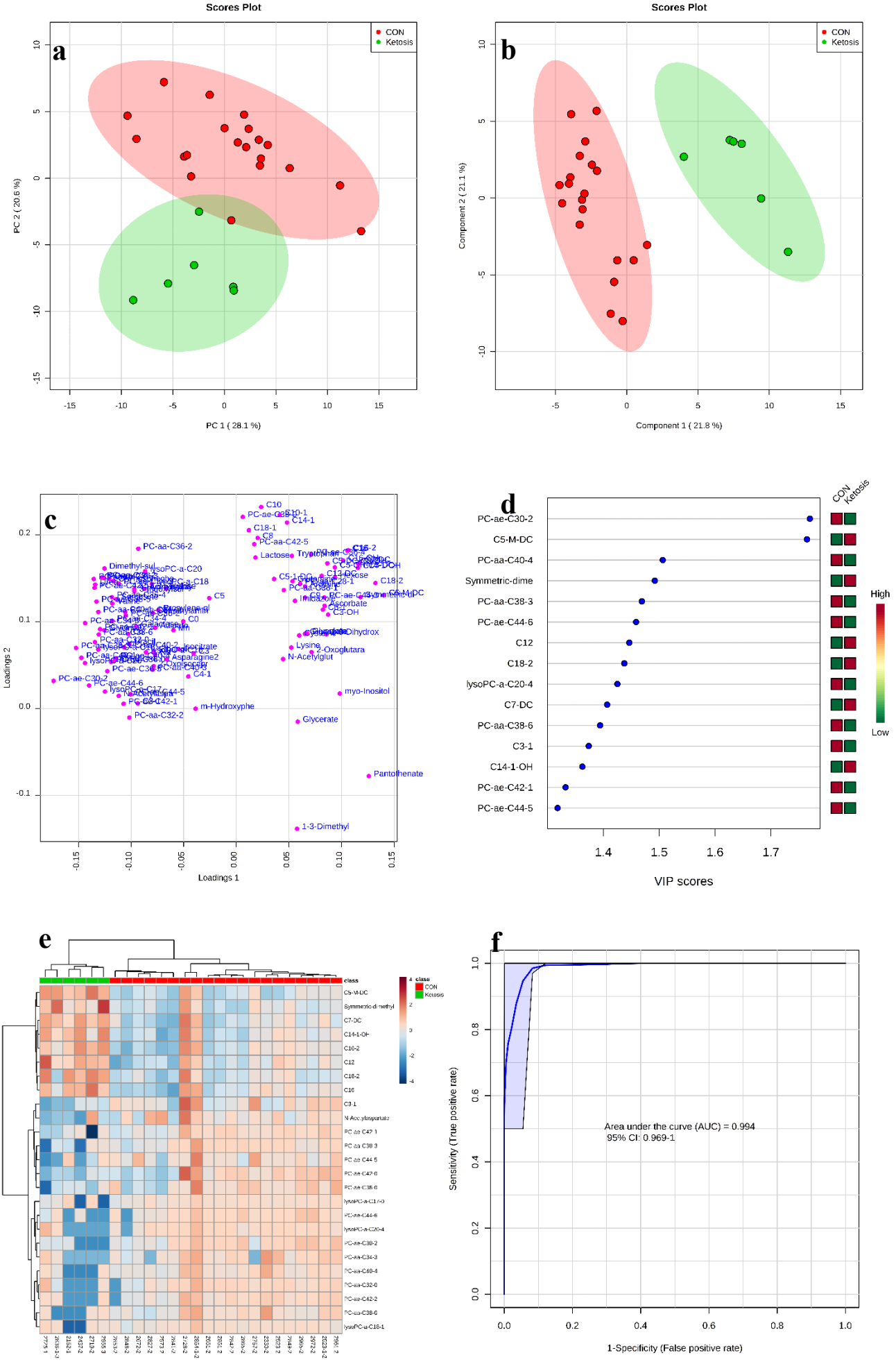

a
Scores Plot
CON
Ketosis
PC 2 (20.6 %)
PC 1 (28.1 %)
b
Scores Plot
CON
Ketosis
Component 2 (21.1 %)
Component 1 (21.8 %)
c
Loadings 2
Loadings 1
PC-aa-C36-2
PC-aa-C42-5
C18-1
C8
C10
C14-1
Lactose
C18-2
C5
Ascorbate
C3-OH
C0
Lysine
N-Acetylglut
2-Oxoglutara
Citrate
C4-1
PC-ae-C30-2
PC-ae-C44-6
myo-Inositol
m-Hydroxyphe
PC-aa-C32-2
Glycerate
Pantothenate
1-3-Dimethyl
d
CON
Ketosis
PC-ae-C30-2
C5-M-DC
PC-aa-C40-4
Symmetric-dime
PC-aa-C38-3
PC-ae-C44-6
C12
C18-2
lysoPC-a-C20-4
C7-DC
PC-aa-C38-6
C3-1
C14-1-OH
PC-ae-C42-1
PC-ae-C44-5
High
Low
1.4
1.5
1.6
1.7
VIP scores
e
class
CON
Ketosis
C5-M-DC
Symmetric-dimethyl
C7-DC
C14-1-OH
C10-2
C12
C18-2
C16
C3-1
N-Acetylglutamate
PC-aa-C42-1
PC-aa-C38-3
PC-aa-C44-5
PC-ae-C42-1
PC-ae-C36-6
lysoPC-a-C17-0
PC-ae-C44-6
lysoPC-a-C20-4
PC-ae-C30-2
PC-aa-C34-3
PC-aa-C40-4
PC-aa-C32-0
PC-aa-C42-2
PC-aa-C38-0
lysoPC-a-C16-1
f
Sensitivity (True positive rate)
1-Specificity (False positive rate)
Area under the curve (AUC) = 0.994
95% CI: 0.969-1

Figure 4-4.

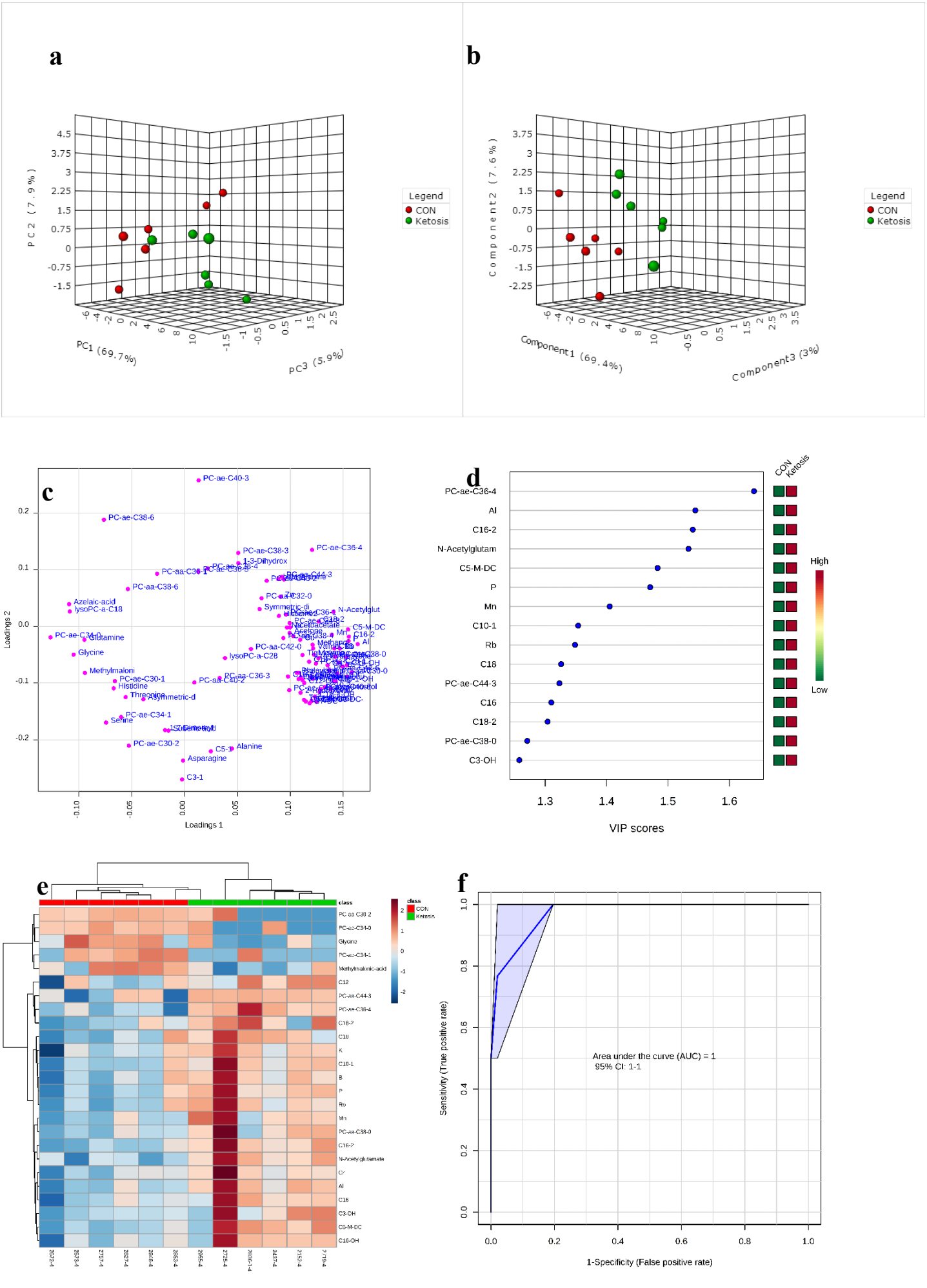
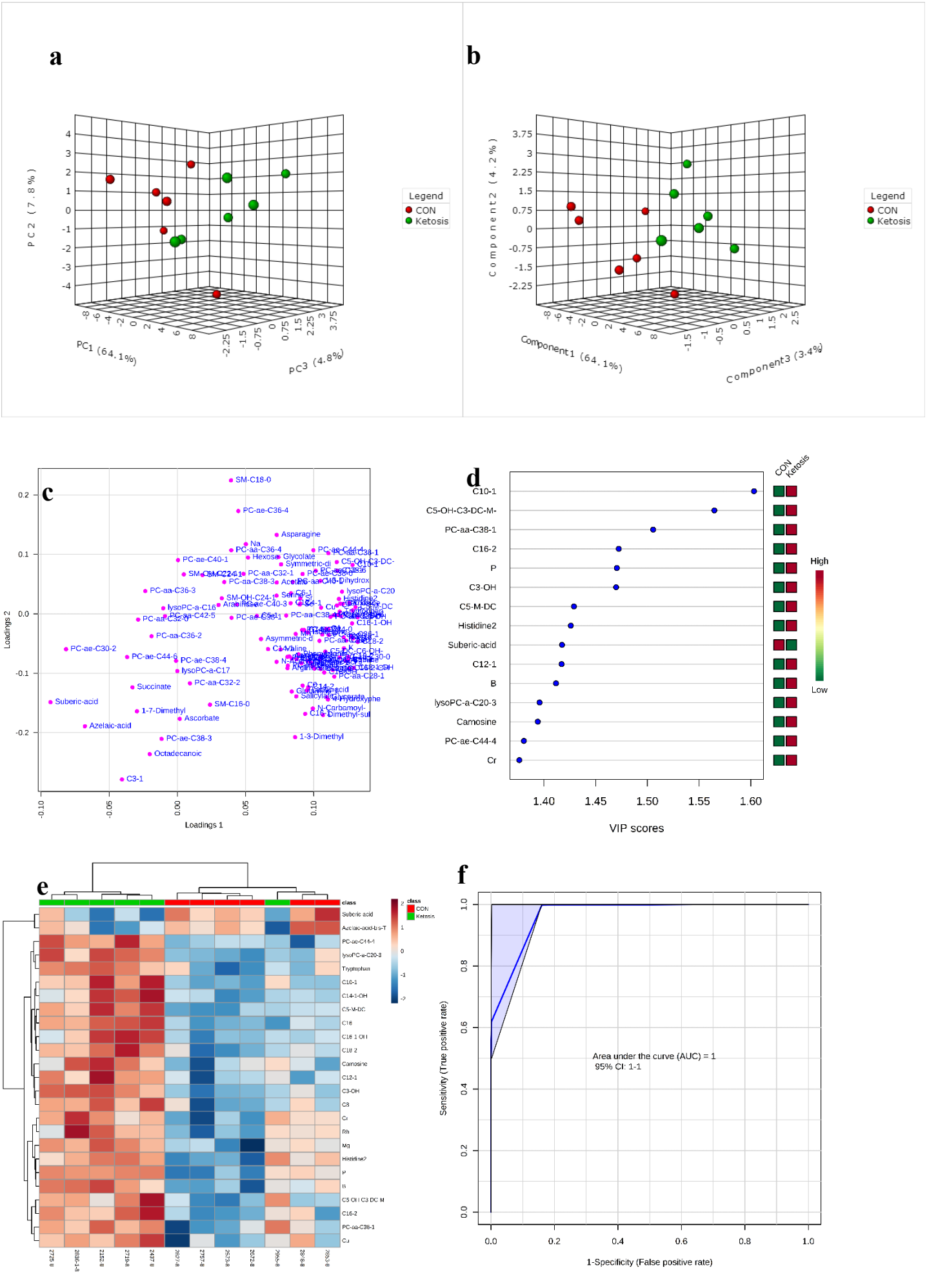

a
PC 2 (7.9%)
PC1 (69.7%)
PC3 (5.9%)
Legend
CON
Ketosis
b
Component2 (7.6%)
Component1 (69.4%)
Component3 (3%)
Legend
CON
Ketosis
c
Loadings 2
Loadings 1
PC-ae-C40-3
PC-ae-C38-6
PC-ae-C38-3
PC-ae-C36-4
1-3-Dihydrox
PC-aa-C36-1
PC-aa-C38-6
Azelaic-acid
lysoPC-a-C18
Symmetric-di
N-Acetylglut
C5-M-DC
PC-ae-C34-0
Glutamine
Glycine
PC-aa-C42-0
lysoPC-a-C28
Methylmaloni
PC-ae-C30-1
Histidine
PC-aa-C36-3
PC-aa-C40-2
Threonine
Asymmetric-d
PC-ae-C34-1
Serine
PC-ae-C30-2
C5-1
Alanine
Asparagine
C3-1
d
CON
Ketosis
High
Low
PC-ae-C36-4
Al
C16-2
N-Acetylglutam
C5-M-DC
P
Mn
C10-1
Rb
C18
PC-ae-C44-3
C16
C18-2
PC-ae-C38-0
C3-OH
VIP scores
e
class
CON
Ketosis
PC-ae-C30-2
PC-ae-C34-0
Glycine
PC-ae-C34-1
Methylmalonic-acid
C12
PC-ae-C44-3
PC-ae-C36-4
C16-2
C10
K
C10-1
B
P
Rb
Mn
PC-ae-C38-0
C18-2
N-Acetylglutamate
Cr
Al
C16
C3-OH
C5-M-DC
C16-OH
f
Sensitivity (True positive rate)
1-Specificity (False positive rate)
Area under the curve (AUC) = 1
95% CI: 1-1

Figure 4-5.


a
PC 2 (7.8%)
PC1 (64.1%)
PC3 (4.8%)
Legend
CON
Ketosis
b
Component 2 (4.2%)
Component 1 (64.1%)
Component 3 (3.4%)
Legend
CON
Ketosis
c
Loadings 2
Loadings 1
SM-C18-0
PC-ae-C36-4
Asparagine
Na
PC-aa-C36-4
PC-ae-C44-4
PC-aa-C38-1
PC-ae-C40-1
Hexose
Glycolate
Symmetric-di
C5-OH-C3-DC-
C16-1
PC-aa-C32-1
PC-aa-C38-3
Acetone
PC-aa-C40-1
Dihydrox
PC-aa-C36-3
lysoPC-a-C20
SM-OH-C24-1
Serine
C6-1
Histidine2
lysoPC-a-C16
Arabinose
C5-1
Cu
PC-aa-C42-5
PC-aa-C38-1
C16-1-OH
PC-aa-C36-2
Asymmetric-d
PC-aa-C28-2
PC-ae-C30-2
C4-Valine
PC-ae-C44-6
PC-ae-C38-4
lysoPC-a-C17
PC-aa-C28-1
PC-aa-C32-2
Succinate
Suberic-acid
SM-C16-0
Salicylic-Glycinate
1-7-Dimethyl
Ascorbate
N-Carbamoyl-
Azelaic-acid
PC-ae-C38-3
1-3-Dimethyl
Octadecanoic
C3-1
d
C10-1
C5-OH-C3-DC-M-
PC-aa-C38-1
C16-2
P
C3-OH
C5-M-DC
Histidine2
Suberic-acid
C12-1
B
lysoPC-a-C20-3
Carnosine
PC-ae-C44-4
Cr
CON
Ketosis
High
Low
VIP scores
e
class
Suberic acid
Azelaic-acid-bis-T
PC-ae-C44-4
lysoPC-a-C20-3
Tryptophan
C10-1
C14-1-OH
C5-M-DC
C16
C16 1 OH
C10 2
Carnosine
C12-1
C3-OH
C8
Cr
Rh
Mg
Histidine2
P
B
C5 OH C3 DC M
C16-2
PC-aa-C38-1
Cu
f
Sensitivity (True positive rate)
1-Specificity (False positive rate)
Area under the curve (AUC) = 1
95% CI: 1-1

Chapter 5 Conclusions

5.1 Alterations of blood and urine variables precede the occurrence of ketosis in dairy cows

In this thesis, ELISA-based immuno-assays as well as NMR and MS-based metabolomics were carried out to investigate selected innate immunity reactants and metabolite profiles of serum and urine along with their alterations before, during, and after the occurrence of ketosis in transition dairy cows. Two major goals of the current study were: 1) to identify newly predictive biomarker candidates for ketosis in the serum and urine of dairy cows that might be useful for screening cows during the dry off period for the potential development of postpartum ketosis; and 2) to elucidate metabolic alterations of serum and urinary profiles in pre-ketotic, ketotic, and post-ketotic cows, to better understand pathophysiologic mechanisms of the onset and progression of disease. Moreover, it was anticipated that this study would be able to elaborate on metabolic pathway perturbations during different stages of ketosis.

5.1.1 A potential role of inflammatory insults in the development of ketosis

In several other studies, we have reported that cows with periparturient diseases such as ketosis, metritis, subclinical mastitis, lameness, milk fever, and retained placenta, had elevated concentrations of pro-inflammatory cytokines and acute phase proteins (APP) during the dry off period starting at -8 wks and -4 wks prepartum, suggesting that activation of a non-specific innate immune response precedes all 6 diseases (Dervish et al., 2015, 2016a&b; Zhang et al., 2014, 2015, 2016). Results of

the present study showed that concentrations of two pro-inflammatory cytokines (i.e., IL-6 and TNF), one APP (i.e., SAA), BHBA, and lactate in the serum were greater in pre-ketotic cows and in ketotic cows compared with CON cows. Given that multiple periparturient diseases of dairy cows share many similarities with regards to enhanced levels of innate immunity variables in the serum during the dry-off period and early lactation, we believe that these series of metabolic or infectious diseases might experience inflammatory insults at early stages of disease. The source(s) of inflammatory insults are beyond the scope of this thesis but are to be addressed in the future.

Elevations of pro-inflammatory cytokines and APPs have been associated with disorders of a wide range of liver functions. In a previous gene network analysis study conducted by Loor et al. (2007), it was suggested that up-regulation of IL-6 in the liver might affect multiple networks related to fatty acid β-oxidation, lipoprotein metabolism, oxidative stress, and protein degradation. Correlations between serum levels of TNF and total lipids in cows with fatty liver as well as insulin resistance has been reported (Ametaj et al., 2005; Ohtsuka et al., 2001). Pro-inflammatory cytokines such as IL-6 and TNF could stimulate the breakdown of body fat stores by depression of feed intake, triggering of insulin resistance, and direct initiation of lipolysis (Kushibiki et al., 2003), all of which are associated with development of ketosis in dairy cows (Ingvartsen, 2006). Additionally, strong positive correlation between APPs (e.g., Hp and SAA) and indicators of lipomobilization (i.e., BHBA and NEFA) have been reported previously (Tóthová et al., 2014).

Another interesting finding described in chapter 2, is that cows with ketosis had greater concentrations of lactate in their serum not only at the wk of ketosis diagnosis but also at -8 wks and -4 wks prepartum. Beside its role as an important precursor for gluconeogenesis, lactate has been shown to have immune suppressor activity by lowering the production of pro-inflammatory cytokines (Goetze et al., 2011; Haas et al., 2015). Results of serum cytokines and APPs give insight into potential involvement of inflammatory insults during the dry-off period in development of ketosis. It is intriguing to note that elevated levels of several serum cytokines, APPs, and lactate were observed in other diseases, besides ketosis. On the basis of our observations, it is suggested that serum levels of pro-inflammatory cytokines (i.e., IL-1, IL-6, and TNF), APPs (i.e., Hp and SAA), and lactate during the dry-off period might be used as non-specific screening biomarkers for multiple periparturient diseases of transition dairy cows. In order to better understand the pathobiology of ketosis and to identify specific biomarkers for the disease, we used a metabolomics approach on both serum and urine samples, which were further described in chapters 3 and 4 of this thesis.

5.1.2 Identification of screening and diagnostic biomarkers in the serum of transition dairy cows

During the last decade, metabolomics science has emerged as a key tool in identification of novel biomarkers of ketosis in several published studies (Klein et al., 2011; Li et al., 2014; Sun et al., 2014; Xu et al., 2015b; Zhang et al., 2013). However, it should be pointed out that in all previous metabolomics studies the focus has been

on identifying metabolites in plasma or milk for diagnosing ketosis during the early lactation and not for screening or prediction of disease. Another characteristic of these studies is that all of them have used non-target metabolomics, which means that all of them have used qualitative but not quantitative analysis. Thus far, there has been no reports with regards to discovering screening or predictive biomarkers of ketosis in dairy cows. In studies described in chapter 3, we utilized DI/LC-MS/MS based metabolomics to identify biomarkers of ketosis by quantification of serum metabolites several weeks before the occurrence of disease. To the best of our knowledge, this research work is the first comprehensive report describing alterations of serum amino acids, acylcarnitines, lipids (e.g., glycerophospholipids and sphingolipids), biogenic amines, and hexose during the dry-off period in relation to future development of ketosis after parturition.

The primary significance of this serum metabolomics study is that alterations (mostly elevated in pre-ketotic and ketotic cows) of serum amino acids, lipids, acylcarnitines, biogenic amines, and hexose profiles preceded occurrence of ketosis in dairy cows. Oscillations in amino acid and lipid metabolites have been previously described in cows affected by multiple diseases by our group (Hailemariam et al., 2014a&b). It has been widely believed that elevated concentrations of amino acids in the blood of dairy cows during the week of ketosis occurrence are related to lower DMI in those cows. Lower feed intake and high milk production during disease establishes a negative energy balance (NEB). The latter stimulates proteolysis and the release of amino acids, which are used in the process of gluconeogenesis (especially

glucogenic amino acids) to produce glucose and balance the energy. Some released amino acids (i.e., ketogenic ones) contribute to production of ketone bodies and as a consequence lead to the development of ketosis (D'Mello, 2003).

Although the hypothesis of NEB deserves credit for the postpartum period it is not adequate for interpreting the increased levels of amino acids in the serum during the dry-off period because there was no difference in the DMI between the healthy cows and pre-ketotic ones. Elevated amino acids during the dry off period might be explained better with a new hypothesis related to the role of amino acids in immune response. This idea is supported by the fact that there is an enhanced utilization of amino acids during inflammatory responses as reviewed previously by Li et al. (2007) and McGaha et al. (2012). Mounting an efficient innate or acquired immune response requires interaction of multiple immunocytes and production of important proteins including immunoglobulins, cytokines, and APPs as well as a series of molecules like anthranilic acid, glutathione, histamine, hydrogen peroxide, nitric oxide, and superoxide (Calder, 2006; Li et al., 2007). The synthesis of the aforementioned proteins and polypeptides is highly dependent on availability of amino acids (Kim et al., 2007). Therefore, elevated levels of multiple amino acids in the serum of pre-ketotic cows in our study might be explained, as suggested in chapter 2, by the need for mounting an immune response, as reflected by the greater concentrations of pro-inflammatory cytokines and APP in those cows.

It has also been reported that acylcarnitines, glycerophospholipids [e.g., lysophosphatidylcholine (lysoPC) and phosphatidylcholine (PC)] as well as

sphingolipids [e.g., sphingomyelin (SM)] play important roles in immune functions, the process of inflammation, and inflammatory diseases (Kabarowski et al., 2002; Maceyka et al., 2014; Sampey et al., 2012; Stremmel et al., 2010). Enhanced levels of acylcarnitines [e.g., decanoyl-L-carnitine (C10)], lysoPC (e.g., lysoPC acyl C17:0), PC (e.g., PC acyl-alkyl C30:1), and SM (e.g., SM (OH) C24:1) in pre-ketotic cows at -8 wks and -4 wks prepartum might be related to an increased demand for activation of immune response the subclinical inflammation during the early stages of ketosis.

Biomarker analysis identified two sets of serum screening biomarker models and one serum diagnostic biomarker model with high sensitivity and specificity. More specifically, a 7-serum metabolite set [i.e., lysine (Lys), lysophosphatidylcholine acyl C17:0 (lysoPC a C17:0), lysophosphatidylcholine acyl C18:0 (lysoPC a C18:0), lysophosphatidylcholine acyl C16:0 (lysoPC a C16:0), isoleucine (Ile), kynurenine, and leucine (Leu)] of biomarker model and a 8-serum metabolite set [i.e., Lys, Ile, Leu, kynurenine, carnosine, arginine (Arg), lysoPC a C17:0, and phosphatidylcholine acyl-alkyl C44:4 (PC ae C44:4)] were identified at -8 wks and -4 wks, respectively, to screen cows for risk of developing ketosis. In addition, another 8-serum metabolite set [Lys, Leu, Ile, lysophosphatidylcholine acyl C28:0 (lysoPC a C28:0), sphingomyelin C20:2 (SM C20:2), kynurenine, hexadecanoyl-L-carnitine (C16), and acetyl-L-carnitine (C2)] for diagnosing ketosis was identified. All three biomarker models were evaluated by receiver operating characteristic (ROC) curve (permutation test attained empirical $P = 0.001$ under 1,000 permutations), showing high AUC (area under the ROC curve) values.

5.1.3 Identification of new diagnostic and predictive metabolites in the urine of pre-ketotic and ketotic cows

In chapter 4, a combination of four metabolomics analytical instruments including DI/LC-MS/MS, ^{1}H-NMR spectroscopy, GC-MS, and ICP-MS were used to identify and quantify metabolites present in the urine of dairy cows. Urine serves as the optimal sample type for metabolomics study given its easy accessibility in large volumes, non-invasiveness, and low content of proteins or lipids that interfere in the analytical process. Our comprehensive urine metabolomics study revealed major fluctuations of metabolites from various analyte groups including acylcarnitines, amino acids, lysoPC, PC, SM, hydroxyl-sphingomyelin [SM (OH)], biogenic amines, hexose, ketones, saccharides, organic acids, alcohols, and other molecules in pre-ketotic, ketotic, and post-ketotic cows.

As described in chapter 4, traditional urinary biomarkers [i.e., ketone bodies such as 3-hydroxybutyric acid (3HBA or BHBA) and acetoacetic acid (AcAc)] for diagnosis of ketosis were confirmed by NMR profiling. However, when it comes to their performance in predicting the disease, they were found to be relatively insensitive biomarkers. In the present study, we identified new urinary biomarkers for screening and diagnosis of ketosis in dairy cows. More specifically, a 7-metabolite biomarker set [i.e., methylglutaryl-L-carnitine (C5-M-DC), octadecadienyl-L-carnitine (C18:2), hydroxypropionyl-L-carnitine (C3-OH), C16, aluminum (Al), hydroxytetradecenoyl-L-carnitine (C14:1-OH), and hexadecadienyl-L-carnitine (C16:2)] at -8 wks, and a 9-metabolite biomarker set [i.e.,

C3-OH, methanol, C16, phosphatidylcholine acyl-alkyl C30:2 (PC ae C30:2), C18:2, Al, N-acetylglutamine, C5-M-DC, and tetradecenoyl-L-carnitine (C14:1)] at -4 wks prepartum was identified as the best combination of urine predictive biomarkers for ketosis. Additionally, five metabolites including PC ae C30:2, C5-M-DC, phosphatidylcholine diacyl C40:4 (PC aa C40:4), symmetric dimethylarginine (SDMA), and phosphatidylcholine diacyl C38:3 (PC aa C38:3) were selected as a new set of diagnostic urine biomarkers for ketosis.

Another interesting finding of this urine metabolic analysis was that several urinary metals were found to fluctuate significantly in pre-ketotic and post-ketotic cows. For example, urine levels of Ca, P, Na, K, Cu, Co, Mg, Mn, Zn, and Cr were greater in pre-ketotic and post-ketotic cows versus helthy CON cows. Since urine samples are collected in a non-invasive manner, the new identified screening and diagnostic urinary biomarkers show remarkable advantages compared to the current 'golden standard' of blood biomarker (e.g. serum BHBA) or urine ketostix test (measurement of acetoacetate).

5.1.4 Identification of metabolic pathways involved in pre-ketotic and ketotic cows

In chapter 3, metabolic pathway analyses and metabolite set enrichment analyses (MSEA) were conducted to confirm several key metabolites and associated metabolic pathway perturbations that might be involved in the pathobiology of ketosis. Results of this experiment showed that multiple metabolic pathways were involved in pre-ketotic and ketotic cows including the lysine degradation pathway, tryptophan

metabolism pathway, valine-leucine-isoleucine degradation pathway, bile acid biosynthesis pathway, histidine and glutathione metabolism pathways, protein biosynthesis pathway, phospholipid biosynthesis pathway, biotin metabolism, and aspartate metabolism. However, it is not possible to measure all metabolites or intermediates in a specific pathways using just one platform or metabolomics tool. Therefore, targeted metabolomics studies of the aforementioned metabolic pathways are warranted to fully clarify alterations and the reason for involvement of those metabolic pathways during the early stages of ketosis.

Besides the metabolic pathway analysis we conducted, multiple databases like 'The Small Molecule Pathway Database' (SMPDB) (Frolkis et al., 2010; Jewison et al., 2014), 'Bovine Metabolome Database' (BMDB) (http://www.cowmetdb.ca/cgi-bin/browse.cgi), 'The Urine Metabolome Database' (UMD) (Bouatra et al., 2013), and 'Kyoto Encyclopedia of Genes and Genomes' (KEGG) (Kanehisa and Goto, 2000) were referenced in trying to better understand altered metabolic pathways during different stages of ketosis. Significant metabolic pathways involved in the onset and progression of ketosis are mainly centralized in fatty acid metabolism (e.g., fatty acid transport, lipid catabolism), glycerophospholipid metabolism (e.g., free fatty acid metabolism and membrane component), sphingolipid metabolism (e.g., membrane component and cell signaling), amino acid metabolism (e.g., arginine and proline metabolism, urea cycle, protein biosynthesis, beta-alanine metabolism, valine-leucine-isoleucine degradation, and glutathione metabolism), ketone body metabolism, glycolysis/gluconeogenesis,

tricarboxylic acid (TCA) cycle, pentose phosphate pathway, and other pathways (e.g., methane metabolism and inositol metabolism).

5.2 Future implications

Based on the significant findings of this study and the state-of-the-art NMR and MS based metabolomics, we believe that metabolomics has shown an enormous potential for identification of screening or diagnostic biomarkers for ketosis in dairy cows. The large number of metabolites identified to change in the serum and urine metabolomes in pre-ketotic, ketotic, and post-ketotic cows suggest that ketosis is a complex disease. Results showed that hyperketonemia, which is the only actual phenotype to characterize ketosis, fails to indicate subclinical changes occurring early in the disease process and cannot fully explain the complex pathobiology of the disease and therefore new preventive or more efficient treatment strategies need to be developed.

The current reductionist approach, as related to the diagnosis and treatment of ketosis by measurement of ketone bodies in various body fluids, needs to be substituted by a broader, more comprehensive systems approach. It should be noted that the cut-off values of serum BHBA for defining ketosis, especially subclinical ketosis, appears to be somewhat arbitrary. Both blood and urine metabolomes are fluctuating over time, and longitudinal temporal evaluation of ketosis during different time points (e.g., dry-off and early lactation) needs to be considered. We like to emphasize that because of the relatively modest number of ketosis cases in our study the findings of this study should be considered preliminary. Therefore, more research

is warranted to validate the biomarkers identified in a larger cohort of transition dairy cows.

Some of the future implications in this area of research include the following. First, validation experiments should be performed in at least four levels including a lab repeatability study, a lab replication study, inter-lab repeatability study, and inter-lab replication study as described by Xia et al. (2013). Second, targeted metabolomics studies of specific pathways covering as many metabolites and intermediates as possible are warranted to validate ketosis-associated metabolic alterations and a better understanding of the pathobiology of the disease. Third, isotopic labeling techniques could be used to track molecular pathways and biological processes of targeted metabolites, which would help to determine the source and end use of the molecules of interest. Fourth, although metabolomics platforms show advantages in biomarker studies, in order to understand the etiology and pathogenesis of ketosis, a system veterinary approach combining different 'omics' technologies (i.e., genomics, transcriptomics, proteomics, and metabolomics) is needed for integration of different tiers of omics data. Thus, the same samples collected from an individual cow would require to be analyzed by different "omics" platforms simultaneously for better interpretation of the findings. Fifth, longitudinal studies with repeated samplings from the same cows at different stages (i.e., before, during, and after diagnosis of ketosis) should be used as a standard experimental design in the future, for biomarker studies and interpretation of disease. Sixth, new preventive and therapeutic strategies combining multi-target interventions need to be developed.

5.3 Conclusions

In summary, using targeted metabolomics and multiple high-throughput instruments including DI/LC-MS/MS, ^{1}H-NMR, GC-MS, and ICP-MS multiple sets of screening and diagnostic biomarkers of ketosis, in the serum and urine, were identified. Moreover, elevated pro-inflammatory cytokines and APPs and evidenced presence of an inflammatory state during the dry off period and diagnosis of disease were observed. It is concluded that metabolomics provides a powerful approach to discover new screening and diagnostic biomarkers of ketosis in transition dairy cows by analyzing global changes in the serum and urine metabolite fingerprints. Once these identified biomarkers are validated as highly specific and sensitive markers of ketosis, rapid, reliable, and inexpensive cow-side tests can be developed. By taking one drop of blood or urine, dairy producers in the near future should be able to identify cows that might be affected by ketosis 4-10 wks before clinical appearance of the disease and take preventive measures to lower the incidence of the disease and related economic and welfare issues. ‘An ounce of prevention is worth a pound of cure’.

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