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Blood plasma biomarkers correlating with hepatic lipidosis in dairy cows

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Summary

Fatty liver disease is a major metabolic disorder of dairy cows in their early lactation. The syndrome is associated with impaired health, reduced immunity which leads to infections, and decreased reproductive performance. Fatty liver disease is recognized as a major herd problem and represents a serious animal welfare concern. The disorder is associated with increased veterinary costs, longer calving intervals and decreased lifetime and, hence, causes huge economic losses worldwide.

The pathogenesis of this disease is closely associated with the transition period between the end of gestation and the beginning of lactation, during which high-performance dairy cows experience a negative energy balance. Although the organism attempts to maintain milk production, the voluntary feed intake declines by 30–40% around calving. To compensate for this lack of energy, fat is mobilized from the adipose tissue in the form of non-esterified fatty acids and transported to different organs.

The liver is able to reconvert non-esterified fatty acids to triacylglycerides and subsequently store them in hepatocytes. When the incorporated non-esterified fatty acids exceed their amount secreted from the liver as triacylglycerides, they accumulate in the liver and fatty liver disease develops. In cattle, the ability to release hepatic triglycerides into the blood as very low-density lipoproteins is extremely low compared to non-ruminant animals. Therefore, high-producing cows are particularly susceptible to the development of this specific illness. Indeed, up to 40% of dairy cows have a tendency to moderate or even severe fatty liver disease.

Serum biochemical abnormalities depend on the severity of the disease and are not reliable for the diagnosis of fatty liver disease. Increased blood levels of liver enzymes reflect hepatic tissue damage, yet fail to display a specificity for fatty liver disease. Therefore, a major challenge for veterinary clinicians is that the fatty liver disease can currently only be diagnosed by liver biopsy.

This PhD thesis addresses the pathogenic background of the fatty liver disease and monitors changes in pathways accompanying this disease. The altered plasma lipidome during the transition period is expected to precede the manifestation of fatty liver disease. To identify potential blood biomarkers as a new diagnostic alternative, plasma of healthy and diseased cows were subjected to untargeted and targeted lipidomic analysis by mass spectrometry. This thesis reveals reduced plasma concentrations of phosphatidylcholines in cows with fatty liver disease. On the basis of these altered lipidomic patterns, it is possible to distinguish between dairy cows with no fatty liver disease and those with different stages of the disorder. Furthermore, plasma of cows during the transition period was analyzed to show how the lipidome develops from the condition two weeks before calving to the stage four weeks after calving. This work shows that triacylglyceride con-

centrations drop after calving whereas phosphatidylcholine concentrations rise continuously after calving. This concomitant shift in the bloodtriacylglycerides and phosphatidylcholines reflects the physiologic adaptation of lipoproteins during the transition period. These results constitute the basis for the development of a new minimally invasive method for the diagnosis of fatty liver disease in dairy cows.

Zusammenfassung

Das Fettlebersyndrom ist eine bedeutende Stoffwechselstörung bei Milchkühen in der Frühlaktation. Das Syndrom hängt oft mit weiteren Erkrankungen zusammen, die zu Infektionen und verminderter Fortpflanzungsfähigkeit führt. Die Krankheit verursacht erhöhte Tierarztkosten, längere Zwischenkalbezeiten, verringerte Lebensdauer und führt zu grossen wirtschaftlichen Verlusten weltweit.

Die Pathogenese ist eng mit der Übergangszeit am Ende der Schwangerschaft bzw. Beginn der Laktation verbunden. Die Futteraufnahme sinkt während dieser Zeit um 30–40% und die Kuh führt dadurch weniger Energie zu als sie verbraucht. Trotz dieser negativen Energiebilanz ist der Organismus fähig durch Mobilisation der Fettreserven Energie für die einsetzende Laktation bereitzustellen. Die Fettreserven werden hierbei als nicht veresterte Fettsäuren im Körper verteilt. Die Leber hat die Eigenschaft, die nicht veresterten Fettsäuren wieder zu Triglyceriden zu verestern und gegebenenfalls in den Hepatocyten zu speichern. Übersteigt die Einlagerung von Triglyzeriden den Abbau und Abtransport derselben, findet eine Verfettung der Leber statt. Für den Transport von Triglyzeriden aus der Leber heraus ins Blut sind Lipoproteine mit sehr geringer Dichte verantwortlich. Diese Fähigkeit besitzen Hochleistungskühe nur in geringen Massen und sind dadurch anfällig für das Fettlebersyndrom. Bis 40% der Kühe in der peripartalen Phase leiden an einer moderaten bis schweren Verfettung der Leber.

Biochemische Veränderungen im Serum sind abhängig vom Schweregrad der Erkrankung und diagnostisch unzuverlässig. Erhöhte Konzentrationen von Leberenzymen weisen zwar auf einen Leberschaden hin, sind jedoch nicht spezifisch für das Fettlebersyndrom. Die definitive Diagnose kann deshalb nur invasiv mit einer Leberbiopsie gestellt werden.

Diese Dissertation beschäftigt sich mit den Veränderungen der Lipidzusammensetzung und deren Mechanismen beim Fettlebersyndrom. Um potentielle Biomarker für eine diagnostische Alternative zu identifizieren, wurde Plasma von gesunden und kranken Kühen mittels ungezielter und gezielter Lipidomics-Analyse massenspektrometrisch untersucht. Es wurde nachgewiesen, dass bei Kühen mit Fettlebersyndrom die Plasmakonzentrationen von Phosphatidylcholinen reduziert sind. Damit ist es nun möglich zwischen gesunden Kühen und Kühen mit unterschiedlichen Schweregraden von Fettlebersyndrom zu unterscheiden. Zusätzlich wurden Kühe während der peripartalen Periode geprüft um deren Lipidzusammensetzung im Blut während der Zeit von zwei Wochen vor bis vier Wochen nach dem Kalben zu untersuchen. Die Analyse zeigt einen Rückgang der Triglyzeriden am Tag des Kalbens während die Phosphatidylcholine nach der Kalbung kontinuierlich steigen. Die Verschiebungen in den Konzentrationen von Triglyzeriden und Phosphatidylcholinen sind Konsequenzen der physiologischen Anpassung der Lipoproteine an die Transitionsperiode. Diese Ergebnisse bilden

die Grundlagen für eine neue Analysemethode zur minimalinvasiven Diagnostik des Fettlebersyndroms bei Milchkühen.

Abbreviations

γ-GT	γ-glutamyl transferase
AA	amino acid
Apo	apolipoprotein
ApoB-100	apolipoprotein B-100
AST	aspartate transaminase
BCS	body condition score
BHB	*beta*-hydroxybutyric acid
CDP	catidine diphosphate
CID	collision-induced dissociation
CNS	central nervous system
CI	cardiolipin
DMI	dry matter intake
ESI	electrospray ionization
FA	fatty acids
FLD	fatty liver disease
GC	gas chromatography
GLDH	glutamate dehydrogenase
HDL	high density lipoproteins
HPLC	high-performance liquid chromatography
IGF-1	insulin-like growth factor-1
IS	internal standard
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoproteins
LPC	lysophosphatidylcholine
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl *tert*-butyl ether
MW	molecular weight
$\boldsymbol{m/z}$	mass-to-charge ratio
NEFA	non-esterified fatty acid
NEG	negative energy balance
NMR	nuclear magnetic resonance
PC	phosphocholine
PE	phosphoethanolamine
PEMT	phosphatidylethanolamine methyltransferase
PG	phosphoglycerol
PI	phosphoinositol
PS	phosphoserine
SAMe	*S*-adenosyl-L-methionine
SDH	sorbitol dehydrogenase

SM	sphingomyelins
SPE	solid-phase extraction
TAG	triacylglyceride
TOF	time of flight mass spectrometer
TLC	thin-layer chromatography
UPLC	ultra-performance liquid chromatography
VFAs	volatile fatty acids
VLDL	very low-density lipoproteins

1 Introduction

1.1 Energy metabolism during transition period

The transition period of dairy cows is referred to as the time frame from three weeks before to three weeks after parturition [Grummer, 1995, Goff and Horst, 1997], involving the passage from pregnancy to postpartal and lactating states. Diary cows undergo several physiological, metabolic, and endocrine changes [Drackley, 1999]. The success of the transition period determines the cost efficiency of the following lactation phase. Energy consumption due to body functions and milk production in lactating cows are about three times higher than before parturition [Drackley et al., 2001]. The intake of nutrients during the early stage of lactation is insufficient because of poor feed consumption, reaching its peak only a few weeks after the climax of the milking yield [Ingvartsen and Andersen, 2000, Grummer et al., 2004, Stengärde et al., 2008]. Therefore, a negative energy balance (NEB) occurs, which is aggravated by a nutritive prioritization of the mammary gland [Leroy et al., 2008, Contreras et al., 2010]. To compensate for this NEB during the early transition period, especially during early lactation, energy reserves from different tissues; particularly fatty tissue, are mobilized [Ingvartsen and Andersen, 2000]. Milk fever, abomasum dislocation, mastitis, ketosis, and metritis have an increased incidence during the time of calving [Goff and Horst, 1997]. Also, 30–50% of dairy cows suffer from metabolic or infectious diseases during the transition period. Nearly all dairy cows undergo insulin resistance, hypocalcemia, and bacterial infection of the uterus [Leblanc, 2010]. All these factors and, additional changes of the circulating concentration of progesterone, estrogen and cortisol cause a decline of the immune system. Different physiological functions have to be preserved during an impaired transition phase to prevent diseases. This includes the adaption of the rumen to a high-energy lactation diet in order to keep calcium blood level and support the immune system [Goff and Horst, 1997].

A reduction of the feed intake by 20–40% during the last three weeks of gestation induces the cow to adapt to the physiological changes of the transition phase. At the beginning of the lactation period, the cow undergoes an even stronger NEB. Ketone bodies influence the gluconeogenesis, thus leading to hypoglycemia [Goff and Horst, 1997]. The body condition score (BCS), a numeric score to estimate body energy reserves, is a possibility to monitor the energy balance during this transition phase. The BCS reflects nutritive and metabolic alterations of the previous weeks. The ideal BCS around the time of gestation is between 3 and 3.5 (on a scale from 1 to 5). Cows losing more than one BCS point in the period of early lactation have a higher risk of disease [Leblanc, 2010, Roche et al., 2013].

The metabolism of ruminants is different from that of monogastric mam-

mals. Ruminants are able to digest cellulose and metabolize non-protein nitrogen by microbial fermentation, whereas the absorption of glucose in the intestinal tract is limited. Although the ruminal microorganisms split cellulose into glucose, all carbohydrates are further fermented to the volatile fatty acids (VFAs) acetate, butyrate, and propionate [Adewuyi et al., 2005]. In ruminants, acetate is the primary substrate for fatty acid synthesis. For the hepatic gluconeogenesis, only propionate is available. The main part of energy consumption is therefore covered by acetate and butyrate and the metabolism of ruminants is designed to use VFAs instead of carbohydrates. This has an impact on the presence of enzymes in liver, adipose tissue, and udder tissues. Since little glucose reaches the liver via the portal vein, it is likely that glycogen synthesis in the ruminant liver plays only a subordinate role. These and other features of the carbohydrate metabolism of ruminants are important for understanding the emergence of metabolic diseases [Geelen and Wensing, 2006]. The difference between the energy absorbed through the gut and the overall glucose consumption of the cow must be compensated through increased gluconeogenesis from intestinally absorbed amino acids (AAs) and endogenous substrates such as AAs, lactate, and glycerol. In ruminants, propionate (from the rumen and to a lesser extent from the large intestinal fermentation) is the most important precursor for gluconeogenesis. All AAs, except leucine and lysine, can contribute to gluconeogenesis. Mainly in the liver, AAs present in excess are catabolized, and nitrogen is converted to urea and finally excreted. Insulin, glucagon, somatotropin and cortisol act as a signal in the endocrine regulation of gluconeogenesis [Drackley et al., 2001]. Glucagon stimulates gluconeogenesis while inhibiting the release of insulin. Glucocorticoids also promote gluconeogenetic processes [Brockman, 1990, Donkin and Armentano, 1995].

The liver, adipose tissue and (during lactation) the mammary gland, are the main locations of fatty acid metabolism. All these tissues can synthesize fatty acids (FAs) and esterify them to triacylglycerides (TAGs). FAs are the main source of energy for most of the body tissues in the context of a NEB. FAs are mostly stored in adipose tissue as TAGs. The mobilization of stored fat is performed via degradation of TAGs by ester hydrolysis, thereby producing non-esterified fatty acids (NEFAs) and glycerol. An increase of NEFAs in the blood, e.g. as a consequence of obesity or early lactation, leads to esterification of NEFAs to TAGs, which can accumulate in other tissues such as the liver or muscle [Vernon, 2005].

1.2 Fatty liver disease

Fatty liver disease (FLD) occurs mainly in high-performance cows in the peripartal transition phase as a consequence of a NEB. This condition is associated with other metabolic and infectious diseases and with reduced fertility [Bobe et al., 2004]. The degree of FLD can be divided into different

stages by biochemical analysis of TAGs in the liver or by measuring the total fat content of the liver. Various authors differentiate between normal, mild, moderate and heavy grade, whereas other authors distinguish five groups, from healthy to highly affected, depending on histological criteria [Gaal et al., 1983, Johannsen et al., 1993, Bobe et al., 2004, Geelen and Wensing, 2006].

FLD occurs when the liver takes up more lipids than it can oxidize or secrete, especially in the first four weeks post-partum. One important reason is the insufficient food intake to meet the rising energy needs shortly before the calving and the onset of milk production. FLD can be defined on the basis of the percentage of TAGs in the liver, and is associated with reduced health status as well as poor productivity and reproductive performance [Goff and Horst, 1997, Bobe et al., 2004, Grummer, 1993, Herdt, 2000].

Bobe et al. [2004] have summarized different incidence studies [Reid, 1980, Gerloff et al., 1986, Jorritsma et al., 2004]. In the first month after calving, 5–10% of all cows develop a high-grade and 30–40% a moderate-grade of FLD, thus up to 50% of dairy cows have an increased risk of suffering from FLD. This incidence has remained constant over the last decades in spite of the scientific work in this area [Mulligan and Doherty, 2008]. Cows with fatty liver syndrome respond poorly to therapy, and mortality rates of up to 50% are observed [Adewuyi et al., 2005]. Most metabolic and infectious diseases of dairy cows occur during this transitional phase from late pregnancy to lactation.

1.2.1 Lipid metabolism

During the transition period, dairy cows typically undergo a condition of NEB. The feed intake during this phase is insufficient to meet the increased demand for glucose, AAs and FAs, considering the ongoing pregnancy and onset of milk production. Therefore, an abrupt shift from anabolic to catabolic metabolism takes place [Hammon et al., 2009]. The liver is the main organ for this adaptation to NEB. The energy suppliers in the body are carbohydrates, fats and AAs. Uptake of carbohydrates via the gastrointestinal tract is limited in ruminants. Circulating glucose in the blood comes mainly from gluconeogenesis in the liver [Rukkwamsuk et al., 1999b, Herdt, 2000]. During periods of NEB, FAs are the main source of energy for most tissues. These FAs are stored in the adipose tissue as TAGs [Vernon, 2005]. The mobilization of fat reserves occurs by means of TAGs in adipose tissue, whereby NEFAs and glycerol are produced. Adaptation to a NEB includes the suppression of lipogenesis, increase of lipolysis in adipose tissue, gluconeogenesis in the liver, and decreased use of glucose in peripheral tissue [Drackley et al., 2001]. Even before calving, free FAs are mobilized from adipose tissue [Gerloff et al., 1986]. The NEFAs released through lipolysis are transported in the blood by binding to albumin and are taken up by the

liver and other tissues. The hepatic uptake is regulated by the NEFA concentration in the blood and the blood flow into the liver [Emery et al., 1992]. The fate of NEFAs in the liver is either β-oxidation to CO_2 and ATP, ketogenesis or esterification to TAGs [Gruffat et al., 1996]. TAGs are either stored in the liver or secreted via very low-density lipoproteins (VLDLs) [Herdt, 2000, Van den Top et al., 2005]. The degradation of NEFAs in the liver in the metabolic pathways of either β-oxidation or TAG synthesis is a key factor in the adjustment of the cow to a NEB. The increased β-oxidation of NEFAs and other substrates provides ATP for the gluconeogenesis and is an important mechanism to remove excess FAs from the liver [Emery et al., 1992, Bell, 1995, Herdt, 2000, Grum et al., 2002]. Secretion of TAGs by VLDLs is lower in ruminants than in other species [Pullen et al., 1990]. VLDLs are synthesized in the liver and are the main carriers of TAG [Katoh and Nakagawa-Ueta, 2001]. The major protein of the VLDLs is apolipoprotein B-100 (ApoB-100) [Gruffat et al., 1996].

Since synthesis and export of VLDLs in hepatocytes are energy- and resource-dependent, any disruption in the supply of the building blocks or of ATP may lead to an inhibition of lipoprotein synthesis or secretion. If the uptake of NEFAs in the liver continues, TAGs accumulate in the cytoplasm. Under normal physiological conditions, the liver begins to accumulate TAGs a few weeks ante partum and reaches a peak around one week post-partum with a fat content of up to 20% (wet weight basis). Post-partum, the TAG content decreases again to less than 5%. The concentration of VLDLs and thus also the TAGs in the blood post-partum are reduced in a period of NEB [Van den Top et al., 1996]. After a fasting or peripartum transition period, the concentration of TAGs in VLDLs increases in the hepatic vein. This indicates that the hepatic secretion of TAGs is increased in order to reduce its content in the liver [Oikawa et al., 2010].

1.2.2 Pathogenesis

When the aforementioned physiological adaptions are insufficient, the organism responds with FLD. This happens when the rate of TAG synthesis in the liver exceeds TAG hydrolysis, NEFA oxidation and the secretion of TAG via VLDL. The result is an accumulation of TAGs in the liver by which the function of the liver cells is affected by the infiltration of fat [Grummer, 1993, Bell, 1995, Vernon, 2005]. This is reflected by the negative correlation of the capacity of the liver to perform β-oxidation with the NEFA concentration in plasma and milk production. However, whether this is the result or the cause of the increased concentration of TAGs in the liver, is not known [Grum et al., 2002]. Furthermore, there is evidence that in cows with increased hepatic fat content, the capacity for gluconeogenesis in the liver and VLDL secretion are reduced [Grummer, 1993, Rukkwamsuk et al., 1999b, Kuhla et al., 2009]. These factors lead to a reduction of the blood

glucose concentration, which in turn exacerbates the NEB and enhances NEFA mobilization [Herdt, 2000].

Increased uptake of NEFAs into the liver results in decreased feed intake [Emery et al., 1992]. Simultaneously, the urea synthesis is reduced in cows suffering from FLD, leading to an increased ammonia blood concentration [Strang et al., 1998, Zhu et al., 2000]. This increased ammonia concentration inhibits gluconeogenesis from propionate and also has an adverse effect on reproduction, immunity and the central nervous system [Drackley, 1999, Overton and Waldron, 2004]. A high NEFA concentration in blood is accompanied by an impaired immune system, which makes the cows susceptible to infection [Rukkwamsuk et al., 1999a]. Impairment of the immune system includes a reduced number of leukocytes [Reid, 1980] and a decreased ability to respond to an inflammatory process [Wensing et al., 1997, Bobe et al., 2004]. Other diseases also lead to a reduction of feed intake [Rukkwamsuk et al., 1999a]. Cows with FLD have reduced fertility [Bobe et al., 2004], because a NEB and thus an increased TAG content in the liver are associated with a prolonged interval from birth to the first ovulation and decreased conception rate [Rukkwamsuk et al., 1999a]. NEFAs in the liver reduce the appetite and enhance TAG synthesis. The ability for β-oxidation and TAG secretion are reduced, more TAGs are stored in the liver and gluconeogenesis and urea synthesis are reduced. Due to the increased ammonia concentration, gluconeogenesis is even more inhibited. The low gluconeogenesis rate and decreased appetite in turn lead to an even deeper NEB.

1.2.3 Diagnosis

The clinical symptoms of FLD are usually unspecific. They almost always include weakness and anorexia. Extended lying periods (downer cow syndrome), decreased rumen activity, little feces and nervous symptoms may occur [Gerloff and Herdt, 1984]. Often, these cows suffer from other peripartal diseases as well and show little to no response to treatment. Without proper treatment, the animals become weaker, suffer from downer cow syndrome and, ultimately, die [Herdt, 1988]. In cows with moderate FLD, clinical signs are less pronounced and if they resume eating, they usually recover within a few days [Radostits et al., 2000]. Subclinical FLD is a problem in many stocks and is associated with reduced health status, reduced fertility, reduced milk production and thus economic losses [Veenhuizen et al., 1991, Herdt, 1988, Bobe et al., 2004].

For a clear diagnosis of FLD, a biopsy from the liver must be taken to determine the fat content. The estimation of the fat content may be made in a histological or a biochemical manner [Gaal et al., 1983]. Histologically, the fat content is determined by stereological methods, the proportion of fat droplets is measured against the volume of hepatocytes [Reid, 1980, Gaal

et al., 1983, Kalaitzakis et al., 2007]. In addition to the TAG content, other relevant values in connection with FLD may be determined by different methods in a liver sample (Table 1). However, taking a liver biopsy is an invasive procedure causing a temporary discomfort to the cow, and represents a risk for infections and bleedings, which can be lethal when a large vessel is damaged. Therefore, liver biopsies are considered impracticable for a diagnosis directly on the farm [Bobe et al., 2008].

Several authors describe biochemical changes in the serum of cows with FLD. However, these biochemical parameters depend on the severity of the disease and are diagnostically unreliable [Acorda et al., 1995]. Increased activities of the liver enzymes aspartate transaminase (AST), glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH) and γ-glutamyl transferase (γ-GT)), as well as increased concentrations of bile acids in serum correlate with an advanced liver damage [Cebra et al., 1997]. AST increases in proportion to the liver TAG content; however, it is not liver-specific, and is also increased in cases of muscle damage. In addition, AST is increased in almost all cows post-partum [Kalaitzakis et al., 2007]. Ammonia and albumin can be used as an indicator of liver dysfunction [Bernabucci et al., 2004]. Hypoglycemia is an indicator of NEB [Adewuyi et al., 2005]. The glucose concentration in serum decreases with increasing TAG content in the liver, but only in cases of serious FLD it falls below the reference range [Cebra et al., 1997, Kalaitzakis et al., 2006]. The serum concentrations of lecithin-cholesterol acyltransferase (LCAT), Apo-B100 and Apo-A1 are reduced in cows with FLD. The same changes were, however, described in other diseases associated with FLD [Marcos et al., 1990, Nakagawa et al., 1997, Katoh and Nakagawa-Ueta, 2001]. LCAT, Apo-B100 and Apo-A1 therefore represent useful markers for early diagnosis of liver diseases in general but are not specific [Katoh, 2002].

Overall, the parameters listed in Table 1 may indicate a serious liver damage and/or hepatic impairment, inflammation or a NEB. A non-invasive, but often inaccurate detection of liver diseases is the use of ultrasound [Braun, 2009]. Digital analysis of the ultrasonography of the liver can potentially be used to determine the severity of TAG infiltration and the TAG content of the liver [Bobe et al., 2008]. Ultrasound-based measurements of liver size and shape can be used to assess the severity of FLD. However, this method is only of limited use for diagnosis, since there are large individual differences between cows [Haudum et al., 2011].

Table 1: Hepatic parameter changes in cows suffering from FLD

Parameter	**Changes**	**Referenzes**
TAG	↑	[Kalaitzakis et al., 2007]
Total lipids	↑	[Kalaitzakis et al., 2007]
		[Gruffat et al., 1997]
Glycogen	↓	[Veenhuizen et al., 1991]
Phospholipids	↓	[Brumby et al., 1975]
		[Geelen and Wensing, 2006]
Cholesterol	↓	[Brumby et al., 1975]
Enzymes of the gluconeogenesis	↓	[Kuhla et al., 2009]
Enzymes of the β-oxidation	↓	[Kuhla et al., 2009]
Enzymes of the glycolysis	↓	[Kuhla et al., 2009]
Enzymes of the TAG-synthesis	↑	[Geelen and Wensing, 2006]

↑ Respective concentration or activity is elevated in cows with FLD
↓ Respective concentration or activity is reduced in cows with FLD

1.3 Lipidomics

Lipids play an important and essential role as building blocks for membranes and the compartmentalization of the cell, for signaling events as well as for energy storage and metabolism [Hannun and Obeid, 2008, Phillips et al., 2009]. The finding that lipids are linked to manifold disorders such as atherosclerosis, diabetes, Alzheimer's disease and cancer, has promoted the study of lipids during the last decade [Wenk, 2005, Shevchenko and Simons, 2010, Di Paolo and Kim, 2011]. Typically, lipids have been analyzed in a targeted fashion. Since the 1960s, the '-omics' revolution has been used as a tool for the molecular profiling of biological systems. During the last decade, lipid research has gained prominence with the development of lipidomics [Seppänen-Laakso and Oresic, 2009]. Lipidomics is defined as the large-scale quantification and identification of lipids within biological systems. In analogy to the broad '-omics' disciplines such as trancriptomics, proteomics and metabolomics, untargeted lipidomics "aims at the comprehensive measurement of the lipids present in a biological matrix and the concomitant detection of the individual lipid responses to various stimuli, for example diseases, pharmaceutical treatment or genetic modification" [Sandra and Sandra, 2013].

New functional interactions have been revealed by lipidomics. Thus, lipids can no longer be regarded solely in the context of simple lipid-protein and lipid-lipid interactions in biochemical systems. On the contrary, they adopt important biochemical roles in the animal physiology.

1.4 Classification of lipids

1.4.1 Fatty acyls

Fatty acyls, also called fatty acids (FAs), are the fundamental building blocks of the lipids. FAs consist of a carboxylic group with a hydrophobic aliphatic tail. This tail is unbranched and can be saturated or unsaturated. FAs are categorized depending on, the chain length, the number, *cis-trans* configuration and position of double bonds, and the possible presence of functional groups [Ohlrogge and Jaworski, 1997]. FAs are mostly composed of an even number of carbons. Plants and bacteria can assemble FAs with an odd number of carbon atoms. In animals, odd-numbered fatty acids resulting from the α-oxidation of fatty acids with even numbered carbon atoms. Oxygen, nitrogen, sulfur and halogen can be linked to the carbon chains of FAs.

1.4.2 Glycerolipids

The category of glycerolipids involves all glycerol-containing lipids. Depending on the number of the substitutions on the glycerol, glycerolipids are named mono-, di- or triglycerides, thus describing how many of the hydroxyl groups of the glycerol are esterified with FAs. Most known are the triacylglycerides (TAGs) where all hydroxyl groups of the glycerol are esterified. Glycerolipids are important for the energy storage in animal tissues. For mobilizing the stored energy, the ester bonds of TAGs are hydrolyzed and the FAs are released [Coleman, 2004].

1.4.3 Glycerophospholipids

Glycerophospholipids are key components of cellular membranes, act as binding sites for cellular and extracellular proteins and also function as secondary messengers involved in proliferation and apoptosis [Cronan, 2003, Peretó et al., 2004]. Depending on the polar head group on the glycerol backbone, glycerophospholipids are grouped into different classes: glycerophosphocholine (PC), glycerophosphoethanolamine (PE), glycerophosphoserine (PS), glycerophosphoinositol (PI), glycerophosphoglycerol (PG) and cardiolipin (CL). Subclasses are further differentiated based on distinct substituents of the glycerol backbone.

1.4.4 Sphingolipids

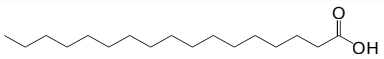
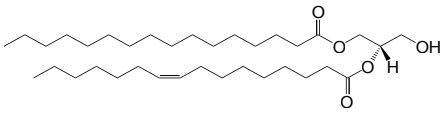
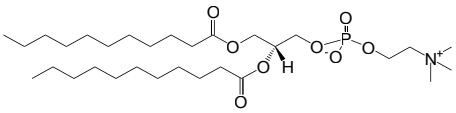
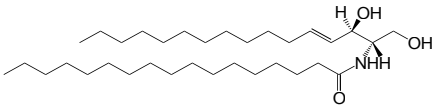
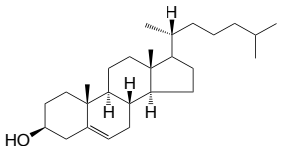
Sphingolipids are important components of the cell membrane and are often enriched in neuronal tissues. Sphingolipids play an important role in signal transduction and interaction between individual cells in the nervous system [Piomelli et al., 2007]. In contrast to the glycerophospholipids, building on glycerol, sphingolipids are derived from the unsaturated amino alcohol sphingosine [Fahy et al., 2005]. Sphingosine is connected via amide bond

with an acyl group such as a fatty acid. The sphingosine backbone is connected via a phosphate moiety by ester bonds with a charged group, such as serine, ethanolamine or choline. An important group includes the sphingomyelins (SM), where the sphingosine backbone is connected to a phosphocholine group.

1.4.5 Sterol lipids

Together with glycerophospholipids and sphingomyelins, sterol lipids are important components of membrane lipids [Bach and Wachtel, 2003]. Sterol lipids play a crucial role in biological processes involving hormones and other signaling molecules. Steroid hormones are separated in several groups depending on the amount of carbon atoms. The C18 steroids include the estrogens, the C19 the androgens. The C21 class includes the progestogens, glucocorticoids and mineralocorticoids [Tsai and O'Malley, 1994].

Table 2: Common lipid classes and representative lipid molecular species [Fahy et al., 2005]

Categories	Structure Examples	Typical Classes
Fatty acyls	 heptadecanoic acid	Fatty acids Eicosanoids Fatty alcohols Fatty esters Fatty amides
Glycerolipids	 1-hexadecanoyl-2-(9Z-hexadecenoyl)-sn-glycerol	Monoacylglycerols Diacylglycerols Triacylglcerols (TAG)
Glycerophospholipids	 1,2-diundecanoyl-sn-glycero-3-phosphocholine	Glycerophosphocholines Glycerophosphoserines Glycerophosphoethanoloamines
Shingolipids	 N-(heptadecanoyl)-hexdecasphing-4-enine	Phosphosphingolipids Sphingoid bases Ceramides
Sterol lipids	 cholest-5-en-3β-ol	Sterols Cholesterol and derivatives Steroids Bile acids and derivatibes

1.5 Analytical methods for lipidomics

The complexity of lipids, due to the enormous number of chemically and structurally distinct lipid species represents a challenge for traditional methods. Previously, separation and analysis of lipids were performed on gels in one- or two-dimensional electrophoresis [Vitić et al., 1981]. Other methods of separation of lipid mixtures into classes are thin-layer chromatography (TLC), liquid chromatography (LC), and solid-phase extraction (SPE) followed by analysis of the lipids. Disadvantages of theses methods are the lack of sensitivity and limitation of resolution by only analyzing a limited set of individual molecular species. They usually require large sample volumes and multi-step procedures for sample preparation. Alternatively, gas chromatography (GC) with mass spectrometric detection has been used broadly. A drawback of the method is the time-consuming procedure of hydrolysis and derivatization of most lipids to make them amenable to GC [Wilson and Lyall, 2002]. Nowadays, high-performance liquid chromatography (HPLC) is applied for versatile separation of neutral fatty acids, neutral lipids, glycerophospholipids, sterols, and lipid head group derivatives [Wenk, 2005]. Normal- and reverse-phase conditions are available to separate the lipids. Normal-phase chromatography separates the phospholipids based on the hydrophilic head groups. Reverse-phase separates phospholipids according to the hydrophobicity of the fatty acyl chains: lipids with shorter fatty acyl chains elute faster than those with longer ones. Lipids with higher degree of unsaturation elute faster than those with low levels of unsaturation.

New methods of mass spectrometry (MS) with soft ionization technologies, coupled to liquid chromatography, enabled rapid and sensitive analyses [Bhardwaj and Hanley, 2014]. Mass spectrometry is an analytical tool to measure the mass-to-charge ratio *(m/z)* of ions. By using the m/z values, the molecular weight (MW) of the measured molecule can be determined. The process of determing the MW involves three steps [Di Girolamo et al., 2013]:

1. conversion of the target molecules into gas-phase ions
2. separation of the ions by their masses and charges
3. detection of the separated ions obtaining signals proportional to their abundance

The major technological advantage of soft ionization methods, in particular electrospray ionization (ESI), has overcome most of the earlier limitations, such that they have become the method of choice for mass spectrometry in lipidomics [Kim et al., 1994, Hu et al., 2009]. In ESI, the sample solution is sprayed trough a capillary held at an electric potential gradient. The solvent of the charged droplets vaporizes and ions are produced in the gas phase. Based on their m/z values, the molecules can be separated and measured in

the spectrometer. Coupling liquid chromatography to ESI-MS increases the number of lipid classes that can be studied in a single analytical run.

Another spectroscopic method is nuclear magnetic resonance (NMR), which is used mostly for analyzing small biochemical substances. NMR spectroscopy requires little sample preparation and is a non-destructive method. It has, however, low sensitivity and can detect only highly abundant analytes [Ling et al., 2014]. The different methods for large-scale lipid analyses are summarized in Table 3.

Table 3: Technologies for lipidomic research

Technology	**Studied lipids**	**Advantages**	**Disadvantages**
Mass spectrometry			
MALDI	many lipids	direct detection by m/z	Matrix backgrounds, ionization suppression
ESI	many lipids	LC combination possible, direct detection by m/z high sensivity and selectivity, high turnover	Absolute quantification involves significant effort, ionization suppression
NMR			
^{1}H	all lipids	Non-destrucive direct measurement	Low sensitivity, spectra dominated by very abundant lipids
^{31}P	phospholipids	Non-destructive, direct measurement, quantitative	Low sensitivity
Chromatography			
Gas chromatography	non-polar compounds	Detection by mass spectrometry, mainly used for FA	Requires derivatization of polar lipids or volatile compounds
HPLC	many lipids	Quantitative, easy automation	Detection by mass detector or refractive index
Thin-layer chromatography	many lipids	Technically easy, minor instrumental investment	Low sensitivity and resolution drawbacks

2 Thesis objectives and outline

Fatty liver disease in cows is a metabolic disorder that arises from the uncoupling of non-esterified fatty acid synthesis and triglyceride catabolism, leading to hepatic triglyceride accumulation. Considering this pathogenic background, lipidomics provides a new powerful tool to obtain a comprehensive view of the state of lipid and carbohydrate pathways in this disease. In particular, fatty liver disease is expected to be preceded by an altered plasma lipidome signature, or fatty acid precursor signature, which can be detected by exploiting advanced lipidomic technologies including quadrupole-time of flight and triple quadrupole mass spectrometry.

The main goal of this study is to gain knowledge of the circulating lipidome and its alterations during different stages of the disease. This information will serve to gain further insights into the metabolic pathogenesis of fatty liver in cows. A long-term aim of this research is to identify and validate novel screening biomarkers for the early and sensitive detection of animals at risk of contracting fatty liver disease. For that purpose, I compared the plasma lipidome of cows affected by fatty liver with that of cows without this disease. In addition, I monitored the lipidome changes in the plasma of dairy during the transition period, i.e., during the time before and after calving.

This project was carried out with existing plasma samples collected from both diseased and healthy control animals using clearly defined inclusion/exclusion criteria as well as from cows during the transition period. A comprehensive analysis of these samples was performed by organic solvent extraction, chromatographic separation followed by mass spectrometric analysis of lipidomic profiles. The raw data files produced by mass spectrometry-based methods were processed using freely available or commercial statistical software packages for filtering (noise reduction), feature detection, alignment and normalization, followed by multivariate statistical methods (e.g. principal component analysis) for visualization and metabolite identification.

The most important benefit of this project is the comprehensive analysis of lipidomic fingerprints associated with fatty liver disease. It provides the basis for an improved transition cow management in herd health programs. Another added value is the development of an early, accurate and sensitive diagnostic test that does not involve invasive procedures. The study should yield basic information about proper sample analysis and further insights into the pathogenesis of fatty liver disease in dairy cows.

3 Altered plasma lipidome profile of dairy cows with fatty liver disease

Altered plasma lipidome profile of dairy cows with fatty liver disease

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Abstract

Fatty liver disease (or hepatic lipidosis) is a common production problem of dairy cows occurring during the transition from pregnancy to lactation. This syndrome develops when the hepatic uptake of non-esterified fatty acids results in their excessive storage as triacylglycerols. Currently, the diagnosis of fatty liver disease requires confirmation through biopsies to determine the hepatic lipid content. In view of this lack of a practical diagnostic tool, we compared the plasma lipidome of diseased dairy cows using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. A subsequent multivariate test involving principal component analyses yielded 20 masses that were able to distinguish between dairy cows with no hepatic lipidosis and those exhibiting different stages of the disease. Based on the chromatography retention time and m/z ratios, two of these masses could be identified as phosphatidylcholines with reduced plasma concentrations in cows with fatty liver disease. These results indicate that the measurement of specific representatives of phosphatidylcholines in plasma may provide a novel diagnostic biomarker of fatty liver disease in dairy cows.

Introduction

The transition time around parturition and early lactation involves critical physiologic changes in dairy cows. An excessive demand for nutrients due to the increased performance required for milk production results in a negative energy balance (Ametaj 2005; Mulligan & Doherty 2008; Hammon et al. 2009; Mullins et al. 2012). One major adjustment consists in the rapid mobilization of energy sources from tissue depots in the form of non-esterified fatty acids (Veenhuizen et al. 1991; Ingvartsen 2006; Geelen & Wensing 2006). Although in cattle the major site of fatty acid synthesis is the adipose tissue, the liver plays a central role in coping with sudden increases in the energy requirement (Kreipe & Deniz 2011). Fatty liver disease develops when, during this critical transition period, the hepatic uptake of non-esterified fatty acids liberated from the adipose tissue exceeds their elimination from the liver, thus causing their hepatic storage as triacylglycerols (Grummer 1993; Bobe et al. 2004). In cattle, the ability to release hepatic triacylglycerols as very low-density lipoproteins (VLDL) circulating in the blood is extremely low compared to other species (Katoh 2002; Pullen et al. 1990). High-producing cows with increased milk yields are, therefore, particularly susceptible to the development of the fatty liver syndrome (Reid & Collins 1980).

Affected dairy cows respond poorly to therapy and the mortality reaches rates of 50% (Adewuyi et al., 2005). The syndrome is associated with impaired health, reduced immunity leading to infections and a decreased reproductive performance. Fatty liver disease is also recognized as a major herd problem and also represents a serious animal welfare concern. The disorder is therefore associated with increased veterinary costs, longer calving intervals and reduced lifetime, thus causing huge economic losses worldwide (Bobe et al. 2004). A major challenge is also the diagnosis of fatty liver disease. With anorexia, reduced rumen motility, weight loss and the coincidence with puerperal diseases, the clinical signs are rather unspecific. Increased blood levels of liver enzymes reflect hepatic tissue damage but fail to display specificity for fatty liver disease (Cebra et al., 1997). To date, the diagnosis of this syndrome can therefore only be confirmed by liver biopsy and direct measurement of the hepatic triacylglycerol content.

Towards the development of an alternative, less invasive and more accurate test to be used both in individual animals and on a herd level, we hypothesized that fatty liver disease is accompanied or preceded by alterations in the plasma lipid composition. To test this hypothesis, we employed a non-targeted mass-spectrometric approach to compare the blood lipidome of dairy cows affected by fatty liver disease with that of animals suffering from other peripartal disorders.

Materials and Methods

Study design

Cows diagnosed with fatty liver disease were selected for this study on the basis of medical records ranging from 2000 to 2011. The degree of hepatic lipidosis was determined by histological examinations of liver tissue obtained through biopsy or necropsy. The animals were classified according to the severity of fatty liver and their archived blood plasma was analyzed for lipidome comparisons. A total of 63 transition period cows (33 Holstein Frisian and 30 Red Holstein) were included.

Plasma samples

Blood was drawn from the jugular vein and, in the presence of heparin, centrifuged at 1862 g for 5 min. The resulting plasma was stored at –80°C. It was demonstrated that, at –80°C, lipids are protected from oxidation and remain stable for up to 10 years (Matthan et al., 2010).

Liver histology

Tissue samples from biopsy or necropsy were fixed in 10% formalin and embedded in paraffin. Sections were prepared with a thickness of 2-3 μm, stained with hematoxylin/eosin and examined by a board-certified pathologist. The histological lesions (excessive triacylglycerol storage) were staged in: grade 0, no or only weak abnormality; grade 1, only cells from the central lobe affected; grade 2, periportal and midzonal areas affected; grade 3, all three liver zones affected; grade 4, all three zones and Kupffer cells affected (Figure 1). To increase the statistical power, the animals were divided in three major groups displaying weak (grades 0 and 1), medium (grade 2) and severe fatty liver disease (grades 3 and 4).

Chemicals

All solvents for mass spectrometry were purchased from Sigma Aldrich (Buchs, Switzerland). The following lipids were used as internal standards: phosphocholin 16:0/16:0 (CAS-Nr. 32448-32-1, product no. 850356; mass 845.687), phosphocholin 23:0/23:0 (CAS-Nr. 112241-60-8, product no. 850372; mass 929.781), lysophosphocholin 19:0 (CAS-Nr. 108273-88-7, product no. 855776; mass 537.379), phosphocholin 13:0/13:0 (CAS-Nr. 71242-28-9, product no. 850340; mass 649.468), lysophosphocholin 17:0 (CAS-Nr. 50930-23-9, product no. 855676; mass 509.348) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and

palmitine acid isotope (1-13C, 99%, CAS-Nr. 57677-53-9, product no. CLM-150-1; mass 257.240) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Lipid extraction

Plasma samples were spiked with the above listed internal standards and extracted as described (Matyash et al. 2008). Briefly, 127 µl of methanol were placed into a 1.5-ml Eppendorf tube and supplemented with 23 µl of a 10-µM internal standard mixture. Next, a 20-µl aliquot of each extracted probe was added and the resulting samples were mixed for 10 s. Thereafter, 500 µl of methyl-*tert*-butyl ether was added and the mixture incubated for 60 min at room temperature in a shaker (Vaudaux-Eppendorf AG, Schönenbuch, Basel) moving at 800 rpm. A phase separation was induced by adding 125 µl of water and mixing. The samples were centrifuged for 10 min at 1000 g and 4°C, and 500 µl of the upper organic phase containing the lipids and other non-polar components was collected. Finally, the organic solvent was evaporated under a nitrogen stream at 30°C in a Techne Dri-Block DB-3D sample concentrator (Bibby Scientific Limited, Staffordshire, UK), redissolved in 500 µl methanol and stored at –20 °C.

UPLC Analysis

UPLC and mass spectrometry was performed according to a previously described protocol (Castro-perez et al. 2010). The nanoAcquity UPLC system (Waters, Milford, MA, USA) was used for chromatographic separation. The column (0.2 mm x 110 mm) was filled by the slurry method with 1.8-µm C_{18} HSS T-3 particles (Waters, Milford, MA, USA). The average column pressure was 5935.54 psi (max. 8549.19 psi) in the positive electrospray ionization (ESI) mode and 5861.79 psi (max. 7784.67 psi) in the negative ESI mode. A binary gradient system was applied with two eluents. Eluent A was acetonitrile and water (60:40, v/v) with 10 mM ammonium acetate and eluent B acetonitrile and isopropanol (10:90, v/v) with 10 mM ammonium acetate. The total running time was 33 min. Initially, the column buffer was held at 65% A and 35% B for 2 min. For the next 20 min, the gradient was changed in a linear fashion to 95% B and held at this composition for 5 min before the system was switched back to the initial gradient (65% A) within 1 min and equilibrated for an another 5 min. The injection volume was 1 µl and the flow rate used for the experiments was 4 µl/min.

Mass Spectrometry

The nanoUPLC was directly connected to a quadrupole time-of-flight (qTOF) mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA, USA) equipped with a dual ESI device (NanoLockSpray; Waters, Milford, MA, USA) operating in positive and negative mode. The source temperature was 80°C with a cone gas flow of 30 L/h and desolvation temperature of 180°C. The capillary voltage was set at 2.5 kV in the positive ESI mode and 1.6 kV in the negative ESI mode, with a sampling cone voltage of 30 V and an extraction cone voltage of 2.50 V. The data acquisition rate was 0.25 s with an interscan delay of 0.024 s. Leucine enkephalin was employed as the lock mass compound and infused straight into the mass spectrometer at a concentration of 2 ng/µL in 5% acetonitrile and 95% water containing 0.1% formic acid at a flow rate of 0.5 µL/min. This lock mass yielded a monoisotopic ion peaks at m/z 556.2771 $[M+H]^+$ and m/z 554.2614 $[M–H]^–$. All mass spectral data were acquired in the MS^E centroid mode with direct lock mass corrections by scanning at the m/z 50-1200 range.

Data processing

The open source program MZmine (Pluskal et al, 2010; http://mzmine.sourceforge.net/; Project GNU free software foundation, Boston, USA) was used for automatic alignment, de-noising, de-convolution and extraction of peaks. Statistical analyses were performed using the open source program R (R_Core_Team (R Foundation for Statistical Computing) 2014). All data were standardized by the median of the internal standards and logarithmically transformed. To graphically illustrate the separation between the different sample groups, the data were subjected to principal component analysis (PCA) and MADE4 package (Culhane et al., 2005) was used for multivariate analysis. This statistical package provides a supervised classification method of the PCA, also known as between-group analysis (Culhane et al., 2002). The between-group analysis distinguishes between entire groups rather than between individual samples. For further investigation, only masses best correlating with the principal components 1, 2 and 3 were further considered. These masses are responsible for the separation of the different disease groups in the BGA. Quantitative data were normalized to the internal standards lysophosphocholin 17:0 in the positive ESI mode and ^{13}C-labeled palmitine in the negative ESI mode and plotted with GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Sixty-three dairy cows with different degrees of fatty liver disease were analyzed. These animals included 33 Holstein Frisian and 30 Red Holstein cows, ranging in age from 2 to 12 years that were referred to the Veterinary Hospital from 58 different farms, thus accounting for heterogeneous diet, housing and management conditions. Samples were taken from these cows during the transition period (2.7 ± 1.9 weeks post partum). Based on the histopathological analysis of their liver tissue, the animals were divided into a group with no or weak signs of fatty liver disease (group "weak", n = 26), a group with medium-grade fatty liver disease (group "medium", n = 21), and a group with severe fatty liver disease (group "severe", n = 16). There were no differences in the average age of animals and time of lactation between these three groups. In 49 out of the 63 cows, further diseases were diagnosed that typically occur during the peripartal period, including left displaced abomasum (n = 15), bronchopneumonia (n = 7), laminitis (n = 2), hypophosphatemia (n = 2), mastitis (n = 5), peritarsitis (n = 2), abomasal ulcer (n = 2), cecal dilation (n = 2) and retained placenta, sole ulcer, myopathy, dermatitis solaris and endometritis (each n = 1).

The plasma lipidome analysis by UPLC coupled to qTOF mass spectrometry revealed 252 variable masses in the positive ESI mode and 112 variable masses in the negative ESI mode. A between-group analysis of these variable masses demonstrated a separation in metabolic patterns among the three major groups of the study, i.e., between the animals with "weak", "medium" and "severe" fatty liver disease (Figure 2). The distinction between these groups was improved if the data from Holstein Frisian cows (Figure 3) or Red Holstein cows (Figure 4) were subjected to separate statistical analyses, particularly with respect to the masses detected in the positive ESI mode. For further investigation, the quantitative levels of the 20 masses that best correlate with the principal component of the overall between-group analysis of Figure 2 were extracted and plotted separately. Based on their chromatographic retention time, their m/z value and corresponding isotope pattern, two of these masses could be identified as representatives of phosphatidylcholines with decreased abundance in cows affected by medium-grade or severe fatty liver disease compared to the control group of animals with no or weak signs of fatty liver disease (Figure 5). Further masses with altered abundance measured in either positive of negative ESI mode could not be identified (Figures 6-9).

Discussion

Fatty liver disease is a metabolic disorder characterized by the accumulation of lipids in liver tissue. Currently, the only diagnostic tool to confirm fatty liver in cows and to assess the severity of lipid accumulation remains the examination of a liver biopsy, which requires surgical procedures. The so far available biomarkers of negative energy balance and damaged liver function are not specific for fatty liver disease (Cebra et al. 1997). Therefore, the purpose of this large-scale screening approach was to provide a hypothesis for the development of a novel diagnostic test based on lipidome profiles that is less invasive and more accurate, thus allowing for the diagnosis of fatty liver disease in individual animals as well as at the level of herd management.

For that purpose, we established an untargeted lipidomic evaluation of the plasma of cows with different levels of fatty liver disease in comparison to animals with poor signs of the disease. Lipidomics is considered a useful tool for the elucidation and characterization of defects in lipid homeostasis (Loizides-Mangold 2013). Such a lipidomic approach had been chosen because we postulated that fatty liver disease is likely to be preceded or at least accompanied by an altered plasma lipid signature that can be detected on advanced metabolomics platforms (Drackley, 1999; Puri et al., 2007). This general assumption that fatty liver disease is characterized by specific alterations of the lipid metabolism is widely supported (reviewed by Bobe et al., 2004). An altered lipid metabolism plays an important role in the pathogenesis of many diseases, in both animals and humans, including diabetes, insulin resistance, Alzheimer's disease, schizophrenia, cancer, atherosclerosis, and toxic manifestation of infectious disease (Oresic et al., 2008). All these conditions were previously evaluated using lipidomics techniques. A general advantage of metabolomics and lipidomics, compared to other large-scale screening strategies like transcriptomics or proteomics, is that they measure metabolites or lipids representing the end product of the interaction of genes or proteins with environmental factors.

The results of the current pilot study confirm the expectation that fatty liver disease is accompanied by changes in the plasma lipidome. In particular, the discovery that phosphatidylcholines may be reduced in fatty liver disease is intriguing. On the one hand, phosphatidylcholines are an important precursor for the synthesis of triacylglycerols (Jacobs et al. 2013) indicating that their level may decrease in response to an enhanced triacylglycerol production. On the other hand, phosphatidylcholines are required for the secretion of hepatic triacylglycerols as VLDL particles, indicating that a reduced phosphatidylcholine level may

directly cause an excessive accumulation of triacylglycerols in the liver. Further studies are now required to confirm a causal relationship between reduced phosphatidylcholin concentrations and fatty liver disease in dairy cows.

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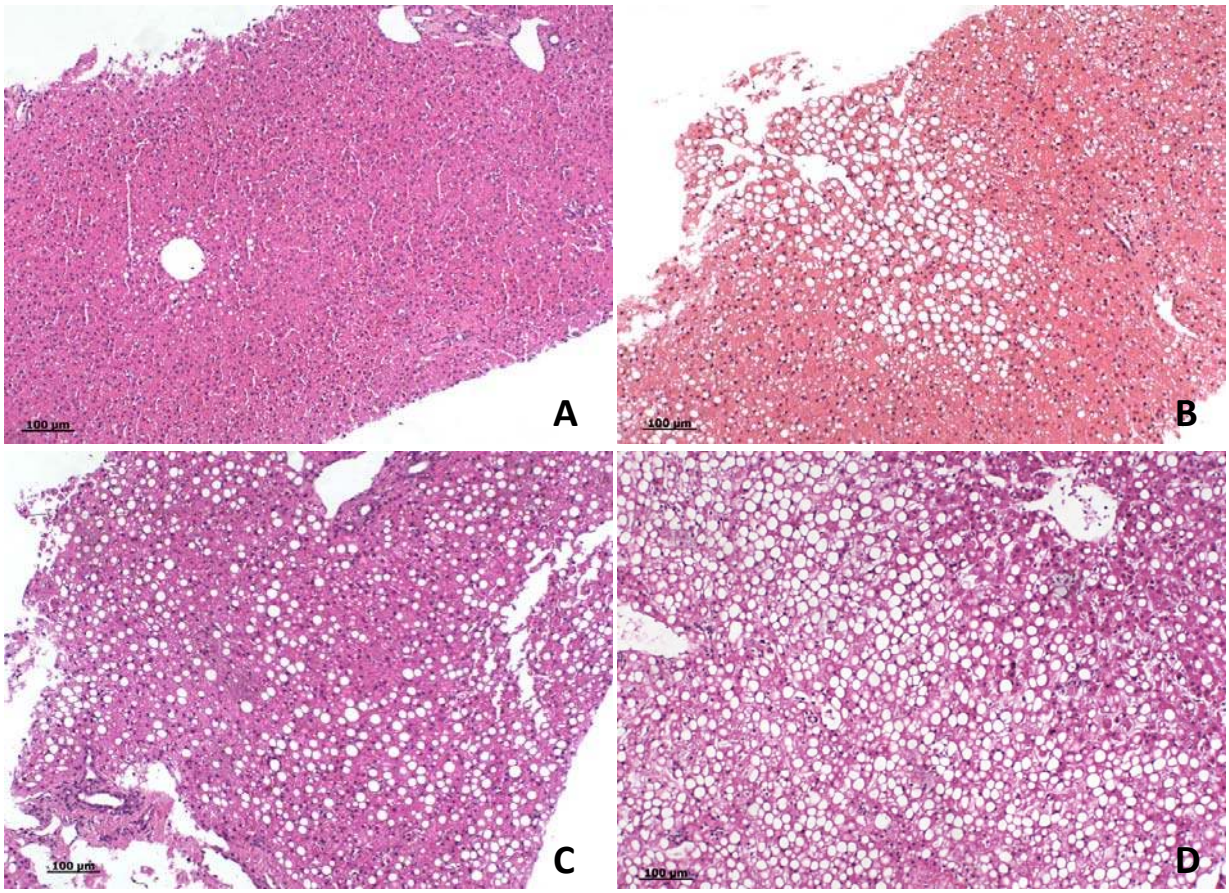


Figure 1. Documentation of different stages of fatty liver disease in histologic sections stained with hematoxylin/eosin and shown in 10-fold magnification. (A) Grade 1: only cells from the central lobe are affected. (B) Grade 2, the periportal and midzone areas are affected. (C) Grade 3, all three liver zones are affected. (D) Grade 4, all liver area contain triacylglycerol depots, including the Kupffer cells.

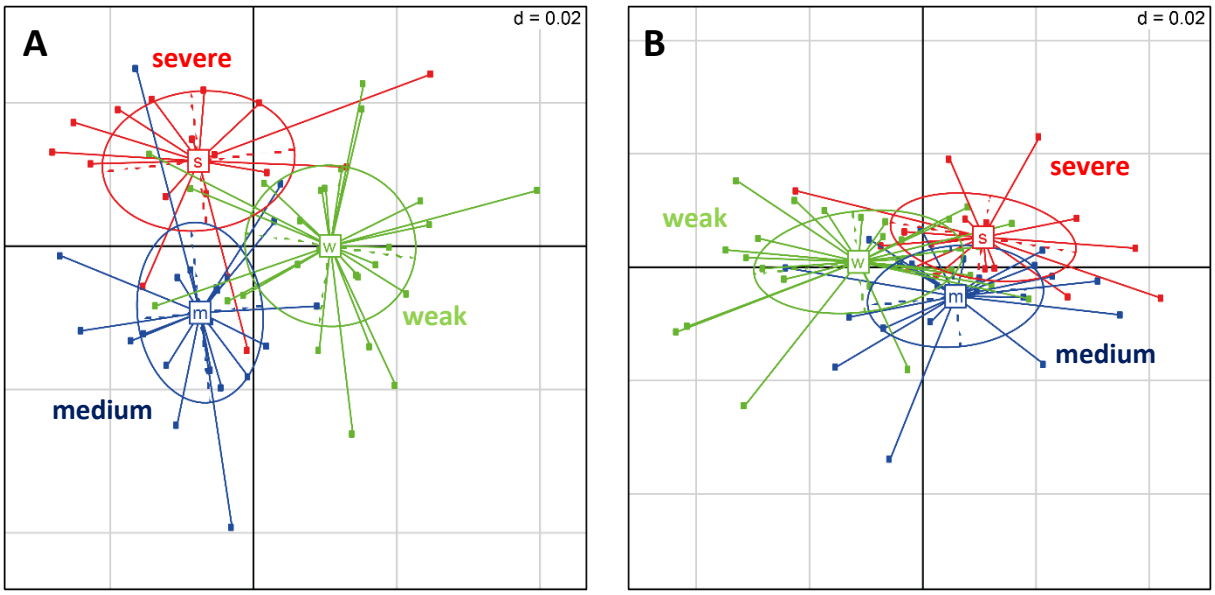
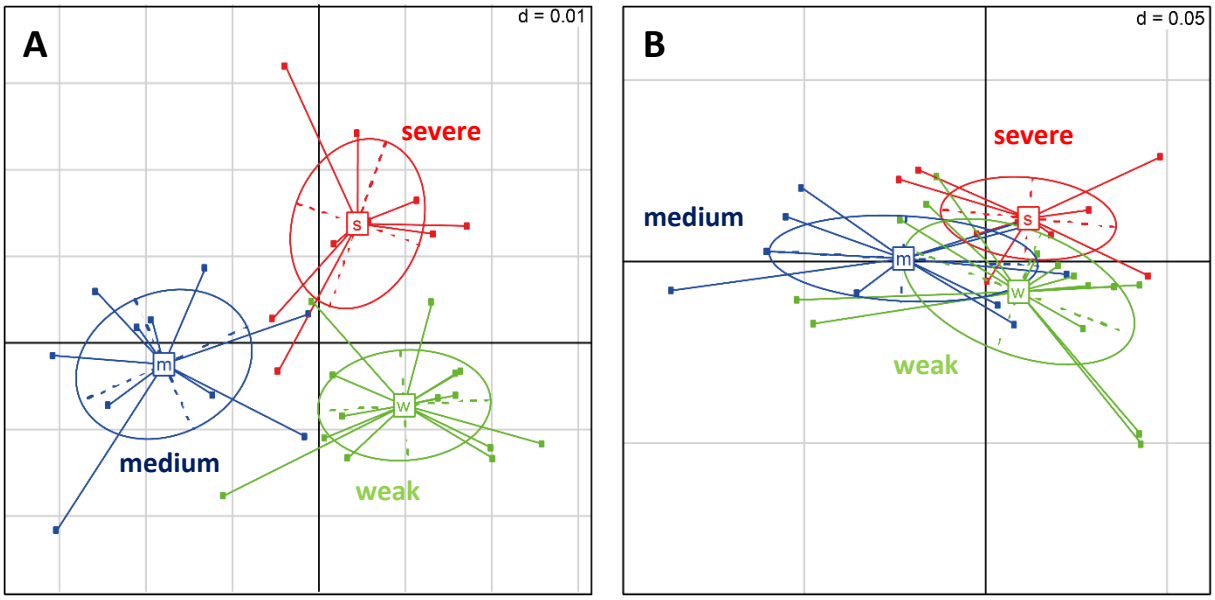


Figure 2. Between-group analysis of the lipidome data of all 63 cows. (A) Positive ESI mode measurement. (B) Negative ESI mode measurement. Green: cows with no or weak signs of fatty liver disease; blue: cows with medium-grade fatty liver disease; red: cows with severe fatty liver disease.



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361 **Figure 3**. Between-group analysis of the lipidome data of the 33 Holstein Frisian cows. (A)
362 Positive ESI mode measurement. (B) Negative ESI mode measurement. Green: cows with no
363 or weak signs of fatty liver disease; blue: cows with medium-grade fatty liver disease; red:
364 cows with severe fatty liver disease.

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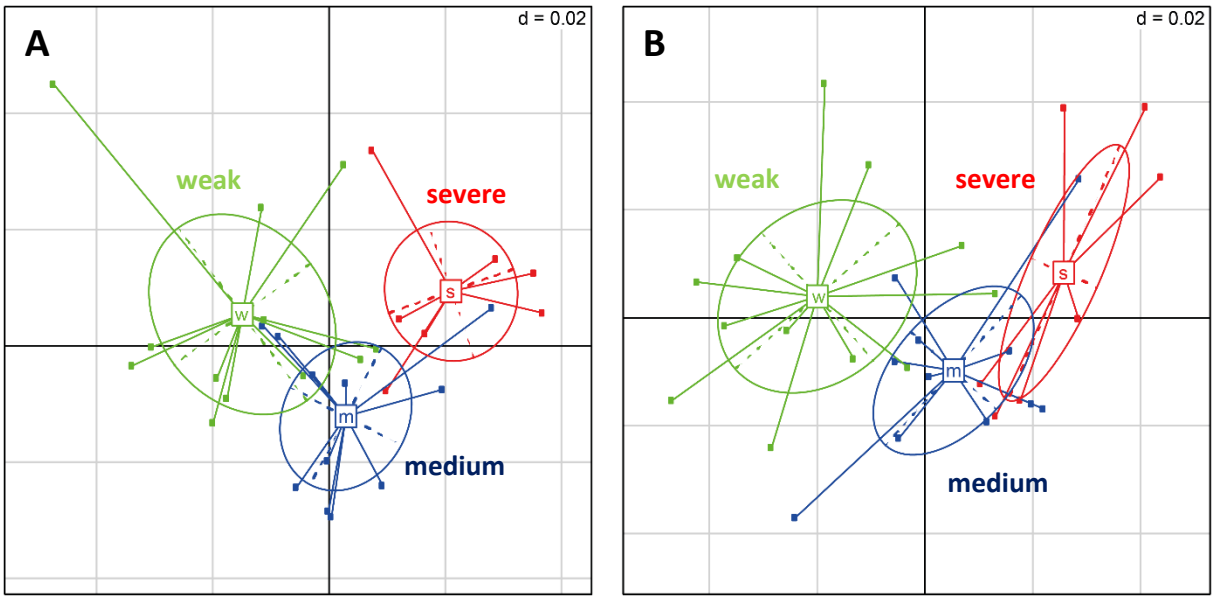

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369 **Figure 4**. Between-group analysis of the lipidome data of the 30 Red Holstein cows. (A)
370 Positive ESI mode measurement. (B) Negative ESI mode measurement. Green: cows with no
371 or weak signs of fatty liver disease; blue: cows with medium-grade fatty liver disease; red:
372 cows with severe fatty liver disease.

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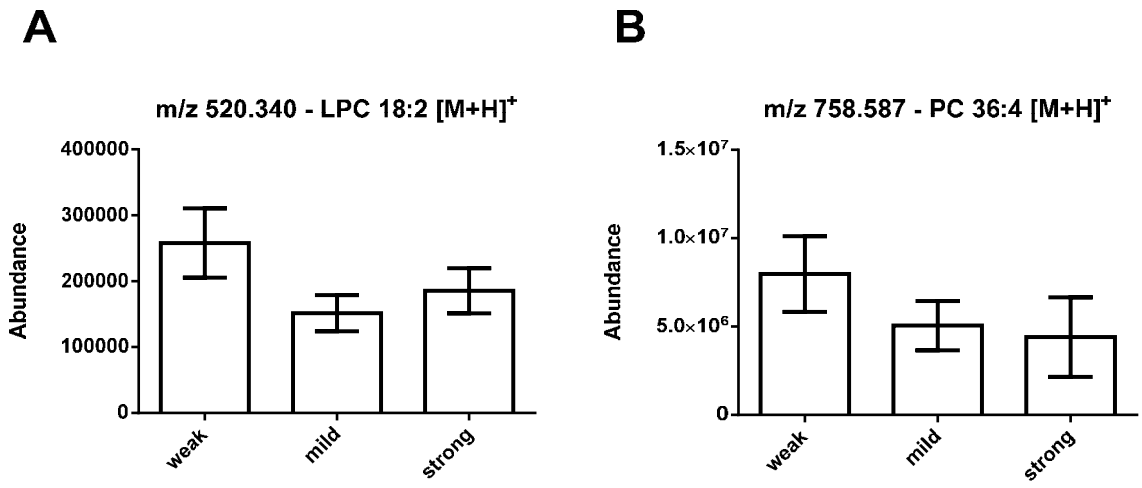

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379 **Figure 5**. Decreased abundance of phosphatidylcholines in cows affected by fatty liver
380 disease. (A) Lyso-phosphatidylcholine 18:2. (B) Phosphatidylcholine 36:4.

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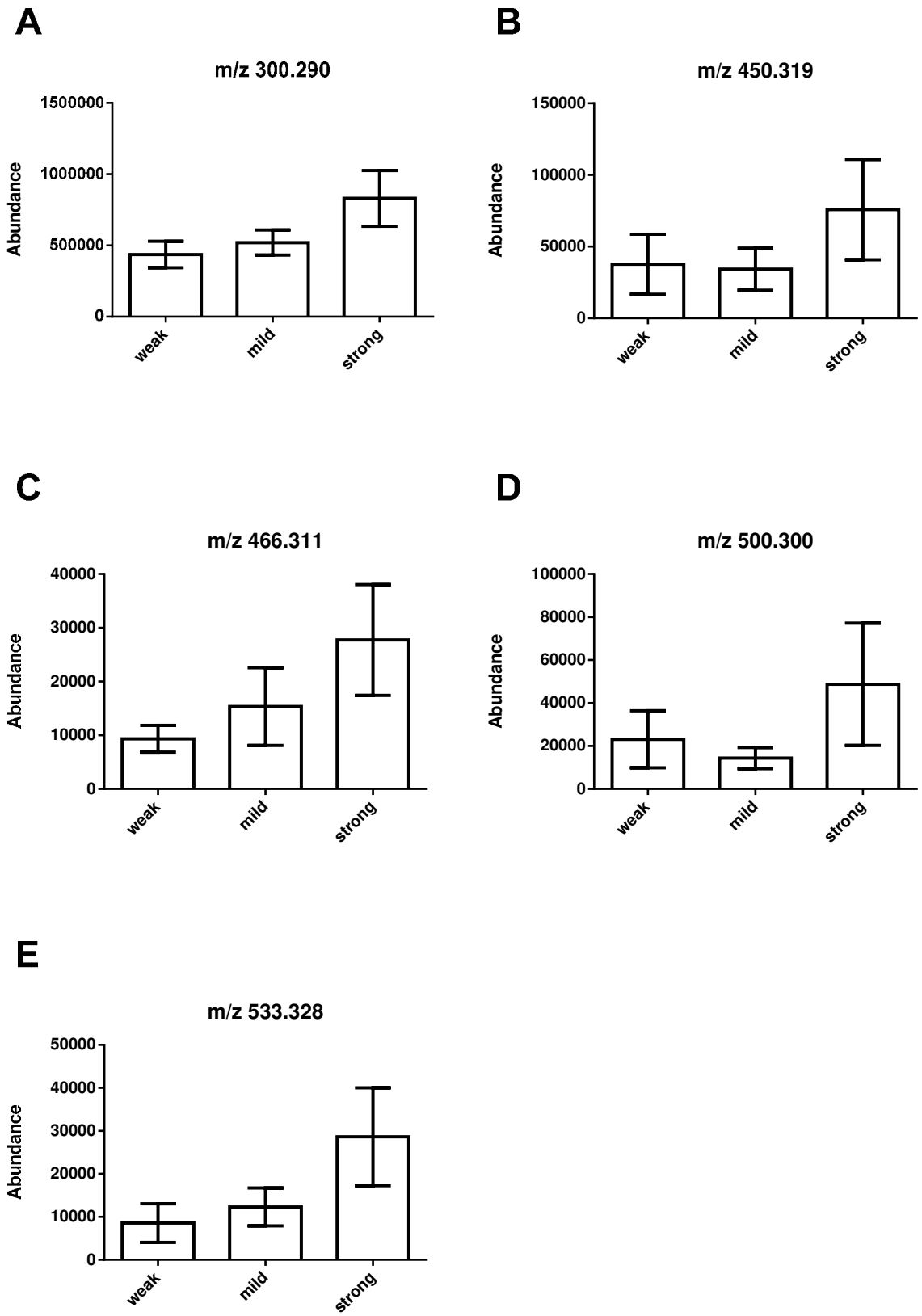

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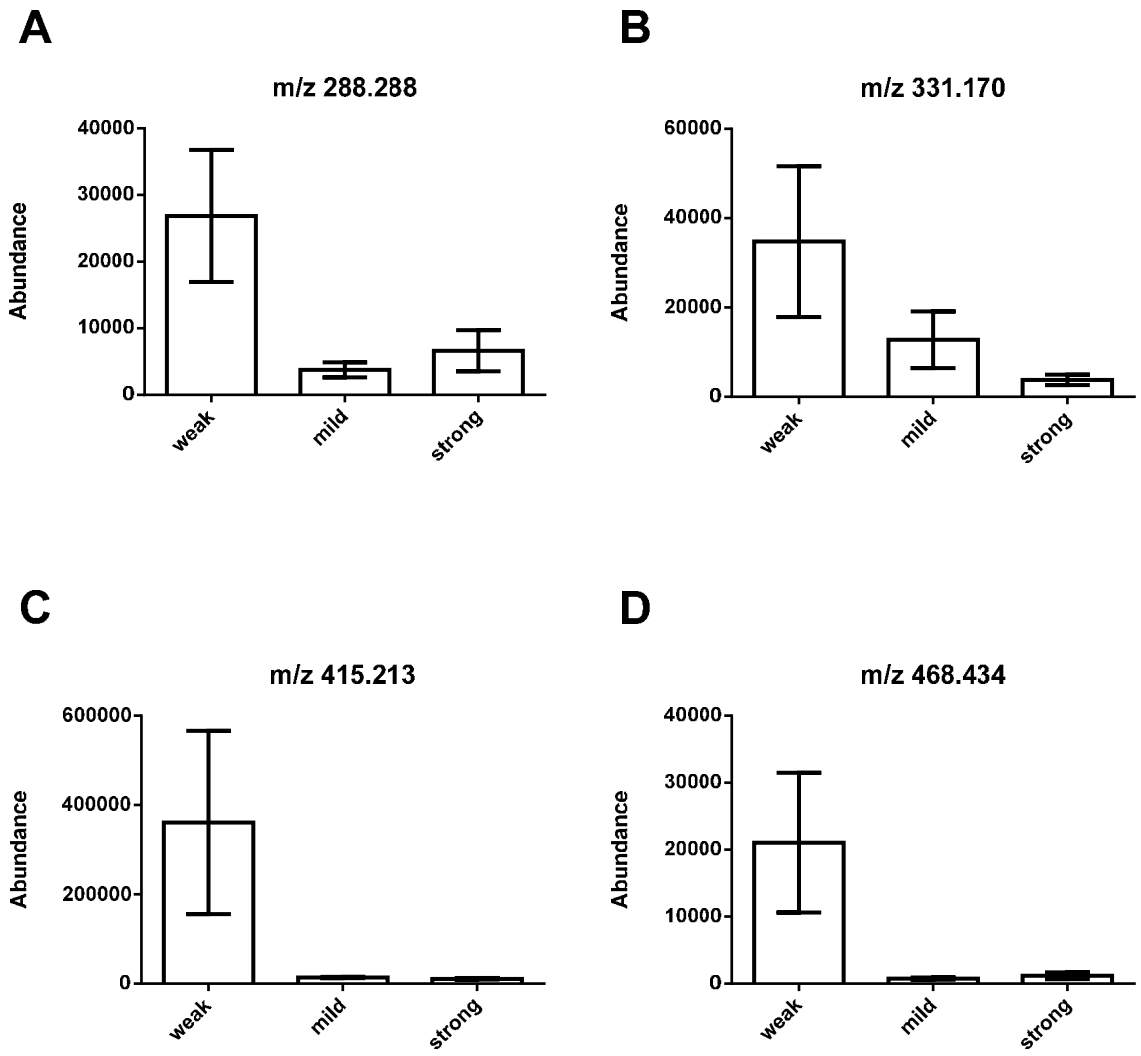
384 **Figure 6**. Increased abundance of masses determined by positive ESI in cows affected by

385 fatty liver disease.



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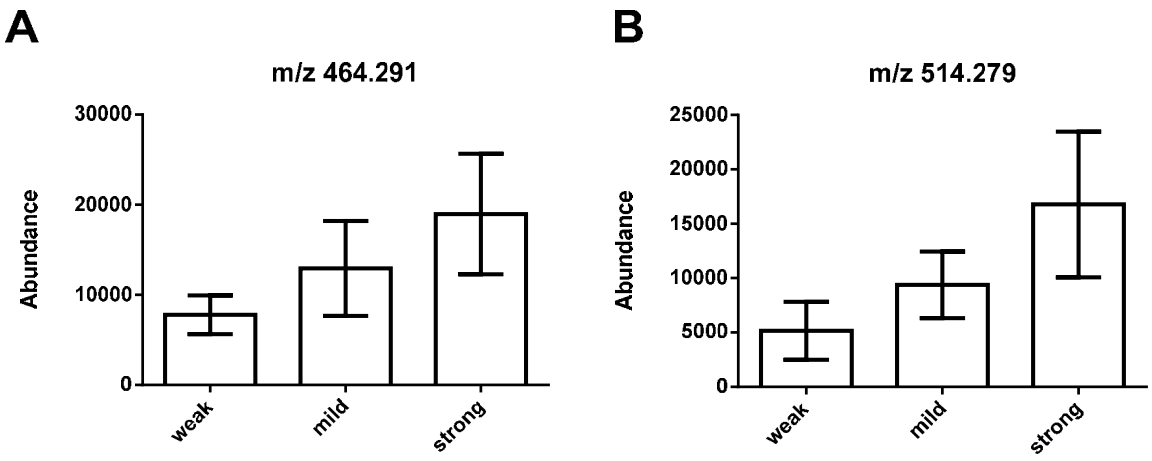
389 **Figure 7**. Decreased abundance of masses determined by positive ESI mode in cows affected

390 by fatty liver disease.



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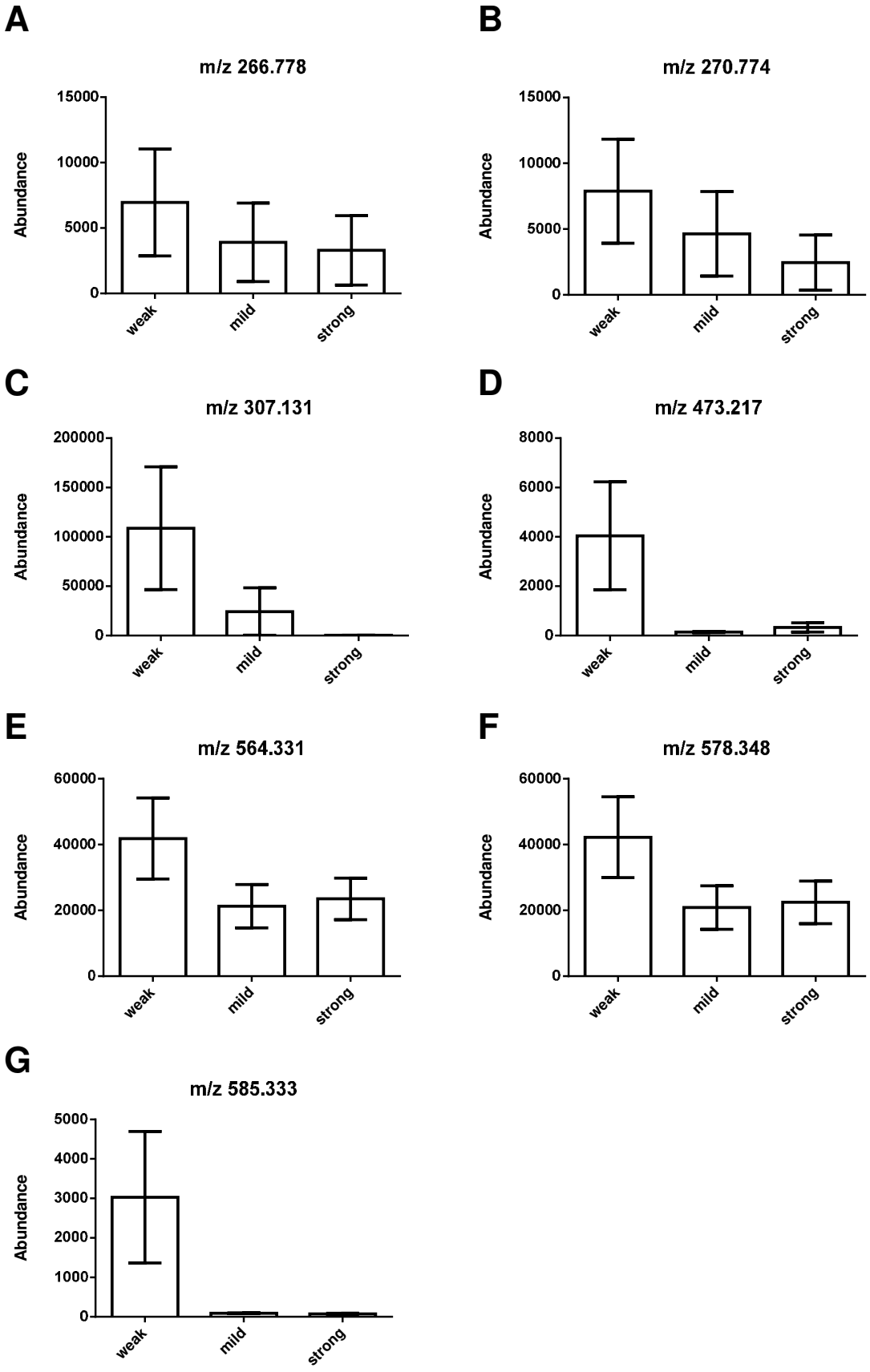
394 **Figure 8**. Increased abundance of masses determined by negative ESI in cows affected by

395 fatty liver disease.



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397 **Figure 9**. Decreased abundance of masses determined by negative ESI in cows affected by
398 fatty liver disease.

4 Blood plasma lipidome profile of dairy cows during the transition period

Blood plasma lipidome profile of dairy cows during the transition period

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Abstract

The transition period of dairy cows, around parturition and the onset of lactation, involves endocrine and metabolic changes to compensate for an increased energy requirement aggravated by reduced feed intake. Transition cows adjust to the resulting negative energy balance with the mobilization of fat from the adipose tissues yielding increased blood levels of non-esterified fatty acids and ketone bodies like β-hydroxybutyrate. To study the biochemical adaptations underlying this physiologic adjustment and possible pathologic derangements, we analyzed the blood plasma lipidome of transition cows by ultra-pressure liquid chromatography coupled to high-resolution quadrupole time-of-flight mass spectrometry. The resulting data were processed by principal component analysis, revealing over 60 lipid masses that change in abundance over the test period ranging from two weeks before calving to four weeks post partum. Further characterization of analytes by tandem mass spectrometry demonstrated that the concentration of triacylglycerides in plasma drops at the day of parturition whereas the plasma level of many phosphatidylcholines and two sphingomyelins increases steadily during early lactation. This newly identified shift in phospholipid composition delivers a potential biomarker to detect aberrant metabolic pathways in transition cows and also provides insights into how to prevent and treat associated disorders like fatty liver disease.

Introduction

During the last few weeks before parturition and the first weeks post partum, dairy cows have to undergo the physiologic transition from pregnancy to lactation, which involves many endocrine and metabolic adaptations related to parturition and the onset of milk production (Grummer et al. 1995; Huzzey et al. 2005). A key problem during this transition period is the dramatic increase in energy requirements for milk production, paralleled by a decreased feed intake, occurring around parturition and the first week after calving (Moyes, Larsen, and Ingvartsen 2013). This condition results in a negative energy balance, to which dairy cows adjust by fat mobilization from the adipose tissue representing a major fuel source during the transition period (Sordillo and Raphael 2013). However, fat mobilization leads to an increase in non-esterified fatty acid (NEFA) concentrations, which are linked to greater incidences of ketosis, displaced abomasum, and retained placenta (Dyk 1995). Many transition period disorders, including fatty liver, also occur in a subclinical form affecting the overall health status, milk production and reproductive performance of dairy cows.

Fatty liver is a multifactorial metabolic disorder resulting from an increased flux of NEFAs to the liver, followed by their reconversion to triacylglycerides and storage as intracellular fat droplets. The development of fatty liver disease in cows with a negative energy balance depends on the level of overall fat mobilization, the rate of fatty acid oxidation in the liver and the efficiency of fatty acid elimination from the liver, either as ketone bodies reflecting the fatty acid breakdown or as full triacylglycerides exported in the form of very low density lipoproteins (VLDLs) (K.L. Ingvartsen 2006). Fatty liver is related to other diseases including retained placenta, uterine infection, milk fever, abomasal displacement and mastitis. These pathological changes affect up to 50% of dairy cows during early lactation (Sejersen et al. 2012; Jorritsma et al. 2001).

A liver biopsy remains the only diagnostic tool to determine the lipid content of the liver and, as a consequence, the extent of the disease is in practice frequently uncertain. A non-invasive and accurate test for the diagnosis of fatty liver disease would, therefore, be helpful for the management of this disorder in individual animals as well as on a herd level. Such a simplified test would also allow to investigate the incidence and risk factors of the disorder (K.L. Ingvartsen 2006). Because the lipid metabolism is a key aspect of the physiology of transition cows (Drackley 1999; Gross et al. 2013), we conducted a comprehensive analysis of the plasma lipidome of cattle during the time before and after parturition. This study reveals

that the physiologic adaptation to an increased energy requirement of transition cows involves a post partum elevation of phosphatidylcholines and sphingomyelins in their blood plasma.

Materials and methods

Animals and management

Twelve dairy cows of the Holstein Frisian breed were included in this study. In order to reduce variability, all cows were selected from the same farm. The animals were housed in an open spaced barn comprising a total of 75 dairy cows. To be included in the study, the cows had to be multiparous, clinically healthy and not receiving any treatments at the beginning of the study. Also, they had to be confirmed pregnant with a known breeding date. Their health status was assessed by the herd veterinarian and clinicians of the Department of Farm Animals. All cows of the study were fed exactly the same died and kept in the same barn. Milking times were 5.30 a.m. and 4.45 p.m. During the preceding dry period, the cows were fed grass silage, straw, hay and a mineral supplement. Two weeks before parturition, the cows were regrouped to the lactating animals receiving increasing amounts of a concentrate (0-1.5 kg). Post partum, the cows were fed grass and corn silage, beet pulp, soybean, grass, mineral supplement, and up to 4 kg concentrate. The body condition score was recorded concurrently with each blood sample collection.

The study was approved by the Veterinary Office of the Kanton Zurich and conducted in accordance with guidelines established by the Animal Welfare Act of Switzerland (permission No. 27/2013)

Collection of blood samples and liver tissue

Samples were obtained from the jugular vein at days –14, –7, 0, +7, +14, +21 and +28 relative to parturition. The samples were drawn always at the same time in the afternoon. Tubes supplemented with lithium heparin (Sarstedt AG and Co., Nümbrecht, Germany) were used to collect 10 ml of blood. The heparinized blood was centrifuged at 4000 g for 5 minutes. Separated plasma was stored in 2-ml tubes at –80°C until analysis. Plasma samples were used for lipidome evaluation and measurement of β-hydroxybutyrate and NEFA.

Liver biopsies were taken as described (Mølgaard et al. 2012) under ultrasonographic control. The biopsies were fixed in 10% formalin and embedded in paraffin. The specimens were sliced at a thickness of 2-3 μm and sections were stained with hematoxylin-eosin. The

histological lesions were staged into four categories: no abnormalities, mild fatty liver (only cells from one liver zone affected by lipid inclusions), moderate fatty liver (the periportal and midzonal areas contained lipid inclusions), severe fatty liver (all three zones affected including the Kupffer cells).

Measurement of NEFA and *β*-hydroxybutyrate

Plasma concentrations of NEFA and *β*-hydroxybutyrate were determined by enzymatic analyses using the Wako NEFA-HR (2) (Wako Chemicals GmbH, Neuss, Germany) and the *β*-Hydroxybutyrate LiquiColor® kit (Stanbio Laboratory, Boerne, TX, USA), respectively. Spectrophotometric measurements were performed for both NEFA and *β*-hydroxybutyrate, using a Cobas Mira S Chemistry Analyzer (Roche, Basel, Switzerland).

Lipid nomenclature

For the designation of lipids, the common standard lipid language described by the Lipidomics Gateway (http://www.lipidmaps.org, National Institute of General Medical Sciences, National Institutes of Health) and by Schmelzer et al. (2007) was applied.

Chemicals and internal standards

All solvents were liquid chromatography-grade. Acetonitrile was purchased from Scharlau (Sentmenat, Spain). Propanol, methanol, ammonium acetate, water and methyl-*tert* butyl ether (MTBE) were purchased from Sigma-Aldrich (Buchs, Switzerland). Leucine-enkephalin was used as the lock mass at a concentration of 1 ng/µl in a solution of acetonitrile/water (50:50, v/v) supplemented with 0.1% formic acid.

For the internal standards, an exogenous mixture of lipids was added to the solvent during the initial extraction step. These standards consisted of 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 17:0/0:0), 1-nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC 19:0/0:0), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (PE 15:0/15:0), 1,2-diheptade-canoyl-*sn*-glycero-3-phosphoethanolamine (PE 17:0/17:0), 1,2-dipentadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt (PG 15:0/15:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (PG 17:0/17:0), 1,2-ditridecanoyl-*sn*-glycero-3-phosphocholine (PC 13:0/13:0), 1,2-ditricosanoyl-*sn*-glycero-3-phosphocholine (PC 23:0/23:0), 1,2-dinona-decanoyl-*sn*-glycero-3-phosphocholine (PC 19:0/19:0) and 1,2-di-(3,7,11,15-tetramethylhexadecanoyl)-*sn*-glycero-3-phosphocholine [PC 16:0 (3me,7me,11me,15me)/16:0 (3me,7me,11me,15me)] purchased from Avanti Polar

Lipids (Alabaster, AL, USA); 1,2,3-tripentadecanoylglycerol (TG 15:0/15:0/15:0) and 1,2,3-trihepta-decanoylglycerol (TG 17:0/17:0/17:0) were from Sigma-Aldrich (Buchs, Switzerland). The concentration of internal standards (350 nM) was calculated relative to the final amount of organic solvent in the extraction tube.

Sample extraction

Lipids were extracted from bovine plasma following a published protocol (Matyash et al. 2008). The extraction was validated by the addition of internal standards. For that purpose, 127 µl methanol was placed into 1.5-ml Eppendorf tubes and 23 µl of the 10 µM internal standard mixture was added (final concentration of 350 nM in the organic phase). A sample aliquot of 20 µl was added to each tube and the mixtures vortexed for 10 s. The mixtures were supplemented with 500 µl of methyl-*tert*-butyl ether and incubated for 60 min at room temperature in a thermo shaker (Vaudaux-Eppendorf AG, Schönenbuch, Basel) at 800 rpm. Then, a phase separation was induced by adding 125 µl of water and further vortexing. The samples were centrifuged for 10 min at 1000 g and 4°C; 300 µl of the upper (organic) phase containing the lipids and non-polar components were collected and transferred into a new tube. The organic phases were dried in a vacuum centrifuge (Savant Speed Vac Plus SC 110A, Savant Instruments Inc., Holbrook, NY, USA), dissolved in 300 µl methanol and stored at –80°C until measurements.

Liquid chromatography

An Acquity UPLC (Waters, Milford, MA, USA) system was used for ultra-pressure chromatographic sample separations. The plasma extracts were injected as triplicates and in a random order onto a HSS T3 column (Waters, Milford, MA, 1.8-µm particle, 100 x 2.1 mm id) heated to 55°C. The average column pressure was 7000 psi. A binary gradient of two solvent mixtures was used for elution. Eluent A consisted of acetonitrile and water (50:40, v/v) with 10 mM ammonium acetate; eluent B consisted of acetonitrile and isopropanol (10:90, v/v) with 10 mM ammonium acetate. Eluent A was used for weak needle washes, whereas isopropanol was used for strong needle washes. The flow rate was 0.4 ml/min and the injection volume 10 µl. A linear gradient was performed for the sample analysis. The initial portion of the gradient was held at 60% A and 40% B. In the next 10 minutes, the composition was changed in a linearly ramped gradient (curve 6) to 100% B and held for 2 minutes. The system was switched back to the initial proportion (60% A, 40% B) and the column was equilibrated for 3 minutes. The total run time was 15 min.

Mass spectrometry

The UPLC inlet was coupled to a quadrupole time-of-flight mass spectrometer (SYNAPT G2 HDMS, Waters, MS Technologies Manchester, U.K.). Mass spectrometry was carried out following the protocol of Castro-Perez and Kamphorst (2010), whereby electrospray ionization (ESI) was employed in the positive and negative mode. A capillary voltage of 3 kV and cone voltage of 30 V were used for both polarities. The desolvation source conditions involved desolvation gas at 700 L/h and a temperature of 400°C. The mass range during acquisition was 50-1200 Da and the signals were acquired in the centroid mode. As internal reference during the measurement, leucine-enkephalin was used to collect reference scans every 10 s lasting 0.3 s. A lock mass calibration with m/z 556.2771 in positive and m/z 554.2615 in negative ion mode was used.

During data acquisition, the first quadrupole was operated in a wide band RF mode, such that all ions were able to enter the T-wave collision cell. In this cell, two acquisition functions were applied. The first function with 5 eV resulted in non-fragmented ion molecules while the second function used a collision energy ramp of 20-30 eV to generate fragmented ions (MS^E method). Argon gas was used for collision-induced dissociation. Using this interleaved acquisition, fragmented and non-fragmented ions could be used for quantification and initial ion identification. For final ion identification, a tandem mass spectrometry (MS/MS) method was applied by setting the energy ramp for collision-induced dissociation at 15-40 eV. The fragmentation pattern resulting from each parental mass was identified using the mass spectrometry database provided online the Lipidomics Gateway (http://www.lipidmaps.org, National Institute of General Medical Sciences, National Institutes of Health).

Data analysis

Descriptive statistics of the concentrations of NEFA and β-hydroxybutyrate was performed using graphpad GraphPad Prism 6 (Graph Pad Software, La Jolla, CA, USA). The open source program MZmine (http://mzmine.sourceforge.net; Project GNU free software foundation, Boston, USA) was used for automatic alignment, denoising, deconvolution and extraction of peaks (Pluskal et al. 2010). All data were standardized using exogenous standards. The follow-up statistics were performed with the open source program R (R_Core_Team; R Foundation for Statistical Computing 2014) and GraphPad Prism 6.

Results

A total of 12 multiparous cows of the Holstein Frisian breed, kept in the same farm, were tested for changes in the blood plasma lipidome occurring during the peripartal transition phase. The selected cows were aged between 3 and 12 years (mean age: 5 ± 2.6 years) and undergoing their 2nd to 10th lactation (mean number of lactations 3 ± 2.3). The mean dry period lasted 64 ± 14.27 days. The animals were clinically healthy at the beginning of the study, but the histologic findings by means of liver biopsies taken four weeks after calving showed that only one animal remained completely devoid of lipid inclusions; 7 cows displayed a mild and 4 cows a moderate degree of lipid deposition in hepatocytes. Importantly, however, none of these animals developed clinical symptoms of fatty liver disease although several cows experienced other health problems after parturition including hypocalcemia (n = 7), retained placenta with subsequent metritis (n = 2), mastitis (n = 1), and lameness (n = 4). Accordingly, the overall body condition score of the 12 cows deteriorated slightly with progression of the transition period (Figure 1).

Clinical chemistry analysis

Blood samples were obtained ante partum (on days –14 and –7) on the day of calving and post partum (on days +7, +14, +21 and +28). The blood plasma concentration of NEFAs increased from low values before calving (for example 0.36 ± 0.19 mmol/l on day –7) to significantly higher values post partum, reaching a maximum at day +7 (0.77 ± 0.16 mmol/l). These NEFA concentrations remained at high levels until at least day +28 (Figure 2). The plasma concentrations of β-hydroxybutyrate displayed some variability among the individual cows. However, a trend of increasing β-hydroxybutyrate levels as the transition period progressed could be detected with significantly higher concentrations in the plasma post partum (for example 1814 ± 1407 µmol/l on day +28) compared to the samples taken ante partum (for example 632 ± 299 µmol/l on day –14) (Figure 3).

Plasma lipidome analysis

The lipid composition of plasma samples obtained at the different time points were compared by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. This analysis yielded over 500 masses detected in the positive ion mode, and over 200 masses in the negative ion mode, representing lipids and other non-polar metabolites. To illustrate the differences in the lipidome profile at different stages of the transition period, from 14 days

ante partum to 28 days post partum, the multivariate data were subjected to principal component analysis (PCA), which is an unsupervised clustering method that provides an overview of the results by reducing the dimensionality of complex findings. The principal components of the data acquired in the positive ion mode were graphically plotted as a score plot (Figure 4), thus revealing that the samples from each time point cluster together forming distinguishable groups. Instead, the masses acquired in the negative ion mode did not shown any such clustering (data not shown).

The data of 40 masses that best correlate with the principal component 1 (PC1), and collections of 20 masses that each correlate best with the principal components 2 and 3 (PC2 and PC3) were extracted. These 80 masses accounted for all the observed separation between the seven time points in the PCA score plot. Due to duplicate representations of the same masses in the different PCs, finally 62 masses were selected for further investigation. From these, 32 masses could be identified by tandem mass spectrometry (Table 1). The score plot was then replicated using the data of these 32 identified masses, yielding a closer clustering of the samples obtained from individual time points (Figure 5). An additional vector plot of the variables evidenced the particular contribution of each of the 32 identified masses to the overall separation between the different time points around parturition (Figure 6).

As the next step, the lipids responsible for this separation were characterized and quantified. In Figure 7, the chromatographic retention time windows of lipid classes are indicated to illustrate how their classification is simplified by the respective position in the elution profile (Ogiso, Suzuki, and Taguchi 2008). One class of lipids that discriminates between the different time points around parturition consisted of triacylglycerides (TGs), including TG 48:3, TG 48:1, TG 49:2, TG 49:1, TG 50:4, TG 50:3, TG 50:2, TG 51:3, TG 51:2, TG 51:1, TG 52:4, TG 52:3, TG 53:3, TG 54:6 and TG 56:6. All these TGs displayed high plasma levels in cows before calving but, on the day of parturition, their levels dropped instantaneously and remained low for up to 28 days post partum (Figures 8-10). Lyso-posphatidylcholine (LPC) and phosphatidylcholine (PC) levels, in contrast, increased progressively post partum. This increment was significant for LPC 16:0, LPC 18:3, LPC 18:2, LPC 18:1, LPC 20:5, PC P-34:2, PC P-36:5, PC P-36:4 and PC 36:6 (Figures 11 and 12). In addition, the sphingomyelines SM 39:1 and 43:3 were increased post partum in the same manner as described for phosphatidylcholines (Figure 13). It should be noted that not all identified phosphatidylcholines showed this same pattern of increased concentrations post partum compared to ante partum. In the case PC 38:1, PC 40:3 and PC 42:6, there was first a drop in plasma levels from day –14 to calving, followed by a slow recovery post partum

(Figure 14). For PC 34:0, we observed an exceptionally sharp increase at the day of calving followed by a progressive reduction to reach the starting level measured at the beginning of the study (Figure 14). Finally, we also identified the two fatty acid amides linoleamide and anandamide, whose plasma levels are transiently depressed only at the time of calving (Figure 15).

Discussion

During the transition from pregnancy to lactation, dairy cows experience a challenging period with increasing energy requirements for maintenance, fetal growth in late pregnancy, mammary tissue remodeling and early lactation (Herdt 2000; Esposito et al. 2014). Despite the enhanced nutritional demands, this period coincides with a reduction in dry matter intake (K L Ingvartsen and Andersen 2000). The resulting negative energy balance is considered a normal occurrence in dairy cows after calving (McArt et al. 2013), but only animals poorly adapting to these changing physiologic conditions are at risk of developing various production disorders.

Several potential biomarkers have already been evaluated for their ability to predict which cows are at risk of diseases related to the transition period. Most tested parameters reflect the negative energy balance and the rate of hepatic fatty acid oxidation, which can be demonstrated by measuring NEFAs and β-hydroxybutyrate, respectively. Increased concentrations of these metabolites ante partum have been associated with higher odds of displaced abomasum, poor lactation and reduced fertility (Chapinal et al. 2012; Ospina et al. 2010). However, there is an urgent need for a non-invasive and accurate test to diagnose early alterations in the fat metabolism leading to fatty liver disease. For that specific purpose, we used a large-scale and untargeted metabolomic approach, based on high-resolution quadrupole time-of-flight mass spectrometry, to analyze the physiologic lipidome of healthy dairy cows. Since metabolic profiles begin to change by the end of gestation (Holtenius et al. 2003; Bossaert et al. 2008), this investigation focused on a period of 6 weeks around parturition.

As expected (Ospina et al. 2010; McArt et al. 2013), the tested animals responded to their negative energy balance by mobilizing large amounts of body fat, leading to increased NEFA concentrations in plasma starting from the day of calving (Figure 2). Similarly, β-hydroxybutyrate levels in plasma increased post partum (Figure 3) as a marker of lipid catabolism. In parallel, the overall lipidome profile changed abruptly on the day of calving

with further intriguing adaptations post partum (Figure 5). Our large-scale approach demonstrates that two main lipid classes undergo significant shifts in their plasma concentration during the transition period of dairy cows, i.e., triacylglycerides and phospholipids. The 15 unequivocally identified triacylglycerides were all characterized by a sharply reduced plasma concentration on the day of calving and post partum compared to ante partum values (see for example Figure 8). This observation reflects the suddenly enhanced energy requirement and is in agreement with previous studies (Van den Top et al. 2005; Kessler et al. 2014). In contrast, the plasma concentration of the vast majority of the identified phospholipids (five lyso-phosphatidylcholines, four phosphathidylcholines and two sphingomyelins) increased steadily with progression of early lactation post partum (see for example Figure 12). A common feature of these phospholipids is their requirement for the secretion of hepatic triacylglycerides as VLDL particles, indicating that a reduced level of phosphatidylcholins and other phospholipids may directly cause an excessive accumulation of triacylglycerides in the liver (Artegoitia et al. 2014; Côté et al. 2014). Further studies are now required to confirm a causal relationship between reduced phospholipid concentrations and fatty liver disease in dairy cows. On that line, we recently reported that dairy cows affected by fatty liver disease during the transition period display lower levels of phosphatidylcholines and sphingomyelins in their blood plasma than control animals (Imhasly et al. 2014). Thus, the abnormal decline of certain specific phosphatidylcholines and sphingomyelins could be regarded as a promising biomarker indicative of fatty liver disease. Conversely, dietary supplementation of biochemical precursors of phosphatidylcholines and sphingomyelins may help to prevent the occurrence of fatty liver disease in dairy cows. Additionally, direct intravenous infusion of missing phosphatidylcholines and sphingomyelins should be tested as a new therapeutic strategy for the management of animals affected by severe-grade fatty liver disease.

Acknowledgements

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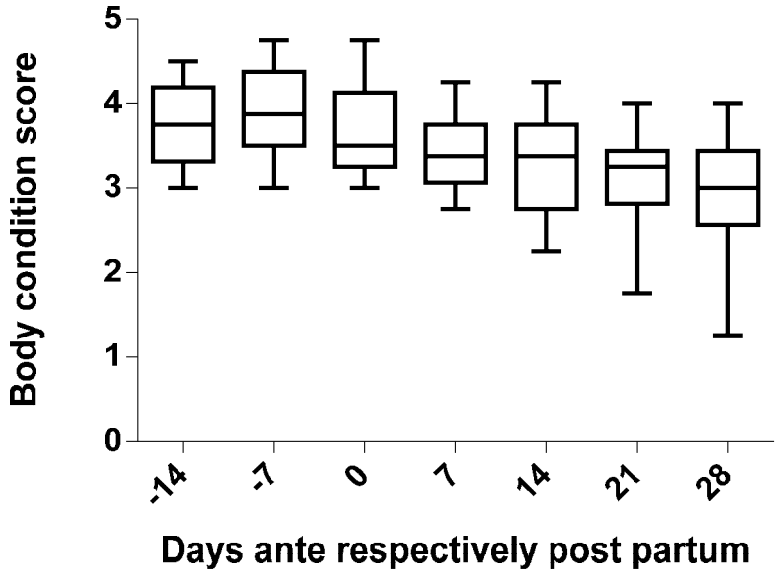


Figure 1: Clinical examination. History of the body condition score of the 12 cows over the experimental period (median values with range). The time point of clinical examination is indicated relative to the date of calving.

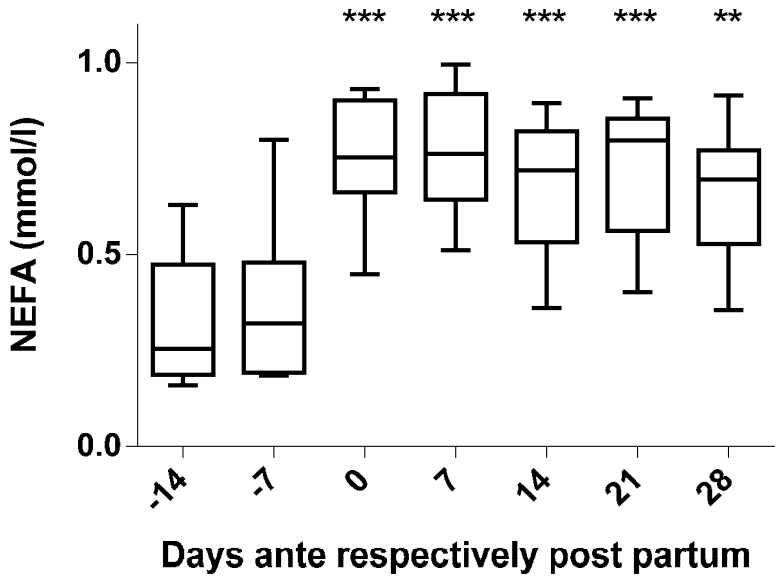
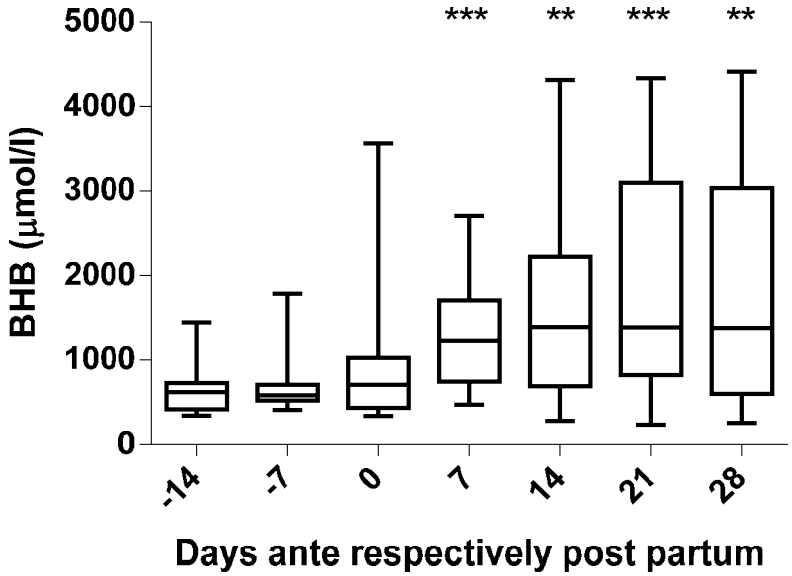


Figure 2: NEFA levels. History of NEFA concentrations in the plasma of the 12 cows over the experimental period (median values with range). The time points are indicated relative to the date of calving. ***P < 0.001 compared to NEFA concentrations at the beginning of the study (day –14).



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14 **Figure 3: Ketone bodies.** History of *β*-hydroxybutyrate (BHB) concentrations in the plasma
15 of the 12 cows over the experimental period (median values with range). The time points are
16 indicated relative to the date of calving. **P < 0.01 and ***P < 0.001 compared to *β*-
17 hydroxybutyrate concentrations at the beginning of the study (day –14).

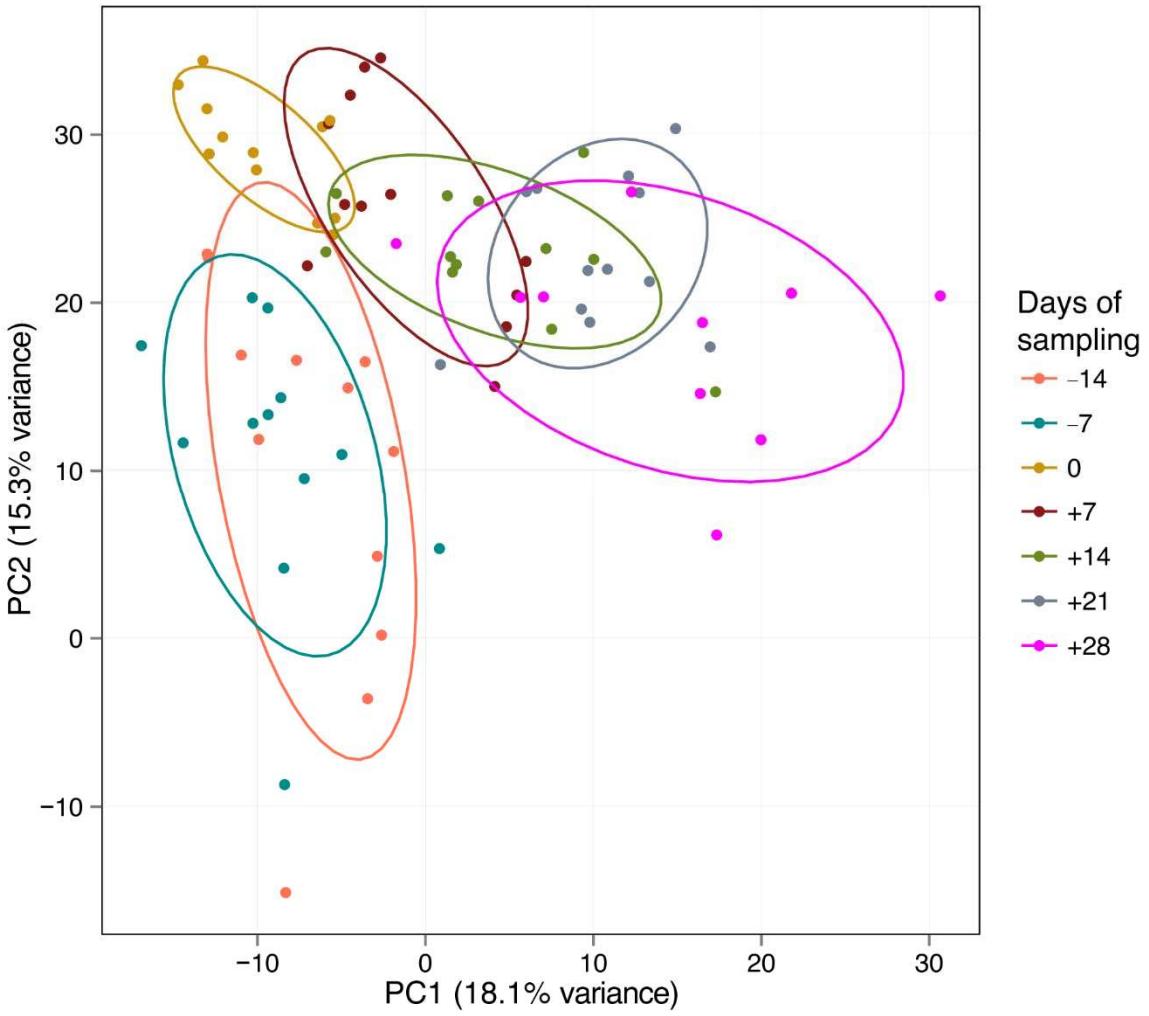


Figure 4: Principal component analysis. Principal components were determined with all 579 detected masses of the 82 measured samples and arranged in 7 groups according to the time of sampling relative to the date of calving (–14 days to +28 days).

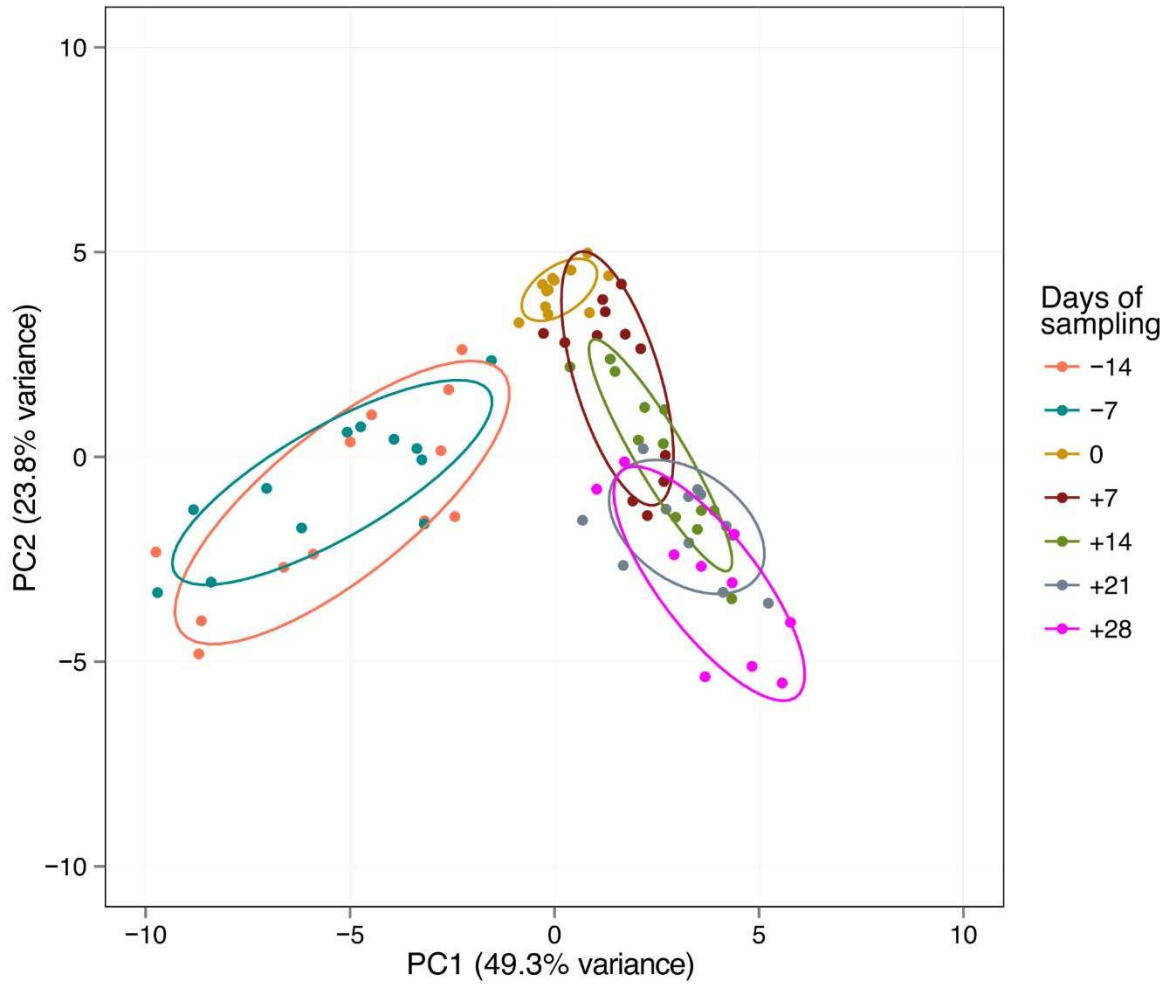
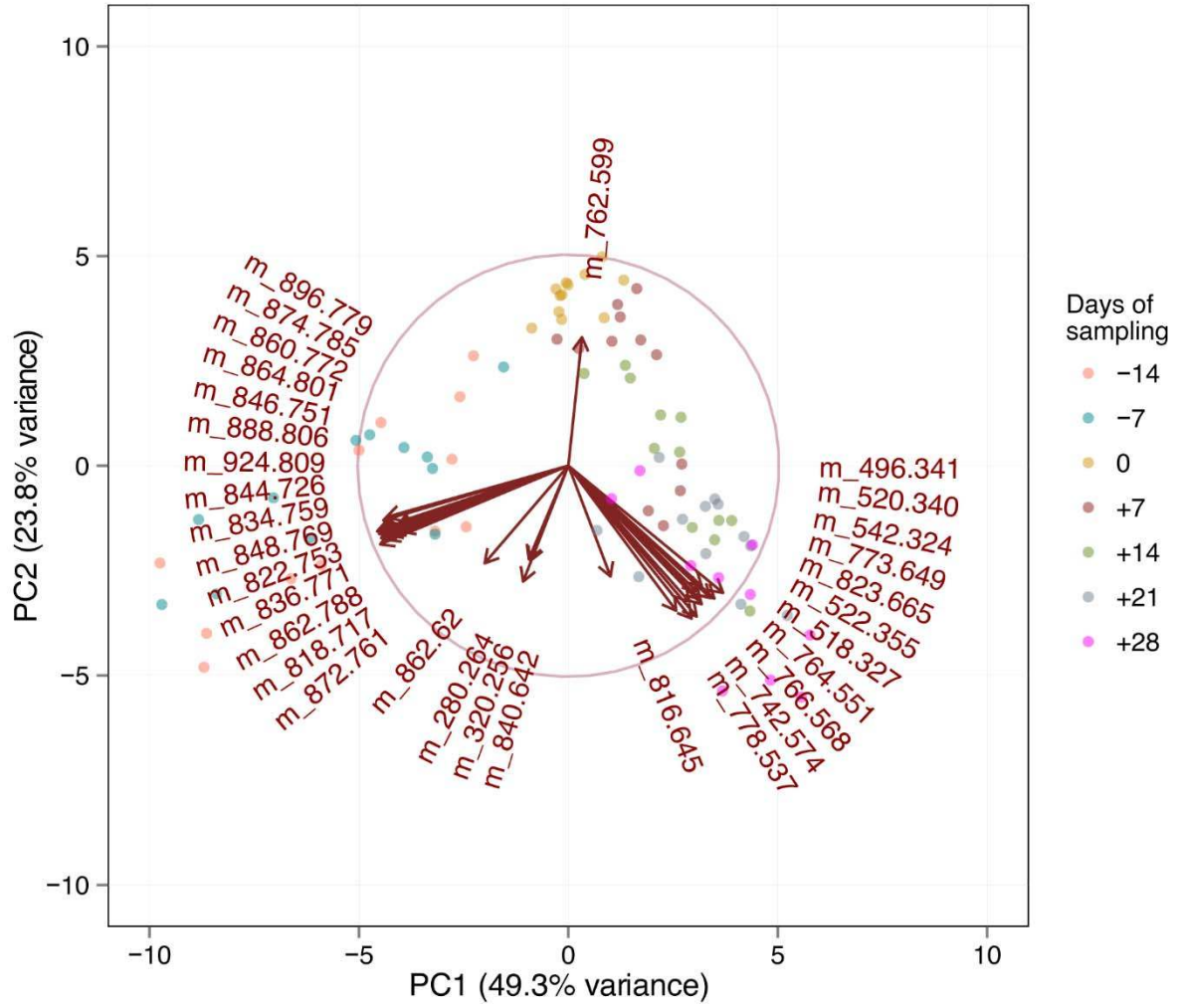


Figure 5: Principal component analysis. Principal components were re-calculated focusing on the 32 identified masses and arranged in 7 groups according to the time of sampling relative to the date of calving (–14 days to +28 days).



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32 **Figure 6: Plot of the variable factors.** Values were extracted from the principal component
33 analysis obtained with 32 identified masses. The time of sampling is indicated relative to the
34 date of calving (–14 days to +28 days).

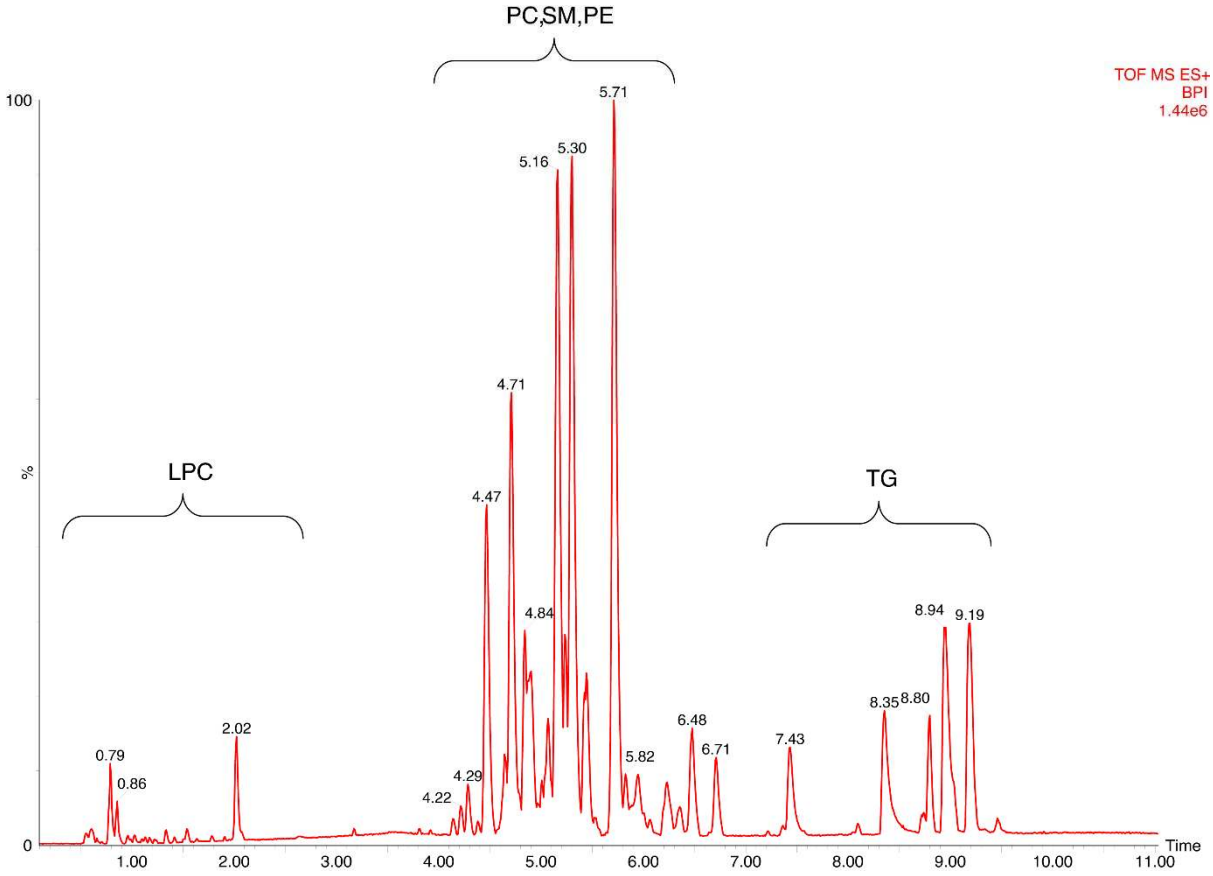


Figure 7: Ultra-pressure liquid chromatogram. This chromatographic profile of a representative sample demonstrates the distinct elution windows of different lipid classes identified by mass spectrometry. LPC, lyso-phosphatidylcholines; PC, phosphatidylcholines; SM, sphyngomyelins; PE, phosphoethanolamines; TG, triacylglycerides.

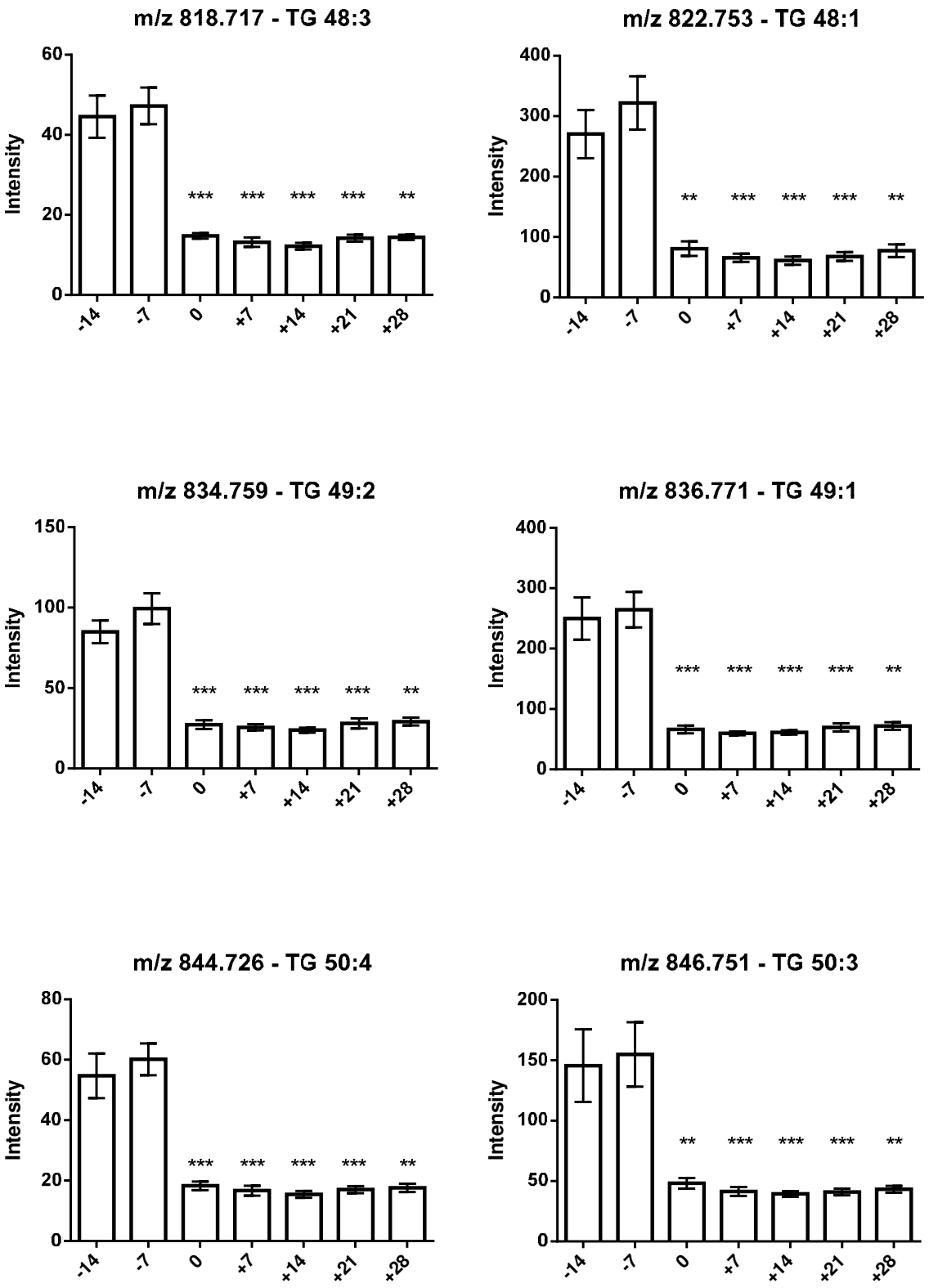


Figure 8: Plasma concentration of triacylglycerides (TGs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; **P < 0.01 and ***P < 0.001 compared to triacylglyceride concentrations at the beginning of the study (day –14).

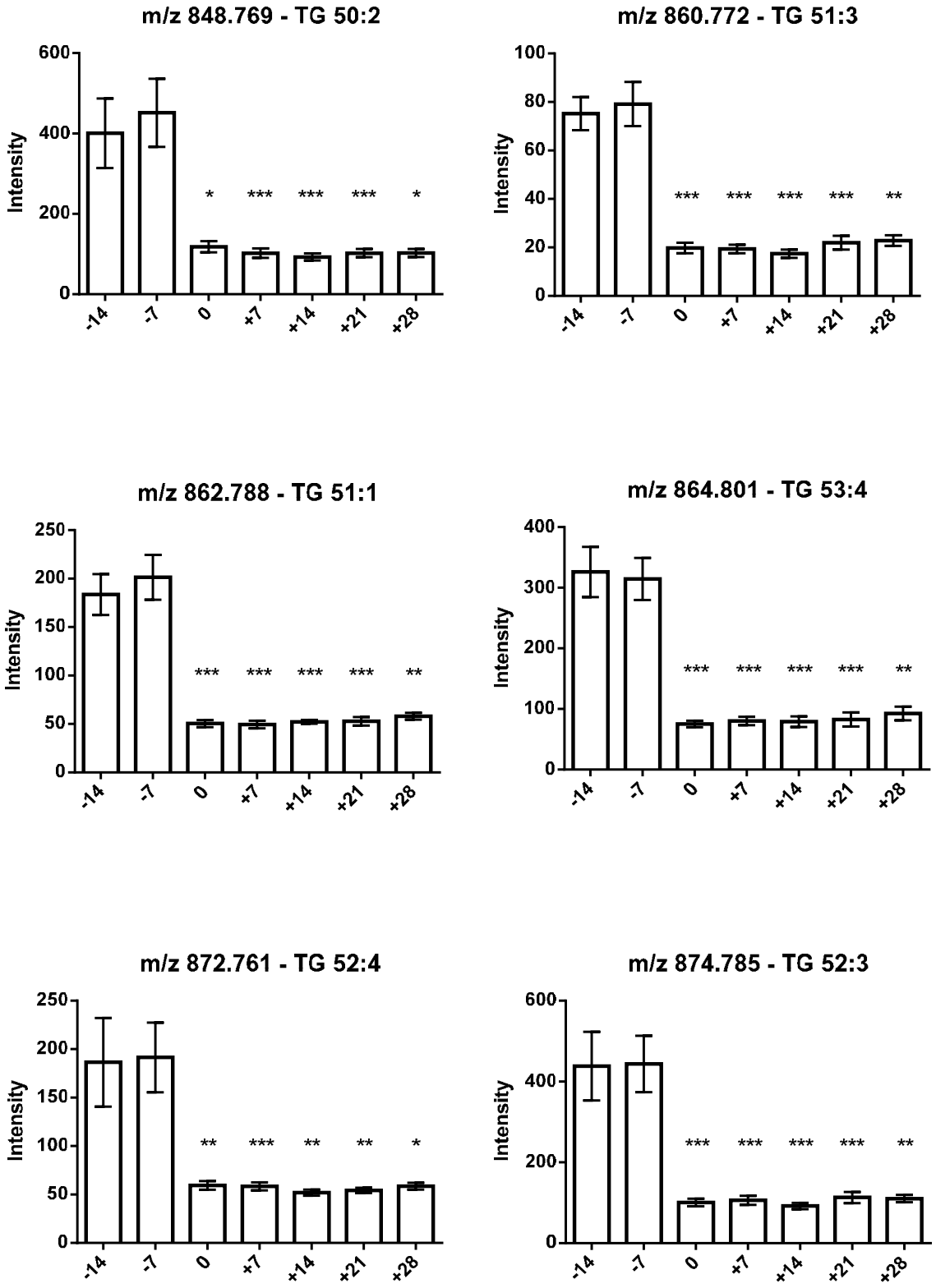


Figure 9: Plasma concentration of triacylglycerides (TGs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

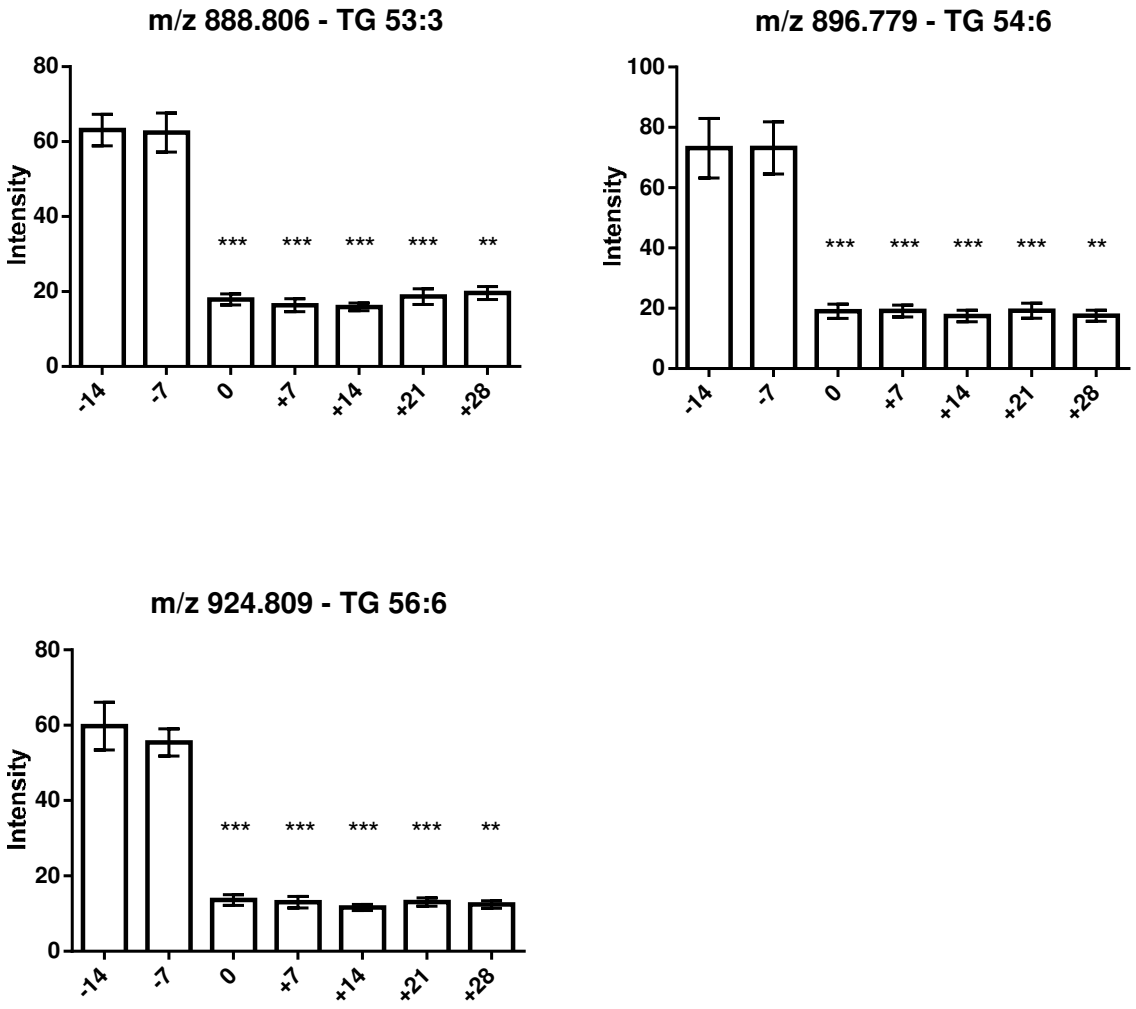


Figure 10: Plasma concentration of triacylglycerides (TGs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

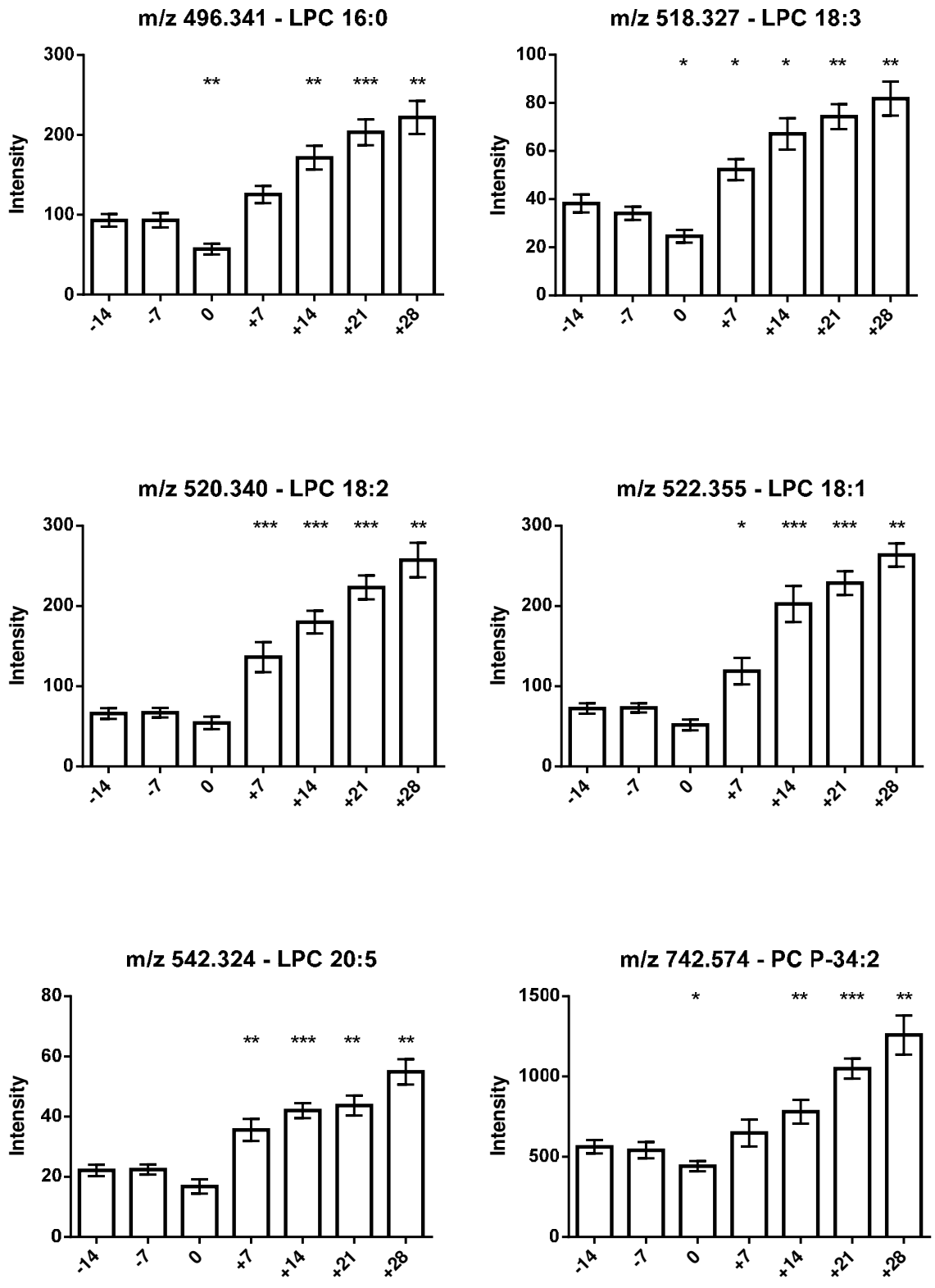


Figure 11: Plasma concentration of lyso-phosphatidylcholines LPCs and phosphatidylcholines (PCs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

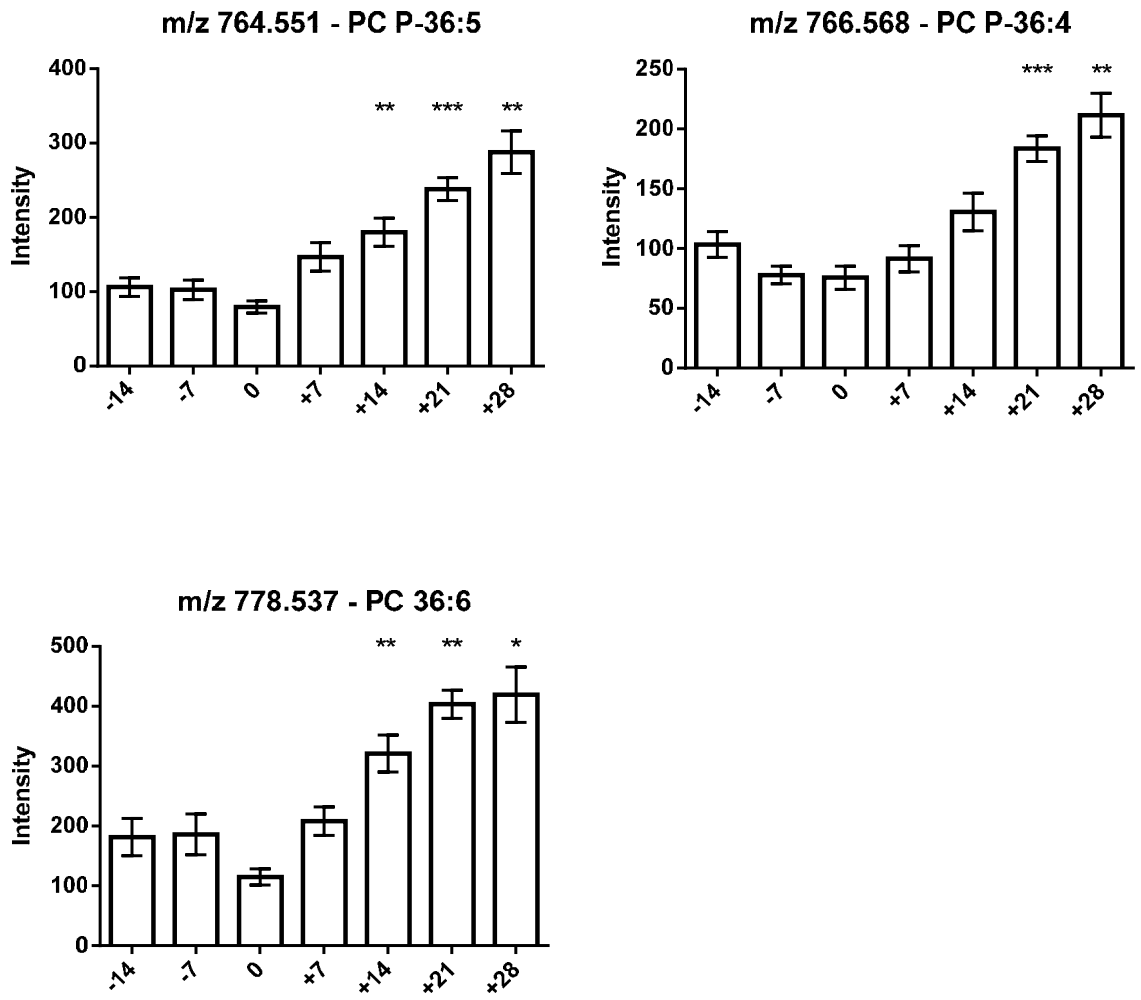


Figure 12: Plasma concentration of phosphatidylcholines (PCs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

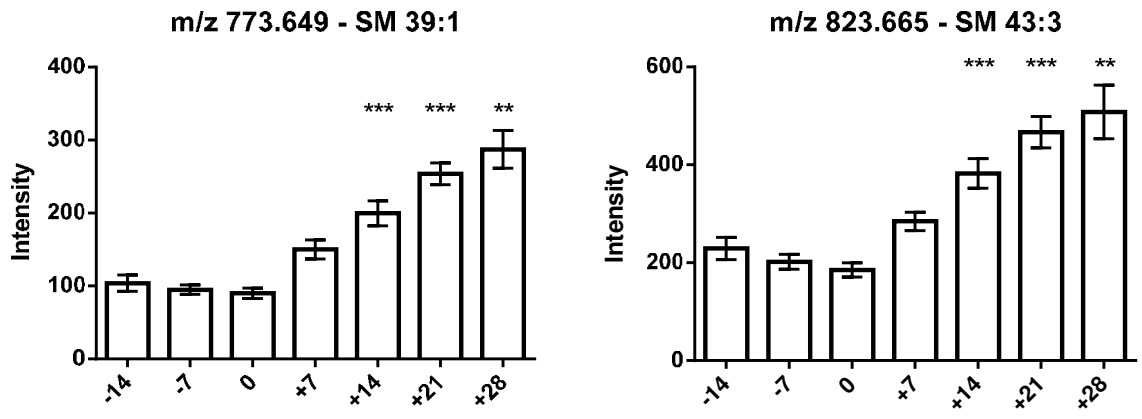


Figure 13: Plasma concentration of sphingomyelines (SMs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

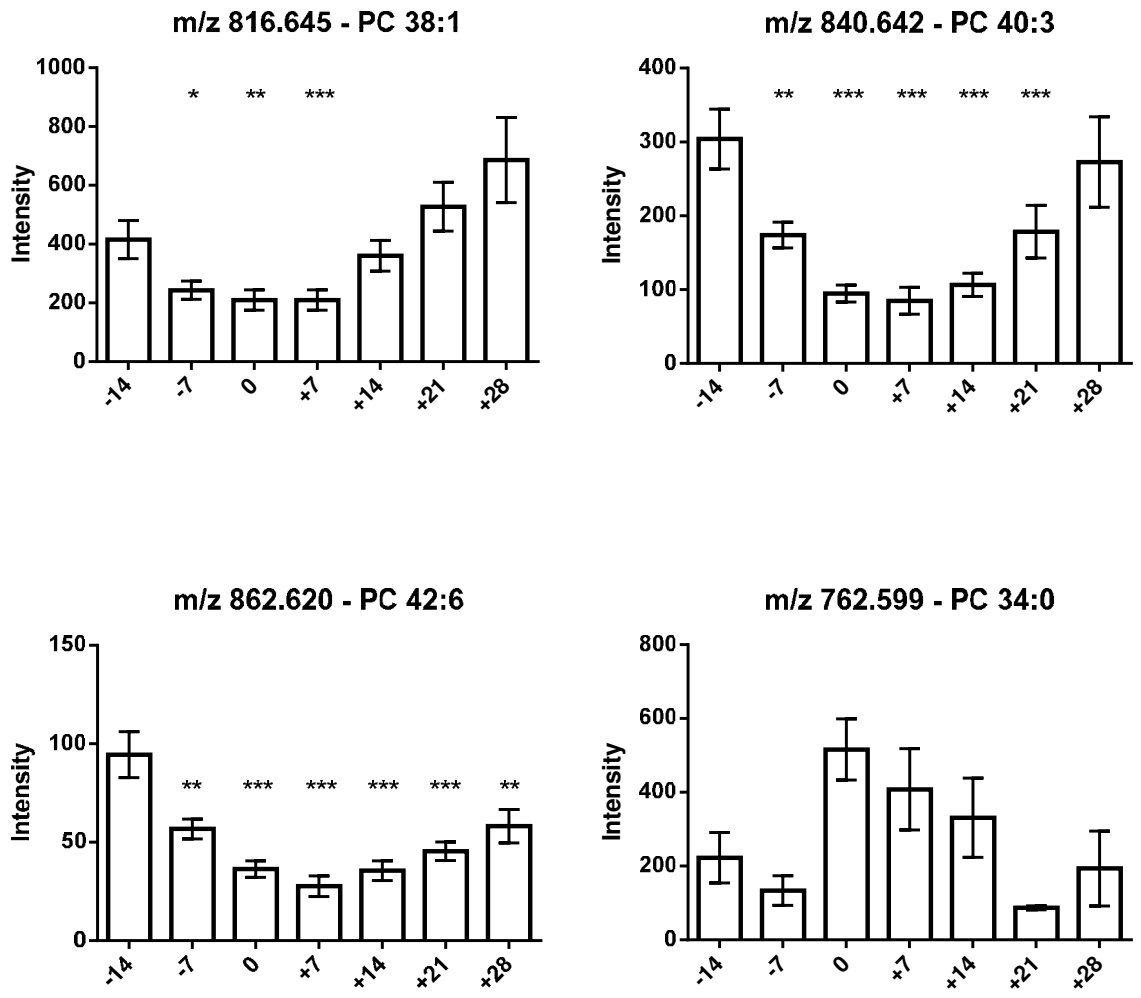
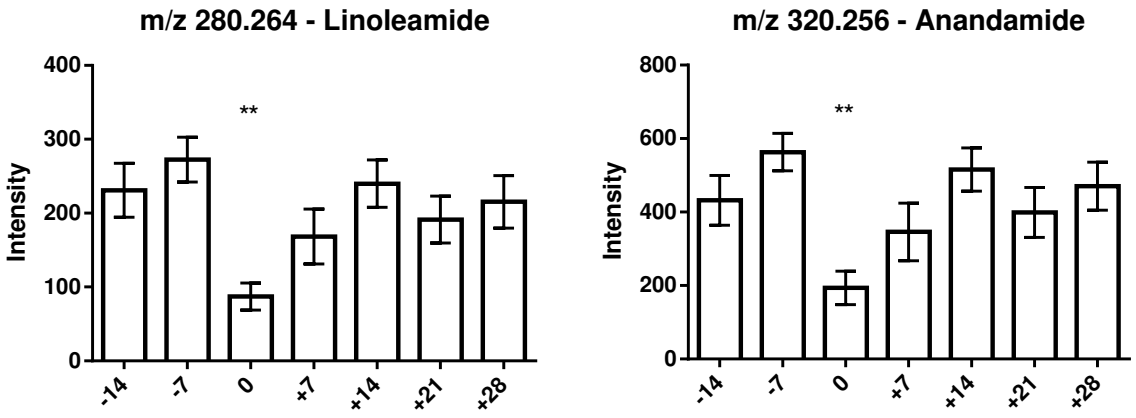


Figure 14: Plasma concentration of phosphatidylcholines (PCs) in the 12 transition period cows. The time of sampling is indicated relative to the date of calving. Mean values ± SEM; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).



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84 **Figure 15: Plasma concentration of fatty acid amides in the 12 transition period cows.** The
85 time of sampling is indicated relative to the date of calving. Mean values ± SEM;
86 **P < 0.01 and ***P < 0.001 compared to the values at the beginning of the study (day –14).

Identified masses

Class	Formula	m/z	Annotation	Ionization mode
lysoPC	$C_{24}H_{50}NO_7P$	496.341	LPC 16:0	$[M+H]^+$
	$C_{26}H_{50}NO_7P$	520.340	LPC 18:2	$[M+H]^+$
	$C_{26}H_{52}NO_7P$	522.355	LPC 18:1	$[M+H]^+$
	$C_{28}H_{48}NO_7P$	542.324	LPC 20:5	$[M+H]^+$
	$C_{26}H_{48}NO_7P$	518.324	LPC 18:3	$[M+H]^+$
PC	$C_{42}H_{80}NO_7P$	742.574	PC P-34:2	$[M+H]^+$
	$C_{42}H_{84}NO_8P$	762.599	PC 34:0	$[M+H]^+$
	$C_{44}H_{78}NO_7P$	764.551	PC P-36:5	$[M+H]^+$
	$C_{44}H_{80}NO_7P$	766.568	PC P-36:4	$[M+H]^+$
	$C_{44}H_{76}NO_8P$	778.537	PC 36:6	$[M+H]^+$
	$C_{46}H_{90}NO_8P$	816.645	PC 38:1	$[M+H]^+$
	$C_{48}H_{90}NO_8P$	840.642	PC 40:3	$[M+H]^+$
	$C_{50}H_{88}NO_8P$	862.620	PC 42:6	$[M+H]^+$
SM	$C_{48}H_{91}N_2O_6P$	773.649	SM 39:1	$[M+H]^+$
	$C_{48}H_{91}N_2O_6P$	823.665	SM 43:3	$[M+H]^+$
Fatty amides	$C_{18}H_{33}NO$	280.264	Linoleamide	$[M+H]^+$
	$C_{20}H_{33}NO_2$	320.256	Anandamide	$[M+H]^+$
TG	$C_{51}H_{92}O_6$	818.717	TG 48:3	$[M+NH4]^+$
	$C_{51}H_{96}O_6$	822.753	TG 48:1	$[M+NH4]^+$
	$C_{52}H_{96}O_6$	834.759	TG 49:2	$[M+NH4]^+$
	$C_{52}H_{98}O_6$	836.771	TG 49:1	$[M+NH4]^+$
	$C_{53}H_{94}O_6$	844.726	TG 50:4	$[M+NH4]^+$
	$C_{53}H_{96}O_6$	846.751	TG 50:3	$[M+NH4]^+$
	$C_{53}H_{98}O_6$	848.769	TG 50:2	$[M+NH4]^+$
	$C_{54}H_{98}O_6$	860.772	TG 51:3	$[M+NH4]^+$
	$C_{54}H_{100}O_6$	862.788	TG 51:2	$[M+NH4]^+$
	$C_{54}H_{102}O_6$	864.801	TG 51:1	$[M+NH4]^+$
	$C_{55}H_{98}O_6$	872.761	TG 52:4	$[M+NH4]^+$
	$C_{55}H_{100}O_6$	874.785	TG 52:3	$[M+NH4]^+$
	$C_{56}H_{102}O_6$	888.806	TG 53:3	$[M+NH4]^+$
	$C_{57}H_{98}O_6$	896.779	TG 54:6	$[M+NH4]^+$
	$C_{59}H_{102}O_6$	924.809	TG 56:6	$[M+NH4]^+$

Table 1: Identified masses by tandem mass spectrometry

5 Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows

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RESEARCH ARTICLE Open Access

Metabolomic biomarkers correlating with hepatic lipidosis in dairy cows

Sandro Imhasly[1], Hanspeter Naegeli[1*], Sven Baumann[2], Martin von Bergen[2,3,4], Andreas Luch[5], Harald Jungnickel[5], Sarah Potratz[5] and Christian Gerspach[6*]

Abstract

Background: Hepatic lipidosis or fatty liver disease is a major metabolic disorder of high-producing dairy cows that compromises animal performance and, hence, causes heavy economic losses worldwide. This syndrome, occurring during the critical transition from gestation to early lactation, leads to an impaired health status, decreased milk yield, reduced fertility and shortened lifetime. Because the prevailing clinical chemistry parameters indicate advanced liver damage independently of the underlying disease, currently, hepatic lipidosis can only be ascertained by liver biopsy. We hypothesized that the condition of fatty liver disease may be accompanied by an altered profile of endogenous metabolites in the blood of affected animals.

Results: To identify potential small-molecule biomarkers as a novel diagnostic alternative, the serum samples of diseased dairy cows were subjected to a targeted metabolomics screen by triple quadrupole mass spectrometry. A subsequent multivariate test involving principal component and linear discriminant analyses yielded 29 metabolites (amino acids, phosphatidylcholines and sphingomyelines) that, in conjunction, were able to distinguish between dairy cows with no hepatic lipidosis and those displaying different stages of the disorder.

Conclusions: This proof-of-concept study indicates that metabolomic profiles, including both amino acids and lipids, distinguish hepatic lipidosis from other peripartal disorders and, hence, provide a promising new tool for the diagnosis of hepatic lipidosis. By generating insights into the molecular pathogenesis of hepatic lipidosis, metabolomics studies may also facilitate the prevention of this syndrome.

Keywords: Biomarker, Fatty liver, Lipidosis, Metabolomics

Background

Hepatic lipidosis (also known as “fatty liver disease” or “fat cow syndrome”) is a common production problem of dairy cows occurring during the critical physiologic transition from pregnancy to lactation [1-3]. During the last decades, dairy cows have undergone an intense genetic selection to increase the milk yield, thereby reaching an enhanced performance level where the excessive demand for nutrients results in a severe energetic deficit at the onset of lactation [4-6]. A major adjustment to counteract this metabolic imbalance is the rapid mobilization of fat depots, thus providing non-esterified fatty acids as an energy source. Hepatic lipidosis develops when, during early lactation, the hepatic uptake of these non-esterified fatty acids and storage in the form of triacylglycerols exceeds their elimination [2,7]. The rate of triacylglycerol production in the liver tissue of ruminants is similar to that found in other species [8]. However, besides their use for energy production through mitochondrial breakdown by oxidation, triacylglycerols are released from hepatocytes as part of lipoproteins, whereby very low-density lipoproteins (VLDL) constitute the largest proportion. In ruminants, the secretion of VLDL from the liver is very limited compared with other species such that the resulting storage of excess lipids in hepatocytes leads to liver damage and depressed liver functions [8,9].

Hepatic lipidosis in cows is associated with ketosis, anorexia, reduced rumen motility, displaced abomasum, weight loss, predisposition to infections and diminished fertility [10,11], although the disease also occurs in a

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subclinical form affecting milk production and the long-term profitability of dairy farms. Indeed, previous reports suggest that this syndrome is a substantial problem for up to 50% of high-yielding cows [12,13] and, hence, hepatic lipidosis is recognized as a serious herd problem and animal welfare concern. Unfortunately, the diagnosis of hepatic lipidosis can only be confirmed by taking biopsies to determine the hepatic lipid content [8]. Biochemical abnormalities like increased liver enzymes and bilirubin concentrations in plasma correlate with advanced tissue damage but are not specific for hepatic lipidosis [14]. Thus, in view of the lack of a practically useful diagnostic tool, we explored an alternative strategy by screening for small-molecule biomarkers. The purpose of this study was to identify serum biomarkers that distinguish cows with hepatic lipidosis from those affected by other peripartal disorders.

Methods

Animal samples

Blood samples were taken from the jugular vein of Holstein-Friesian and Red-Holstein cows that contracted hepatic lipidosis or other diseases that typically occur during the peripartal period, including displaced abomasum, retained placenta or mastitis (Table 1). Plasma for clinical chemistry was prepared by supplementing blood samples with either heparin or EDTA. For metabolomic analyses, serum was collected and stored at −80°C. The diagnosis of hepatic lipidosis was obtained by liver biopsy and histologic inspection. The stage of disease was classified as described [15] according to the different extension of lipid deposition across the morphologic liver zones (periportal, transition zone and pericentral): group 1 (no lipid deposition), group 2 (only one zone affected), group 3 (all three zones affected), group 4 (Kupffer cells affected in addition to all three zones). Ethics approval was not requested and not needed because samples were taken from diseased animals during routine diagnostic investigations when hepatic lipidosis was suspected in peripartal dairy cows.

Clinical chemistry

Biochemical parameters were determined in plasma using the Cobas Integra 800 instrument (Roche Diagnostic, Rotkreuz, Switzerland). Aspartate aminotransferase (ASAT) was measured according to recommendations of the International Federation of Clinical Chemistry [16] with intra-assay and inter-assay coefficients of variance (CV) of 1.4% and 1.7%, respectively; γ-glutamyl transferase (GGT) was measured by a colorimetric assay [17] with intra-assay and inter-assay CV of 1.8%; further colorimetric assays [18,19] were employed for the determination of glutamate dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH) activities. For GLDH, the intra-assay and inter-assay CV were 0.8% and 1.2%, respectively; the intra-assay and inter-assay CV for SDH measurements were 3.9% and 3.2%. Bilirubin levels (intra-assay CV of 2.4%) were assessed by a diazo method [20]. Total protein (intra-assay CV and inter-assay CV of 6.6% and 11.5%) and fibrinogen levels (intra-assay CV of 11.1%) were measured by refractometry [21].

Metabolite quantification

The Absolute-IDQ platform (Kit p150, Biocrates, Innsbruck, Austria) was employed for targeted metabolite profiling as described by the manufacturer. This platform detects a total of 163 metabolites, including 14 amino acids, 41 acylcarnitines (Cx:y), hydroxylacylcarnitines [C (OH) x:y] and dicarboxylacylcarnitines (Cx:y-DC), the sum of hexoses, 15 sphingomyelins (SMx:y) and sphingomyelin derivatives [SM (OH) x:y], as well as 15 lyso-phosphatidylcholines and 77 phosphatidylcholines (PC). The latter were further differentiated with respect to the presence of ester ("a") and ether ("e") bonds in the glycerol moiety, whereby two letters "aa" (=diacyl) and "ae" (=acyl-alkyl) indicate that two glycerol positions are bound to a fatty acid residue, while a single letter "a" (=acyl) indicates the presence of a single fatty acid residue. The lipid side chain composition is abbreviated with "Cx:y", whereby "x" denotes the number of carbons in the side chain and "y" the number of double bonds. A detailed list of all analyzed metabolites is presented elsewhere [22].

The assay was performed on a double-filter 96-well plate containing stable isotope-labeled internal standards. All chemicals were from Sigma-Aldrich (Steinheim, Germany). Briefly, the serum samples (10 μl) were pipetted onto the upper filter spots of the 96-well plate and

Table 1 Periparturient dairy cows of the study, their disease, mean age (years) and lactation stage (weeks postpartum)

Group	N	Diseases (number of affected animals)	Age years ± S.D.	Lactation weeks ± S.D.
1	6	Displaced abomasum (3), bronchopneumonia (2), ileus (1)	7.2 ± 1.8	1.8 ± 0.7
2	10	Low grade hepatic lipidosis (10) and, in addition, displaced abomasum (3), bronchopneumonia (1), retained placenta (1), mastitis (1)	5.1 ± 1.7	2.9 ± 2.1
3	7	Medium grade hepatic lipidosis (7) and, in addition, displaced abomasum (2) and bronchopneumonia (1)	5.2 ± 2.4	2.3 ± 0.7
4	5	Severe hepatic lipidosis (5) and, in addition, displaced abomasum (2) and bronchopneumonia (1)	5.5 ± 0.8	4.2 ± 1.3

The 28 animals were from 27 different farms, i.e., only two cows of group 3 came from the same farm.

phenylisothiocyanate was added for derivatization of amino acids. Next, the samples were dried under a nitrogen stream, extracted with 5 mM ammonium acetate in methanol, centrifuged through the filter membranes and diluted with chromatographic solvent. Finally, the extracts were injected into the Agilent 1100 Series HPLC (operated by the Analyst 1.4.2 software) coupled to an API 4000 triple quadrupole mass spectrometer (ABSciex) through electrospray ionization. A standard flow injection with two 20-μl aliquots (one for the positive and one for the negative ion mode) was applied to all measurements. Quantification was achieved by multiple reaction monitoring (MRM) detection using the MetIQTM software package, which is an integral part of the AbsoluteIDQ kit. This method is in conformity with the proof of reproducibility outlined in the Guidance for Industry–Bioanalytical Method Validations issued by the FDA [23]. The analytical variability, in terms of intra-assay coefficient of variance (CV), was 7.3%. For statistical analyses, only metabolites were chosen for which all values exceeded the detection limit, thus restricting the profile to a total of 80 metabolites (5 amino acids, 62 phosphatidylcholines, 8 sphingomyelins and 5 sphingomyelin derivatives). These metabolites were: glutamine, glycine, phenylalanine, proline, serine, PC aa C24:0, PC aa C26:0, PC aa C28:1, PC aa C30:0, PC aa C30:2, PC aa C32:0, PC aa C32:1, PC aa C32:2, PC aa C32:3, PC aa C34:1, PC aa C34:2, PC aa C34:3,PC aa C34:4, PC aa C36:1, PC aa C36:2, PC aa C36:3, PC aa C36:4, PC aa C36:5, PC aa C36:6, PC aa C38:3, PC aa C38:4, PC aa C38:5, PC aa C38:6, PC aa C40:2, PC aa C40:3, PC aa C40:4, PC aa C40:5, PC aa C40:6, PC aa C42:1, PC aa C42:2, PC ae C30:1, PC ae C30:2, PC ae C32:1, PC ae C32:2, PC ae C34:0, PC ae C34:1, PC ae C34:2, PC ae C34:3, PC ae C36:0, PC ae C36:1, PC ae C36:2, PC ae C36:3, PC ae C36:5, PC ae C38:1, PC ae C38:2, PC ae C38:3, PC ae C38:4, PC ae C38:5, PC ae C38:6, PC ae C40:2, PC ae C40:3, PC ae C40:5, PC ae C40:6, PC ae C42:2, PC ae C42:3, lysoPC a C16:0, lysoPC a C16:1, lysoPC a C18:0, lysoPC a C18:1, lysoPC a C26:0, lysoPC a C28:0, lysoPC a C28:1, SM C16:0, SM C16:1, SM C18:0, SM C18:1, SM C24:0, SM C24:1, SM C26:0, SM C26:1, SM (OH) C14:1, SM (OH) C16:1, SM (OH) C22:1, SM (OH) C22:2 and SM (OH) C24:1.

The mass spectrometry data of this study were deposited in the PRIDE database using the mzML format (accession number 1-20130722-115242).

Statistics

A multivariate processing of metabolomics data was carried out using the statistical package SPSS + (version 12.0.2G). First, each sample was standardized to the mean of the control (set to 100%) and normalized using z-score values. Then, a MANOVA (multivariate analysis of variance) was used for compound selection [24,25]. All 29 variables that showed a significant group difference ($p \leq 0.006$) were selected for a principal component analysis-linear discriminant function model. The principal component analysis (without rotation) was performed to achieve data reduction and the resulting factors were used for a post-hoc linear discriminant analysis as described elsewhere [26] for group separation. This linear discriminant analysis model resulted in three factors accounting for 100% of the observed variance in the system. The linear functions for these three discriminant factors were: $F(x_1) = 1.45 \bullet VAR1 + 2.67 \bullet VAR2 - 0.99 \bullet VAR3 - 2.263 \bullet VAR4 + 1.11 \bullet VAR5 + 0.298 \bullet VAR6 + 1.15 \bullet VAR7 + 1.05 \bullet VAR8 - 0.94 \bullet VAR9 - 0.083 \bullet VAR10 - 1.08 \bullet VAR11$; $F(x_2) = -1.19 \bullet VAR1 + 0.59 \bullet VAR2 + 0.024 \bullet VAR3 - 0.774 \bullet VAR4 - 0.72 \bullet VAR5 + 0.18 \bullet VAR6 - 0.46 \bullet VAR7 + 0.71 \bullet VAR8 + 0.32 \bullet VAR9 + 0.42 \bullet VAR10 + 0.61 \bullet VAR11$; $F(x_3) = -0.113 \bullet VAR1 - 0.22 \bullet VAR2 + 0.57 \bullet VAR3 - 0.120 \bullet VAR4 - 0.11 \bullet VAR5 - 0.22 \bullet VAR6 + 0.57 \bullet VAR7 - 0.21 \bullet VAR8 + 0.41 \bullet VAR9 - 0.26 \bullet VAR10 + 0.64 \bullet VAR11$. The discriminant function $F(x_1)$ separates the fatty liver group 3 from control animals (group 1) as well as from group 2. $F(x_1)$ also discriminates between groups 1 and 2. The discriminant function $F(x_2)$ separates controls (group 1) from group 4, whereas $F(x_3)$ discriminates between groups 2 and 4. A detailed description of the equations for VAR1 to VAR11 is included [see Additional file 1]. By this method, all samples were classified correctly in the corresponding hepatic lipidosis groups defined by histopathological findings. The performance of this discriminant model was subsequently verified by applying the "leave-one-out" cross-validation formalism [27,28].

Results

A total of 28 diseased cows (from 27 different farms) were tested to identify metabolic biomarkers distinguishing hepatic lipidosis from other peripartal disorders. Following liver biopsy and histologic examination, the 28 early lactating cows were partitioned into 4 categories: group 1 (constituting the reference group of 6 animals displaying no hepatic lipidosis), group 2 (10 animals with low grade hepatic lipidosis), group 3 (7 animals with medium grade hepatic lipidosis) and group 4 (5 animals with severe hepatic lipidosis). The animals of group 1 were presented to the veterinary hospital because of displaced abomasum, bronchopneumonia or ileus. Some of these disorders, besides retained placenta or mastitis, were also encountered in the animals of groups 2–4 in addition to their different stages of hepatic lipidosis (Table 1).

Clinical chemistry analysis

In dairy cows, the excess storage of triacylglycerols in the liver causes progressive hepatocyte damage and, consequently, membrane leakage that results in the increased

release of liver enzymes and bile constituents into the blood [2,9]. However, clinical chemical parameters like aspartate aminotransferase (ASAT), γ-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH) and bilirubin levels in the blood, being non-specific biomarkers of organ injury, were already elevated in the reference group 1 of cows whose biopsies did not reveal typical features of hepatic lipidosis but that were admitted to the veterinary hospital for other disorders (Table 2). As a general trend, some of these conventional clinical chemistry values (GGT, GLDH and SDH) further increased from group 1 to group 4 (Figure 1), but without being able to discriminate between distinct disease etiologies. Conversely, we observed decreasing plasma fibrinogen concentrations correlating with the gradually enhanced severity of fatty liver disease in groups 2–4 relative to group 1 (Figure 2A), although the overall protein level remained in the normal range (Figure 2B). In summary, these selected clinical chemistry parameters fail to display specificity for the appearance of hepatic lipidosis and, hence, are not sufficient to confirm the diagnosis of this particular disease.

Metabolomic analysis

To expand the spectrum of biomarkers that accompany hepatic lipidosis in dairy cows, the AbsoluteIDQ system was applied in conjunction with triple quadrupole mass spectrometry to compare the serum metabolites of animals that did not show any lipid storage in liver cells (reference group 1) and the corresponding groups 2–4 displaying various stages of the syndrome. The AbsoluteIDQ metabolomic platform allows for the accurate identification and quantitative measurement of 163 endogenous metabolites from different compound classes, including amino acids, carbohydrates, carnitines, sphingolipids and phosphatidylcholines [29-31]. A multivariate statistical model revealed 29 metabolites, listed in Table 3, who could be used to separate the hepatic lipidosis groups 2 to 4 from the reference group 1. To graphically illustrate the clear separation between groups achieved by this set of metabolic endpoints, the data were subjected to principal component and linear discriminant analyses (Figure 3). Using the loading factors for linear discriminant function 1 outlined in the "Methods" section, we identified 6 phosphatidylcholines that contributed the most to the observed separation and could therefore be regarded as a promising predictive biomarkers of hepatic lipidosis: PC aa C30:2, PC aa C32:2, PC aa C36:3, PC aa C38:3, PC aa C36:4 and PC ae C36:2).

Two of the 29 discriminating metabolites turned out to be the amino acids glycine (Figure 4A) and glutamine (Figure 4B) with diminished blood levels in the diseased animals of groups 2–4 relative to group 1. However, the majority of metabolites able to discriminate between the reference group 1 and the different stages of fatty liver disease were phosphatidylcholines (PC), i.e. the diacylphosphatidylcholines PC aa C30:2, PC aa C32:2, PC aa C36:2, PC aa C36:3, PC aa C36:4, PC aa C38:3, PC aa C38:4, PC aa C38:6, PC aa C40:2, PC aa C40:3, PC aa C40:4 and PC aa C42:2, as well as the acyl-alkylphosphatidylcholines PC ae C34:1, PC ae C36:2, PC ae C36:3, PC ae C38:2, PC ae C38:3, PC ae C38:4, PC ae C40:2 and PC ae C40:3 (Table 3). Additional discriminating components in the tested sera included the sphingomyelins SM C18:0 (the only saturated lipid), SM C18:1, SM C24:1 and SM C26:1 as well as the hydroxysphingomyelines SM(OH) C22:1, SM (OH) C22:2 and SM (OH) C24:1. As exemplified by PC aa C40:3 in Figure 5A, generally these metabolites showed lower levels in cows with fatty liver disease (groups 2–4) than in the reference animals of group 1. Exceptions were the diacylphosphatidylcholines PC aa C30:2 and PC aa C32:2, for which higher levels could be measured in cows with hepatic lipidosis compared to the reference animals (Figures 5B and 5C). All lyso-phosphatidylcholines and the sum of hexoses did not show any significant difference between the four groups of early lactating cows.

Discussion

All cows of this study displayed increased serum levels of enzymes and bilirubin that are generally indicative of liver damage or injuries to other tissues like cardiac or skeletal muscle [21]. Therefore, these canonical clinical chemistry values were unable to distinguish between hepatic lipidosis and other peripartal disorders. A novel observation of this study is the reduction of the fibrinogen serum conten in periparturient cows suffering from hepatic lipidosis. As this fibrin precursor is produced in

Table 2 Key clinical chemistry values in the blood of the reference group 1 (without hepatic lipidosis)

Parameter	Normal range	Measured values in the reference animals (group 1)		
		Median	Mean	Standard error of the mean
ASAT	57-103 U/l	568 U/l	600 U/l	202 U/l
GGT	13-32 U/l	34 U/l	48 U/l	16 U/l
GLDH	4-18.2 U/l	73 U/l	164 U/l	86 U/l
SDH	4-7.4 U/l	19 U/l	57 U/l	33 U/l
Total bilirubin	1.5-2.9 μmol/l	14.4 μmol/l	18.6 μmol/l	7.0 μmol/l

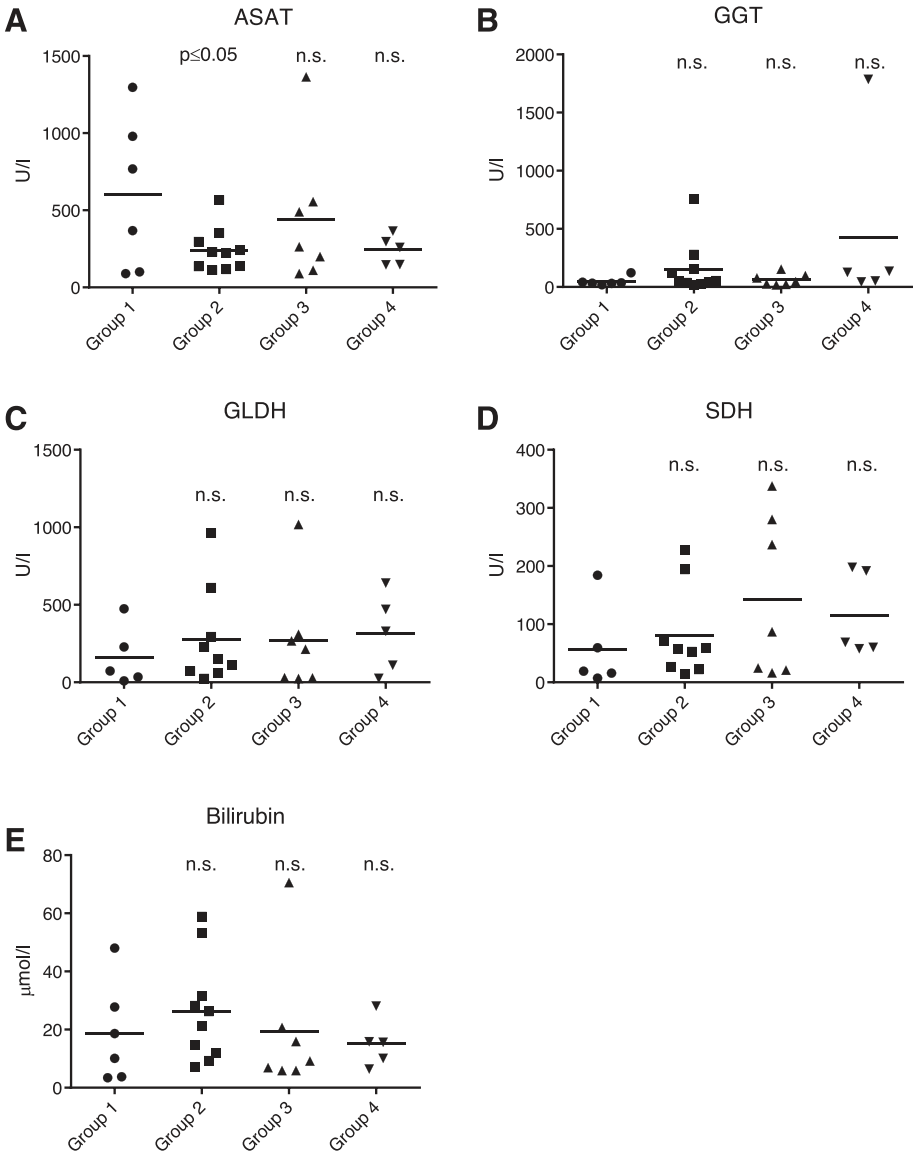

Figure 1 Comparison of clinical chemistry parameters between the reference animals of group 1 (without hepatic lipidosis) and groups 2–4 (with progressive stages of hepatic lipidosis). The parameters examined were **(A)** Aspartate aminotransferase (ASAT), **(B)** γ-glutamyl transferase (GGT), **(C)** glutamate dehydrogenase (GLDH), **(D)** Sorbitol dehydrogenase (SDH) and **(E)** Bilirubin. $P \leq 0.05$ indicates a significant reduction of ASAT activity in the animals of group 2 relative to group 1; n.s., not significant.

the liver, its lower level may represent a direct consequence of hepatocyte dysfunction due to excessive lipid deposition, but may also result from coagulopathy linked to liver disease [32]. In fact, hyperfibrinolysis leading to hypofibrinogenemia is known to arise from a poor hepatic clearance of tissue plasminogen activator or from reduced hepatic production of fibrinolysis inhibitors. However, there was no clinically apparent coagulopathy in the cows of the current study.

In view of the lack of specificity of canonical clinical chemistry values indicative of hepatic lipidosis, including the aforementioned fibrinogen levels, the purpose of our pilot study was to establish a targeted metabolomics platform to discover correlations between this disorder and molecular changes detectable in blood samples of periparturient dairy cows. Instead of comparing groups of healthy and diseased animals, we employed a more practical situation encountered in the veterinary hospital environment, where hepatic lipidosis has to be differentiated from other peripartal diseases. As a consequence, the reference group in our study consisted of animals whose clinical chemistry parameters revealed considerable injuries to the liver or other tissue damages (Figure 1). Nevertheless, a targeted metabolomics approach led to the

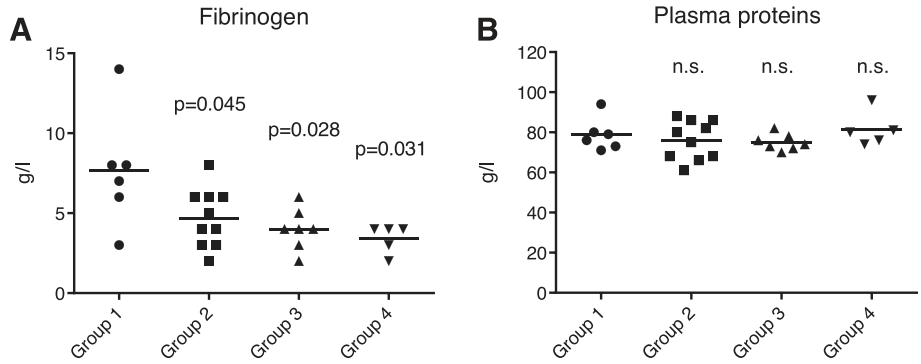

Figure 2 Level of fibrinogen in plasma and total plasma protein concentration in the different groups of dairy cows (mean values of 5–10 animals). (A) Plasma fibrinogen. **(B)** Total plasma protein. Group 1: no hepatic lipidosis; groups 2–4: progressive stages of hepatic lipidosis. The *P* values indicate significant differences with the reference group 1; n.s., not significant.

Table 3 List of metabolites with significant changes between the groups upon MANOVA (normalized mean values ± S.D.)

Metabolite	Category	Group 1	Group 2	Group 3	Group 4	*P* value
Glutamine	Amino acids	100 ± 28.7	50.1 ± 24.3	49.6 ± 13.4	30.8 ± 5.9	≤ 0.0004
Glycine		100 ± 21.9	66.6 ± 23.4	52.0 ± 11.8	49.0 ± 9.3	≤ 0.0004
PC aa C30:2	Phosphatidyl-cholines	100 ± 15.7	87.7 ± 24.1	118.4 ± 32.9	139.0 ± 16.2	0.004
PC aa C32:2		100 ± 17.3	81.7 ± 38.4	121.0 ± 62.0	159.0 ± 39.7	0.002
PC aa C36:2		100 ± 15.0	66.2 ± 33.8	52.8 ± 13.9	40.8 ± 10.8	0.002
PC aa C36:3		100 ± 15.2	58.4 ± 20.7	50.5 ± 14.4	50.0 ± 13.0	≤ 0.0004
PC aa C36:4		100 ± 11.5	60.8 ± 21.7	50.2 ± 13.4	51.6 ± 14.4	≤ 0.0004
PC aa C38:3		100 ± 14.5	58.3 ± 20.7	49.1 ± 14.7	45.9 ± 10.3	≤ 0.0004
PC aa C38:4		100 ± 11.7	70.7 ± 22.2	55.3 ± 21.0	59.5 ± 14.9	0.002
PC aa C38:6		100 ± 15.5	70.1 ± 16.0	79.1 ± 12.4	78.5 ± 12.7	0.006
PC aa C40:2		100 ± 17.7	70.6 ± 39.5	51.0 ± 21.4	34.1 ± 12.0	0.004
PC aa C40:3		100 ± 18.3	45.0 ± 18.4	40.1 ± 12.6	32.3 ± 6.9	≤ 0.0004
PC aa C40:4		100 ± 10.6	68.0 ± 26.6	55.1 ± 17.5	49.2 ± 10.2	0.001
PC aa C42:2		100 ± 11.2	76.4 ± 35.9	64.7 ± 25.9	36.6 ± 9.4	0.005
PC ae C34:1		100 ± 12.7	71.4 ± 23.9	62.0 ± 10.3	58.1 ± 10.6	0.001
PC ae C36:2		100 ± 15.8	63.7 ± 24.5	58.6 ± 10.6	55.7 ± 12.1	0.001
PC ae C36:3		100 ± 17.8	61.3 ± 14.3	58.3 ± 12.8	60.8 ± 8.8	≤ 0.0004
PC ae C38:2		100 ± 14.8	73.9 ± 31.8	54.3 ± 16.6	42.6 ± 7.5	0.001
PC ae C38:3		100 ± 11.7	56.9 ± 18.2	50.0 ± 15.8	41.3 ± 8.0	≤ 0.0004
PC ae C38:4		100 ± 17.8	67.4 ± 14.1	68.8 ± 17.1	63.7 ± 14.2	0.002
PC ae C40:2		100 ± 7.6	62.5 ± 17.4	62.9 ± 9.9	57.1 ± 12.6	≤ 0.0004
PC ae C40:3		100 ± 11.5	61.7 ± 13.2	65.1 ± 10.3	75.2 ± 10.2	≤ 0.0004
SM C18:0	Sphingomyelins	100 ± 22.4	67.2 ± 34	49.2 ± 48.6	14.2 ± 20.5	0.003
SM C18:1		100 ± 21.9	66.9 ± 33.3	53.5 ± 20.2	34.0 ± 10.2	0.002
SM C24:1		100 ± 13.7	52.2 ± 16.5	62.3 ± 15.5	47.8 ± 9.7	≤ 0.0004
SM C26:1		100 ± 14.8	45.9 ± 16.3	57.8 ± 12.9	42.2 ± 7.9	≤ 0.0004
SM (OH) C22:1	Hydroxy-sphingomyelins	100 ± 13.0	52.1 ± 18.1	68.8 ± 16.6	67.7 ± 12.8	≤ 0.0004
SM (OH) C22:2		100 ± 10.6	48.0 ± 15.4	56.5 ± 15.9	55.6 ± 11.9	≤ 0.0004
SM (OH) C24:1		100 ± 6.0	56.3 ± 21.8	74.2 ± 15.2	58.0 ± 13.9	≤ 0.0004

Group 1, without hepatic lipidosis; groups 2–4, progressive stages of hepatic lipidosis.

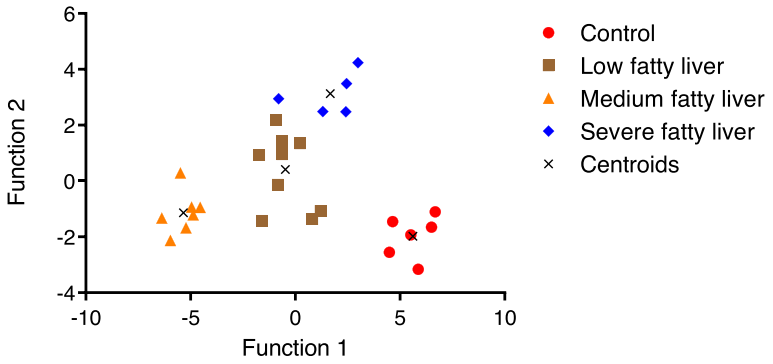

Figure 3 Combined principal component and linear discriminant analysis. This model reveals that 29 endogenous metabolites, in conjunction, distinguish dairy cows with different stages of hepatic lipidosis (groups 2–4) from reference animals (group 1) lacking the characteristic lipid deposition in their liver. The centroid, or center of mass, were computed from the coordinates of all points in each group. The linear functions for the two main discriminant factors [F (x_1) and F (x_2)] are laid down in the "Methods" section.

identification of an endogenous molecular profile that appears to be specific for hepatic lipidosis, thus distinguishing this disease from other peripartal problems. Two of the 29 discriminating metabolites were the amino acids glycine and glutamine that have already been linked to metabolic disorders and chronic inflammatory conditions [33-37], which are both key hallmarks of hepatic lipidosis. From the discriminant function coefficients of the variables we found, however, that the following six phosphatidylcholines contributed the most to the observed separation and could therefore be regarded as a potential set of predictive biomarkers of hepatic lipidosis: PC aa C30:2 (Figure 5B), PC aa C32:2 (Figure 5C), PC aa C36:3, PC aa C38:3, PC aa C36:4 and PC ae C36:2. Although changes of phosphatidylcholine and sphingomyelin blood levels have previously been associated with metabolic disorders [38] or chronic liver diseases [39], the possible link of the listed phosphatidylcholines with hepatic lipidosis is intriguing. Because they constitute an important precursor for the synthesis of hepatic triacylglycerols [40], phosphatidylcholines may be reduced in peripartal dairy cows as a direct consequence of an enhanced triacylglycerol production [2,7,8]. Conversely, hepatic phosphatidylcholines are required for the assembly and secretion of VLDL implying that a reduced phosphatidylcholine content may aggravate the accumulation of triacylglycerols in the liver by limiting their export from hepatocytes [41]. In our study, the observed changes of lipid levels involve an increase of those phosphatidylcholines that carry relatively short fatty acid moieties (PC aa C30:2 and PC aa C32:2), whereas the quantity of phosphatidylcholines containing larger fatty acid components (≥34 carbons) was reduced. This shift in phosphatidylcholine composition suggests a possible prophylactic or therapeutic approach based on the modulation of phosphatidylcholine biosynthesis by appropriate feed supplementation [42]. In any case, the observed shift in phosphatidylcholine distribution is a novel finding that needs to be further investigated mechanistically to understand if it has a causal relationship with the disease or rather constitutes a consequence thereof.

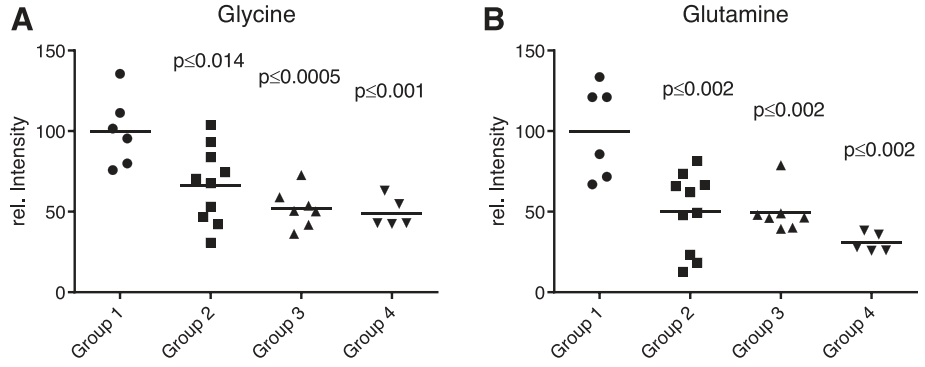

Figure 4 Typical examples of metabolites whose concentrations decreased with progressing hepatic lipidosis. Group 1: no hepatic lipidosis; groups 2–4: different stages of hepatic lipidosis (mean values of 5–10 animals). **(A)** Level of glycine in the serum of dairy cows. **(B)** Level of glutamine in the serum of dairy cows. The *P* values indicate significant differences with the reference group 1.

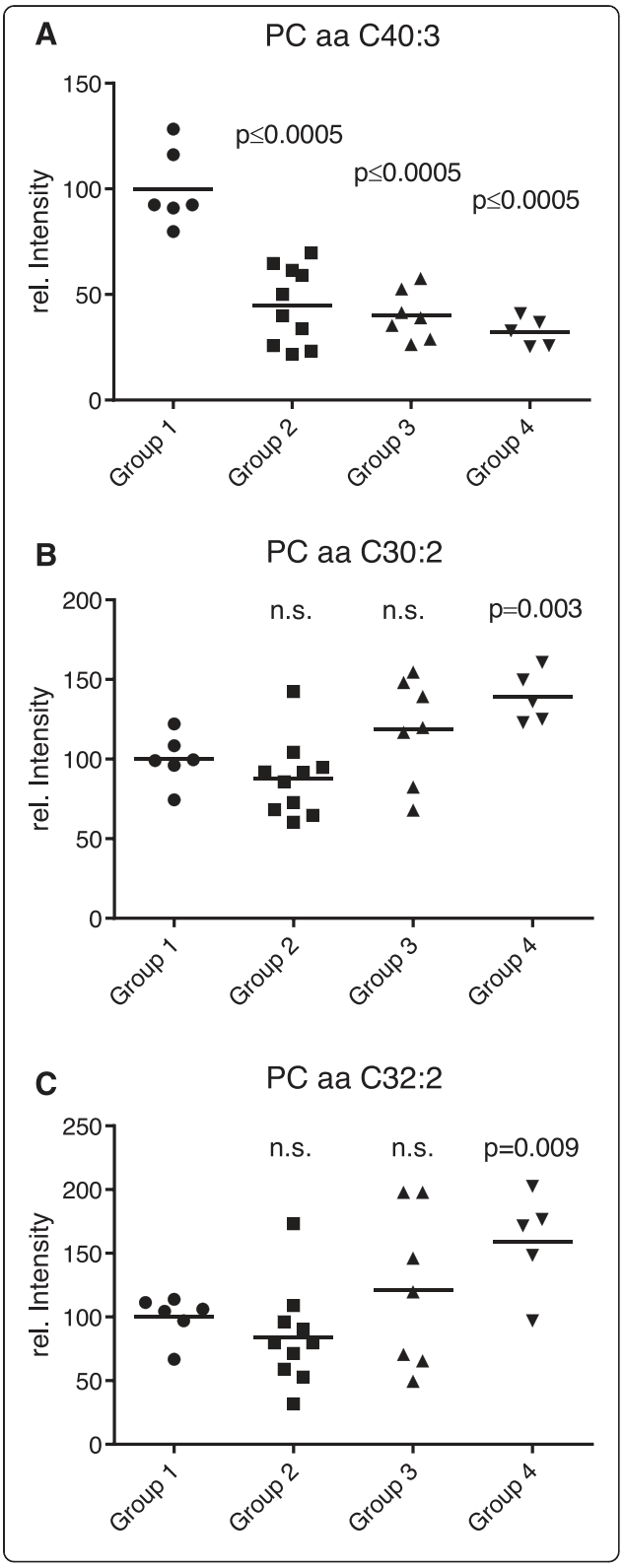

Figure 5 Serum concentration of diacyl-phosphatidylcholines (mean values of 5–10 animals). (A) Example of metabolite (PC aa C40:3) whose concentration decreased with progressing hepatic lipidosis. Group 1: no hepatic lipidosis; groups 2–4: different stages of hepatic lipidosis. **(B)** and **(C)** Diacyl-phosphatidylcholines whose serum concentrations increased with progressing hepatic lipidosis. The *P* values indicate significant differences with the reference group 1; n.s., not significant.

Conclusion

New biomarkers of hepatic lipidosis are urgently needed to facilitate diagnostic procedures, identify distinct stages of the disease, monitor the response to treatment regimens and allow for the design of prevention strategies. An important new paradigm in biomarker discovery research is to consider entire sets of molecular changes, instead of single parameters, that correlate with a particular disease [43]. In the present study, we have exploited the fact that serum metabolite concentrations provide a direct readout of disturbed biochemical pathways [29]. This approach led us to employ multivariate statistics, based on 29 key metabolites, to recognize deranged metabolic patterns that correlate specifically with distinct stages of hepatic lipidosis in dairy cows, thus distinguishing this disease from other peripartal disorders. Future studies with larger animal groups are needed to confirm the findings of this study, to validate the newly identified metabolic profile and explore its clinical application to the diagnosis and treatment of diseased animals.

Additional file

Additional file 1: Equations for VAR1 to VAR11.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SI was involved in the metabolomics measurements and statistical analysis, HN, CG and AL initiated and organized the study, SB, MvB, HJ and SP participated in the metabolomics analysis and statistical evaluation, HJ and HN wrote the manuscript that was subsequently reviewed by all co-authors. All authors read and approved the final manuscript.

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Additional information

Equations for variables VAR1 to VAR11 (see main text for abbreviation of the different metabolites):

VAR1 = 0.039 • Gln + 0.037 • Gly + 0.042 • PC aa C36:3 + 0.042 • PC aa C36:4 + 0.042 • PC aa C38:3 + 0.041 • PC aa C40:3 + 0.038 • PC ae C36:3 + 0.041 • PC ae C38:3 + 0.41 • PC ae C40:2 + 0.031 • PC ae C40:3 + 0.30 • SM (OH) C22:1 + 0.036 • SM(OH) C22:2 + 0.035 • SM(OH) C24:1 + 0.039 • SM C24:1 + 0.037 • SM C26:1 + 0.039 • PC aa C40:4 + 0.042 • PC ae C34:1 + 0.042 • PC ae C36:2 + 0.040 • PC ae C38:2 – 0.008 • PC aa C32:2 + 0.041 • PC aa C36:2 + 0.038 • PC aa C38:4 + 0.039 • PC ae C38:4 + 0.039 • SM C18:1 + 0.030 • SM C18:0 – 0.009 • PC aa C30:2 + 0.037 • PC aa C40:2 + 0.034 • PC aa C42:2 + 0.30 • PC aa C38:6

VAR2 = 0.011 • Gln – 0.041 • Gly + 0.023 • PC aa C36:3 + 0.013 • PC aa C36:4 + 0.006 • PC aa C38:3 + 0.021 • PC aa C40:3 + 0.070 • PC ae C36:3 + 0.015 • PC ae C38:3 + 0.43 • PC ae C40:2 + 0.114 • PC ae C40:3 + 0.132 • SM (OH) C22:1 + 0.105 • SM(OH) C22:2 + 0.087 • SM(OH) C24:1 + 0.078 • SM C24:1 + 0.088 • SM C26:1 – 0.047 • PC aa C40:4 – 0.008 • PC ae C34:1 + 0.020 • PC ae C36:2 – 0.076 • PC ae C38:2 + 0.190 • PC aa C32:2 – 0.045 • PC aa C36:2 – 0.011 • PC aa C38:4 + 0.035 • PC ae C38:4 – 0.066 • SM C18:1 – 0.093 • SM C18:0 + 0.193 • PC aa C30:2 – 0.080 • PC aa C40:2 – 0.068 • PC aa C42:2 + 0.115 • PC aa C38:6

VAR3 = 0.046 • Gln + 0.048 • Gly – 0.078 • PC aa C36:3 – 0.060 • PC aa C36:4 – 0.093 • PC aa C38:3 – 0.137 • PC aa C40:3 – 0.058 • PC ae C36:3 – 0.154 • PC ae C38:3 + 0.019 • PC ae C40:2 – 0.131 • PC ae C40:3 – 0.107 • SM (OH) C22:1 – 0.191 • SM(OH) C22:2 + 0.022 • SM(OH) C24:1 + 0.032 • SM C24:1 – 0.138 • SM C26:1 + 0.001 • PC aa C40:4 + 0.136 • PC ae C34:1 + 0.078 • PC ae C36:2 + 0.086 • PC ae C38:2 + 0.399 • PC aa C32:2 + 0.132 • PC aa C36:2 - 0.129 • PC aa C38:4 – 0.108 • PC ae C38:4 – 0.004 • SM C18:1 – 0.282 • SM C18:0 + 0.303 • PC aa C30:2 + 0.221 • PC aa C40:2 + 0.312 • PC aa C42:2 + 0.152 • PC aa C38:6

VAR4 = – 0.695 • Gln – 0.890 • Gly + 0.085 • PC aa C36:3 + 0.078 • PC aa C36:4 + 0.031 • PC aa C38:3 – 0.237 • PC aa C40:3 + 0.010 • PC ae C36:3 + 0.145 • PC ae C38:3 + 0.068 • PC ae C40:2 – 0.465 • PC ae C40:3 + 0.254 • SM (OH) C22:1 + 0.132 • SM(OH) C22:2 + 0.136 • SM(OH) C24:1 – 0.057 • SM C24:1 – 0.086 • SM C26:1 + 0.114 • PC aa C40:4 + 0.105 • PC ae C34:1 + 0.127 • PC ae C36:2 + 0.050 • PC ae C38:2 – 0.059 • PC aa C32:2 +

0.046 • PC aa C36:2 + 0.260 • PC aa C38:4 – 0.121 • PC ae C38:4 + 0.097 • SM C18:1 + 0.361 • SM C18:0 + 0.247 • PC aa C30:2 + 0.185 • PC aa C40:2 – 0.003 • PC aa C42:2 + 0.255 • PC aa C38:6

VAR5 = 0.114 • Gln + 0.191 • Gly + 0.025 • PC aa C36:3 – 0.004 • PC aa C36:4 + 0.051 • PC aa C38:3 – 0.046 • PC aa C40:3 + 0.197 • PC ae C36:3 – 0.132 • PC ae C38:3 – 0.309 • PC ae C40:2 – 0.726 • PC ae C40:3 – 0.006 • SM (OH) C22:1 – 0.020 • SM(OH) C22:2 + 0.003 • SM(OH) C24:1 + 0.084 • SM C24:1 – 0.080 • SM C26:1 – 0.312 • PC aa C40:4 – 0.068 • PC ae C34:1 – 0.027 • PC ae C36:2 – 0.035 • PC ae C38:2 + 0.405 • PC aa C32:2 + 0.020 • PC aa C36:2 + 0.242 • PC aa C38:4 + 1.120 • PC ae C38:4 + 0.273 • SM C18:1 + 0.469 • SM C18:0 + 0.545 • PC aa C30:2 – 0.133 • PC aa C40:2 – 0.058 • PC aa C42:2 – 0.831 • PC aa C38:6

VAR6 = – 0.357 • Gln + 0.495 • Gly – 0.244 • PC aa C36:3 + 0.061 • PC aa C36:4 – 0.101 • PC aa C38:3 – 0.091 • PC aa C40:3 – 0.591 • PC ae C36:3 – 0.611 • PC ae C38:3 – 0.270 • PC ae C40:2 + 0.647 • PC ae C40:3 + 0.612 • SM (OH) C22:1 + 0.003 • SM(OH) C22:2 + 0.253 • SM(OH) C24:1 – 0.304 • SM C24:1 – 0.368 • SM C26:1 + 0.745 • PC aa C40:4 – 0.063 • PC ae C34:1 – 0.232 • PC ae C36:2 – 0.192 • PC ae C38:2 – 0.174 • PC aa C32:2 – 0.106 • PC aa C36:2 + 0.170 • PC aa C38:4 + 0.199 • PC ae C38:4 + 0.436 • SM C18:1 + 0.139 • SM C18:0 + 0.486 • PC aa C30:2 + 0.190 • PC aa C40:2 – 0.411 • PC aa C42:2 + 0.066 • PC aa C38:6

VAR7 = – 1.021 • Gln + 0.705 • Gly – 0.239 • PC aa C36:3 + 0.219 • PC aa C36:4 + 0.562 • PC aa C38:3 + 0.826 • PC aa C40:3 + 1.024 • PC ae C36:3 – 0.427 • PC ae C38:3 + 0.288 • PC ae C40:2 – 0.171 • PC ae C40:3 + 0.676 • SM (OH) C22:1 + 0.474 • SM(OH) C22:2 – 1.319 • SM(OH) C24:1 + 0.176 • SM C24:1 – 0.020 • SM C26:1 – 0.596 • PC aa C40:4 – 0.031 • PC ae C34:1 – 0.683 • PC ae C36:2 – 0.647 • PC ae C38:2 + 0.050 • PC aa C32:2 – 0.506 • PC aa C36:2 – 0.415 • PC aa C38:4 – 0.167 • PC ae C38:4 – 0.389 • SM C18:1 – 0.005 • SM C18:0 + 0.152 • PC aa C30:2 + 0.684 • PC aa C40:2 + 0.429 • PC aa C42:2 – 0.014 • PC aa C38:6

VAR8 = 0.167 • Gln – 0.128 • Gly – 0.328 • PC aa C36:3 + 0.108 • PC aa C36:4 – 1.161 • PC aa C38:3 + 0.035 • PC aa C40:3 – 0.013 • PC ae C36:3 – 0.263 • PC ae C38:3 + 1.562 • PC ae C40:2 – 0.370 • PC ae C40:3 – 0.299 • SM (OH) C22:1 + 0.447 • SM(OH) C22:2 + 0.112 • SM(OH) C24:1 + 0.058 • SM C24:1 – 0.479 • SM C26:1 + 1.349 • PC aa C40:4 + 0.209 • PC

ae C34:1 – 0.053 • PC ae C36:2 – 0.121 • PC ae C38:2 + 0.592 • PC aa C32:2 – 0.145 • PC aa C36:2 – 0.711 • PC aa C38:4 + 1.002 • PC ae C38:4 – 1.728 • SM C18:1 + 0.640 • SM C18:0 – 0.586 • PC aa C30:2 – 0.816 • PC aa C40:2 – 0.530 • PC aa C42:2 + 0.216 • PC aa C38:6

VAR9 = – 1.628 • Gln + 1.637 • Gly + 0.436 • PC aa C36:3 – 1.827 • PC aa C36:4 – 0.494 • PC aa C38:3 – 0.932 • PC aa C40:3 + 0.487 • PC ae C36:3 + 4.216 • PC ae C38:3 – 0.301 • PC ae C40:2 – 0.080 • PC ae C40:3 – 0.278 • SM (OH) C22:1 + 0.612 • SM(OH) C22:2 + 0.222 • SM(OH) C24:1 – 0.591 • SM C24:1 – 0.067 • SM C26:1 + 0.997 • PC aa C40:4 – 2.169 • PC ae C34:1 – 0.554 • PC ae C36:2 + 1.568 • PC ae C38:2 + 0.282 • PC aa C32:2 + 0.186 • PC aa C36:2 – 1.279 • PC aa C38:4 + 0.634 • PC ae C38:4 + 0.124 • SM C18:1 – 1.133 • SM C18:0 + 0.759 • PC aa C30:2 + 0.777 • PC aa C40:2 – 0.848 • PC aa C42:2 – 0.149 • PC aa C38:6

VAR10 = – 3.605 • Gln + 1.801 • Gly – 1.177 • PC aa C36:3 + 2.571 • PC aa C36:4 + 0.045 • PC aa C38:3 – 2.049 • PC aa C40:3 – 0.598 • PC ae C36:3 + 1.801 • PC ae C38:3 + 1.496 • PC ae C40:2 + 0.426 • PC ae C40:3 – 1.291 • SM (OH) C22:1 – 4.051 • SM(OH) C22:2 – 0.437 • SM(OH) C24:1 + 1.199 • SM C24:1 + 3.403 • SM C26:1 + 0.773 • PC aa C40:4 + 2.225 • PC ae C34:1 – 3.358 • PC ae C36:2 – 0.127 • PC ae C38:2 + 2.038 • PC aa C32:2 – 2.004 • PC aa C36:2 + 0.416 • PC aa C38:4 + 0.522 • PC ae C38:4 + 2.633 • SM C18:1 + 0.965 • SM C18:0 – 1.247 • PC aa C30:2 – 2.999 • PC aa C40:2 + 1.766 • PC aa C42:2 + 1.222 • PC aa C38:6

VAR11 = 1.085 • Gln – 1.378 • Gly – 7.162 • PC aa C36:3 – 2.398 • PC aa C36:4 – 3.430 • PC aa C38:3 – 0.279 • PC aa C40:3 + 1.574 • PC ae C36:3 + 0.674 • PC ae C38:3 – 1.073 • PC ae C40:2 + 1.353 • PC ae C40:3 – 1.061 • SM (OH) C22:1 + 9.071 • SM(OH) C22:2 + 0.395 • SM(OH) C24:1 – 3.948 • SM C24:1 + 1.832 • SM C26:1 – 2.765 • PC aa C40:4 + 5.387 • PC ae C34:1 – 6.698 • PC ae C36:2 + 4.872 • PC ae C38:2 – 0.717 • PC aa C32:2 – 2.206 • PC aa C36:2 + 4.876 • PC aa C38:4 – 0.256 • PC ae C38:4 – 0.76 • SM C18:1 – 1.541 • SM C18:0 + 0.487 • PC aa C30:2 – 0.863 • PC aa C40:2 + 1.063 • PC aa C42:2 – 0.522 • PC aa C38:6

6 Discussion and perspective

The goal of my thesis was to overcome the invasive step of liver biopsy by providing the basis for a minimally invasive procedure like blood sampling. Since lipids play a key role in the metabolic changes underlying energy production and distribution during the peripartal period, I focused my investigations on the lipidome of blood plasma. Alterations of lipid metabolism play an important role in several disorders such as cancer, atherosclerosis, Alzheimer's disease and schizophrenia [Oresic et al., 2008]. Lipidomics offers excellent analytical methods. Conceptually, an advantage over other '-omics' techniques, such as proteomics or metabolomics, is that lipidomics can measure the end-products of the interactions of genes and proteins with environmental factors. In my thesis, this concept has been applied to the the lipid dynamics occurring during the peripartal period of dairy cows.

Cows experience a negative energy balance (NEB) during the time from pregnancy to lactation [Herdt, 2000]. The feed uptake is not sufficient to compensate for the energy consumed for fetal growth in late pregnancy, mammary tissue remodeling, basic maintenance, and early lactation [Esposito et al., 2014]. The missing energy is retrieved by the breakdown of fat tissue. Energy reserves in form of triacylglycerides (TAGs) in adipocytes are degraded and released as non-esterified fatty acids (NEFAs), transported in the blood stream by binding to albumin, and finally taken up by the tissues [Ingvartsen et al., 2003]. NEFAs are also taken up by the liver where they are oxidized to CO_2 or reesterified to TAGs. The produced TAGs are stored in the hepatocytes or secreted via very low-density lipoproteins (VLDLs) [Côté et al., 2014]. Fatty liver occurs if the esterification of NEFAs to TAGs exceeds their degradation and export from hepatocytes.

The most important predisposing factor for fatty liver is a condition of antepartal obesity after an extended dry period [Rukkwamsuk et al., 1998, Radostits et al., 2000]. Also, underfeeding in early lactation [Reid and Collins, 1980] and a genetically determined tendency to rapidly lose fat after calving, are predisposing factors causing an elevated liver fat content [Schulz et al., 2014]. Normally, the accumulation of TAGs in the liver starts a few weeks before the end of gestation, with the peak about one week before calving. The fat content of the liver reaches a level of 20% (on a wet weight basis), decreasing post-partum to less than 5%.

In the case of an insufficient physiological adaption to the extended fat mobilization, the fat content of the liver exceeds the physiological level and, as a result, the cow will suffer from fatty liver disease (FLD). A severe FLD increases the morbidity and mortality of other ordinary peripartal health problems significantly [Breukinki and Wensing, 1997]. The reason for this may be in the liver itself, because an elevated liver fat accumulation leads to disruption of liver functions and thus increases the risk of liver failure [West, 1990]. Other studies have shown that an increase of the fat concentration in

the liver leads to reduced urea production and an increase in the ammonia concentration in the blood. Because it results in a toxic metabolic product, this process can play a role in the increased morbidity in cattle displaying fatty liver [Strang et al., 1998, Zhu et al., 2000]. Another reason is the depressive effect of FLD on the immune system. As a consequence, the cows are more receptive to infections due to the limited immune system and acquired diseases take mostly a more dramatic course [Zerbe et al., 2000].

FLD-inflicted problems consist of unspecific clinical symptoms including weakness„ anorexia, reduced milk production, little or thick feces and neurological abnormalities, accompanied by an increased ketone body formation. Biochemical changes in the serum of cows with FLD are diagnostically unreliable. As an example, an increased activity of aspartate transaminase (AST) indicates chronic liver injuries as well as muscle damage [West, 1989]. In addition, AST is increased in almost all cows post-partum as a result of injuries during calving [Kalaitzakis et al., 2007]. For a definitive diagnosis of FLD, a biopsy of the liver tissue must be taken. From the obtained sample, the fat content has to be estimated either by histological or a biochemical method.

In my first study, we could show changes of the lipid profile in plasma of cows affected by different stages of FLD using large-scale lipidomic approach. The abundance of certain phosphatidylcholines in cows affected by mild and strong FLD is lower than in cows with physiological normal concentrations of fat in the liver. Besides apolipoproteins, phosphatidylcholines constitute the major building block of blood lipoproteins. These Lipoproteins, most notably VLDLs, are the main carriers of TAGs from the liver to other organs of the body [Herdt et al., 1988]. In addition to VLDL, other lipoproteins such as low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) are important carriers of lipids. It is known, that the concentration of VLDLs decreases post-partum probably due to the increase of VLDL-catabolism in the udder [Moore and Christie, 1979]. The massive transport of fat across the whole body at the beginning of lactation is paired with an increased need of phospholipids. It has been proven that a decrease of phosphatidylcholine biosynthesis has an impact in the secretion of VLDL in hepatocytes after calving [Cole et al., 2012]. Therefore, this first screening study allowed us to formulate the hypothesis that cows suffering from FLD have a reduced phosphatidylcholine biosynthesis and therefore are not able to build sufficient VLDLs for TAG depletion from the liver.

To understand the casual relationship between reduced phosphatidylcholine concentrations and FLD in dairy cows, I carried out further large-scale lipidomic studies. To reveal more metabolic changes during the transition period, twelve dairy cows from the same farm were monitored over a defined time frame during the peripartal period. Blood samples were obtained at days -14, -7, 0, +7, +14, +21, and +28 relative to parturition. A liver biopsy was taken at day +28. A new, quicker, and more reliable

method for measuring the lipidome in plasma was used according to Castro-Perez and Kamphorst [2010] . Major advantages of this method include the short running times and stable retention times during all measurements. By the use of a tandem mass spectrometry (MS/MS) instrument, fragmentation patterns resulting from each parental mass was used to annotate structures to the measured masses.

First, the lipidomic measurement showed a change in the concentration of TAGs during the peripartal period. The plasma concentrations of TAGs decreases abruptly on the day of calving and then remains constant at the same low level until the end of the study. This observation reflects the suddenly increased energy consumption for the onset of lactation [Van den Top et al., 2005]. Second, the concentration of a majority of the annotated phospholipids increased steadily postpartum. Four phosphatidylcholines, five lysophosphatidylcholines and two sphingomyelins were increased after parturition. With this study, I could support the hypothesis that phosphatidylcholine biosynthesis has an impact in the lipid balance and plays an important role in the metabolic changes at the beginning of lactation.

A targeted approach was finally taken , using the same plasma samples as in the first untargeted study, to explore the suspected relationship between phosphatidylcholines and FLD. For this purpose, plasma samples from cows with different grade of fatty liver were measured using a commercial lipidomic platform. This study confirmed the previous findings that phosphatidylcholines are connected to the deranged lipid metabolism in cows displaying fatty liver. The targeted and highly quantitative lipidomic platform demonstrated that cows suffering from FLD have lower phosphatidylcholine concentrations in the blood plasma than control cows. Several phosphatidylcholines could be identified as potential plasma biomarkers for FLD.

One possible explanation for the observed lower concentration of phosphatidylcholines is the use of these molecules as a precursor for the synthesis of hepatic TAGs. The sudden need of TAGs in the mammary gland for milk production may enhances the conversion of phosphatidylcholines to TAGs in the liver [Jacobs et al., 2013]. As a consequence, phosphatidylcholine concentrations are reduced in peripartal dairy cows. The phospholipid synthesis may be the limiting step for VLDLs production and the elimination of hepatic TAGs is consequently reduced, leading to hepatic lipidosis. An excessive reduction of phosphatidylcholine levels would then be a major cause for the development of FLD.

Hepatic phosphatidylcholine synthesis takes place by two distinct pathways. About 70% of the hepatic phosphatidylcholine is synthesized via the CDP-choline pathway. As an initial substrate, choline is processed by three enzymes: choline kinase, CTP:phosphocholine cytidylyltransferase and choline-phosphate transferase. Previous studies have proven the importance of choline supplies for the synthesis of phosphatidylcholines via this CDP-choline pathway. For example, rats fed 3 days with a choline-deficient

diet had 25% lower hepatic phosphatidylcholines but 6.5 fold higher amounts of TAGs compared to choline-supplemented rats [Yao and Vance, 1990]. Already in 1932, Best and Huntsman [1936] showed that a choline-deficient diet results in hepatic lipidosis in monogastric animals. Unfortunately, choline is extensively degraded in the rumen. Cooke et al. [2007] proved the same effect in ruminants by feeding prepartum dairy cows with rumen-protected choline. The findings of Cooke et al. implied that an adequate restriction in feed intake in conjunction with rumen-protected choline could alleviate fatty liver. Similar experiments with rumen-protected choline were carried out in dairy cows 25 days prepartum until 80 days postpartum [Lima et al., 2012]. These authors reported increased synthesis of phosphatidylcholines and VLDLs accompanied by a reduction in lipid infiltration of the liver.

Hepatocytes possess a uniquely high capacity to convert phosphoatidylethanolamine (PE) to phosphatidylcholine. About 30% of the hepatic phosphatidylcholines are produced by the phosphatidylethanolamine methyltranferase (PEMT)-pathway [Cole et al., 2012]. If choline supply is limiting for phosphatidylcholine synthesis via the CDP-choline pathway, the PEMT pathway increases its activity. Also, a high demand of phosphatidylcholines due to the production and secretion of VLDL as well as phosphatidylcholine secretion into the bile stimulates the PEMT pathway [Vance et al., 2007]. PEMT converts phosphatidylethanolamine to phosphatidylcholine by three sequential methylation reactions on the amine. For each step of the reaction, one *S*-adenosyl-L-methionine (SAMe) is used as methyl donor. Jacobs et al. [2013] showed in mice that abnormally high and low levels of SAMe can lead to hepatic lipidosis . However, further studies are necessary to establish the potential of a feed supplementation with SAMe on hepatic phosphatidylcholine synthesis in ruminants.

Finally, the observed shift in phosphatidylcholines concentration and feeding behavior needs further investigation to understand the physiological changes and mechanism during the peripartal period in relation to FLD. However, my own studies are in line with previous reports highlighting the importance of phospholipids, particularly phosphatidylcholines, in the lipid metabolism during the peripatal period of dairy cows. In combination with the clinical chemistry values, lipid profile analyses of blood plasma is likely to represent a powerful additional tool for a minimally invasive diagnostic approach in peripartal cows. The advantages of the presented lipidomic test are easy sample handling and extraction either by commercial available kits or in-house lipid extraction, large throughput due to automation and short running times.

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