Institute of Animal Science

Department of Veterinary Medicine

Importance of supplying dairy cows with essential fatty acids (EFA) and conjugated linoleic acids (CLA) during the transition period on metabolism and health

Dissertation

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“Dedicated to all of my teachers, especially

my mother,

my father,

my brother,

and my professors.

My gratitude never ceases for your love, support, and help.”

English abstract

Ketogenesis and steatosis are common sequelae of the severe mobilization of fat reserves necessary at the onset of lactation when the energy expenditures for milk and maintenance exceed the energy provided by voluntary feed intake in dairy cows. This situation along with a common state of systemic inflammation during early lactation makes dairy cow vulnerable to further metabolic and infectious diseases which impact their profitability. A periparturient supplementation with essential fatty acids (**EFA**) and conjugated linoleic acids (**CLA**) has been shown to influence different aspects of lipid and energy metabolisms as well as markers of immune homeostasis in dairy cows. This study aimed at investigating the synergistic effects of EFA, particularly α-linolenic acid (ALA, n-3 fatty acid (**FA**), and CLA on the liver and plasma proteome profile of dairy cows during the time spanning 3 weeks before to 9 weeks after parturition.

Late-gestation Holstein cows were infused from 9 wk ante partum (AP) to 9 wk post partum (PP) into the abomasum with either coconut oil (CTRL, n = 8, 38 & 76 g/d during dry period and lactation, respectively) or a mixture of EFA (Linseed + safflower oil) and CLA (Lutalin, BASF) (EFA+CLA, n = 8, 60 and 120 g/d during dry period and lactation, respectively). An untargeted shotgun proteomics approach based on liquid chromatography coupled with tandem mass spectrometry was performed in liver biopsies and plasma samples collected at days -21, +1, +28, and +63 relative to calving. At each timepoint, differentially abundant proteins (**DAP**) between treatment groups were identified as the proteins at the intersection between a multivariate supervised Partial Least Squares Discriminant Analysis (PLS-DA) and an univariate analysis (proteins with P-value <0.05 according to a t-test and fold change ≥ 1.3).

In liver a total of 1680 proteins was identified at each timepoint, of which 100 DAP between groups were assigned to the metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, drug metabolism - other enzymes, arachidonic acid metabolism, cholesterol metabolism and bile secretion, pyruvate metabolism, steroid hormone biosynthesis, glycolysis/gluconeogenesis, glutathione metabolism, and citrate cycle at all timepoints. At each timepoint, cytochrome P450, as a central hub to these annotated pathways, was related to specific CYP enzymes comprising CYP51A1, CYP1A1, CYP4F2, and CYP4V2. A total of 241 unique proteins was identified in the plasma, in which a cluster of apolipoproteins (APO) containing APOC3, APOA1, APOA4, and APOC4 was increased in response to EFA+CLA according to the stage of lactation. Overabundant APO were annotated by GO (gene ontology) terms related to triglyceride (TAG) homeostasis, cholesterol and lipoprotein metabolisms, inflammation, and innate immune response.

Altogether, these findings provided novel insights into the molecular mechanisms involved in cytochrome P450, cholesterol, and TAG metabolism by which dietary supplemented EFA+CLA influences metabolic and immune adaptation around parturition. Our results suggest that EFA+CLA could synergistically attenuate hepatic TAG accumulation and marginally mitigate inflammation. Nevertheless, further research is necessary to confirm our results at the other (metabolome) levels.

Kurzfassung

Bei Milchkühen sind Ketogenese und Steatose häufige Folgen der starken Mobilisierung von Fettreserven, die zu Beginn der Laktation erforderlich ist, wenn der Energieaufwand für die Milchproduktion und den Erhaltungsbedarf die Energiezufuhr durch die freiwillige Futteraufnahme übersteigt. Zusammen mit einem allgemeinen Zustand systemischer Inflammation während der frühen Laktation macht dies die Milchkuh anfällig für weitere Stoffwechsel- und Infektionskrankheiten, was ihre Rentabilität stark beeinflusst. Es hat sich gezeigt, dass eine peripartale Supplementierung mit essentiellen Fettsäuren (EFA) und konjugierten Linolsäuren (CLA) verschiedene Aspekte des Lipid- und Energiestoffwechsels sowie Marker der Immunhomöostase bei Milchkühen beeinflussen kann. Ziel dieser Studie war es, die synergistischen Effekte von EFA, insbesondere α-Linolensäure (ALA, n-3-Fettsäure (FA)), und CLA auf das Leber- und Plasmaproteomprofil von Milchkühen im Zeitraum von 3 Wochen vor bis 9 Wochen nach der Geburt zu untersuchen.

Spättragenden Holstein-Kühen wurde von 9 Wochen ante partum (AP) bis 9 Wochen post partum (PP) entweder Kokosöl (CTRL, n = 8, 38 & 76 g/d während der Trockenperiode bzw. Laktation) oder eine Mischung aus EFA (Leinsamen + Distelöl) und CLA (Lutalin, BASF) (EFA+CLA, n = 8, 60 und 120 g/d während der Trockenperiode bzw. Laktation) in den Labmagen infundiert. In Leberbiopsien und Plasmaproben, die an den Tagen -21, +1, +28 und +63 relativ zur Abkalbung entnommen wurden, wurde ein ungezielter Shotgun-Proteomics-Ansatz auf der Grundlage von Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie durchgeführt. Zu jedem Zeitpunkt wurden die unterschiedlich häufigen Proteine (DAP) zwischen den Behandlungsgruppen als die Proteine identifiziert, die den Schnittpunkt zwischen einer multivariaten überwachten Partial Least Squares Discriminant Analysis (PLS-DA) und einer univariaten Analyse bilden (Proteine mit einem P-Wert <0,05 gemäß einem t-Test und einer fachen Veränderung (fold change) ≥ 1,3).

In der Leber wurden zu jedem Zeitpunkt insgesamt 1680 Proteine identifiziert, von denen 100 DAP zwischen den Gruppen dem „Metabolismus von Xenobiotika durch Cytochrom P450“, dem „Arzneimittelmetabolismus - Cytochrom P450“, dem „Arzneimittelmetabolismus - andere Enzyme“, „Arachidonsäure-Stoffwechsel“, „Cholesterin-Stoffwechsel und Gallensekretion“, „Pyruvat-Stoffwechsel“, „Steroidhormon-Biosynthese“, „Glykolyse/Glukoneogenese“, „Glutathion-Stoffwechsel“ und „Citrat-Zyklus“ zu allen Zeitpunkten zugeordnet werden konnten. Zu jedem Zeitpunkt stand Cytochrom P450 als zentraler Knotenpunkt dieser annotierten Stoffwechselwege mit spezifischen CYP-Enzymen (CYP51A1, CYP1A1, CYP4F2 und CYP4V2) in Verbindung. Insgesamt wurden 241 DAP im Serum identifiziert, wobei eine Gruppe von Apolipoproteinen (APO), die APOC3, APOA1, APOA4 und APOC4 enthält, als Reaktion auf EFA+CLA je nach Stadium der Laktation erhöht war. Diese höher konzentrierten APO wurden mitlels *Gene Ontology* (GO) Begriffen mit der Triglycerid-(TAG) Homöostase, dem Cholesterin- und Lipoprotein-Stoffwechsel, Entzündungen und der angeborenen Immunreaktion in Verbindung gebracht.

Insgesamt lieferten diese Ergebnisse neue Einblicke in die molekularen Mechanismen, die in den Cytochrom-P450-, Cholesterin- und TAG-Stoffwechsel involviert sind, und die durch eine Nahrungsergänzung mit EFA+CLA die metabolische und immunologische Anpassung um die Geburt herum beeinflussen. Unsere Ergebnisse deuten darauf hin, dass EFA+CLA die hepatische TAG-Akkumulation synergistisch abschwächen und Entzündungen geringfügig lindern können. Dennoch sind weitere Forschungen erforderlich, um unsere Ergebnisse auf den anderen (Metabolom-) Ebenen zu bestätigen.

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Abbreviations

ARA	Arachidonic acid (C20:4 n-6)
ABCA1	ATP-Binding cassette transporter 1
ABCD	ATP-binding cassette transporters of subfamily D
ACACA	Acetyl-CoA Carboxylase Alpha
ACACA	Acetyl-Coa carboxylase alpha
ACC	Acetyl-CoA carboxylase
ACSL	Acyl-CoA Synthetase Long Chain Family Member 1
ACSS1	Acyl-Coa Synthetase Short Chain Family Member 1
ADIPOQ	Adiponectin
ADP	Adenine dinucleotide phosphate
AFLP	Amplified Fragment Length Polymorphism
AGPAT6	1-Acylglycerol-3-phosphate O-acyltransferase 6
AIF	apoptosis-inducing factor
ALA	A-Linolenic acid C18:3 n-3
Alb	Albumin
ALR	Adiponectin: leptin ratio
APO	Apolipoproteins
APOB	Apolipoprotein B
APP	Acute phase proteins
ASCA	Analysis of variance – simultaneous component analysis
AT	Adipose tissue
BA	Bile acid
BDH1	β-hydroxybutyrate dehydrogenase
BHB	β-hydroxybutyrate
BHBA	B-Hydroxybutyric acid
BME-UV1	Immortalized bovine mammary epithelial cells
BRCFA	Branched-chain fatty acid
CAC	Carnitineacylcarnitine carrier
CK	Clinical ketosis
CLA	Conjugated linoleic acids
CM	Chylomicron
CM-R	Chylomicron remnants
CoA	Acetyl-Coenzyme A
COX	Cyclooxygenase
CRP	C reactive protein
CYP4F	Cytochrome P450 Family 4 Subfamily F Member
DAG	Diacylglycerol
DEFA	Deficiency in essential fatty acid (project conducted at FBN)
DGAT1	Diacylglycerol O-Acyltransferase 1
DMI	Dry matter intake
EFA	Essential fatty acids
EpDPE	Epoxydocosapentaenoic acid (19,20-EDP)
EpETE or EEQ	Epoxyeicosatetraenoic acid (17,18-EEQ)
ER	Endoplasmic reticulum
FA	Fatty acids
FABP	Fatty Acid binding proteins

FABP4	Fatty acid binding protein 4
FAD	Flavin adenine dinucleotide
FADH2	Reduced FAD
FASN	Fatty Acid Synthase
FASN	Fatty acid synthase
FFA	Free fatty acids
FGA	Fibrinogen
FGF21	Fibroblast growth factor 21
FOXA2	Forkhead box A2
GC	Gas chromatography
GH	Growth hormone
GPAM	Glycerol-3-Phosphate Acyltransferase, Mitochondrial
GPAT	Glycerol phosphate acyl transferase
GSH	Glutathione
HDL	High-Density lipoproteins
HETE	Hydroxy-Eicosatetraenes
HMGCL	HMG-CoA lyase
HMGCS2	3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 2
HP	Haptoglobin
HSL	Hormone sensitive lipase
IGFALS	IGFBP Acid Labile Subunit
IGFBP2	IGF-Binding protein
IL	Interleukin
IR	Insulin resistance
KB	Ketone bodies
LA	Linoleic acid C18:2 n-6
LCFA	Long chain fatty acids
LC-MS	Liquid chromatography-mass spectrometry
LD	Lipid droplets
LDL	Low-Density lipoproteins
LEPR	Leptin receptor
LPL	Lipoprotein lipase
LPL	Lipoprotein lipase
LT	Leukotriene
LT	Leukotrienes
LX	Lipoxin
LX	Lipoxins
MCFA	Medium chain fatty acids
MCT1/2	Monocarboxylic acid transporters 1/2
MDA	Malondialdehyde
MFD	Milk fat depression
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NADPH	Nicotinamide adenine dinucleotide phosphate
NEB	Negative energy balance
NEL	Net energy of lactation

NF-κB	Nuclear factor κ-light-chain enhancer of activated B cells
NR1H2	Nuclear Receptor Subfamily 1 Group H Member 2
Nrf2	Nuclear factor E2-related factor
NRS	NAD(H) redox shuttles
OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase
OXPHOS	Oxidative phosphorylation
PCA	Principal component analysis
PCYOX1	Prenylcysteine Oxidase 1
PG	Prostaglandins
PG	Prostaglandins
PGI	Prostacyclin
PL	Phospholipids
PLSDA	Partial least squares discriminant analysis
PON1	Paraoxonase
PPAR	Peroxisome proliferator-activated receptors
PPARG	Peroxisome proliferator-activated receptor gamma
PPARα	Peroxisome proliferator activated receptor α
PPARγ	PPAR-Gamma
PUFA	Polyunsaturated fatty acids
RBP	Retinol binding protein
ROS	Reactive oxygen species
RQUICKI	Revised Quantitative Insulin Sensitivity Check Index
RXRA	Retinoid X Receptor Alpha
SAA2	Serum amyloid A2
SAGE	Serial analysis of gene expression
SCD	Stearoyl-CoA Desaturase
SCD1	Stearoyl-Coa desaturase 1
SI	Systemic inflammation
SIRT3	Sirtuin 3
SK	Subclinical ketosis
SLC16A6	Solute carrier family 16 (monocarboxylic acid transporter), member 6
SLCO1B3	Bile acid transporters
sPLS	Sparse partial least squares
SREBP	Sterol regulatory element binding proteins
SREBP-1	Sterol Regulatory Element Binding Transcription Factor 1
TAG	Triacylglycerols
TCA	Tricarboxylic acid
TCA	Tricarboxylic acid
THRSP	Thyroid hormone responsive spot 14 protein
TLR4	Toll-Like receptor 4
TNF	Tumor necrosis factor
TX	Thromboxane
VFA	Volatile fatty acids
VLCFA	Very-long-chain fatty acid
VLCFA	Very-Long-Chain fatty acid
VLDL	Very low-density lipoproteins

Introduction

1.1 Dietary Fats in ruminant

Fat supplements are traditionally considered as a source of energy to increase the energy density in dairy cows' diets and thus the net energy of lactation (NEL) intake (Jenkins and McGuire, 2006; Weiss et al., 2011; Lock et al., 2013). Production responses of dairy cows to fat supplements are dose-dependent and the range of responses can differ depending on the total amount, fatty acid (FA) composition (chain length and degree of saturation), and chemical form (free FA, calcium salts of FA, and triacylglycerides (TAG)), which determine their gross energy content and digestibility (Weiss et al., 2011). In addition, fat supplements can affect the digestibility of other dietary components, and dry matter intake (DMI) (Weiss et al., 2011). In most situations, unsaturated FA supplements tend to decrease DMI, whereas saturated fatty acids have less effect on DMI as reviewed by (Allen, 2000). Besides serving as an energy supply, polyunsaturated fatty acids (PUFA) influence a wide variety of physiological processes, including growth, lactation, and reproduction (Wathes et al., 2007; Spector and Kim, 2015).

Mammalian cells can synthesize longer chain n-6 and n-3 PUFA *de novo* only if their precursors linoleic acid (C18:2 n-6, LA) and α-linolenic acid (C18:3 n-3, ALA) are supplied by the diet (Jeyapal et al., 2018). Therefore, LA and ALA are regarded as "essential fatty acids" (EFA)(Russo, 2009). These EFA are also precursors of lipid mediator molecules, i.e., eicosanoids such as prostaglandins (PG) and leukotrienes (Figure 1), and are incorporated into the phospholipid (PL) fraction of cell membranes, which influence the structural and functional properties of the cells (Cholewski et al., 2018). As shown in Figure 1, arachidonic acid (C20:4 n-6, ARA), the derivative of LA, can be converted into the pro-inflammatory PG 2-series and the 4-series leukotrienes. On the other side, eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA), the derivatives of ALA, give rise to the anti-inflammatory PG 3-series and 5-series leukotrienes (Patterson et al., 2012). These hormones which are termed eicosanoids, function in both the promotion and inhibition of inflammation (Contreras et al., 2012).

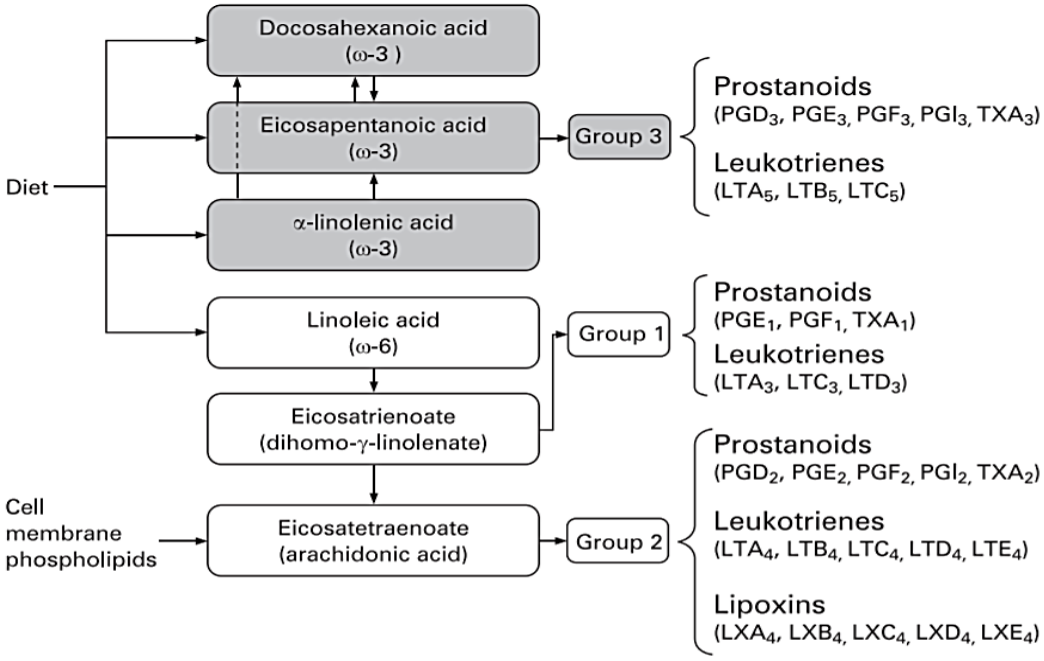

Figure 1. Metabolic fate and products of essential fatty acids.
PG, prostaglandins; PGI, prostacyclin; TX, thromboxane; LT, leukotriene; LX, lipoxin (Yashodhara et al., 2009).

In ruminants, gut microbes extensively biohydrogenate and reduce the availability of dietary PUFA for absorption (Lanier and Corl, 2015). Trans-FA originating from biohydrogenation are biologically active and have been shown to act as metabolic modifiers of lipid metabolism, milk fat synthesis, and reproductive efficiency (Harvatine and Allen, 2006). In this regard, conjugated linoleic acids (CLA) are naturally occurring stereo and positional isomers of LA with the arrangement of the double bonds in the molecule (Churruca et al., 2009). In other words, CLA isomers are derived from a group of octadecadienoic acids (18:2) whose double bond pairs are arranged differently and have different geometrical configurations (Delmonte et al., 2004). The steps of the biohydrogenation of LA and ALA are presented in Figure 2.

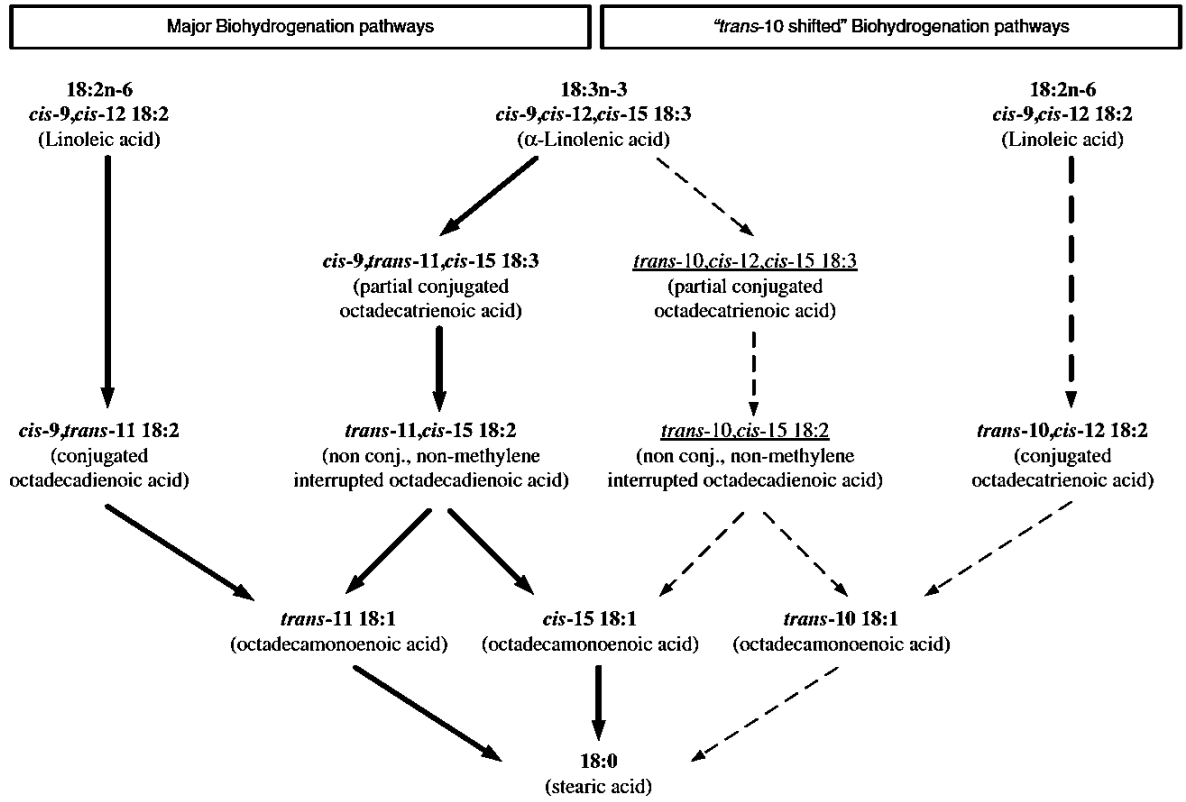

Figure 2. Steps in the biohydrogenation pathways of linoleic acid (18:2n-6) and a-linolenic acid (18:3n-3). Arrows with solid lines describe the main known biohydrogenation pathways and dashed lines describe the proposed pathways (Alves and Bessa, 2014).

There are more than 28 isomers of CLA, but the most abundant isomers of CLA are *cis*-9, *trans*-11, and *trans*-10, *cis*-12. The *cis*-9, *trans*-11 CLA is known for its anti-cancerogenic effect (Dhiman et al., 2000), and the *trans*-10, *cis*-12 is mainly related to milk fat depression (MFD) in dairy animals (Giesy et al., 2002). During digestion, generated CLA through ruminal biohydrogenation are absorbed and accumulate in peripheral tissues and milk fat, which makes ruminant products the primary source of CLA for humans (Lehnen et al., 2015). Rumenic acid (*cis*-9, *trans*-11 CLA) is the predominant CLA isomer (70–90% of total CLA isomers) in dairy products (Lock and Bauman, 2004). There is growing interest in using PUFA in dairy cows' diets in order to alter body energy partitioning during the transition from late pregnancy to early lactation which will be addressed in the following sections.

1.2 Lipid metabolism in dairy cows

The current model of absorption and metabolism of FA in dairy cows relies on information generated in monogastric species and was modified according to data produced in dairy cows

(reviewed by (Bionaz et al., 2020)) (Figure 3). There is a great similarity between monogastric and ruminant for lipid absorption in the small intestine.

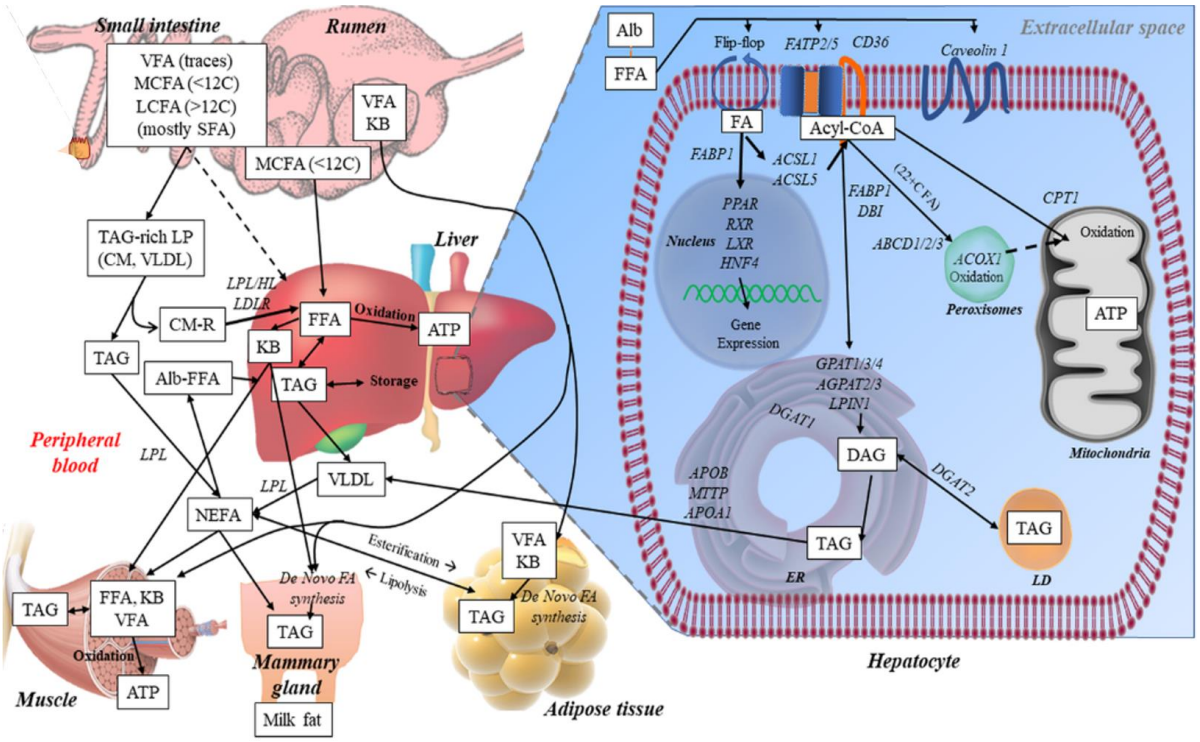

Figure 3. Enzymes and other proteins/complexes involved in the absorption and utilization of fatty acids by enterocytes and by peripheral tissues (especially liver) in dairy cows. Alb: albumin; CM: chylomicron; CM-R: chylomicron remnants; DAG: diacylglycerol; ER: endoplasmic reticulum; FA: fatty acids; FFA, free fatty acids; KB: ketone bodies; LCFA: long chain fatty acids; LD: lipid droplets; LP: lipoprotein(s); LPL: lipoprotein lipase; MCFA: medium chain fatty acids; NEFA: non-esterified fatty acids; TAG: triacylglycerol; TAG-rich LP: TAG-rich lipoproteins; VFA: volatile fatty acids; VLDL: very low density lipoproteins (adapted from (Bionaz et al., 2020)).

As reviewed in more detail by (Bionaz et al., 2020), the process of FA absorption in dairy cows can be divided into (a) uptake into the enterocyte, (b) intracellular processing, and (c) transport into the circulation. Most FA reach the outer membrane of enterocytes in the form of micelles which facilitate their transfer across the intestinal epithelial cell membrane. Once the FA reach the outer membrane of enterocytes, they are bound by FA binding proteins (FABP), transported into cell, and reesterified to triacylglycerols (TAG) by combining with glycerol. The TAG are then packaged into lipoprotein particles in combination with cholesterol, PL, and specific proteins (called apolipoproteins, APO) for transportation in the lymph to the blood. Through the lymph and later bloodstream, the lipoprotein particles are delivered to peripheral tissues (especially liver) where TAG are broken down to free FA by an enzyme called lipoprotein

lipase. The free FA then enter the hepatocytes mainly through the membrane-embedded protein CD36, where they can be formed back into TAG or undergo oxidation (Bionaz et al., 2020), which is reviewed later.

As mentioned, lipoproteins enable the transport of FA throughout the body and play a crucial role in the metabolism of FA (Kwiterovich, 2000). The four major classes of circulating lipoproteins include chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) and all have their unique lipid and surface marker APO composition (German et al., 2006) (Figure 4). Chylomicrons and VLDL are predominately transporting intracellularly accumulated TAG from the enterocytes and the hepatocytes, respectively (German et al., 2006). On the other side, LDL and HDL are mainly known as cholesterol carriers which deliver their lipid cargo from the liver to the peripheral tissues and then bring it back to the liver (reverse cholesterol transport), respectively (Kessler et al., 2014).

Most APO are made by the hepatocytes and enterocytes; both cell types are essential in lipid modification and absorption (Bionaz et al., 2020). The APO are considered as the "brain" of the lipoprotein system directing them to the adequate site of metabolism (German et al., 2006). The four major classes of APO including A, B, C, and E, and their occurrence in lipoproteins are graphed in Figure 4. It is worth mentioning that APO are multifunctional proteins with immunomodulatory potential, for instance, ApoE was reported to exert an anti-inflammatory effect (Tudorache et al., 2017). Lipoproteins, besides APO, carry other enzymes and exchangers such as lipoprotein lipase, phospholipases, cholesterol ester transport protein, and phospholipid exchange proteins which direct their own metabolism (Ståhlman et al., 2008).

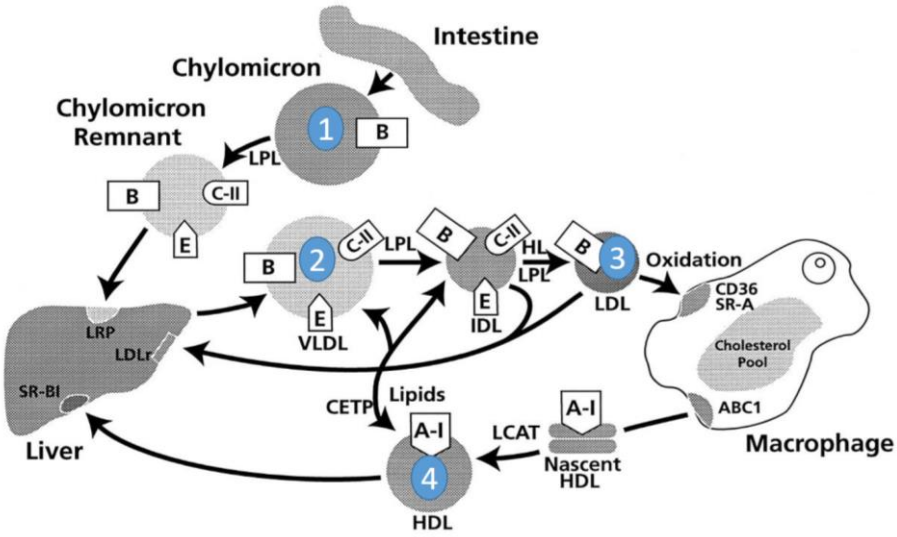

Figure 4. Schematic of lipoprotein metabolism: (1) intestinal cells package dietary fats on chylomicrons, a process that requires apolipoprotein B (B); (2) very low-density lipoprotein (VLDL) is secreted from the liver and contains triglycerides (TG), apo B, and apo C-II; (3) low-density lipoprotein (LDL) enzymatically formed from VLDL which contains only apo B; (4) low-density lipoprotein (HDL) particles synthesized by the liver and intestine which contain mainly phospholipid and apo A-1. The cholesterol content of HDL is then returned to the liver, reverse cholesterol transport. IDL: intermediate-density lipoprotein. HL: hepatic lipase, LCAT: lecithin cholesterol acyltransferase, CETP: cholesteryl ester transfer protein, LRP: lipoprotein-like receptor protein, (adapted from (Kwiterovich, 2000)).

Under normal physiological conditions, mitochondrial and to a much lesser content peroxisomal β-oxidation of FA, is the predominant cellular oxidation pathway which catalyzes the formation of acetyl-Coenzyme A (CoA), in a four-step process: dehydrogenation, hydration, dehydrogenation, and thiolytic cleavage. Processing of very-long-chain FA (VLCFA, 24-26 carbon atoms) is limited to peroxisomal β-oxidation, in which some enzymatic steps differ from mitochondrial β-oxidation (Fransen et al., 2017) (Figure 5). The generated acetyl-CoA in mitochondria can further be used for generating ATP via the mitochondrial electron transport chain in the tricarboxylic acid (TCA) cycle (Natarajan and Ibdah, 2018).

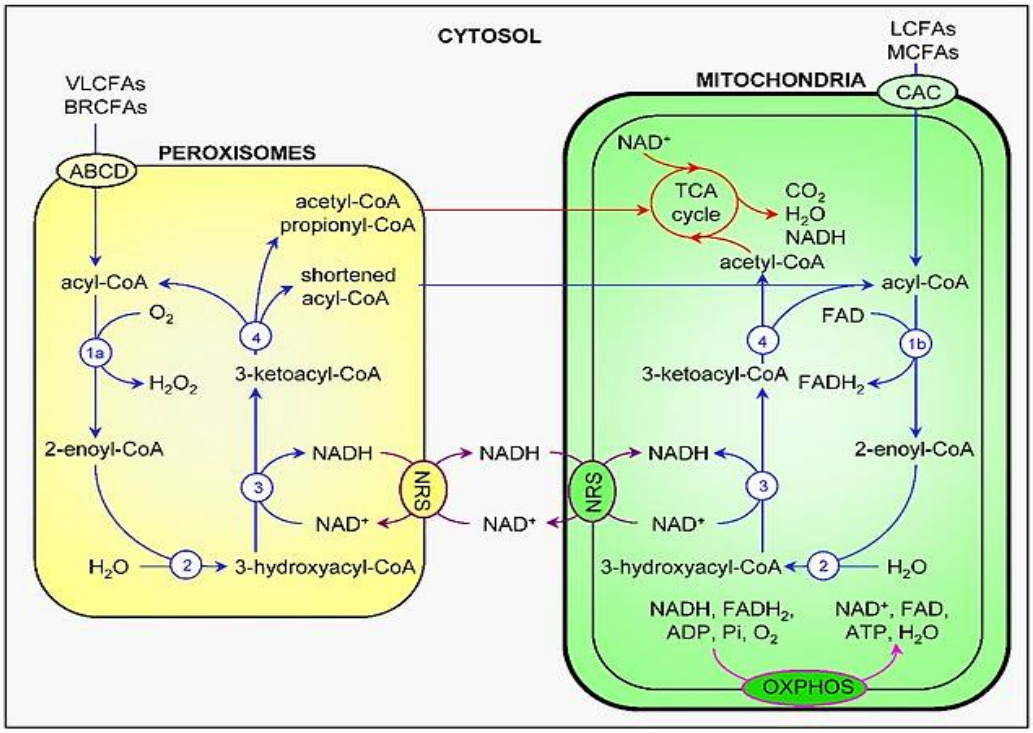

Figure 5. Peroxisomal and mitochondrial β-oxidation of long chain fatty acid involves 1) dehydrogenation, 2) hydration, 3) dehydrogenation, and 4) thiolytic cleavage, actcivated by the enzymatic function of 1a, acylCoA oxidase; 1b, acyl-CoA dehydrogenase; 2, enoyl-CoA hydratase; 3, 3-hydroxyacyl-CoA dehydrogenase; 4, 3-ketoacyl-CoA thiolase.
ABCD: ATP-binding cassette transporters of subfamily D; ADP: adenine dinucleotide phosphate; BRCFA: branched-chain fatty acid; CAC: carnitineacylcarnitine carrier; FAD: flavin adenine dinucleotide; FADH2: reduced FAD; LCFA: long-chain fatty acid; MCFA: medium-chain fatty acid; NAD: nicotinamide adenine dinucleotide; NADH: reduced NAD; NRS: NAD(H) redox shuttles; OXPHOS: oxidative phosphorylation; TCA: tricarboxylic acid; VLCFA: very-long-chain fatty acid (Fransen et al., 2017).

Besides β oxidation, α- and ω-oxidation can also contribute to the metabolism of some specific FA and other molecules. In this regard, 3-Methyl-branched FA, such as phytanic acid, can be degraded by β-oxidation only after passing peroxisomal α-oxidation in which they are shortened by 1 carbon atom from carboxylic end (Casteels et al., 2003). Knowledge concerning the ω-oxidation of FA in dairy cows is scarce, and the existing information mostly relies on the finding of other species. Briefly, ω-oxidation refer to the process of FA hydroxylation to hydroxy FA and dicarboxylic acid, which is catalyzed by the enzymatic function of the hemoprotein cytochrome P450 superfamily (Munro et al., 2018). Most cytochrome P450 enzymes contributing to ω-oxidation are from the cytochrome P450 family 4 subfamily F member (CYP4F) and belong to the membrane-bound protein of the endoplasmic reticulum (Wanders et al., 2011; Han et al., 2017). Accumulated FA and 3-hydroxy FA are transferred to

the endoplasmic reticulum to first pass the ω-oxidation, and further peroxisomal β-oxidation (Han et al., 2017). Figure 6 summarizes the interplay among α-, β-, and ω-oxidation of FA.

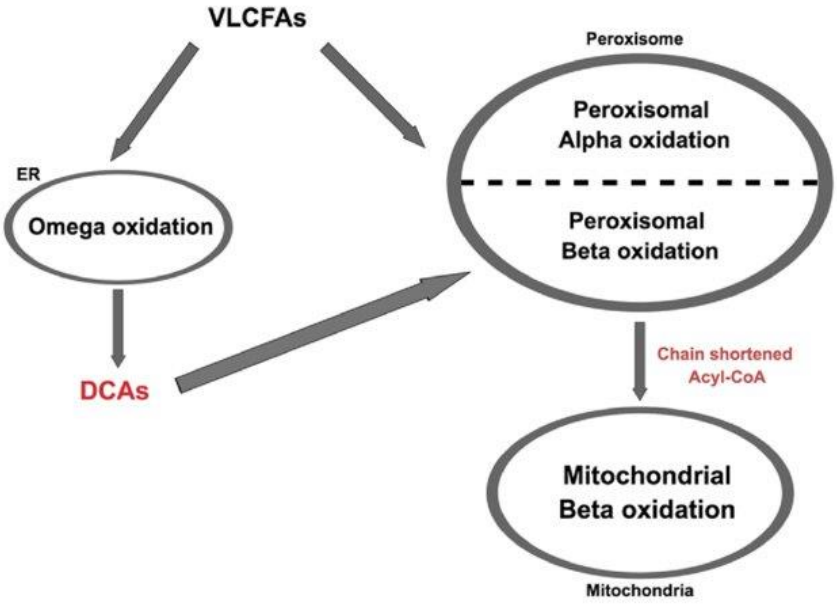

Figure 6. Interplay among α-, β-, and ω-oxidation of fatty acids. Very long chain fatty acids need to pass either α- or ω-oxidation to be able to be further oxidized by mitochondrial β-oxidation. DCA: dicarboxylic acid; VLCFA: very long chain fatty acid; ER: endoplasmic reticulum (Watson et al., 2021).

1.2.1 Lipid metabolism in dairy cows during the transition period

The transition period, which spans from three weeks before to three weeks after parturition (Drackley, 1999), is a critical physiological stage, in which dairy cows undergo extensive endocrine, metabolic, and immunological changes for adapting to lactation (Duffield, 2000). Within this time frame, substantially elevated energy and nutrient demands along with decreased feed intake commonly lead to a state of negative energy balance (NEB) in high-yielding dairy cows (Grummer et al., 2004; Ingvartsen, 2006). In a state of NEB, stored energy in a form of TAG are mobilized from adipose tissue (AT) by the hydrolytic action of hormone sensitive lipase (HSL), hydrolase adipose triglyceride lipase, and monoglyceride lipase, similarly functioning enzymes (Contreras et al., 2017). Hydrolysis of TAG liberates non-esterified FA (NEFA) into the circulation and to the liver, where they undergo hepatic oxidation either to CO_2 (complete oxidation) or ketone bodies (incomplete oxidation), or re-esterification to form TAG which are then exported as VLDL (Figure 7) (Humer et al., 2016).

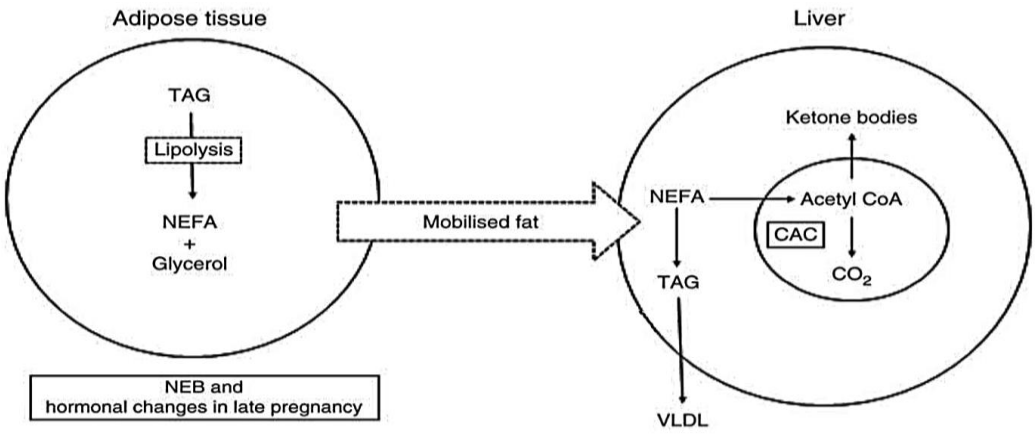

Figure 7. Schematic of lipid metabolism in adipose tissue and liver during negative energy balance (NEB). NEFA: non-esterified fatty acids, VLDL: very low density lipoproteins, TAG: triacylglycerols. Adapted from (Drackley, 1999; Shahsavari et al., 2016).

Excessive lipolysis in the adipose tissues may overload the hepatocytes' capacity to oxidize NEFA resulting in incomplete oxidation and accumulation of ketone bodies such as β-hydroxybutyric acid (BHBA) in blood (Chilliard et al., 2000) and accumulation of TAG in the hepatocytes, which may result in the development of metabolic disorders such as ketosis and fatty liver (Ospina et al., 2010b). The process of hepatic ketogenesis and ketone body utilization in dairy cows is illustrated in Figure 8.

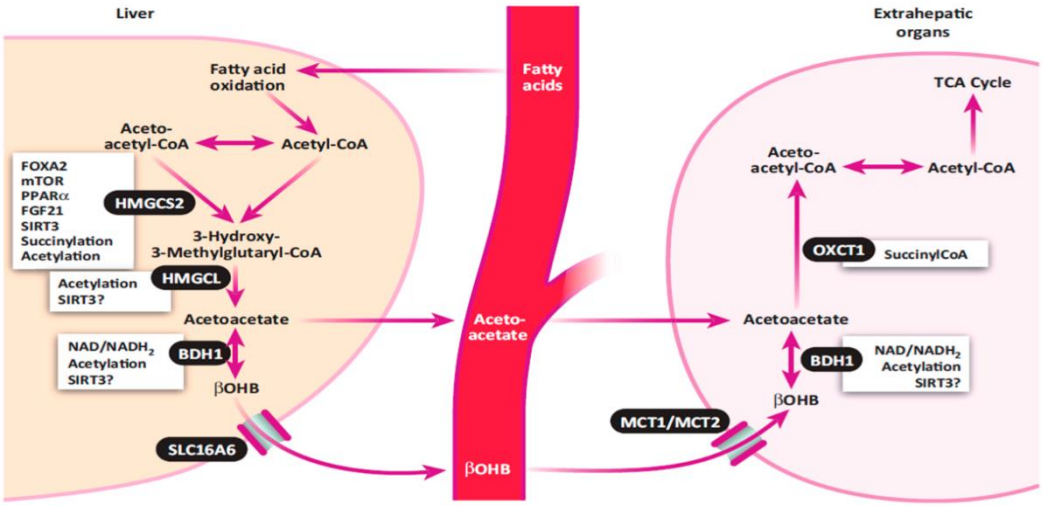

Figure 8. Schematic of hepatic ketogenesis and ketone body utilization in dairy cows.
Abbreviations: BDH1, β-hydroxybutyrate dehydrogenase; βOHB, β-hydroxybutyrate; FGF21, fibroblast growth factor 21; FOXA2, forkhead box A2; HMGCS2, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 2; HMGCL, HMG-CoA lyase; MCT1/2, monocarboxylic acid transporters 1/2; mTOR, mechanistic target of rapamycin; OXCT1, succinyl-CoA:3-ketoacid coenzyme A transferase; PPARα, peroxisome proliferator activated receptor α; SIRT3, sirtuin 3; SLC16A6, solute carrier family 16 (monocarboxylic acid transporter), member 6; TCA cycle, tricarboxylic acid cycle (adapted from (Zhang and Ametaj, 2020)).

Both NEFA and BHBA concentrations can be measured as robust indicators of lipolysis and ketogenesis, respectively. They are routinely used as a herd-level indicator for the incidence of metabolic disorders (Ospina et al., 2010a). Indeed, the incidence rate of subclinical ketosis is high in the early lactation dairy cows and causes substantial economic losses in the dairy industry (Zhang and Ametaj, 2020). This is mainly due to the fact that ruminant's liver is not capable of secreting VLDLs effectively (Kleppe, Aiello et al. 1988).

The mechanisms controlling lipolysis and ketosis are complex, although recent Omics-based studies provided a comprehensive view of the metabolic fingerprinting of the underlying pathways (Wu et al., 2020; Zhang and Ametaj, 2020). This new era of a systems biology approach not only expands our knowledge about metabolic adaptation and diseases but could also could be beneficial for identifying biomarker candidates in blood that may distinguish cows at greater risk of developing metabolic disorders. For instance, metabolomics results revealed that LDL, VLDL, alanine, formate, lysine, tyrosine, citrate, glutamate, and glutamate had higher, and acetate, creatine, glycine, leucine, acetoacetate, and choline had lower abundance in ketotic cows compared to healthy cows (Sun et al., 2014). These metabolites were enriched in pathways including amino acid metabolism, gluconeogenesis, glycolysis, lipolysis, and TCA (Figure 9).

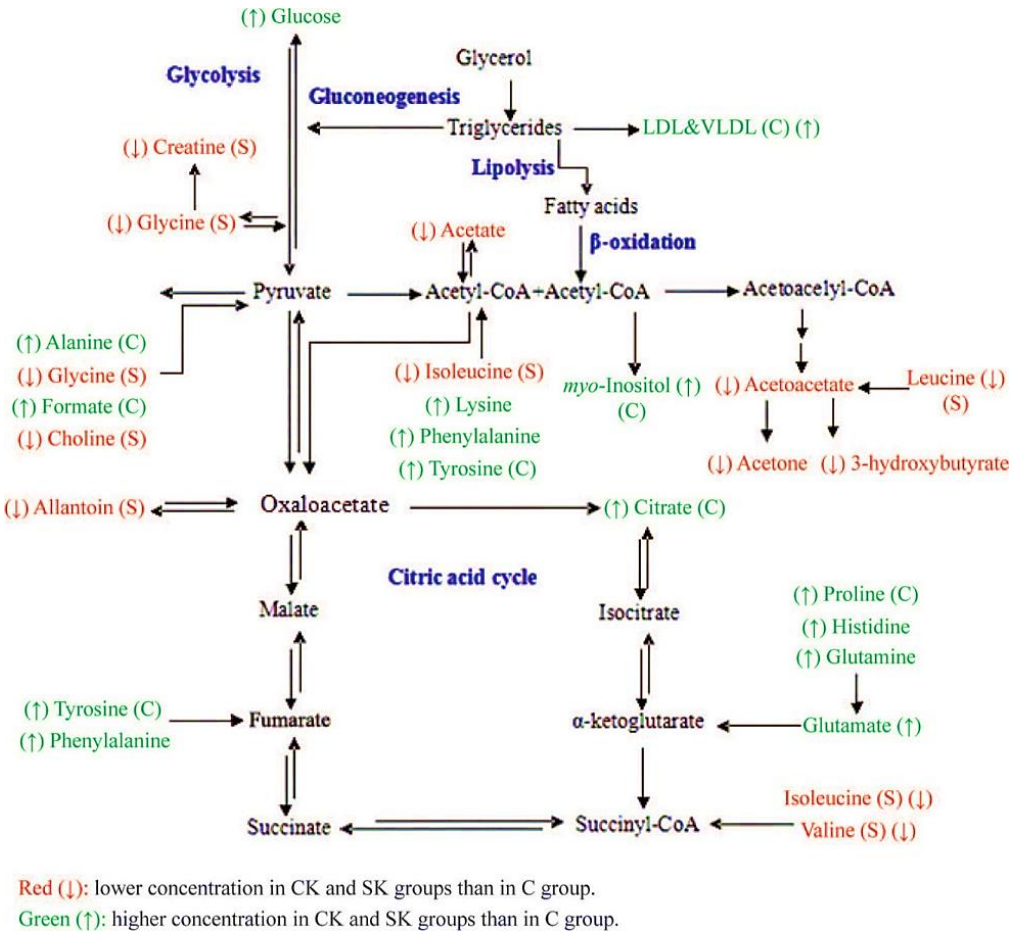

Figure 9. Metabolomics analysis revealed the relevant metabolic differences among the clinical ketosis (CK), subclinical ketosis (SK), and control groups (C). Parenthetical notation (C, S) represents the changes only found in clinical ketosis and subclinical ketosis, respectively. LDL = low-density lipoprotein; VLDL = very low density lipoprotein (Sun et al., 2014).

1.3 Importance of supplementing EFA and CLA during the transition period

As mentioned earlier, during the transition from late pregnancy to early lactation, dairy cows are susceptible to metabolic and immune disorders (Contreras et al., 2017). This is mainly due to a natural state of systemic inflammation (SI) which is induced by the tissue remodeling to adapt to a new physiological status (Horst et al., 2021). Also, a natural state of oxidative stress, initiated by excessive hepatic lipid oxidation and production of numerous free radicals beyond the scavenging capacity of their antioxidant systems (Spears and Weiss, 2008). Therefore, a smooth and safe transition to lactation is an important determinant for the subsequent production and reproductive performance of dairy cows. On the other hand, poor transition often coincides with increasing incidences of health problems and consequently economic losses to dairy farmers (Wankhade et al., 2017).

Nutritional strategies aim to avoid excessive fat accumulation in late pregnancy, to reduce fat mobilization, to accelerate the hepatic removal of NEFA, optimize milk production, improve oxidative capacity, and reduce immunosuppression in transition cows (Sun et al., 2016). In dairy cows, dietary supplementation of appropriate dosages of EFA and CLA can be a nutritional strategy to improve energy balance (EB) and immune responses. Transition cows are fed EFA (Zachut et al., 2010) and/or CLAs (Moore et al., 2004) with an objective to induce MFD. Since milk fat synthesis is the most energy-consuming step in milk production, MFD could spare more energy for milk production and secretion of milk protein and/or an improvement in EB at the onset of lactation (Bauman et al., 2011; Pappritz et al., 2011).

There are two main sources of n-3 PUFA to enrich the ruminants' diet: flaxseed, as a botanical source of ALA, and fish oil, which is rich in EPA and DHA (Moallem, 2018). Many studies reported that feeding a source of n-3 PUFA (e.g. extruded flaxseed (*Linum usitatissimum*)) during the transition period decreased milk fat content and thus improved EB in lactation dairy cows although the results on dry matter intake were controversial (for review see (Benninghoff et al., 2015; Moallem, 2018)). Moreover, as previously described, n-3 PUFA are involved in the production of eicosanoids like PG and exert anti-inflammatory properties (Calder, 2013). Feeding camelina (*Camelina sativa*) meal (rich in ALA) reduced the relative expression of pro-inflammatory cytokines (interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α) in immune cells from dairy cows (Rezamand et al., 2016). This is due to the conversion of ALA into the more biologically active EPA, DPA, and DHA which are known to exert positive immune modulatory effects (Moallem, 2018).

Numerous studies have shown that CLA supplements reduced milk fat yield, alleviated energy demands, and improved EB in dairy cows (Pappritz et al., 2011; Vogel et al., 2020). In this regard, the *trans*-10, *cis*-12 CLA was identified to induce MFD (Baumgard et al., 2000), whereas *cis*-9 *trans*-11 CLA may act as an antioxidant, and attenuate inflammatory responses during the periparturient period (Bauman et al., 2008; Galamb et al., 2017). These features of EFA and CLA in the transition period are the topic of interest for this research.

Just recently, studies started to investigate the synergistic effect of these FA. The following studies are based on a project named DEFA “deficiency in EFA in dairy cows”. In DEFA, an animal experiment was performed at the Research Institute for Farm Animal Biology (FBN), testing the effects of abomasal infusions of EFA and CLA alone and in combination (EFA+CLA) from late gestation until mid-lactation in dairy cows. Working on this study,

Haubold et al. (Haubold et al., 2020) focused on mid-lactating dairy cows' performance, systemic and hepatic antioxidative status, inflammatory responses, and fatty acids profiles. CLA treated cows had a higher n-6/n-3 FA ratio in milk, while this ratio was lower in EFA treated cows. CLA supplementation in both groups receiving CLA alone or in combination with EFA, decreased energy-corrected milk yield, milk fat content, milk protein content, and milk urea concentration while the concentration of milk citrate was increased. Moreover, plasma Glutathione peroxidase activity was higher, and EB tended to be higher in CLA and EFA+CLA groups.

In a further study from this experiment, Vogel et al. 2020 reported that CLA and EFA+CLA supplementation in transition dairy cows induced MFD, increased body fat mass, and improved EB in late and early lactation. On the other side, EFA supplementation had no effect on EB. CLA but not EFA+CLA elevated the milk citrate concentration in early lactation and reduced milk urea during the whole period, whereas EFA+CLA supplementation increased the acetone concentration. Postpartum NEFA concentration and liver TAG were lower in CLA and EFA+CLA groups as compared to the CTRL group. The authors concluded that CLA and EFA+CLA supplementation but not EFA alone improved the metabolic status.

In the third study by Gnott et al. (2020a) (Gnott et al., 2020), the effects of EFA and CLA on plasma lipid status and inflammatory response in dairy cows around parturition were investigated. CLA supplementation increased *cis*-9, *trans*-11 CLA in PL, and in the erythrocyte membranes. The EFA treatment decreased the plasma concentration of haptoglobin and increased IL-1β. Plasma paraoxonase which is an indicator of liver function and health was higher in EFA+CLA on d 49 after calving. The hepatic mRNA abundances of haptoglobin (HP), serum amyloid A2 (SAA2), fibrinogen (FGA), C reactive protein (CRP), paraoxonase (PON1), IL-1α (IL1A), I L-1β (IL1B), TNF, Toll-like receptor 4 (TLR4), cyclooxygenase 1 and 2 (COX1, COX2) were not affected by treatment. All these findings imply a more distinct anti-inflammatory response in EFA and EFA+CLA than CLA treatment alone during the transition period.

Each of these studies provides some clues regarding the metabolic and immune shifts in response to EFA and/or CLA supplementation at metabolite and mRNA levels. However, the exact underlying mechanism is still unclear.

1.4 Fatty acids and metabolic homeostasis

This chapter reviews the involvement of EFA and CLA in the metabolic and hormonal regulation of homeostasis and gluconeogenesis with a focus on the physiological adaptations during the transition period. There are inconsistencies in results between animal studies due to the ratio of fat in a diet, the FA composition, and even the time and duration of supplementation.

1.4.1 Effects of EFA and CLA on blood metabolites, hormones, and biomarkers of homeostasis

Metabolic adaptations are mediated by changes in the pattern and responsiveness of tissues to hormones. As such, a part of the homeorhetic metabolic regulation is mediated by reduced insulin sensitivity and responsiveness in distinct peripheral tissues (Hammon et al., 2009). Low plasma insulin levels of early lactation induce interdependent changes in the insulin / insulin-like growth factor 1 (IGF-I) /glucose signaling pathway which in turn accelerates the uptake of blood glucose into the mammary gland, a tissue that is not insulin-responsive in early lactation (Butler et al., 2003). A low IGF-1 level at the onset of lactation increases the production of growth hormone (GH), which boosts the energy supply for mammary lactogenesis by stimulating hepatic gluconeogenesis and lipolysis (Kim, 2014). Also, GH antagonizes insulin actions leading to insulin resistance (IR), and reduces the rate at which liver, muscle, and AT can utilize glucose (De Koster and Opsomer, 2013). Despite all these homeorhetic adaptations, the glucose demand is not completely met in high yielding animals resulting in a hypoglycemic state which is associated with immunosuppression and infertility problems (Wankhade et al., 2017). As mentioned previously, supplementing an effective dosage of EFA and CLA is a strategy to repartition the energy and alleviate the NEB in order to sustain metabolic homeostasis (Zapata et al., 2015).

In this context, CLA have been shown to improve the metabolic status (Baumgard et al., 2002; Benninghoff et al., 2015; Galamb et al., 2017) and modulate the repartitioning of nutrients and energy (glucose) in postpartum dairy cows (Harvatine et al., 2009b; Grossen-Rosti et al., 2018). Dietary CLA and n-3 PUFA at early lactation induced lesser body weight loss and lowered blood NEFA and BHBA concentrations (Odens et al., 2007; Ballou et al., 2009; Hutchinson et al., 2011; Qin et al., 2018). Qin et al. (2018) demonstrated that postpartum dietary supplementation with CLA or PUFA decreased body fat mobilization, increased glucose concentration, and reduced insulin sensitivity in dairy cows. In an earlier study, cows receiving CLA supplements prepartum experienced a more negative EB, while plasma glucose concentration was higher immediately after calving (Hotger et al., 2013). CLA did not affect

insulin concentration but reduced endogenous glucose production in week 3 after parturition (Hotger et al., 2013). These inconsistent results may reflect the differences between prepartum and postpartum FA supplementation.

In addition to insulin, various signaling molecules of the AT termed "adipokines" modulate inflammation and IR (Rabe et al., 2008). Leptin is one of the important ones, which is, along with insulin, involved in the regulation of glucose homeostasis (Paz-Filho et al., 2012). Insulin acts to increase leptin production (Leury et al., 2003), whereas leptin inhibits insulin mRNA transcription and secretion (Havel, 2004). The n-3 PUFA, but not CLA, increased the plasma leptin concentration at the onset of lactation (Qin et al., 2018). In another study, the expression of leptin receptor (LEPR) was not affected by n-3 PUFA treatment (Elis et al., 2016). Moreover, CLA supplementation in the prepartum period kept plasma IGF-1 and leptin concentration at higher levels in week 5 postpartum (Csillik et al., 2017). The underlying mechanism of elevated circulating IGF-I levels seemed to be independent of energy balance and may probably be mediated by hepatic sensitivity to insulin (Castaneda-Gutierrez et al., 2007). The IGF-I and GH are regulated in a negative feedback loop, which means induced insulin levels result in an increase in the liver GH receptor number cause increased plasma IGF-I concentrations (Butler et al., 2003; Weber et al., 2017). Adiponectin is another adipokine involved in IR when released by adipocytes inhibits lipolysis and stimulates FA oxidation in the liver (Yamauchi et al., 2001). Diet enriched with n-3 PUFA increased the AT expression of adiponectin (ADIPOQ) gene in postpartum dairy cows (Elis et al., 2016). In contrast, CLA supplementation decreased serum adiponectin concentrations which then act towards prolongation of IR and drain of nutrients towards milk synthesis (Singh et al., 2014). However, Saremi et al. (2014) reported that CLA had no significant effect on ADIPOQ expression in AT (Saremi et al., 2014). In humans, it has been suggested that the adiponectin: leptin ratio (ALR) is an effective and reliable marker of insulin sensitivity (Inoue et al., 2005) and metabolic syndrome (Mirza et al., 2011), but in dairy cows, the ALR did not provide any further information (Singh et al., 2014). The reason for these inconsistent results is not clear. It was suggested that prepartum EB had marginal effects on leptin concentrations but not on adiponectin concentrations (Mann et al., 2018).

Moreover, the method ''Revised Quantitative Insulin Sensitivity Check Index'' (RQUICKI) was adopted from human studies as an indirect measurement of systemic insulin sensitivity in dairy cows (Holtenius and Holtenius, 2007). In this regard, Grossen-Rösti (2018) reported that cows receiving CLA had lower values for RQUICKI, although the concentration of basal insulin was not significantly changed, but tended to be higher. It was also reported that CLA

supplementation reduced RQUICKI by 8%, which was attributed to greater insulin concentrations (Saremi et al., 2014).

From the literature, it could thus be postulated that FA supplementation mainly acts on repartitioning of nutrients probably in favor of improved EB but the underlying hormonal and metabolic mechanisms are yet to be elucidated. In addition, the inconsistent results point to the importance of dose, duration, and isomeric composition of supplemented FA.

1.4.2 Fatty acids and transcriptional regulation of metabolism

It has been shown that PUFA regulate the expression of genes mainly via ligand-dependent transcription factors, sterol regulatory element binding proteins (SREBP), and peroxisome proliferator-activated receptors (PPAR) (Nakamura et al., 2004). These two nuclear receptors are the master regulators of lipid and energy metabolic pathways, by inducing the genes for mitochondrial and peroxisomal FA oxidation as well as those for ketogenesis in mitochondria (Nakamura et al., 2004). Milk fat synthesis implies a complex interactive crosstalk between liver and mammary tissue which is also controlled by numerous transcriptional and posttranscriptional regulations including SREBP and PPAR (Figure 10, (Bu et al., 2017).

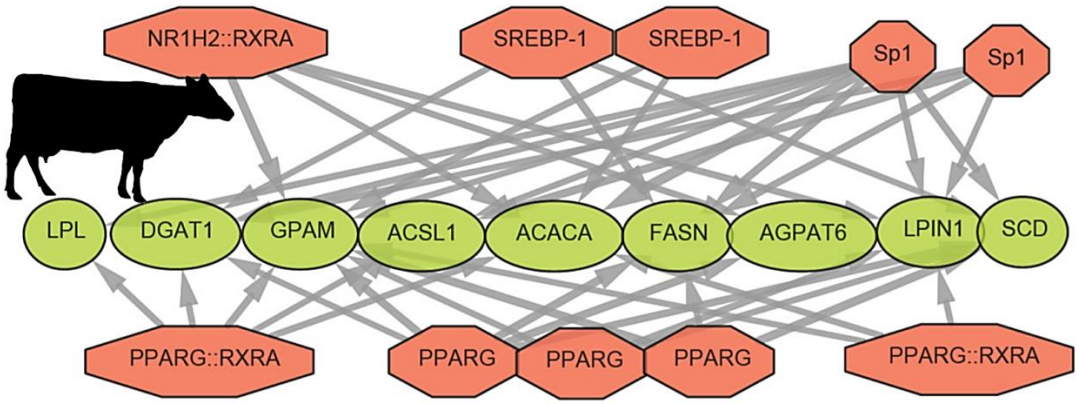

Figure 10. Transcriptional and posttranscriptional regulation of milk fat synthesis (Osorio et al., 2016). NR1H2: Nuclear Receptor Subfamily 1 Group H Member 2; RXRA: Retinoid X Receptor Alpha; SREBP-1: Sterol Regulatory Element Binding Transcription Factor 1; SP: Specificity Protein; LPL: Lipoprotein Lipase; DGAT1: Diacylglycerol O-Acyltransferase 1; GPAM: Glycerol-3-Phosphate Acyltransferase, Mitochondrial; ACSL: Acyl-CoA Synthetase Long Chain Family Member 1; ACACA: Acetyl-CoA Carboxylase Alpha; FASN: Fatty Acid Synthase; AGPAT6: Glycerol-3-Phosphate Acyltransferase 4; SCD: Stearoyl-CoA Desaturase; PPARG: Peroxisome proliferator-activated receptor gamma.

During a diet-induced MFD in dairy cows, the proportion of *de novo* synthesized FA is drastically decreased (Harvatine et al., 2014). The suggested mechanisms involve the alteration in lipogenic capacity by reducing the expression of key lipogenic factors (SREBP1 and thyroid hormone responsive spot 14 protein (THRSP, or Spot14, S14)), and lipid synthesis enzymes (including fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD1), lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4), and glycerol phosphate acyl transferase (GPAT)), as reviewed by (Harvatine et al., 2009a). SREBP1 is a master regulator of lipid synthesis, which belongs to a family of nuclear transcription factors controlling genes involved in *de novo* FA synthesis, FA desaturation, long-chain FA uptake, and TAG esterification (Li et al., 2014). There is some evidence that a dairy cow's SREBF1 expression is downregulated in the mammary gland in response to CLA supplementation (Harvatine and Bauman, 2006; Gervais et al., 2009; Han et al., 2012; Vyas et al., 2013; Harvatine et al., 2018). Furthermore, THRSP, as a transcriptional regulator (Schering et al., 2017), can affect the activity of PPAR-gamma (PPARγ), SREBP1, and lipogenic enzymes (Cui et al., 2015). THRSP expression was downregulated in response to CLA supplementation both *in vitro* (Peterson et al., 2004) and *in vivo* (Harvatine and Bauman, 2006; Harvatine et al., 2018). These two latter (SREBF1 and THRSP*)* are considered as primary transcriptional regulation mechanisms of CLA-induced MFD (Harvatine et al., 2018).

For what concerns EFA, n-3 PUFA are not only the ligand activator of nuclear receptors but also impact intracellular signaling processes, gene expression, and the production of lipid mediators by altering the FA composition of the cell membrane, as reviewed by (Calder, 2012). In dairy cows, dietary n-3 PUFA (linseed and grass source) supplementation reduced the expression of the lipogenic genes acetyl-CoA carboxylase alpha (ACACA), FASN, LPL, and SCD in AT (Hiller et al., 2011; Corazzin et al., 2013). Furthermore, the relative expression of lipogenic genes involved in the SREBP signaling pathway including FA activation (ACSS1; Acyl-CoA Synthetase Short Chain Family Member 1), FA *de novo* synthesis (ACACA), and FA esterification (AGPAT6; 1-acylglycerol-3-phosphate O-acyltransferase 6) was downregulated in mammary tissue of fish oil supplemented ewes (Carreno et al., 2016; Suarez-Vega et al., 2017; Toral et al., 2017). These data suggest that this nuclear transcription factor is the key messenger in translating nutritional stimuli into changes in gene expression and metabolism.

In cattle, both liver x receptor (LXR) (Oppi-Williams et al., 2013; Harvatine et al., 2014) and its coactivator (PPARγ) (Shi et al., 2014) participate in *de novo* FA synthesis, although the

mechanism remains largely unexplored (Liu et al., 2016). In mammalian cells, the PPARs are the main regulators of lipid metabolism and adipogenesis; they are activated by natural ligands such as PUFAs (Bionaz et al., 2013). Particularly, PPARγ is considered as a key regulator of insulin sensitivity and its target genes comprise ADIPOQ, IL-6, and TNF-a (Rosen and MacDougald, 2006; van Doorn et al., 2006; Tontonoz and Spiegelman, 2008). Harvatine et al. (2014) reported that neither the LXR isoform nor PPARGC1A expression in the mammary gland was modified by CLA treatment. Also, mammary expression of the ATP-binding cassette subfamily A member 1 (ABCA1), a target gene of LXR, was not affected during CLA-induced MFD (Harvatine et al., 2014). These results suggest that PPARγ may either be responsive to other FA or to longer duration of CLA-induced MFD (Harvatine et al., 2018). The transcriptional regulation of genes involved in metabolic homeostasis is probably the major pathway regulatated by FA, although more studies are needed to fully address the underlying pathways.

1.5 Fatty acids and modulation of immune response

Beneficial effects of EFA and CLA on immune and inflammatory responses were the subject of many animal studies (Lessard et al., 2003; Bontempo et al., 2004; He et al., 2007; Yao et al., 2012; Garcia et al., 2014; Didara et al., 2015). There is evidence that EFA and CLA are capable to attenuate inflammation by modulating inflammatory mediators such as cytokines, eicosanoids, resolvins, PG, leukotrienes, and immunoglobulins (for review see (Calder et al., 2011)). The following section provides an overview of the latest studies performed to investigate the immune modulatory effect of EFA and CLA supplementation in dairy cows. The mechanisms discussed below are mostly interlinked which might regulate each other through positive and negative feedback loops, although the full extent of these interactions is yet to be elucidated.

1.5.1 Fatty acids and inflammatory mechanisms

A state of inflammation-like condition without any clinical symptom is typical in early lactation dairy cows (Bertoni et al., 2008). It is attributed to tissue damage and remodeling in the mammary gland, liver, adipose tissue, and placenta in response to the new physiological situation (Horst et al., 2021). This condition is accompanied by increased production of pro-inflammatory cytokines which stimulate the acute phase response (Fleck, 1989). The acute phase response is characterized by induction of positive acute phase proteins (APP), e.g., Hp

(Robinson et al., 2002) and SAA (Cohen et al., 1997), and reduction of negative APP (e.g., albumin, apolipoproteins, retinol binding protein (RBP), transferrin, and transthyretin) (Ceciliani et al., 2012).

The PUFA are regarded to play a pivotal role in inflammation and associated metabolic disorders, mainly through one of the following general mechanisms (as reviewed by (Calder et al., 2011)) and summarized in Figure 11.

• Altering the concentrations of lipoproteins, immune-associated metabolites, and hormones;

• Affecting the expression and activation of inflammatory factors such as PPARs and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB);

• Altering signal transduction pathways (e.g., protein kinases);

• Integrating into complex lipids such as cell membrane PL, and thus affecting cellular fluidity and function;

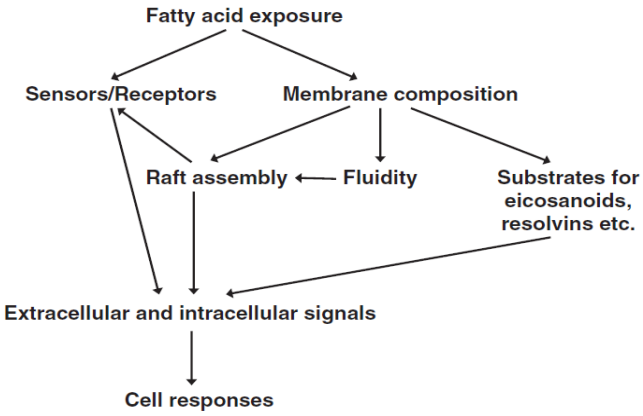

Figure 11. The mechanisms by which polyunsaturated fatty acids can influence inflammatory cell function (Calder et al., 2011).

The n−3 FA were clearly shown to exert anti-inflammatory properties which inhibit some aspects of inflammation, through previously explained mechanisms in particular by reducing the expression of inflammatory genes by inhibition of the pro-inflammatory transcription factor NF-κB, and activation of the anti-inflammatory transcription factor PPARγ (for review (Calder, 2015)).

Among the CLA isomers, the *cis*-9, *trans*-11 CLA, and the *trans*-10, *cis*-12 CLA isomer received much attention for their function in immune regulatory mechanisms. Elevated glucose and depressed BHB concentrations were observed in an experiment investigating the response of CLA-treated dairy cows during a bacterial lipopolysaccharide inflammatory challenge (Gross et al., 2018). Glucose is a preferable source of energy for immune cells (Ingvartsen and

Moyes, 2015), and thus elevated glucose means feasible fuel for immune cells which consequently improved immune response during an inflammatory process (Ingvartsen and Moyes, 2015). CLA may also increase glucose concentrations by repartitioning energy and stimulating immune cells to preferentially use BHB as a source of energy (Gross et al., 2018). As mentioned earlier, PPARγ exerts anti-inflammatory effects by interfering with NF-κB function and preventing NF-κB translocation into the nucleus, thus inhibiting the production of pro-inflammatory cytokines (Tak and Firestein, 2001). NF-κB is the major transcription factor that regulates the expression of pro-inflammatory cytokines in T cells (Lawrence, 2009). Recently, Dipasquale et al. (2018) compared the anti-inflammatory effect of EFA and CLA in immortalized bovine mammary epithelial cells (BME-UV1) (Dipasquale et al., 2018). In this study, PPARγ had higher and PPARα had lower expression in cells treated with EFA and *trans*-10, *cis*-12 CLA which resulted in decreased expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6). Consistent with these results, other reports demonstrated the involvement of EFA and CLA in modulating the secretion of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-10, and interferon-γ (Song et al., 2005; Calder, 2011; de Jong et al., 2014), as well as apoptosis and extracellular respiratory burst (Ávila et al., 2020). Also, the inflammatory response (IL-1β and IL-6) of neonatal calves was affected by maternal supplementation of EFA and CLA, in which EFA exert anti-inflammatory properties while CLA were proinflammatory (Liermann et al., 2021). In contrast, Saremi et al. (2014) reported that CLA supplementation in primiparous cows decreased PPARγ2 mRNA abundance in the mammary gland (Saremi et al., 2014). There is another study in which PPARγ expression was not affected by CLA treatment (Kadegowda et al., 2009). These inconsistent results are probably due to methodological differences in the experimental designs.

The interaction of dietary FA with APP to affect inflammation is also of interest. Alterations in the concentrations of APP are indicative of the acute phase response occurring during inflammation (Cray et al., 2009). In contrast to cytokines that are hormone-like proteins with signaling functions, APP act as effector molecules with higher concentration and a broad spectrum of functions (Malle et al., 2009). The APP are primarily synthesized by hepatocytes but also from other peripheral tissues with the general goal of reestablishing homeostasis and promoting healing (Ceciliani et al., 2012). In cattle, Hp, SAA, and ceruloplasmin are the major positive APP (Humblet et al., 2006). CLA supplementation did not alter serum Hp concentration and hepatic Hp mRNA abundance (Saremi et al., 2012), but decreased plasma ceruloplasmin was reported (Qin et al., 2018). In contrast, n-3 PUFA (fish oil) induced higher plasma concentrations of both Hp and ceruloplasmin (Qin et al., 2018). However, for Hp and

SAA mRNA abundance, the effects of CLA were only marginal and also inconsistent when comparing different tissues of dairy cows (Saremi et al., 2012).

Furthermore, EPA and DHA can also be converted by cytochrome P450 epoxygenases to anti-inflammatory metabolites such as epoxyeicosatetraenoic acid (EpETE or 17,18-EEQ) and epoxydocosapentaenoic acid (EpDPE or 19,20-EDP), respectively (Ostermann et al., 2019) (Figure 12).

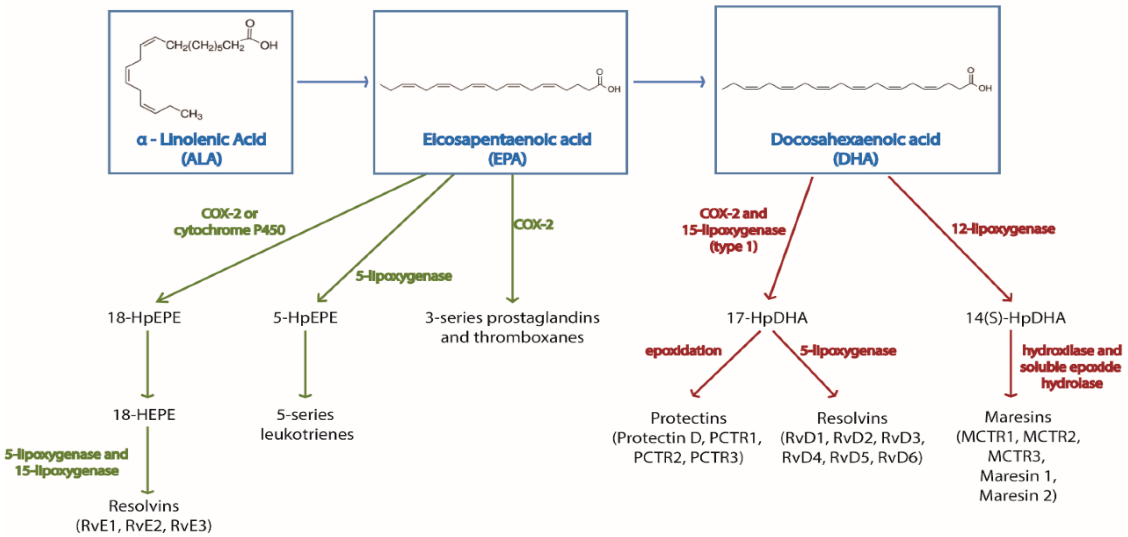

Figure 12. Possible mechanisms by which n-3 fatty acids turn into immune-related metabolites (Gutiérrez et al., 2019). The figure shows α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), their downstream metabolites, and the enzymes regulating their synthesis. The n-3 fatty acids are substrates for COX and cytochrome P450 epoxygenases which can convert them into 3–5 series of eicosanoids, resolvins, prostaglandins, leukotrienes, and thromboxanes. PCTR: protectin conjugates in tissue regeneration, RvE: resolvin E, RvD: resolvin D, MCTR: maresin conjugates in tissue regeneration.

1.5.2 Fatty acids and oxidative damage

As previously described, dairy cows are particularly susceptible to oxidative stress and immune disruption during the transition period, due to excessive lipid mobilization that accentuates the production of reactive oxygen species (ROS) (Bernabucci et al., 2005). The excessive ROS production activates the NF-κB transcription factor which in turn stimulates the expression of various pro-inflammatory mediators, including cytokines (Sordillo and Raphael, 2013).

Generally, PUFA are susceptible to non-enzymatic oxidation by ROS forming α, β-polyunsaturated lipid aldehydes such as malondialdehyde (MDA) which play an important role in many cellular processes (Bochkov et al., 2017). These aldehydes are considered xenobiotic-like metabolites which should be detoxified by phase I and phase II drug metabolism in

cytochrome P450 pathways, otherwise their accumulation would turn into mitochondrial dysfunction and apoptosis (Rogero et al., 2020).

A large body of literature has already been established in terms of immune induction of FA, however, up to now, just a few and somehow conflicting studies exist in regard to the antioxidant capacity of EFA and CLA in dairy cows. In a recent study it has been shown that the CLA isomers containing *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 reduced the production of ROS in BME-UV1 cells when compared with EFA (Dipasquale et al., 2018). Earlier, Basirico et al. (2017) reported that treatment of BME-UV1 with EFA and CLA improved antioxidant capacity through induction of higher intracellular glutathione (GSH) content, nicotinamide adenine dinucleotide phosphate (NADPH) concentration, and γ-glutamyl-cysteine ligase (γGCL) activity and reduced intracellular MDA levels (Basirico et al., 2017). These results are in line with other findings indicating protective effects of EFA and CLA isomers against oxidative stress and lipid peroxidation in animal models (Arab et al., 2006; Andreoli et al., 2010; Chinnadurai et al., 2013; Basirico et al., 2015; Espinosa-Diez et al., 2015). The reduced concentration of MDA, as a biomarker of lipid peroxidation (Ayala et al., 2014), demonstrate the protective effect of FA against lipoperoxidation (Basirico et al., 2017). It was also suggested that replacing CLA with ARA reduced the amount of MDA produced by lipid peroxidation without reducing relative lipid peroxidative activity (Livisay et al., 2000). Earlier, Basirico (Basirico et al., 2015) also reported that the antioxidant properties of CLA generate a high redox status in BME-UV1. A similar effect was reported in an *in vivo* study, showing that CLA supplemented animals exhibited a lower concentration of MDA, whereas the mean serum concentration of hydroperoxides, as an indicator of oxidative stress, remained unchanged (Hanschke et al., 2016).

Therefore, FA can counteract oxidative stress mainly through scavenging ROS and decreasing lipid peroxidation. In addition, results from i*n vitro* studies indicated that CLA have a more powerful free radical quenching activity than LA and ALA (Fagali and Catala, 2008).

1.6 Focusing on CLA and EFA during the transition period by application of "Omics" technologies

This section describes emerging high-throughput technologies termed "Omics", which permit simultaneous monitoring of cells, tissues, organs, and the whole organism at the molecular level. With the advent of these technologies, understanding the complex biological systems has

become increasingly dependent on data generated at these levels. The suffix “Omics” applies for technologies to study the entity of genes (genomics), mRNA/microRNA (transcriptomics/microRNAomics), proteins (proteomics), or metabolites (metabolomics/lipidomics) in a specific biological sample (Horgan and Kenny, 2011).

Genomics stands for mapping the whole genome and performing sequencing to provide insights into the coding gene heterogeneity (Morrison et al., 2014). Transcriptomics is the next level, which simultaneously profiles mRNA/miRNA abundance. Common technologies for transcriptomics are cDNA microarray, oligonucleotide microarray, cDNA-AFLP (Amplified Fragment Length Polymorphism), SAGE (Serial analysis of gene expression), and RNAseq (Wang et al., 2009; Ozsolak and Milos, 2011).

Proteomics is the comprehensive, quantitative description of protein expression patterns and abundance, and post-translational modifications under the influence of biological (such as disease) or environmental conditions. Common methods for proteomics are 2-Dimensional gel electrophoresis or using mass spectrometry (MS) based proteomics such as liquid chromatography-mass spectrometry (LC-MS), MS-MS (Feist and Hummon, 2015).

Metabolomics is the next level at which a large group of metabolites can be analyzed in biological samples. Currently, technologies available for analyzing the metabolome are MS, nuclear magnetic resonance, LC, gas chromatography (GC), LC-MS, and GC-MS (Gowda and Djukovic, 2014). The integration of these technologies allows for the simultaneous monitoring of thousands of molecules.

Evaluation of the complex data obtained by omics data and assigning them to cellular pathways is then determined by means of system biology (Romero et al., 2006). Over the last decades, the use of these technologies has greatly advanced our understanding of the regulation of many physiological and pathophysiological processes (Li et al., 2017).

The application of omics technologies in dairy cattle has grown steadily during the past years in different aspects of dairy cows research (nutrition, physiology, disease, and so on) (Mansor et al., 2013; Min et al., 2016; van Altena et al., 2016; Zachut et al., 2016). Applying Omics technologies to transition period-related disorders will be beneficial to the identification of pathways and biomarkers for future management strategies (Figure 13) (reviewed by (Ceciliani et al., 2018)). It should be noted that Omics technologies are rather for generating new hypotheses than answering (unsolved) biological concerns/issues.

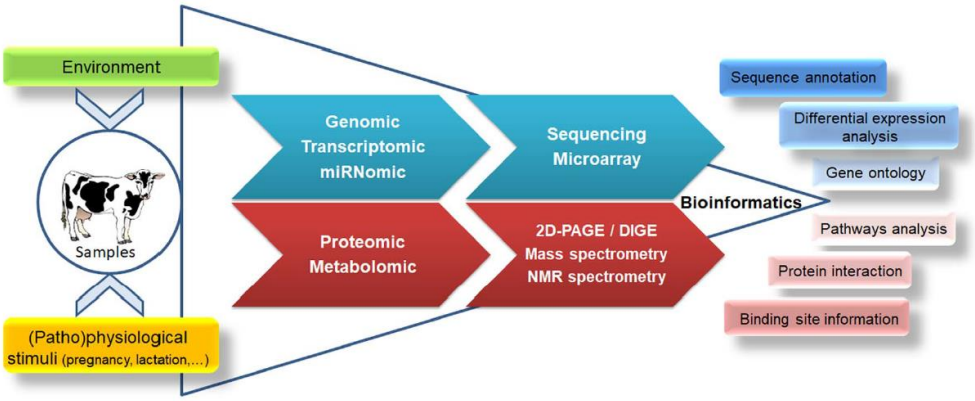

Figure 13. Application of Omics technologies in dairy cows (Ceciliani et al., 2018).

2. Objectives

The preceding chapter provides an overview and general description of the synthesis and metabolism of biologically active FA including EFA (with the focus on n-3 FA) and CLA. The suggested mechanisms by which FA could affect metabolism and immunity in dairy cows with an emphasis on the transition period were addressed. From the literature, it could be postulated that supplementation of dairy cows diet with EFA and CLA typically impacted metabolism and energy repartitioning, and milk fat content, but only marginally affected inflammation and the immune system. These features of FA could be beneficial to attenuate metabolic pressure and immunosuppression in transition dairy cows.

To date, most ruminant studies about FA supplementation focused on performance and milk fat composition, and a growing number of studies highlighted their importance on metabolism, inflammation, and oxidative status, yet the underlying mechanisms have not been fully discovered. The main purpose of this study was to investigate the synergistic effect of CLA and EFA in transition cows using proteomic analyses to get deep insights into the molecular mechanisms by which supplemented FA interact with metabolic and immune homeostasis. With regards to the main objective, in this thesis we investigated:

The effect of EFA and CLA on (1) the plasma and (2) the liver proteome of dairy cows at various time points around parturition.

The Omics-based study has no prior (formal) hypothesis, but interpretations were made based on hypotheses previously described in the DEFA project (listed below). This study was designed based on the DEFA animal experiment in which dairy cows were fed the same basic diet supplemented with either coconut oil which contains a low amount of PUFA, or a combination of EFA (linseed and safflower oil as a source of EFA, in particular, n-3 FA) and CLA (a mixture of *cis-9, trans-11* and *trans- 10, cis-12* isomers), whith the hypotheses that:

- ✓ A combined EFA and CLA supplementation affects performance and energy utilization during late gestation and early lactation.
- ✓ A combined EFA and CLA supplementation has an impact on lipid metabolism in the transition dairy cow.
- ✓ A combined EFA and CLA supplementation affects the endocrine regulation of nutrient partitioning regarding the somatotropic axis.

We also integrated serum proteome profiles, metabolites, and hormones to extract novel information regarding the metabolite–protein networks. Applying a proteomics approach through our defined experimental design not only advanced our understanding of the major metabolic pathways affected by FA but also provided a big database for future studies in the field of physiology of the transition period.

3. Article 1 (Journal of Proteomics, Volume 252, 10 February 2022, 104436)

Liver proteome profiling in dairy cows during the transition from gestation to lactation: effects of supplementation with essential fatty acids and conjugated linoleic acids as explored by PLS-DA

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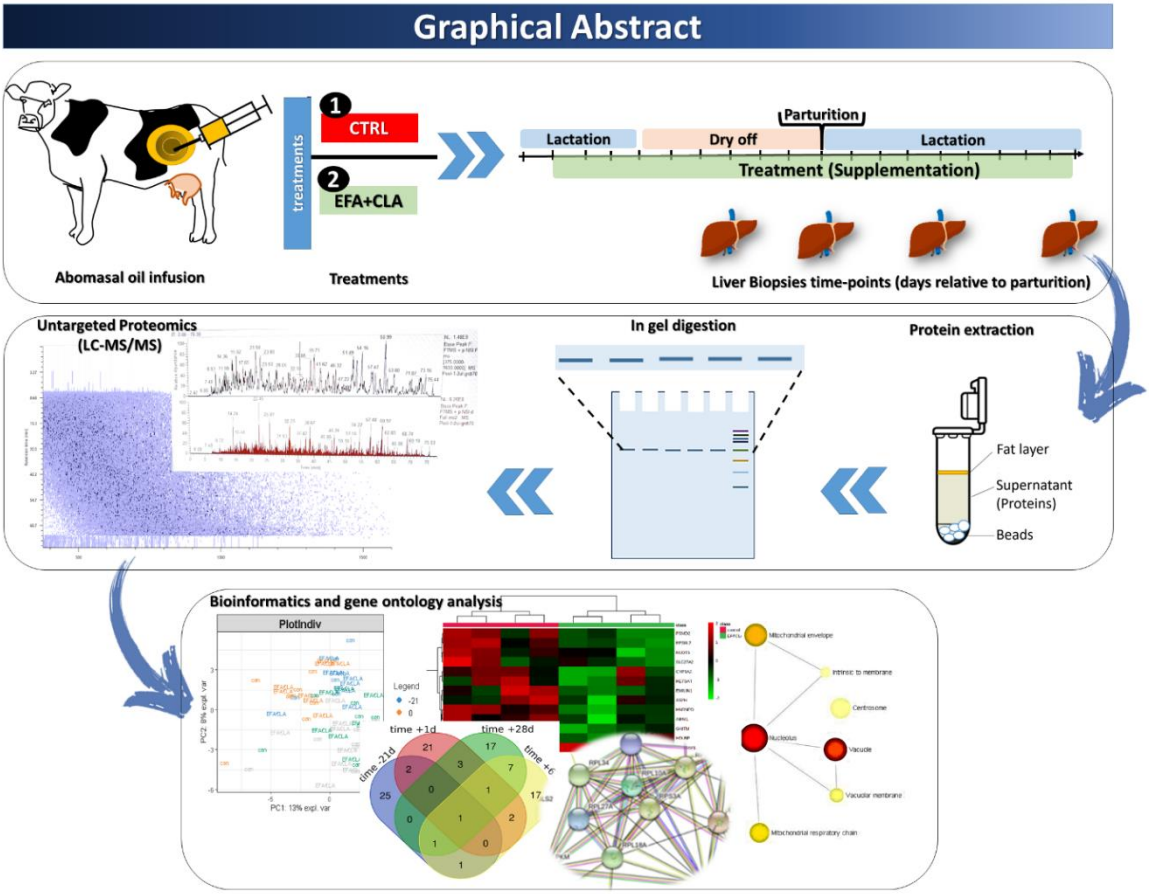
Highlights

1. Supplementation with fatty acids affected the liver proteome in dairy cows
2. Out of 1680 proteins identified, 96 were differentially abundant
3. The key pathways involved were Cytochrome P450 and ω-oxidation of fatty acids
4. Specific cytochrome P450 (CYP) enzymes were identified at each timepoint

Graphical abstract



Abstract

This study aimed at investigating the synergistic effects of essential fatty acids (EFA) and conjugated linoleic acids (CLA) on the liver proteome profile of dairy cows during the transition to lactation. 16 Holstein cows were infused from 9 wk antepartum to 9 wk postpartum into the abomasum with either coconut oil (CTRL) or a mixture of EFA (linseed + safflower oil) and CLA (EFA+CLA). Label-free quantitative proteomics was performed in liver tissue biopsied at days -21, +1, +28, and +63 relative to calving. Differentially abundant proteins (DAP) between treatment groups were identified at the intersection between a multivariate and a univariate analysis. In total, 1680 proteins were identified at each timepoint , of which between groups DAP were assigned to the metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, steroid hormone biosynthesis, glycolysis/gluconeogenesis, and glutathione metabolism. Cytochrome P450, as a central hub, enriched with specific CYP enzymes comprising: CYP51A1 (d -21), CYP1A1 & CYP4F2 (d +28), and CYP4V2 (d +63). Collectively, supplementation of EFA+CLA in transition cows impacted hepatic lipid metabolism and enriched several common biological pathways at all timepoints that were mainly related to ω-oxidation of fatty acids through the Cytochrome p450 pathway.

Keywords: Liver Proteome, negative energy balance, postpartum, cytochrome p450, fatty acid oxidation, gene ontology

Significance

In three aspects this manuscript is notable. First, this is among the first longitudinal proteomics studies in nutrition of dairy cows. The selected timepoints are critical periods around parturition with profound endocrine and metabolic adaptations. Second, our findings provided novel information on key drivers of biologically relevant pathways suggested according to previously reported performance, zootechnical, and metabolism data (already published elsewhere). Third, our results revealed the role of cytochrome P450 that is hardly investigated, and of ω-oxidation pathways in the metabolism of fatty acids with the involvement of specific enzymes.

Introduction

Most mammals enter a state of negative energy balance (NEB) at the onset of lactation when the needs for lactation and maintenance cannot be met by feed intake. This metabolic status leads to mobilization of body reserves, mainly from adipose tissue in the form of non-esterified fatty acids (NEFA) to meet the energy requirements for lactation (Shen et al., 2018). In high-yielding dairy cows, the liver plays a crucial role in metabolic homeostasis and energy production by metabolizing NEFA via precisely regulated signaling and cellular pathways (Moran et al., 2016). However, hepatic lipid metabolism is impaired at the onset of lactation when uptake of NEFA by the liver exceeds their oxidation and the export capacity via lipoproteins and may thus result in a fatty liver syndrome (Li et al., 2013).

Essential fatty acids (EFA), including linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3), affect the energy and FA metabolism, inflammation, and immune responses through activation of nuclear receptors (Pires and Grummer, 2008; Vaughan et al., 2012; Hussein et al., 2013). Conjugated Linoleic Acids (CLA) which are stereo-isomers of LA have been reported to induce milk fat depression (MFD), thus partitioning energy by sparing milk energy for other organs (Bichi et al., 2013; Suárez-Vega et al., 2019). Energy spared from reduced milk fat synthesis was shown to affect energy partitioning, as toward adipose tissue fat stores (Harvatine et al., 2009; Thering et al., 2009) and consequently to decrease plasma NEFA concentration and the risk for fatty liver (Vogel et al., 2020). The shift in dairy farming towards modern indoor production systems went along with a change from using pasture (grass) to feed rations that are largely based on so-called total mixed rations (TMR), in which the roughage component is mainly corn silage in many countries. The decreased or lacking consumption of fresh grass leads to a drop in the intake of ω-3 FA and CLA production (Gómez-Cortés et al., 2009; Mohammed et al., 2009; Bernard et al., 2018). A large body of work has highlighted the increased body deposition of n-3 FA and CLA in dairy cows fed with fresh grass in comparison to corn silage (for example (White et al., 2001)).

Assessing the effects of specific FA in different feeding practices is complex. Using an experimental model in which dairy cows receiving a corn-silage-based ration without any grass, the EFA and CLA's effects were tested by abomasal supplementation avoiding microbial degradation in the forestomaches (Gnott et al., 2020; Vogel et al., 2020; Vogel et al., 2021). The results showed that the FA marginally improved metabolic health by induction of MFD, which increased energy balance and reduced plasma concentration of triglycerides and NEFA. In addition, paraoxonase, a hepatic antioxidant enzyme, was elevated postpartum (PP) by the FA application. Although some of these impacted metabolites and proteins were directly or

indirectly related to the liver, EFA and CLA-driven hepatic responses remain to be investigated.

Improvements in proteomics in the last decade have increased our understanding of the biological pathways impacted by various physiological conditions and diseases (Yates, 2019). Characterization and comprehensive proteome profiling of the liver as a central organ in energy and lipid metabolism could open up new insights into the regulatory metabolic pathways influenced by different nutritional supplements. Proteomics results allow better understanding and predicting the metabolism and help define rapid biomarkers for use in the early diagnosis of steatosis or other metabolic diseases associated with liver metabolic health (Veyel et al., 2020). In this regard, there are several studies in dairy cows entailing the liver proteome for investigating feed efficiency (Fonseca et al., 2019), fatty liver (Sejersen et al., 2012), and heat stress (Skibiel et al., 2018; Ma et al., 2019). In the current study, untargeted proteomics was applied on liver samples from dairy cows supplemented or not with EFA and CLA to investigate metabolic responses during several critical timepoints around parturition. To the best of our knowledge, this is the first proteomics report considering the longitudinal response of EFA and CLA in dairy cows during the transition from late pregnancy to early lactation.

Material and methods

Animals, Treatments, and Experimental Design

The trial was carried out as described previously (Vogel et al., 2020) with 16 multiparous (second lactation) German Holstein cows at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. The experimental animal procedures were evaluated and approved by the German Animal Welfare Act (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany; LALLF M-V/TSD/7221.3-1-038/15). More details on housing, feeding, feed intake, performance, and milk production of studied cows were presented earlier (Vogel et al., 2020). Briefly, dairy cows housed in a free-stall and abomasally injected with 1-control, the coconut oil (CTRL, n = 8; Bio-Kokosöl #665, Kräuterhaus Sanct Bernhard, KG, Bad Ditzenbach, Germany) or 2-EFA+CLA, a combination of linseed oil (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany), safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany) and Lutalin® (CLA, n = 8; *cis*-9, *trans*-11, 10 g/d *trans*- 10, *cis*-12 CLA, BASF SE, Ludwigshafen, Germany) for 18 weeks started from d 63 antepartum (AP) until d 63 PP (Figure 1 A). Supplements were injected twice daily at 0700 and 1630 h in equal portions through abomasal infusion lines (Teflon tube [i. d. 6 mm]

with 2 perforated Teflon flanges [o.d. 120 mm], placed in rumen cannulas (#2C or #1C 4'', Bar Diamond Inc., Parma, ID). The amount and FA composition of the lipid supplements is given in Supplementary, Table S1.

The cows were fed a conventional corn silage-based total mixed ration (TMR), formulated using the equation published by the German Society for Nutrition Physiology (2001 , 2008 , 2009) and Deutsche Landwirtschaftliche Gesellschaft (DLG, 2013) , for AP and PP. The basal diet was provided ad libitum at 0600 h, with free access to water and trace-mineralized salt blocks. The ingredients and chemical composition of the experimental diets are presented in Supplementary Table S2.

Liver biopsies

Liver tissue samples were obtained using a biopsy needle (outer diameter of 6 mm) under local anesthesia on d -21 AP, d 1 and d 28 PP, and after slaughtering the cows on d 63 PP as previously described (Weber et al., 2013) (Figure 1 A). The specimens were immediately frozen in liquid nitrogen and stored at −80 °C until protein extraction.

Liver Preparation for Proteomics Analysis

Frozen samples were first ground mechanically using a mortar and pestle chilled in liquid nitrogen. Eighty mg of tissue powder were placed in a reinforced 2-mL tube containing six ceramic beads (Dutscher, United Kingdom) and mixed with 1 mL of freshly prepared Laemmli sample buffer (50 mM Tris pH 6.8, 2% SDS, 5% glycerol, 2 mM DTT, 2.5 mM EDTA, 2.5 mM EGTA, H2O 920 µLl, 2x phosphatase inhibitors tablets (Perbio, Thermo Fischer, Hercules, California, USA), 1x protease inhibitor (Roche, Boulogne-Billancourt, France), 4 mM sodium orthovanadate, and 20 mM sodium fluoride). Subsequently, liver tissue was homogenized in a Precellys® 24 homogenizer (PEQLAB Biotechnology GmbH, Erlangen, Germany) at 6800 rpm, 3 x 30 sec (30-sec break between each cycle) at room temperature (RT). Immediately after the homogenization step, tubes were boiled for 10 min in 100 °C boiling water, followed by centrifugation for 15 min at 16000 g at RT. The supernatant was carefully separated and stored at −80 °C until proteomics analysis. An aliquot of the lysate was used to measure the total protein concentration using the bicinchoninic acid (BCA, Pierce, Rockford, IL) assay. For peptide preparation, 100 µg of protein were first concentrated in 1D SDS-PAGE gel containing 5-15% acrylamide for stacking and resolving gel, respectively. Once the proteins enter the resolving gel, the electrophoresis was stopped and a small piece of gel containing a major band was cut. After reduction and alkylation, proteins were subjected

to in-gel digestion with 10 ng/µL porcine trypsin (Promega, Madison, Wisconsin, United States) overnight (Figure 1 B).

Nano-LC-MS/MS Analysis

After digestion, the liver peptides mixture was analyzed using nano-scaled liquid chromatography (LC) in Ultimate 3000 RSLCnano system (Dionex) coupled to an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) for mass spectrometry (MS), adopting the methods previously described by (Santos et al., 2019). To reduce between-group variability, the LC-MS/MS was performed on all 64 samples consecutively and samples were randomly injected without any order related to time or treatment.

Briefly, a reversed-phase LC was carried out by loading 1 µL of the resuspended peptide mixture onto a trapping column (pre-column 5 mm length X 300 µm; Acclaim PepMap C18, 5 µm, 100 Å) equilibrated with trifluoroacetic acid 0.05% in water, at a flow rate of 30 µL/min. After 6 min, the pre-column was switched in-line with the analytical column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å, Dionex), equilibrated with 96% solvent A (99.5% H2O, 0.5% formic acid) and 4% solvent B (99.5% ACN, 0.5% formic acid).

Peptides were eluted at a 400 nL/min flow rate according to their hydrophobicity using a 4 to 20% gradient of solvent B for 60 min. Briefly, the analytical column was first equilibrated with 96% A solvent and 4% B solvent for 6 min, followed by a gradual increase of the B solvent to 20% for 70 min. Then, to clear the system from hydrophobic peptides, the B gradient rose from 20 to 80% in one min (at 77 min) and remained constant for further 5 minutes. Subsequently, the concentration of solvent B was decreased to 4% within 0.1 min and kept constant for 8 min to prepare the system for the next injection.

The nanoelectrospray ion source (Proxeon) was used as a connecter between the LC and Q Exactive HF-X mass spectrometer (Thermo Scientific). Eluates of LC step electro sprayed in positive-ion mode at 1.6 kV through a nanoelectrospray ion source heated to 250 °C. The Orbitrap Q Exactive HF-X MS used in HCD top 18 modes (i.e. 1 full scan MS and the 18 major peaks in the full scan selected for MS/MS). The mass spectrometry method duration was set to 79 min, the polarity was positive, and the default charge was 2.

On the MS1 scan, the parent ions were selected in the orbitrap Fourier transform mass spectrometry (FTMS) at the following parameters: a resolution of 60,000, an injection time of 50 ms. mass ranges from 375 to 1600 m/z and the Automatic gain control (AGC) target is set on 3 × 106 ions. Each MS analysis was followed by 18 data-dependent MS2 scans with an

analysis of MSMS fragments at a resolution of 15,000, 1 × 105 AGC, and an injection time of 100 ms. The HCD collision energy set to 28% NCE, and ~15 s dynamic exclusion.

Processing of raw mass spectrometry data

The processing of raw Peptide MS/MS spectra was performed in Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) using automatic alignment to the reference sample automatically defined by the software with the default parameter settings (maximum allowable ion charged set to 5 and Ions ANOVA p-value < 0.05). The mass generating function (mgf) list containing the detected and the quantified peptide ions were directly exported to MASCOT (version 2.5.1) interrogation engine and searched against a Bos taurus decoy database (Uniprot, download date: 2019/11/07, a total of 37,513 entries). The search criteria were set as follows: an enzyme digest of a protein set to trypsin, tryptic specificity required (cleavage C-terminal after lysine or arginine residues); 2 missed cleavages were allowed; carbamidomethylation (C) and oxidation (M) set as variable modification. The mass tolerance was set to 10 ppm for precursor ions, 0.02 Da for fragment ions, and FDR < 0.01. The identified peptides from the database search were imported back to Progenesis QI, and the corresponding proteins were identified and quantified based on the intensities of the specific validated peptides. Strict exclusion criteria (deamidated, carbamidomethyl, and oxidation contaminant proteins, having at least two peptides and two unique peptides, and presence in at least 50% of the samples in each treatment group/timepoint) were applied before analysis.

Data pre-processing

Statistical analyses were performed using the normalized intensity values combined with some in-house developed, EnhancedVolcano, MetaboAnalystR 3.0, and mixOmics R-packages in R statistical software (R version 4.0.0). Before the analyses, the following modifications were applied to proteins, in very severe filtrations: proteins with less than two unique peptides or having zero values in more than 50% of the replicates were not included in the analysis. After filtration, the log10 transformation and auto-scaling (z-transformation), which is mean-cantered and divided by the standard deviation of each variable applied to normalized intensities. The missing or zero values (indicated the peak did not reach the detectable thresholds) were imputed and replaced with the small values (half of the smallest positive value in the dataset). The PCA scatter plot was used to visualize the 2-D cross-section of hyperspace between samples and to distinguish the samples located far away from the treatment clusters

(potential outliers). One cow (from the CTRL group in timepoint -21d AP) considered an outlier by both principal component analysis and hierarchical clustering was removed from the analysis

Statistical analyses

The selection of the most important proteins (VIP) involved in the discrimination of the CTRL and EFA+CLA groups at each timepoint was based on the intersection of two complementary analyses.

A) Multivariate analysis

Firstly, PCA analysis was done to reduce the dimension of data and to visualize clustering of samples regardless of treatment groups. Partial Least Square Discriminant Analysis (PLS-DA) analysis (mixOmics package in R) ranked proteins importance in projection scores of the first two components (PC1 and PC2) in each timepoint . This step aims to rank the most discriminative proteins that contribute to cluster separation between treatment groups. A permutation test (defined to 100 random computations) was applied to disprove the over-fitting of the PLS-DA model. Since the permutation test indicated over-fitting in all timepoints, we performed the second filtration step according to univariate analysis. Although this study aimed to compare different treatments, not assessing populations parameter or identifying predictive model, therefore, permutation test's significance was not the case.

B) Univariate analysis

Secondly, from those proteins that were top VIP-ranked (score > 1.5), only ones with P-value < 0.05, and log2 (fold change) >1.3 (metaboanalyst R package) were considered as differentially abundant proteins (DAP) for further analysis. The P-value was assessed either by Student's t-test (parametric) or Wilcoxon Mann-Whitney test (non-parametric), according to the normality distribution of each protein (Shapiro-Wilk-Test) as previously described (Bazile et al., 2019).

C) Intersection between multivariate and univariate analyses to identify discriminative and differentially abundant proteins (DAP)

The intersection between the results from the two methods was chosen to reduce the list of relevant proteins involved in the treatment effect. Thus, we considered two filters, and we selected the proteins that passed through both by choosing the intersection between the two complementary methods. Hierarchical clustering Heat map analysis was performed to approve and visualize DAP (Figure 1 C).

Bioinformatics analysis of differentially abundant proteins

Before bioinformatics analysis, proteins' accession was converted into Gene ID using the UniProt (retrieve/ID mapping) database conversion tool, and undefined proteins were blasted and replaced with their Gene ID in Bos taurus and Homo sapiens. Then, the gene ontology (GO) analysis containing Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathways enrichment analysis of the DAP were performed in STRING web tool version 11.0 in Cytoscape and ProteINSIDE (version 1.0) constructed specifically under B. taurus interactions map. Only pathways with adjusted P-value < 0.05 (corrected to false discovery rate with Benjamini-Hochberg method) and having at least two hits in each pathway were considered as significantly enriched (Figure 1 C). REVIGO web server (http://revigo.irb.hr/) was used to summarize BP terms. Generated GO terms were submitted to Cytoscape version 3.8.2 and Networkanalyst.ca version 3.0 to build the interaction networks. Protein protein interaction networks was constructed by inputting the DAP in each timepoint to STRING and visualized in cytoscape software, in which nodes and edges represent proteins and their interactions, respectively (Veshkini, 2021).

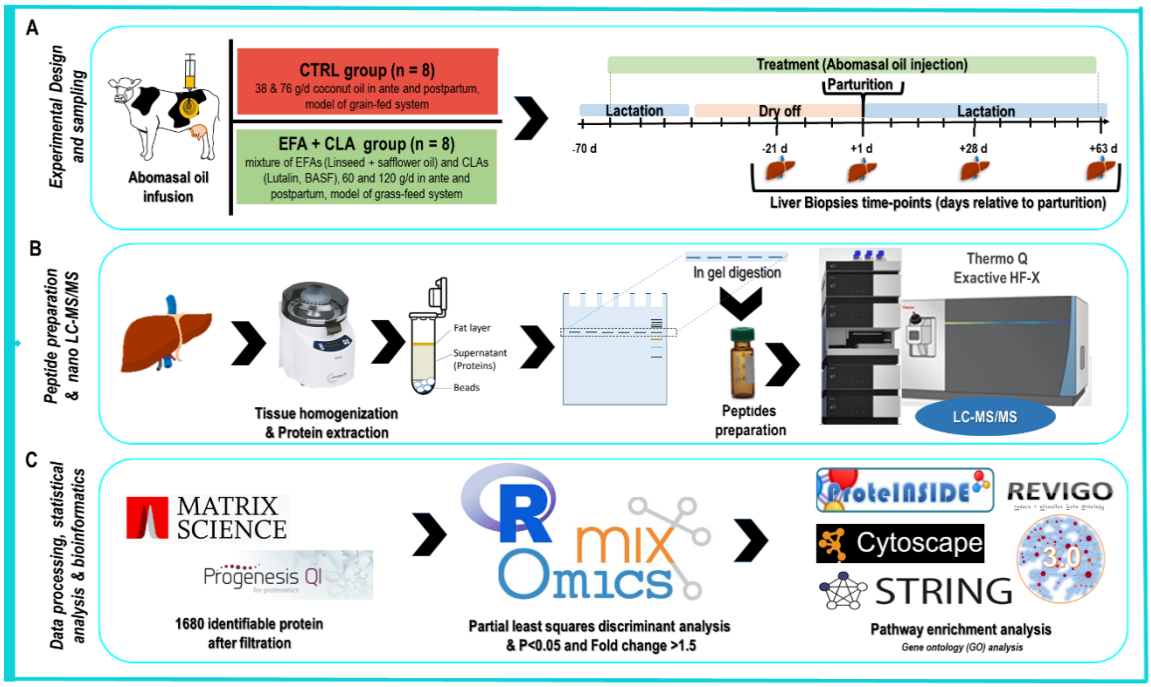


Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow, and (C) bioinformatics pipeline. (A) Timeline of treatments supplementation (from -63d ante to +63d postpartum) and liver biopsy collection (-21 d, +1 d, +28 d, and +63 d relative to parturition). Bold lines indicate liver biopsy sampling timepoints. (B) Protein extraction, purification, reduction, alkylation, and digestion; peptides were analysed by high-resolution LC-MS/MS, (C) Peptides alignment (progenesis), and protein identification (mascot) procedure were performed by Progenesis software coupled with the Mascot search engine, statistical analysis was based on Partial least squares discriminant analysis (PLS-DA) merged with $P < 0.05$ and Fold change > 1.5, followed by bioinformatics analysis (protein-protein interaction and Gene Ontology (GO) enrichment analysis.

Results

Cows performance data

A summary of cows performance and plasma metabolites data from the CTRL and EFA+CLA group was extracted from (Gnott et al., 2020; Vogel et al., 2020; Vogel et al., 2021) and provided in supplementary S3 and S4. In brief, EFA+CLA supplementation increased plasma concentration of these FA, decreased PP NEFA and TAG content, induced MFD, increased energy balance, and slightly affected markers of ketogenesis and hepatic inflammation (i.e., haptoglobin and paraoxonase). Dry matter intake, body weight, milk yield, and net energy intake were not affected by treatment.

Liver proteome profile

Out of 2720 identified proteins, a total of 1680 proteins at each timepoint were maintained for statistical analysis after applying the exclusion criteria (Veshkini, 2021). Of the 1680 proteins,1614 proteins were annotated by GO terms related to 907 BP, as well as 111 KEGG,

and 270 Reactome pathways that covered a diverse range of metabolic pathways related to metabolism (carbohydrate, energy, lipid, nucleotide, amino acid, glycan, vitamin, and xenobiotic metabolism), genetic information processing (translation and folding, sorting and degradation), cellular process (transport and catabolism and cell growth and death), and organismal systems (immune system and endocrine system).

Differentially abundant proteins and functional enrichment at day 21 antepartum (Figure 2 A),

From the total identified proteins, 29 proteins were differentially abundant on 21 d AP (Table 1), in which the relative abundance of 19 proteins was increased with a fold change that ranged from 1.43 - 3.92 (P-value < 0.05), and ten proteins were decreased (ranging from 0.38 - 0.70 fold, P-value < 0.05) in the EFA+CLA group when compared to the CTRL group. The DAP were further approved by clustered Heat map and are presented in Figure 2 (A, B, and C).

The overabundant proteins were annotated by GO terms related to "cholesterol biosynthetic process (GO:0006695)" and "lipid metabolic process (GO:0006629)" (Figure 2 D, details in (Veshkini, 2021)). Underabundant proteins were not annotated by any GO terms.

Considering all DAP, "steroid biosynthesis (bta00100)", "metabolism of xenobiotics by cytochrome P450 (bta00980)", "drug metabolism - cytochrome P450 (bta00982)", "retinol metabolism (bta00830)", "metabolic pathways (bta01100)" were mapped to KEGG metabolic pathways (Figure 2 E, details in (Veshkini, 2021)).

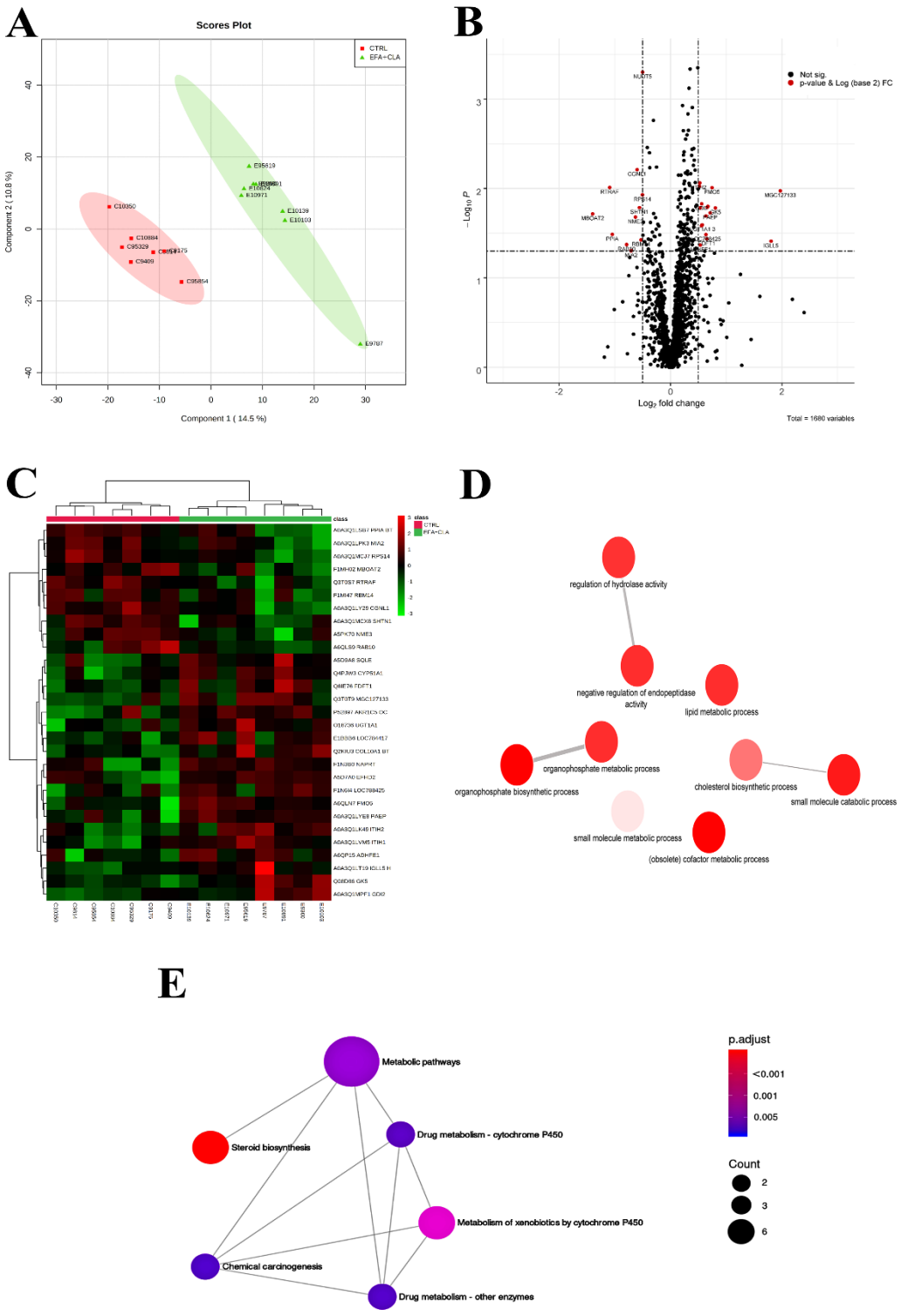


Figure 2) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) on day 21 antepartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>than 0.58 in a log scale that means a fold change of 1.3). C. Hierarchical clustering heat map analysis of differentially abundant proteins; Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Biological Process Ontology for the differentially abundant proteins (DAP). Fold enrichment (Bars, –log10 (adjusted P-value)) refers to the number of relevant gene names represented in each category relative to random expression of all genes in the *Bos taurus* genome. The line between pathways represents their dependence. E. KEGG pathways map of DAP. The colour of the nodes represents the –log10 (adjusted P-value); the size of the dots represents the number of DAP in the pathway. The line between pathways represents their dependence (larger figures are provided in appendix 1).

Differentially abundant proteins, interaction network, and functional enrichment of day 1 postpartum,

On the day after parturition, 12 proteins were differentially abundant between treatment groups (Table 1), including nine increased proteins (with a fold change that ranged from 1.50 - 4.16, P-value < 0.05), and three decreased proteins (ranging from 0.37 - 0.67) in the EFA+CLA group. The DAP are shown in a Volcano plot, and their expression was plotted by heat maps (Figure 3 A, B, C).

Also, the DAP were annotated by KEGG pathways, including “drug metabolism - cytochrome P450 (bta00982)” and “metabolism of xenobiotics by cytochrome P450 (bta00980)” (Figure 3 D) and Reactome pathway “metabolism of lipids (bta556833)” (Figure 3 E, details in (Veshkini, 2021)).

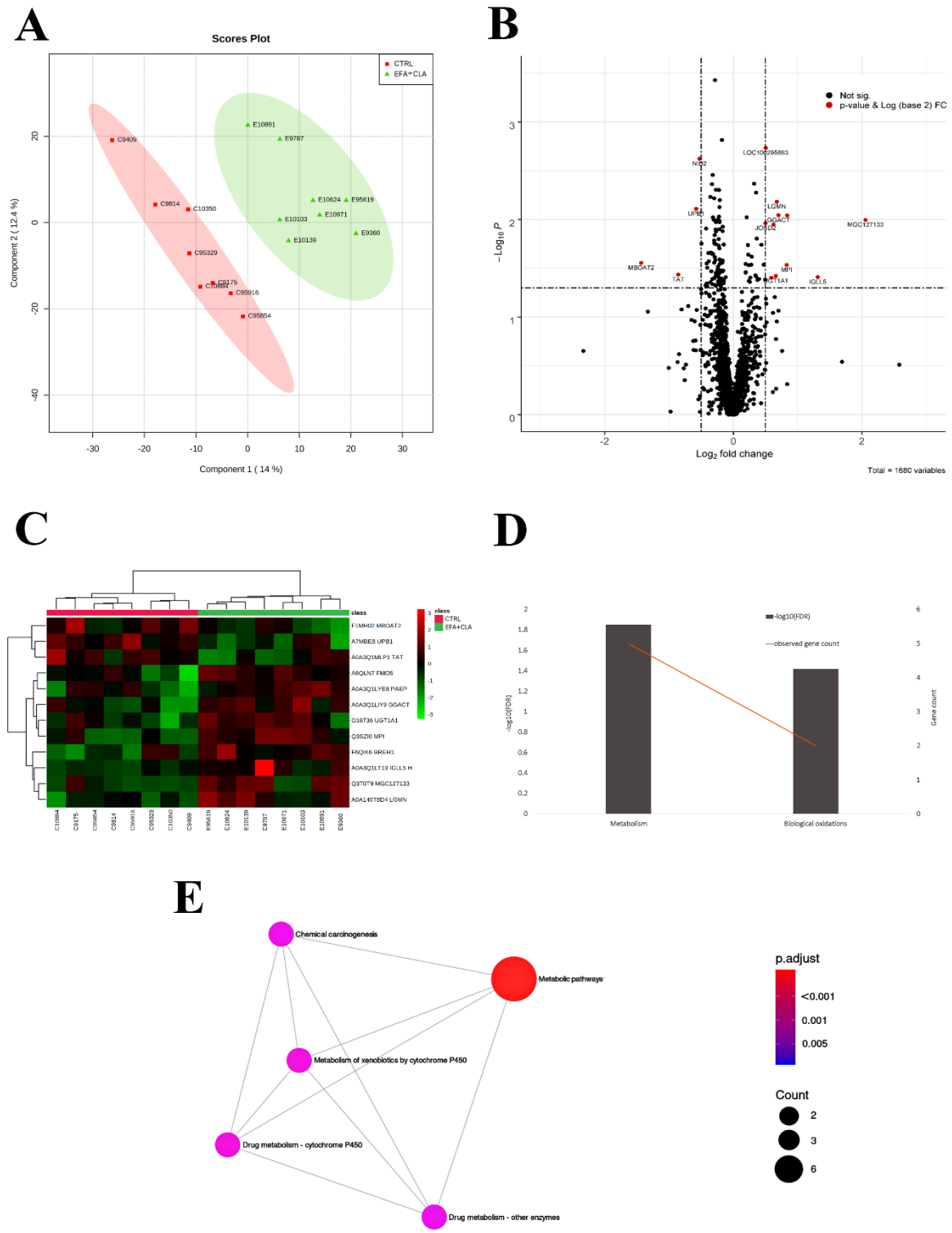


Figure 3) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 1 of postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>1.5). C. Hierarchical clustering heat map analysis of differentially abundant proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Reactome enrichment analysis (x-axis), fold enrichment (bars, left y-axis); the number of significant genes in each pathway (–log10, adjusted P-value) is represented by the lines on the right y-axis) represent. E. KEGG pathways map of differentially abundant proteins (DAP). The colour of the nodes represents the –log10 (adjusted P-value); the size of the dots represents the number of DAP in the pathway. The line between pathways represents their dependence (larger figures are provided in appendix 2).

Differentially abundant proteins, interaction network, and functional enrichment at day 28 postpartum,

At this timepoint , the relative abundance of 27 proteins was different between treatments (Table 1), of which 21 proteins were increased (with a fold change that ranged from 1.50 - 4.70, P-value < 0.05) and 6 proteins decreased (ranging from 0.57 - 0.66) in the EFA+CLA group as compared to the control group (Figure 4 A, B, C). Twenty-three BP have annotated (adjusted P-value < 0.05) by increased proteins, of which "cellular iron ion homeostasis (GO:0006879)", "apoptotic mitochondrial changes (GO:0008637)", "mitochondrial transport (GO:0006839)", "regulation of lipid metabolic process (GO:0019216)", "membrane organization (GO:0061024)", "apoptotic process (GO:0006915)", and "regulation of cellular process (GO:0050794)" (Figure 4 D, details in (Veshkini, 2021)). Moreover, the GO term "ferric iron-binding (GO:0008199)" in the MF category has been annotated. The proteins were localized in the "mitochondrial intermembrane space (GO:0005758)", "lysosome (GO:0005764)", and "cytoplasm (GO:0005737)", respectively ((Veshkini, 2021)).

Also, the KEGG pathways were linked to "ferroptosis (bta04216)", "mineral absorption (bta04978)", "porphyrin and chlorophyll metabolism (bta00860)", "drug metabolism - cytochrome P450 (bta00982)", "metabolism of xenobiotics by cytochrome P450 (bta00980)", "chemical carcinogenesis (bta05204)", "arachidonic acid metabolism (bta00590)", and "metabolic pathways (bta01100)" (Figure 4 E).

Decreased proteins were annotated by KEGG pathways related to "steroid hormone biosynthesis (bta00140)", "metabolism of xenobiotics by cytochrome P450 (bta00980)", and "chemical carcinogenesis (bta05204)" ((Veshkini, 2021)). Reactome enriched pathways included "arachidonic acid metabolism BTA-2142753", "cytochrome P450 - arranged by substrate type BTA-211897" and "metabolism of lipids BTA-556833" (Figure 4 F).

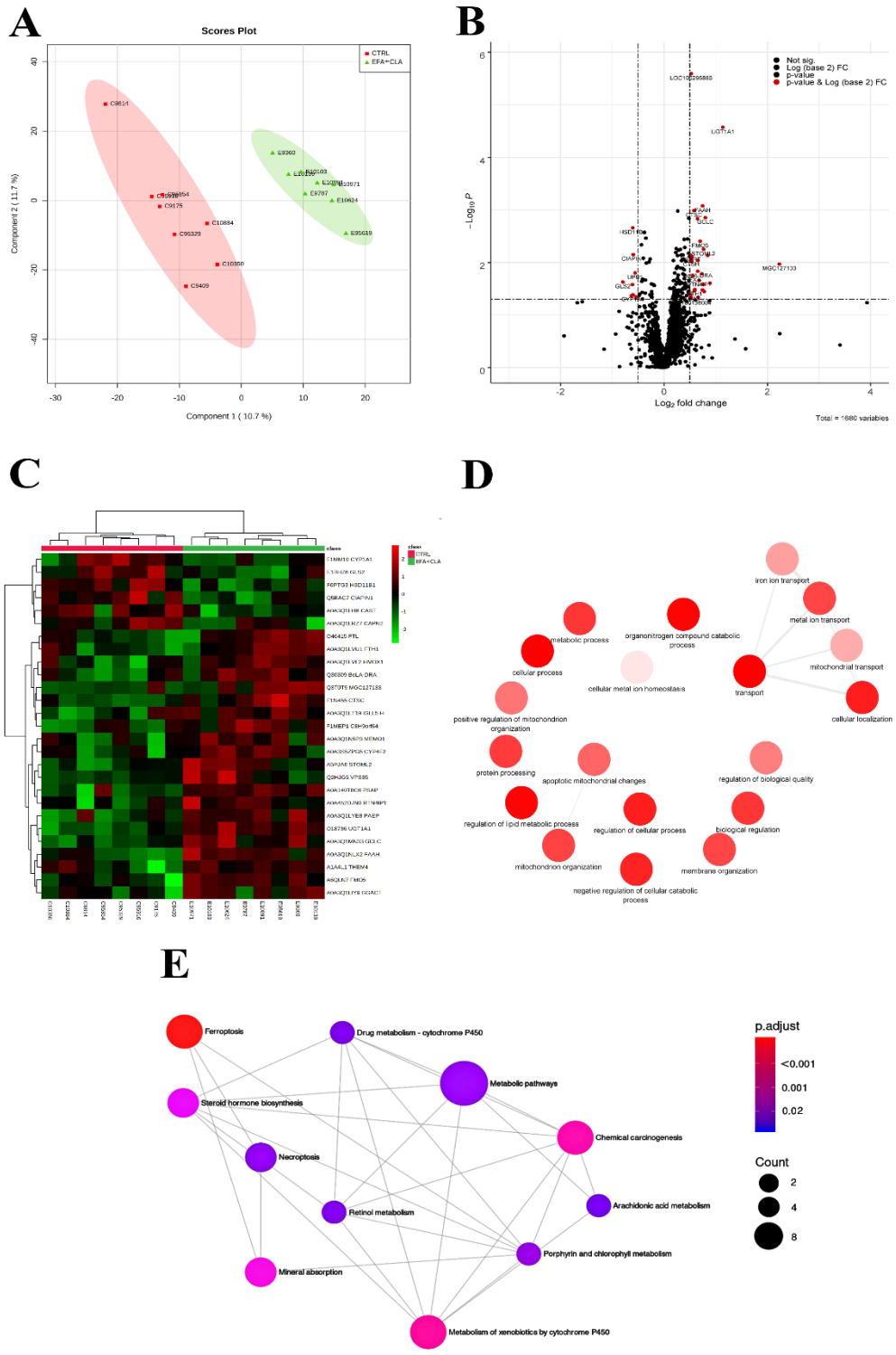


Figure 4) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 28 of postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased (top left) proteins were highlighted in red ($P<0.05$ and fold change>1.5). C. Hierarchical clustering heat map analysis of differentially abundant proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Biological Process Ontology for the differentially abundant proteins (DAP). The fold enrichment (adjusted P-value) is coloured in red according to the degree of significance, refers to the number of relevant gene names represented in each category relative to random expression of all genes in the Bos taurus genome. The line between pathways represents their dependence. E. KEGG pathways map of DAP. The colour of the nodes represents the –log10 (adjusted P-value); the size of the dots represents the number of DAP in the pathway. The line between pathways represents their dependence (larger figures are provided in appendix 3).

Differentially abundant proteins, interaction network, and functional enrichment at day 63 postpartum,

At the last time-point, 26 proteins were considered as DAP (Table 1), among which 16 proteins were upregulated (with a fold change ranging from 1.49 - 4.16, P-value < 0.05), and 10 proteins were downregulated (ranged from 0.11-0.67) in the treatment group as compared to the CTRL group (Figure 5 A, B, C).

The decreased proteins annotated by KEGG pathways belong to “drug metabolism - cytochrome P450 (bta00982)”, “metabolism of xenobiotics by cytochrome P450 (bta00980)”, “chemical carcinogenesis (bta05204)”, and “metabolic pathways (bta01100)” (Figure 5 D, details in (Veshkini, 2021)). Interestingly, the same pathways were also enriched by the upregulated proteins (details in (Veshkini, 2021)). Moreover, DAP were annotated by Reactome terms to “metabolism BTA-1430728”, “Phase II - conjugation of compounds BTA-156580”, “glutathione conjugation BTA-156590” (Figure 5 E).

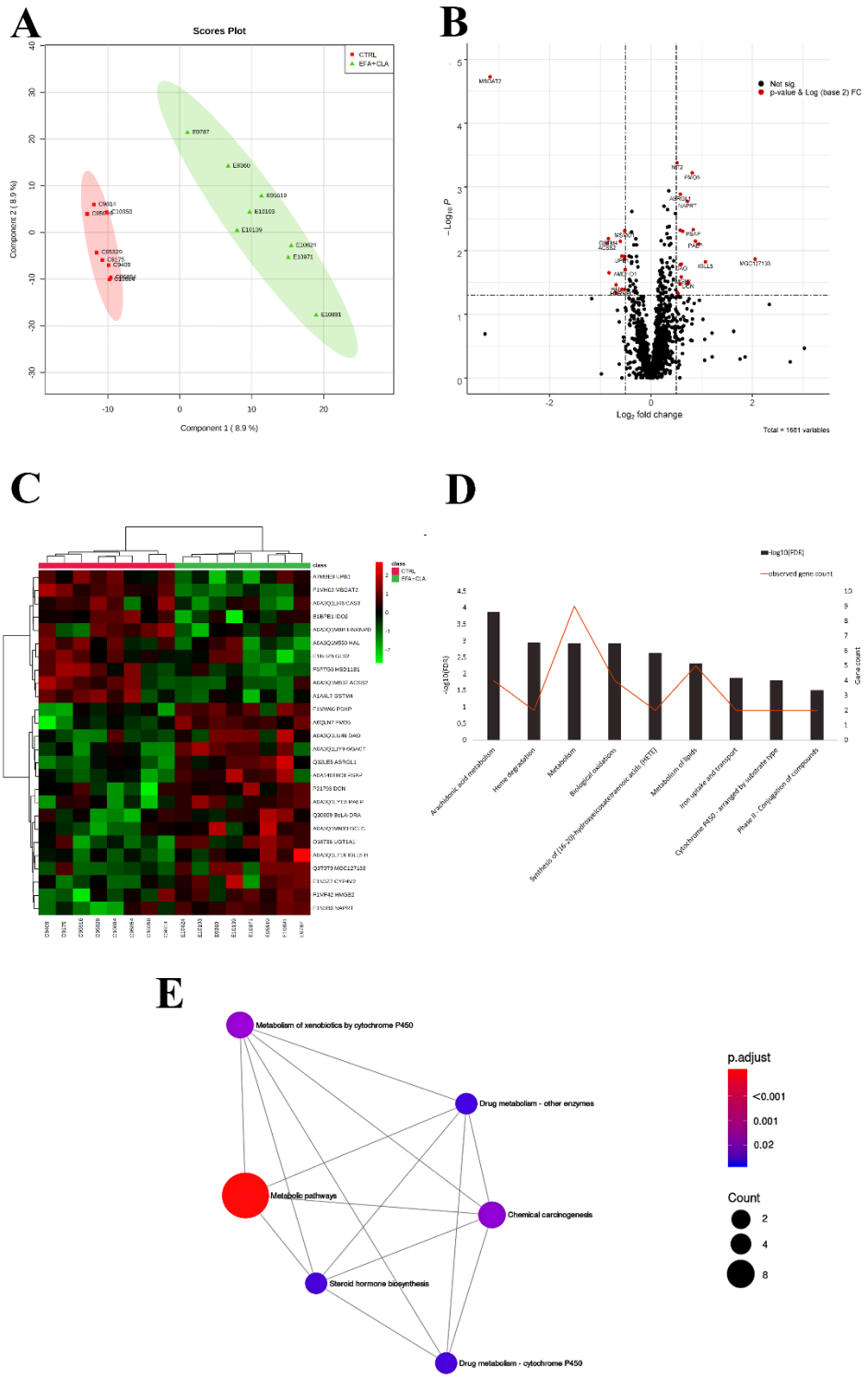


Figure 5) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 63 of postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>1.5). C. Hierarchical clustering heat map analysis of differentially abundant proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Reactome enrichment analysis (x-axis), fold enrichment (bars, left y-axis); the number of significant genes in each pathway (–log10, adjusted P-value) is represented by the lines on the right y-axis) represent. E. KEGG pathways map of differentially abundant proteins (DAP). The colour of the nodes represents the –log10 (adjusted P-value); the size of the dots represents the number of DAP in the pathway. The line between pathways represents their dependence (larger figures are provided in appendix 4).

Common differentially abundant proteins along time

As illustrated in the Venn diagram (Figure 6), the DAP pattern was time-specific, probably due to substrates (i.e. supplemented FA, NEFA, and accumulated intermediates) abundance. The relative abundance of 5 common proteins including 20-beta-hydroxysteroid dehydrogenase-like (Q3T0T9, GN: MGC127133), lipocln_cytosolic_FA-bd_dom domain-containing protein (A0A3Q1LYE8, GN: PAEP), Ig-like domain-containing protein (A0A3Q1LT19, GN: IGLL5), dimethylaniline monooxygenase [N-oxide-forming] (A6QLN7, GN: FMO5), and UDP-glucuronosyltransferase (O18736, GN: UGT1A1) were affected by EFA+CLA treatment during all timepoints (Figure 6). Moreover, seven common proteins including glutamate-cysteine ligase catalytic subunit (A0A3Q1MN33, GN: GCLC), glutaminase 2 (E1BHZ6, GN: GLS2), calpain-2 catalytic (A0A3Q1LRZ7, GN: CAPN2), calpastatin (A0A3Q1LI46, GN: CAST), boLA-DR-alpha (Q30309, GN: BoLA-DRA), prosaposin (A0A140T8C6, GN: PSAP), and hydroxysteroid 11-beta dehydrogenase 1 (F6PTG3, GN: HSD11B1) were affected by EFA+CLA treatment on days 28 and 63.

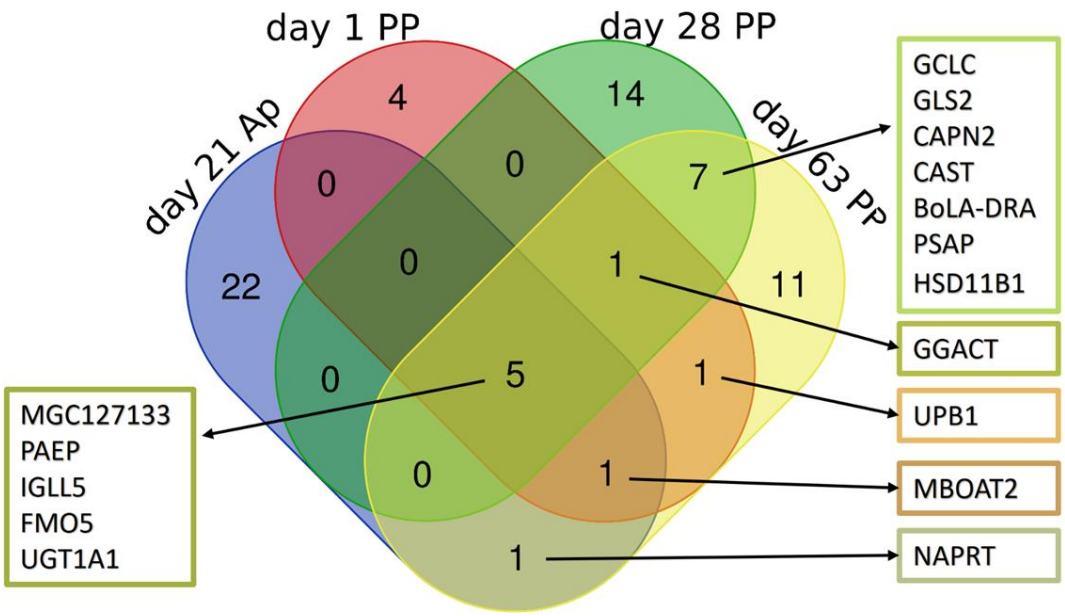


Figure 6) Venn diagram represent common and specific differentially abundant proteins identified in -21, +1, +28, and +63 days relative to parturition.

Discussion

This study aimed to investigate the metabolic adaptation in dairy cows supplemented with a combination of EFA and CLA during the transition from pregnancy to lactation by applying proteomics in liver tissue samples. The synergistic effect of these two FA on performances and

"classical" parameters including energy metabolism, the somatotropic axis signaling pathway, plasma fatty acids profile, and markers of inflammation was recently presented (Gnott et al., 2020; Vogel et al., 2020; Vogel et al., 2021). The present study complements previously published works on the hepatic metabolic adaptations as it pointed out proteins and pathways that are part of the molecular signatures elicited by supplementation with EFA and CLA, the latter representing a model for feeding on grass.

Common pathways identified antepartum and postpartum

The relative abundance of MGC127133, PAEP, IGLL5, FMO5, and UGT1A1 was affected by EFA+CLA regardless of time (Figure 6). These proteins were annotated by KEGG pathways related to drug metabolism - cytochrome P450, metabolism of xenobiotics by cytochrome P450, and retinol metabolism, all belonging to the lesser-studied "cytochrome P450 epoxidation/hydroxylation" pathways involved in ω-oxidation of FA. Unfortunately, less information is available regarding these enzymes' specific functions or their associated pathways in dairy cows, especially in in vivo models. Nevertheless, in many species, particularly humans and mice, cytochrome P450 and xenobiotic metabolism regulate the cross-talk between the immune system and metabolism (Nebert et al., 2013).

Cytochrome (CYP) refers to a superfamily of heme-containing membrane-associated enzymes, regulating several functions related to cholesterol and FA metabolism, detoxification of xenobiotic substances, steroid metabolism, drug and pro-carcinogen deactivation, and catabolism of exogenous compounds, located primarily in the liver, but also in all other tissues (Stavropoulou et al., 2018). In this context, along with the α- and β-oxidation of FA, hepatic ω-oxidation of FA (CYP P450) can help utilize PUFA and prevent hepatic lipid overload (Wanders et al., 2011). ω-oxidation of FA is an alternative pathway when mitochondrial β-oxidation is deficient and involves the oxidation of the ω-carbon of FA in the endoplasmic reticulum to provide succinyl-CoA (Miura, 2013). CYP isoforms may have different functions, activities, and substrates (Elfaki et al., 2018); therefore, their inhibition and induction are regulated indirectly by ligand activation of xenobiotics to nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) (Ng et al., 2007) and pregnane X receptor (PXR) (Wang et al., 2012). In this respect, xenobiotics are defined as natural components such as diet-derived compounds (e.g., lipids) or synthetic drugs considered foreign to the body and therefore being subjected to the liver metabolism primarily to increase their polarity and make them easier to excrete (Patterson et al., 2010).

Previously, in a precise activity-based protein profiling technique, it has been shown that a commercial high-fat diet (based on lard) decreased P450 activity in mouse liver, which led to obesity, obesity-induced chronic inflammation, increased risk for hepatotoxicity, and metabolic disease (Sadler et al., 2018). Herein, we suppose that EFA and CLA or their intermediates acted as xenobiotic substances and oxidized through cytochrome P450 pathways. It is worth pointing out that any alteration to the average physiological level of CYP activities may cause disease, being their activity required to detoxify drugs, neutral components, or biochemical intermediates to avoid impeding critical metabolic pathways. Taken together, the low level of PUFA or n-3 to n-6 ratio in the CTRL group may negatively influence the functional capacity of xenobiotic-metabolizing P450. Herein, the results indicated specific and different isoforms (isoform-specific manner) of CYP affected by treatment during the transition period.

Recent studies using knockout Fmo5−/− mice revealed that FMO5 not only functions as a xenobiotic-metabolizing enzyme but also has been implicated as a regulator of glucose and lipid homeostasis, metabolic ageing, and insulin sensitivity (Gonzalez Malagon et al., 2015; Scott et al., 2017). In addition, FMO5 acts as NADPH oxidase, lowering NADPH which is the electron source in lipid and cholesterol biosynthesis. In this regard, downregulation of FMO5 in mice has been associated with reduced fat deposition and lower plasma cholesterol (Gonzalez Malagon et al., 2015; Scott et al., 2017). Thus, the increased expression of this protein is probably induced by a xenobiotic-like function of supplemented FA.

Metabolic adaptation in the antepartum period

On d 21 AP, 4 proteins were annotated by enriched GO term related to cholesterol metabolism. In addition to FMO5 which is a DAP identified at all timepoints, squalene monooxygenase (A5D9A8, GN: SQLE, unreviewed proteins in Bos taurus) and squalene synthase (Q6IE76, GN: FDFT1, unreviewed proteins in Bos taurus) and CYP51A1 were increased in the EFA+CLA group. Previously in a human study, a significant association of CYP51A1 gene expression with lower blood total cholesterol and LDL cholesterol levels, but not with TAG and HDL-cholesterol, has been reported in women in their second trimester of pregnancy (Lewinska et al., 2013). The CYP51 protein is very conserved between species (NCBI homology, https://www.ncbi.nlm.nih.gov/); therefore, the same function of this protein in dairy cows can be supposed. However, in this study, total cholesterol and LDL cholesterol concentrations were not affected by treatment in the AP period (Figure S4).

Moreover, SQLE, FDFT1, and CYP51A1 are all involved in the cholesterol biosynthesis pathways through the Sterol regulatory element-binding proteins (SREBP)-activated mevalonate pathway (Kondo et al., 2017; Chen et al., 2019). In this pathway, FDFT1 initiates the conversion of farnesyl-pyrophosphate to squalene, which is the first stage of liver cholesterol synthesis (Grünler et al., 1994), followed by the synthesis of lanosterol from squalene catalyzed by SQLE, and the final step is the conversion of lanosterol to cholesterol by the action of CYP51A (Xue et al., 2020). Cholesterol homeostasis is crucial for normal cellular and physiological functions and is strictly controlled by nuclear receptors, mammalian target of rapamycin (mTOR)/SREBP2 pathway (Eid et al., 2017) and Liver X Receptors (LXR) (Zhang et al., 2012) which induce and inhibit its synthesis, respectively. In dairy cows, insufficiency of cholesterol metabolism and acceleration of body fat degradation before parturition was reported to be associated with developing ketosis PP (Akamatsu et al., 2007). On the other side, chronic hepatic expression of SREBP2 and excessive cholesterol storage has been shown to cause fatty liver disease (steatosis), hypertriglyceridemia, and insulin resistance in non-ruminant species (Shimano, 2009). Fortunately, no differences were observed in the plasma concentration of total cholesterol, TG, LDL, HDL (Figure S4), and hepatic expression of HMGCS2 between treatment groups before parturition (Vogel et al., 2020; Vogel et al., 2021), which possibly points towards the feedback regulation that synthesized cholesterol was used to maintain its homeostasis crucial in pregnant cows. Indeed, as a structural component of the cellular membrane and precursor for steroid hormones, cholesterol esters, and bile acids (BA), cholesterol is essential for the normal development of the dam and the fetus. In humans and rodents with a hemochorial or hemoendothelial placenta type, the fetus depends on exogenous cholesterol sources obtained from the maternal circulation transported across the placenta, mainly through lipoproteins (Woollett, 2011). It is not known whether this applies for species with an epitheliochorial placenta type, such as most farm animals. Also, BA are incorporated into lipoproteins and may induce hepatocytes to secrete and export the accumulated lipids from the liver (for review (Wang et al., 2018)). Intrahepatic cholestasis and elevated BA and/or transaminases are considered as a liver disease (Marschall, 2015).

Moreover, antepartum and around parturition, the membrane-bound O-acyltransferase 2 (F1MH02, GN: MBOAT2, also known as lysophosphatidylcholine acyltransferase 4), a newly discovered member of the MBOAT family (Shindou et al., 2009) was decreased in the EFA+CLA group. This conserved enzyme catalyzes the production of glycerophospholipids in the mammalian cell membrane, particularly phosphatidylcholine and phosphatidylethanolamine, which determine membrane intrinsic curvature and fluidity (Eto et

al., 2020). This is the first study reporting the expression of MBOAT2 in dairy cows' hepatocytes, and it is probably involved in modulating the ratio of PUFA in cellular membranes.

Metabolic adaptation in lactation

The day after parturition, along with cytochrome P450 pathways, the catabolic process and proteolysis, and bile secretion KEGG pathways were annotated by DAP in the EFA+CLA group (identified by PLS-DA analysis). The upregulated solute carrier organic anion transporter family member 1B3 (F1MYV0, GN: SLCO1B3) enzyme not only incorporates with activation of BA secretion (Malagnino et al., 2019) but also in the uptake of endogenous and xenobiotic compounds (Hagenbuch and Gui, 2008). Apart from already discussed mechanisms, BA has been reported to play novel roles as signaling molecules regulating energy homeostasis, TAG concentrations, and glucose (Guo et al., 2016; Hao et al., 2017; Pathak et al., 2017). In this regard, in a transcriptomic study, the BA synthesis pathway reduction was reported in dairy cows with severe compared to mild negative energy balance(McCabe et al., 2012).

The liver is the main site regulating BA synthesis (Sadler et al., 2018), primarily through the cholesterol/lipid homeostasis pathway (Kakiyama et al., 2019). The activated mevalonate pathway thereby increased cholesterol synthesis that was discussed for the last time-point, probably induced the downstream pathway, BA synthesis, and may explain why cholesterol concentration was not different between treatments. More interestingly, converting cholesterol to BA, is regulated by cytochrome P450 (CYP7a1 and CYP8b1) pathways (Sadler et al., 2018), although neither CYP7a1 abundance nor CYP8b1 were affected by treatment. This may propose other pathways besides cytochrome P450 to regulate this conversion in dairy cows. Nevertheless, no remarkable differences in performance and metabolite were observed between treatments. The difference in energy balance between treatment groups (Vogel et al., 2020) may indicate that the more negative energy balance in the CTRL group had impaired cholesterol and BA synthesis.

On day 28 PP, cytochrome P450 family 4 subfamily F member 2 (A0A3S5ZPG5, GN: CYP4F2) and cytochrome P450 family 1 subfamily A member 1 (F1MM10, GN: CYP1A1) had higher and lower abundance in the EFA+CLA group, respectively. In this regard, a study in mice reported decreased CYP4F2 protein in the liver upon feeding a high-fat diet associated with impaired hepatic lipid metabolism α-tocopherol pathways (Bartolini et al., 2017). In general, the CYP4 members are tissue-specific and involved in FA metabolism, maintaining the concentration of FA and FA-derived bioactive molecules within a normal physiological

range (Jarrar and Lee, 2019). CYP4F2 (Cui et al., 2000) and CYP4V2 (Nakano et al., 2012) are two important members of this family and are highly abundant in the liver. Arachidonic acid, lauric acid, vitamin K, and leukotriene are the specific substrates for the CYP4F2 enzyme (Edson et al., 2013; Jarrar et al., 2013). We observed a significant difference in the plasma concentration of FA on day 28 PP with lesser values in the EFA+CLA group. The greater FA concentration in the CTRL group may have impaired mitochondrial function, reduced ATP synthesis, and potentially triggered lipotoxicity (Weinberg, 2006). On the other hand, an overabundance of CYP4F2 in the EFA+CLA group has been reported in humans to amplify the capacity of hepatocytes to oxidize excess FA (Hsu et al., 2011), which may support our proteomic results. Induction of CYP4F2 expression is proposed to be mediated by the ligand activation of nuclear receptors with supplemented FA and in response to activated AMPK and SREBP pathways, which then augment the capacity of cytochrome P450 to oxidize xenobiotics (Hsu et al., 2011). However, regulation may be at the level of enzyme activity rather than of protein abundance, since enrichment of these two pathways was not observed in the present study. In other words, during the negative energy balance, when the liver is stressed by the excessive FA supply from lipogenesis that may cause lipotoxicity, the activation of CYP4F2, which removes FA, is logic and may explain how the EFA+CLA group accomplish the inhibition of steatosis.

On the other side, the members of the CYP1 family use endogenous sex hormones such as progesterone and testosterone, amine hormones like melatonin, vitamins, FA such as linoleic acid, and phospholipids as substrates (Lu et al., 2020), which under specific circumstances activate compounds that react with DNA leading to an imitation of the mutagenic process (Santes-Palacios et al., 2016). Furthermore, it has been reported both in in vivo (Huerta-Yepez et al., 2020) and in vitro (Choudhary et al., 2004; Schwarz et al., 2004; Fer et al., 2008) studies that CYP1A1 is involved in PUFA metabolism.

Previously, the xenobiotic-like potential of fish oil in the induction of CYP1A1 mRNA expression in primary cultured bovine hepatocytes was reported (Guruge et al., 2009). Also, there is emerging evidence that induction of CYP1A1 leads to non-alcoholic fatty liver disease and the development of oxidative stress in humans, which is another molecular support for hepatic metabolic imbalance in our CTRL group (Huang et al., 2018). The exact mechanism of how CYP1A1 was inhibited in EFA+CLA is not yet precisely known, although based on a study in mice (Shi et al., 2017), it could be speculated that transcriptional regulation of CYP450 through activation of PPARα is likely a possible pathway.

During the PP period (d +28 and +63), 11β-hydroxysteroid dehydrogenase type 1 (F6PTG3, GN: HSD11B1) and glutamate-cysteine ligase catalytic subunit (A0A3Q1MN33, GN: GCLC) increased, and phosphate-activated mitochondrial glutaminase (E1BHZ6, GN: GLS2) decreased in EFA+CLA (Figure 5). Among them, GCLC and GLS2 are involved in the glutamine and glutamate metabolic processes and the glutathione (GSH) system. In GSH biosynthesis, GLS2 catalyzes the conversion of glutamine to glutamate (Yelamanchi et al., 2016), and GCLC is a rate-limiting enzyme in converting glutamate to GSH (Han et al., 2018). The combination of the above-noted enzymatic changes would be expected to result in glutamate regulation. Glutamate, as one of the most abundant amino acids in the liver, is considered to be at the crossroads of hepatic metabolism, where it is mainly involved in the TCA cycle, gluconeogenesis, FA oxidation (Yang and Brunengraber, 2000), and electron transport from the cytoplasm into the mitochondria via the malate-aspartate shuttle (Meijer, 2003).

The HSD11B1 is an endoplasmic reticulum-located reductase that activates cortisone to cortisol, thereby modulating hepatic gluconeogenesis (Kang et al., 2017). It also plays a crucial role in glucocorticoid receptor (GR) activation, which in turn is involved in the regulation of anti-stress and anti-inflammatory pathways (Huang et al., 2020). It has been previously shown that liver synthetized BA inhibit the HSD11B1 (Maeda et al., 2010), which may be related to the downregulation of HSD11B1.

On day 63 PP, cytochrome P450 family 4 subfamily V member 2 (F1N3Z7, GN: CYP4V2) was more abundant in the EFA+CLA than in the CTRL group. CYP4V2 has the same characteristic as the CYP4 classes but preferably metabolizes arachidonic acid, lauric acid, eicosapentaenoic acid, docosahexaenoic acid, and medium-chain FA as substrates (Nakano et al., 2009; Nakano et al., 2012; Yi et al., 2017). The greater abundance of different CYP isomers between d 28 and 63 PP, probably related to FA concentration, may compete with EFA and CLA for ligand activation of nuclear receptors (substrate dependent). At this timepoint that coincides with returning to positive EB, the previously enriched cytochrome P450 pathways and steroid hormone biosynthesis were affected by both downregulations of HSD11B1, glutathione S-transferase Mu 4 (A1A4L7, GN: GSTM4), and upregulation of MGC127133 and UGT1A1. Moreover, enrichment of several KEGG pathways in the EFA+CLA group was observed by PLS-DA-identified DAP related to pentose and glucuronate interconversions, starch and sucrose metabolism, pyruvate metabolism, glutamate metabolic process, and glycolysis/gluconeogenesis. These pathways are intimately interconnected and are associated with energy metabolism. Therefore, we considered these alterations to restore metabolic

adaptation to the normal metabolism in positive EB status. The EFA+CLA cows turned back to a positive EB around 21 days earlier than the CTRL group (Vogel et al., 2020). Therefore, the activated metabolic adaptive processes in response to the NEB were also switched off or returned to normal functions faster.

Conclusion

The results indicated that EFA+CLA supplementation altered the proteome profile of the liver in transition dairy cows. Bioinformatics analysis of DAP revealed enriched pathways related to hepatic cholesterol biosynthesis, drug metabolism - cytochrome P450, metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis, arachidonic acid metabolism, TCA cycle, and BA synthesis. Furthermore, in each timepoint , the relative abundance of CYP enzymes affected by EFA+CLA supplementation in a time-dependant manner slightly impacted the capacity of hepatic ω-oxidation. The results also suggest that EFA+CLA supplementation might be in support of preventing hepatic steatosis during the transition period. Altogether, these findings provided novel information regarding the underlying molecular mechanism by which hepatic metabolism responds to supplemented FA. Nonetheless, further investigation with more accurate measures of hepatic steatosis is needed to replicate these findings in different populations and physiological statuses.

Acknowledgements

The authors acknowledge A. Delavaud (INRAE) for technical assistance in protein extraction, quantification, and concentration for mass spectrometry analyses.

Supplementary information

Supplementary files were deposited at the INRAe portal as “Gene ontology of hepatic differentially abundant proteins in Holstein cows supplemented with essential fatty acids and conjugated linoleic acids”.

Table heading

Table 1. The differentially abundant proteins identified between CTRL and EFA+CLA in -21, +1, +28, and +63 days relative to parturition and their associated gene names.

Num.	Protein	Associated gene name	Timepoint
1	20-beta-hydroxysteroid dehydrogenase-like	MGC127133	1, 2, 3, 4*
2	Progestagen Associated Endometrial Protein	PAEP	1, 2, 3, 4
3	Dimethylaniline monooxygenase [N-oxide-forming]	FMO5	1, 2, 3, 4
4	Immunoglobulin Lambda Like Polypeptide 5	IGLL5	1, 2, 3, 4
5	UDP-glucuronosyltransferase	UGT1A1	1, 2, 3, 4
6	Membrane bound O-acyltransferase domain containing 2	MBOAT2	1, 2, 4
7	Gamma-glutamylaminecyclotransferase	GGACT	2, 3, 4
8	Nicotinate phosphoribosyltransferase	NAPRT	1, 4
9	Beta-ureidopropionase 1	UPB1	2, 4
10	Glutamate-cysteine ligase catalytic subunit	GCLC	3, 4
11	Glutaminase 2	GLS2	3, 4
12	Calpain-2 catalytic subunit	CAPN2	3, 4
13	Calpastatin	CAST	3, 4
14	BoLA-DR-alpha	BoLA-DRA	3, 4
15	Prosaposin	PSAP	3, 4
16	Hydroxysteroid 11-beta dehydrogenase 1	HSD11B1	3, 4
17	Squalene epoxidase	SQLE	1
18	FDFT1 protein	FDFT1	1
19	Lanosterol 14-alpha demethylase	CYP51A1	1
20	Cytochrome P450 4A25-like	LOC784417	1
21	Inter-Alpha-Trypsin Inhibitor Heavy Chain 2	ITIH2	1
22	Peptidylprolyl Isomerase A	PPIA	1
23	Aldo-keto reductase family 1, member C5	AKR1C5	1
24	Putative glycerol kinase 5	GK5	1
25	Shootin 1	SHTN1	1
26	RNA Transcription, Translation And Transport Factor	RTRAF	1
27	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	1
28	EF-hand domain-containing protein D2	EFHD2	1
29	Nucleoside diphosphate kinase	NME3	1
30	Aldo_ket_red domain-containing protein	LOC788425	1
31	Melanoma inhibitory activity protein 2	MIA2	1
32	RNA-binding protein 14	RBM14	1
33	Rab GDP dissociation inhibitor	GDI2	1
34	Ribosomal Protein S14	RPS14	1
35	Hydroxyacid-oxoacid transhydrogenase, mitochondrial	ADHFE1	1
36	Collagen Type X Alpha 1 Chain	COL10A1	1
37	Ras-related protein Rab-10	RAB10	1
38	Cingulin like 1	CGNL1	1
39	Legumain	LGMN	2
40	Tyrosine aminotransferase	TAT	2
41	Mannose-6-phosphate isomerase	MPI	2
42	Carboxylic ester hydrolase	BREH1	2
43	Cytochrome P450 Family 1 Subfamily A Polypeptide 1	CYP1A1	3
44	Ferritin light chain	FTL	3
45	Mediator Of Cell Motility 1	MEMO1	3

46	Ferritin	FTH1	3
47	Cathepsin C	CTSC	3
48	Heme oxygenase 1	HMOX1	3
49	Fatty acid amide hydrolase	FAAH	3
50	Thioesterase Superfamily Member 4	THEM4	3
51	Cytochrome P450 Family 4 Subfamily F Member 2	CYP4F2	3
52	Cytokine Induced Apoptosis Inhibitor 1	CIAPIN1	3
53	Reticulon-4-interacting protein 1, mitochondrial	RTN4IP1	3
54	Stomatin (EPB72)-like 2	STOML2	3
55	Queuosine salvage protein	C8H9orf64	3
56	Glutathione S-transferase Mu 1	GSTM4	3
57	Acyl-CoA synthetase short chain family member 2	ACSS2	4
58	Pyridoxal phosphate phosphatase	PDXP	4
59	Histidine ammonia-lyase	HAL	4
60	D-amino acid oxidase	DAO	4
61	Cytochrome P450 Family 4 Subfamily V Member 2	CYP4V2	4
62	High Mobility Group Box 2	HMGB2	4
63	Indoleamine 2,3-dioxygenase 2	IDO2	4
64	Decorin	DCN	4
65	Heterogeneous nuclear ribonucleoprotein D	HNRNPD	4
66	Asparaginase And Isoaspartyl Peptidase 1	ASRGL1	4
67	VPS35 Retromer Complex Component	VPS35	4

*1, 2, 3, and 4 correspond to days -21, +1, +28, and +63 relative to parturition, respectively.

Supplementary Material

Supplementary S1

Table S1. Amounts of daily abomasally infused supplements[1].

Supplementation	treatment			
	CTRL[2]	EFA+CLA		
	Coconut oil[3]	Linseed oil[4]	Safflower oil[5]	Lutalin®[6]
Daily infused oils (g/d)				
Dosage lactation	76	78	4	38
Dosage dry period	38	39	2	19
Daily infused fatty acids (g/d) at the lactation dosage[7]				
18:3 cis-9, cis-12, cis-15	0.00	39.9	0.01	0.00
18:2 cis-9, cis-12	1.39	12.4	2.48	1.34
18:2 cis-9, trans-11	0.00	0.00	0.01	10.3
18:2 trans-10, cis-12	0.00	0.02	0.01	10.2

1Cows were supplemented daily with coconut oil (CTRL), or a mixture of linseed, safflower oil (EFA), and Lutalin® (CLA, c9, t11 and t10, c12), (EFA+CLA).

2Addition of vitamin E (0.06 g/d), Covitol 1360 (BASF, Ludwigshafen, Germany), to compensate for the vitamin E in linseed oil (0.07%) and

safflower oil (0.035%).

3Sanct Bernhard, Bad Ditzenbach, Germany

4DERBY, Derby Spezialfutter GmbH, Münster, Germany

5GEFRO, Memmingen/Allgäu, Germany

6BASF, Ludwigshafen, Germany

7The lactation dosage was halved during the dry period.

Supplementary S2

Table S2. Ingredients and chemical compositions of the diets.

Item (g/kg of DM)	Diet	
	Dry period[1]	Lactation
Ingredients	421	457
Corn silage	223	97
Straw		
Compound feed DEFA[2] (granulated)	-	446
Dried sugar beet pulp	163	-
Extracted soybean meal	99	-
Grain of rye	75	-
Mineral-vitamin mixture[3]	10	-

Urea[4]	9	-
Chemical composition		
NEL (MJ/kg DM)[5]	6.2	7.1
Crude fat	21	23
Crude fiber	219	173
Crude protein	141	146
Utilizable protein[5]	141	143
NFC	379	432
NDF	423	346
ADF	249	197
RNB[5,6]	0.0	0.5

[1] The dry period diet was fed from wk 6 to wk 1 before calving.

[2] Ceravis AG, Malchin, Germany Ingredients: 46.5% dried sugar beet pulp, 25.3% extracted soybean meal, 23.8% grain of rye, 1.4% urea, 1.1% premix cow, 1.00% calcium, 0.37% phosphorus, 0.42% sodium, vitamins A, D3, E, copper, ferric, zinc, manganese, cobalt, iodine, selenium Chemical composition: 44.4% NFC, 24.1% crude protein, 21.6% NDF, 12.4% ADF, 9.3% crude fiber, 8.2% crude ash, 1.8% crude fat, 7.9 MJ NEL/kg DM

[3] KULMIN®MFV Plus (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany): 8.5% magnesium, 7.5% phosphorus, 6.5% sodium, 3.5% HCl insoluble ash, 1.5% calcium, additives: vitamins A, D3, E, B1, B2, B6, B5, B3, B12, B9, H, zinc, manganese, copper, cobalt, iodine, selenium, and Saccharomyces cerevisiae

[4] Piarumin® (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany): 99% urea, 46.5% total nitrogen

[5] Society of Nutrition Physiology (GfE, 2001, 2008, 2009) and Deutsche Landwirtschaftliche Gesellschaft (DLG, 2013)

[6] RNB = ruminal nitrogen balance

Supplementary S3.

Table S3. Performance data of day 21 ante, and days +1, +28, and +63 postpartum of cows supplemented abomasally with coconut oil (CTRL; n = 8), or the combination of linseed and safflower oil (EFA) and conjugated linoleic acid (CLA) (EFA+CLA; n=8) from wk 9 antepartum until wk 9 postpartum, Adapted from (Vogel et al., 2020).

		treatment		Fixed effect, P-value		
		CTRL	EFA+ CLA	EFA+ CLA	time	EFA+CLA*time
NEL intake, MJ NEL/d	late lactation	120.2 ± 4.6	113.8 ± 3.9	0.7	0.12	
	Dry period	80.6 ± 3.1	84.1 ± 2.8	0.8	0.001	
	Transition period	93.9 ± 3.3	93.4 ± 2.9	0.7	0.001	
	Postpartum	120.8 ± 3.8	115 ± 3.5	0.3	0.001	
	Entire Study	106.6 ± 3.3	104 ± 2.9	0.6	0.001	
FEMY, kg milk/kg DMI	late lactation	0.96 ± 0.11	0.98 ± 0.09	0.19	0.001	
	Early lactation	2.25 ± 0.1	2.43 ± 0.09	0.7	0.001	
FEECM, kg ECM/kg DMI	late lactation	1.08 ± 0.1	0.95 ± 0.09	0.2	0.001	

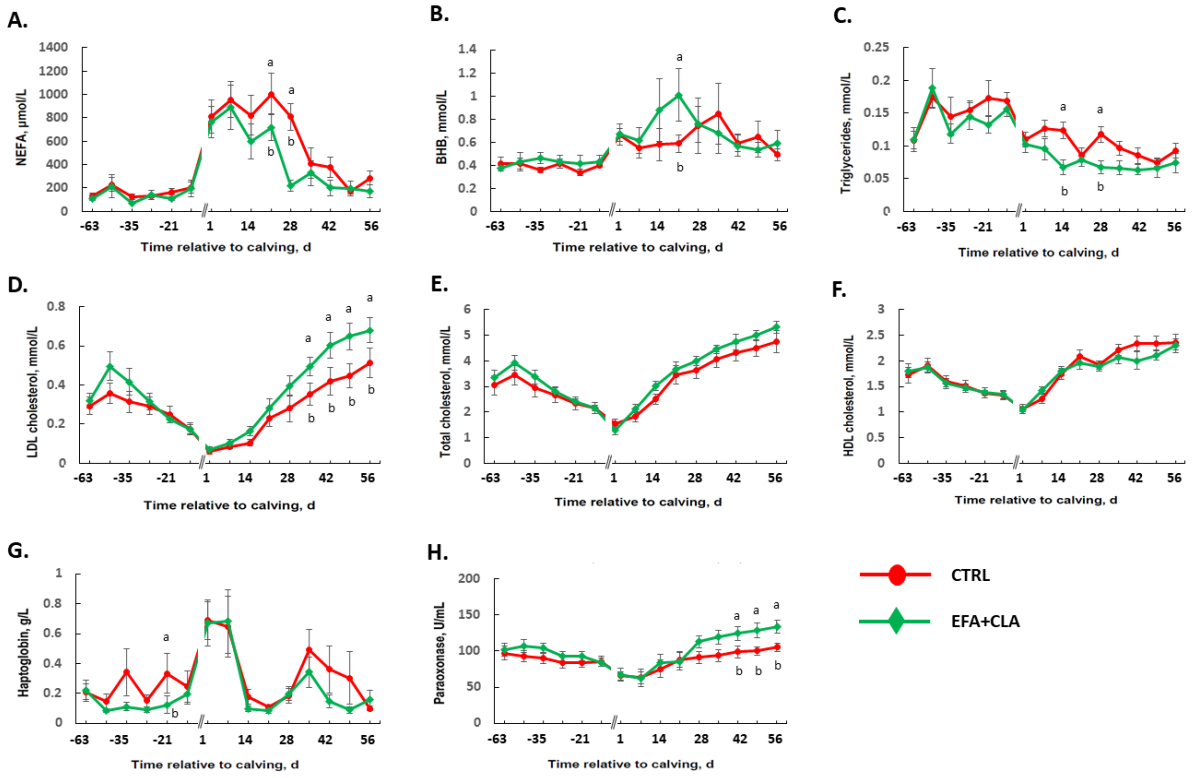
	Early lactation	2.31 ± 0.11	1.95 ± 0.1	0.5	0.001
BW, kg	late lactation	701 ± 21	670 ± 19	0.5	0.001
	Dry period	742 ± 22	718 ± 20	0.2	0.001
	Transition period	690 ± 20	672 ± 18	0.3	0.001
	Postpartum	634 ± 18	621 ± 17	0.4	0.001
	Entire Study	685 ± 20	665 ± 18	0.3	0.001
BCS	late lactation	3.62 ± 0.11	3.29 ± 0.1	0.7	0.001
	Dry period	3.72 ± 0.12	3.62 ± 0.11	0.9	0.001
	Transition period	3.54 ± 0.12	3.5 ± 0.11	1	0.001
	Postpartum	3.12 ± 0.11	3.1 ± 0.1	0.8	0.001
	Entire Study	3.43 ± 0.11	3.31 ± 0.1	0.8	0.001
BFT, mm	late lactation	13.4 ± 1	11.3 ± 0.9	0.8	0.001
	Dry period	15.3 ± 1.1	14.6 ± 1	0.9	0.001
	Transition period	14.7 ± 1.1	14.5 ± 1	0.8	0.001
	Postpartum	12.1 ± 1	12.6 ± 0.9	0.8	0.001
	Entire Study	13.5 ± 1	13 ± 0.9	0.9	0.001

1Values are presented as the LSM ± SE.

2FEMY = feed efficiency for milk production; FEECM = feed efficiency for ECM production; BFT = back fat thickness.

Supplementary S4.

Plasma concentrations of (A) non-esterified fatty acids (NEFA), (B) β-hydroxybutyrate (BHB), (C) triglycerides, (D) low-density lipoprotein (LDL), (E) total cholesterol, (F) high-density lipoprotein (HDL), (G) haptoglobin, and (H) paraxonase from 83 d before until 63 d after calving in cows supplemented daily with coconut oil (○ CTRL; n = 8), or a combination of linseed and safflower oil and Lutalin (cis-9,trans-11 and trans-10,cis-12 CLA; BASF, Ludwigshafen, Germany; ◆EFA+CLA; n = 8). Changes in plasma metabolites concentrations were analyzed using the MIXED procedure by repeated-measures ANOVA. Data are presented as the least squares means (LSM) and their standard errors (SE) (LSM ± SE), LSM with different superscripts (a, b) differ ($P < 0.05$) at the respective timepoint . Statistically significant ($P < 0.05$) effects for (A) NEFA concentration during the entire study (time; EFA+CLA × time interaction). Statistically significant ($P < 0.05$) effect for (B) BHB, (C) triglycerides, (D) LDL, (E) total cholesterol, (F) HDL, (G) haptoglobin, and (H) paraxonase concentration during the time. Adapted from (Gnott et al., 2020; Vogel et al., 2020; Vogel et al., 2021).



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4. Article 2 (Journal of Proteomics, Volume 252, 10 February 2022, 104435)

Longitudinal liver proteome profiling in dairy cows during the transition from gestation to lactation: investigating metabolic adaptations and their interactions with fatty acids supplementation via repeated measurements ANOVA-simultaneous component analysis

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Highlights

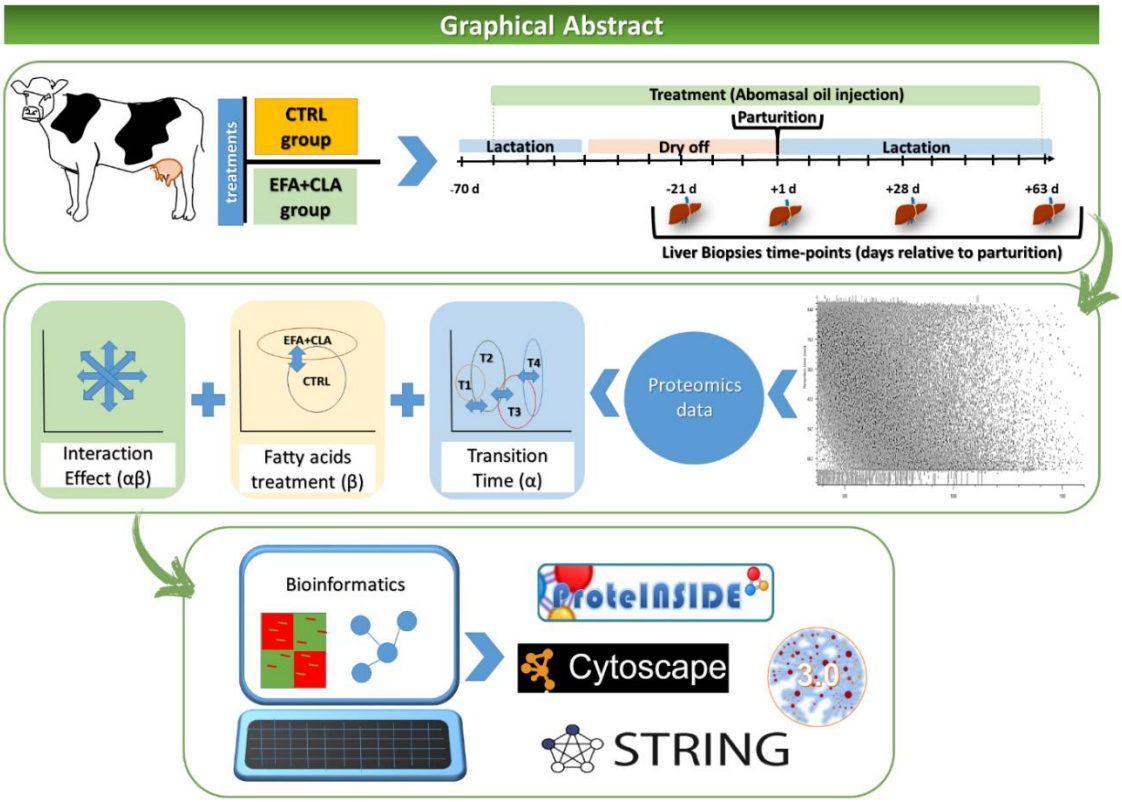
1- ANOVA-simultaneous component analysis applied to time course experimental design

2- Oxidation capacity as a signature of hepatic metabolic adaptation to lactation

3- Fatty acid (FA) supplementation amplified hepatic FA oxidation

4- Ligand-activated nuclear receptors primary regulate hepatic FA oxidation

Graphical abstract



Abstract

Repeated measurements analysis of variance – simultaneous component analysis (ASCA) has been developed to handle complex longitudinal omics datasets and combine novel information with existing data. Herein, we aimed at applying ASCA to 64 liver proteomes collected at 4-timepoints (day -21, +1, +28, and +63 relative to parturition) from 16 Holstein cows treated from 9 wk antepartum to 9 wk postpartum (PP) with coconut oil (CTRL) or a mixture of essential fatty acids (EFA) and conjugated linoleic acid (CLA) (EFA+CLA). The ASCA modelled 116, 43, and 97 differentially abundant proteins (DAP) during the transition to lactation, between CTRL and EFA+CLA, and their interaction, respectively. Time-dependent DAP were annotated to pathways related to the metabolism of carbohydrates, FA, and amino acid in the PP period. The DAP between FA and the interaction effect were annotated to the metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, retinol metabolism, and steroid hormone biosynthesis. Collectively, ASCA provided novel information on molecular markers of metabolic adaptations and their interactions with EFA+CLA supplementation. Bioinformatics analysis suggested that supplemental EFA+CLA amplified hepatic FA oxidation; cytochrome P450 was enriched to maintain metabolic homeostasis by oxidation/detoxification of endogenous compounds and xenobiotics.

Keywords: ASCA; liver biopsy; LC-MS/MS; fatty acids; transition cows; cytochrome p450

Significance

This report is among the first ones applying repeated measurement analysis of variance–simultaneous component analysis (ASCA) to deal with longitudinal proteomics results. ASCA separately identified differentially abundant proteins (DAP) in 'transition time', 'between fatty acid treatments', and 'their interaction'. We first identified the molecular signature of hepatic metabolic adaptations during postpartum negative energy balance; the enriched pathways were well-known pathways related to mobilizing fatty acids (FA) and amino acids to support continuous energy production through fatty acid oxidation, TCA cycle, and gluconeogenesis. Some of the DAP were not previously reported in transition dairy cows. Secondly, we provide novel information on the mechanisms by which supplemented essential FA and conjugated linoleic acids interact with hepatic metabolism. In this regard, FA amplified hepatic detoxifying and oxidation capacity through ligand activation of nuclear receptors. Finally, we briefly compared the strengths and weaknesses of the ASCA model with PLS-DA and outlined why these methods are complementary.

Introduction

A state of negative energy balance (NEB) during the transition from late gestation to early lactation initiates a series of profound metabolic and physiological adaptations in dairy cows to meet the energy demands for milk production (Goff and Horst, 1997). During NEB, fatty acids (FA) are mobilised from adipose tissue to be oxidized in the liver for supplying energy (Weber et al., 2013). Therefore, the major adaptive mechanism is shifting towards the use of non-esterified fatty acids (NEFA) by hepatocytes, where they are further metabolized via various pathways (Wankhade et al., 2017). Numerous transcription factors and coactivators, such as peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element-binding proteins (SREBPs), control these regulatory mechanisms (Rui, 2014). Also, various nutritional treatments, e.g., FA that are not only substrates for generating energy but also natural ligands for nuclear receptors (Bionaz et al., 2013), may interact and impact metabolic adaptations (Zachut et al., 2010; Chandler et al., 2017).

Over the past decades, mass spectrometry (MS)-based proteomics technology has emerged and matured as a powerful approach to discern the key factors contributing to systemic metabolic homeostasis and health in many species, including ruminants (Brockman and Chen, 2012; Kuhla and Ingvartsen, 2018; Fonseca et al., 2019; Ghaffari et al., 2020). With a growing interest that has been paid to proteomics studies, it is not uncommon to have an intricate experimental design with different timepoints (or 'longitudinal'), treatments (multi-group, e.g. different dose groups), and multi-subject (containing data of multiple animals) (Smilde et al., 2005). For instance, in vivo longitudinal animal studies frequently deal with random physiological states (such as lactation, pregnancy, and growth), which could stand as the primary source of variation, especially if the treatment effect is negligible. Such intricate experimental designs call for specific multivariate analysis with predefined matrices of additive effects.

One approach would be the principal component analysis (PCA) which is designed for reducing the dimensionality of large datasets while increasing interpretability (Ringner, 2008; Wood et al., 2018). However, the straightforward use of PCA without predefining the factors may come up with clusters in which the primary sources of variation may be due to the longitudinal effect instead of the treatment effect (Xu et al., 2014). Combining the analysis of variance with PCA led to the development of the ANOVA-simultaneous component analysis (ASCA) method (developed by Smilde (Smilde et al., 2005)). This method is particularly suited for time-resolved studies and has the advantages of decomposing variability separately within the

'treatment' and 'time' and between 'time and treatment' (interaction of time and treatment). Subsequently, PCA is performed on each defined source of variation independently (for review (Bertinetto et al., 2020)).

The ASCA design has been previously reported in some studies, including a metabolomics intervention study, in which guinea pigs were treated with varying doses of vitamin C, and their urine metabolite profiles were analyzed using NMR spectroscopy at several points in time (Smilde et al., 2005). The application of this design is not limited to metabolomics (Timmerman et al., 2015), but there is no report on other omics-based datasets yet.

Previously, we have investigated in detail the effect of supplementation with essential FA (EFA) and conjugated linoleic acids (CLA) on the liver proteome of dairy cows in several timepoints from the ante (AP) to the postpartum (PP) period without considering time as a fixed effect (since it was not the main focus of our study (Veshkini et al., 2021)). This routine procedure had pointed out and emphasized on the treatment effect, and was complemented by our specific longitudinal design for its potential for revealing yet undiscovered aspects: i.e., exploring the shift of proteins within the transition from gestation to lactation could be particularly informative in understanding the physiology of adaptation and lactation as a secondary purpose of a study. Moreover, relatively little is known about hepatic metabolic adaptation in transition dairy cows in response to supplemented FA (interaction effect) at the proteome level.

In this study, we aimed at recruiting the repeated measures ACSA design to reuse our proteomics results and assess differentially abundant proteins (DAP) within the transition from gestation to lactation as an initial objective of this study. A further goal was to investigate how supplemented FA may interact with metabolic adaptations. To the best of our knowledge, this is the first report using the repeated measures ACSA on comprehensive untargeted longitudinal liver proteomics data set for interpreting the metabolic shifts related to FA supplementation in dairy cows during the transition period.

Material and methods

Experimental design, sampling, and peptide preparation

The study used raw LC-MS/MS results from our previously reported liver proteomics study (Veshkini et al., 2021). Briefly, 16 multiparous Holstein dairy cows (11,101 ± 1,118 kg

milk/305 d in second lactation and BW of 662 ± 56 kg; means ± SD) were abomasally injected with either a control fat (coconut oil; CTRL, n = 8; Bio-Kokosöl #665, Kräuterhaus Sanct Bernhard, KG, Bad Ditzenbach, Germany) or EFA+CLA supplement, containing a combination of linseed oil (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany), safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany) and Lutalin® (CLA, n = 8; 10 g/d *cis*-9, *trans*-11, *trans*- 10, *cis*-12 CLA, BASF SE, Ludwigshafen, Germany) from d 63 AP until d 63 post PP (Figure 1 A). The experimental procedures were carried out at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany and approved by German Animal Welfare Act (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany; LALLF M-V/TSD/7221.3-1-038/15). Liver tissues were obtained from each animal on d -21 AP, and d 1, 28, (by biopsy) and 63 PP after slaughtering the cows (Figure 1 A). More information regarding diet ingredients, chemical composition, performance, and plasma metabolite data can be found elsewhere (Vogel et al., 2020).

Liver sample preparation and proteomics analysis

The preparation steps were previously explained in more detail in (Veshkini et al., 2021). Briefly, extracted liver proteins were subjected to in-gel digestion and the peptide mixtures were then analyzed using high-resolution nano-liquid chromatography (Ultimate 3000 RSLC nano-system (Dionex)) coupled to an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) (Veshkini et al., 2021) (Figure 1 B). It is important to point out that some steps were considered to reduce between-group variability and increase the power of analysis. In this regard, LC-MS/MS was performed on all 64 samples consecutively but randomly without any order related to time or treatment using the same setting and unique analytical columns. Before and after MS analysis, LC-MS/MS efficiency (quality of liquid chromatography separation and mass spectrometry performance) was checked using the Pierce ™ HeLa Protein Degradation Standard (catalogue number: 88328 Thermo Scientific ™). For more details, see (Veshkini et al., 2021).

Data processing

Peptides MS/MS spectra were aligned to the reference sample automatically defined by Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastel upon Tyne, UK). It has to be highlighted that the reference sample is defined regardless of time or treatment, and alignment is done to all samples to obtain a set of comparable peaks. After peptide quantification, the identified/quantified peptide ions were searched against a Bos taurus decoy database (Uniprot, download date: 2019/11/07, a total of 37,513 entries) using MASCOT (version 2.5.1) interrogation engine. The specific validated peptides were then inferred to corresponding proteins and their intensities in Progenesis QI software (Figure 1B). A total of 1681 proteins were maintained for analysis after applying strict exclusion criteria (deamidated, carbamidomethyl, and oxidation contaminant proteins, having at least two peptides and two unique peptides, and presence in at least 50% of the samples in each treatment group/timepoint) (Veshkini, 2021).

Decomposing matrices for ASCA and statistical analysis

Statistical analysis was performed on the normalized log-transformed and auto-scaled (z-transformation) intensity values with the metaboAnalystR 3.0 package in R statistical software (R version 4.0.0). The missing intensities were imputed and replaced with the small values (half of the smallest positive value in the dataset).

The ASCA method was described in detail previously (Jansen et al., 2005; Smilde et al., 2005); here, we have briefly illustrated its design for our proteomics dataset. In this study, a balanced experiment was structured by ASCA. Our proteomics dataset comprised 64 distinct proteomes that were organized as described below (Figure 1C):

Individuals: 16 cows were included in the experiments.

(α) Time: four timepoints days -21, +1, +28, and +63 relative to parturition were considered as time variable (16 individual* 4 timepoints).

(β) Treatment: The two treatment groups, including control and EFA+CLA, were inputted into the model as treatment variables (32 individuals * 2 treatment groups)

(αβ) Interaction of time and treatment: possible mixtures = 8 individuals * 2 treatment groups* 4 timepoints.

The first step was to perform a two-way repeated-measures Analysis of variance (ANOVA) on each variable described above individually, according to equation 1,

(1) $x_{ijkp}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+ + S_{k(j)}+\varepsilon_{kij}$.

Equation (1) indicates a series of j ANOVAs where μ is an overall offset, αi the effect of the first factor (transition from gestation to lactation), βj the effect of treatment (supplemented FA), (αβ)ij the interaction between them, Sk(j) is the random effect of the kth subject and εkij the residuals.

Then, applying PCA to each score sub-matrix in (1) (indicated by T α, T β, and T αβ) and submodel loadings (are given by matrices P α, P β, and P αβ) and examining estimated effects for all variables simultaneously by

(2) $X=Xm+TaPaT+TbPbT+TabPabT+Xe$,

where T and P - as mentioned before- represent the scores and loadings matrices for each corresponding factor or interaction, respectively, and Xe defines the residual or deviations of each individual replicate from the average effects. The performed operation (2) was a Simultaneous Component Analysis (given the name ASCA) or repeated PCA on a common set of measured variables allocated to predefined matrices. The following criteria were set to decompose the ASCA model in R: Leverage threshold= 0.9, and alpha threshold= 0.05.

Bioinformatics analysis of differentially abundant proteins

Before bioinformatics analysis, proteins' accession was converted into Gene ID by the UniProt (retrieve/ID mapping) database conversion tool, and undefined proteins were blasted and replaced with their Gene ID in other species. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis of DAP were performed with STRING (version 11.0) and proteINSIDE (version 1.0) setting B. Taurus genome as background, and only pathways enriched with P-value < 0.05, corrected to FDR with Benjamini-Hochberg method (p-adjust < 0.05) and at least two hits in each term were considered significantly enriched (Figure 1 C). Protein-protein interaction networks were constructed and visualized by inputting the DAP identified on main effects (time, treatments,

and their interaction) to Cytoscape version 3.8.2 software, in which nodes and edges represent proteins and their interactions, respectively (Veshkini, 2021).

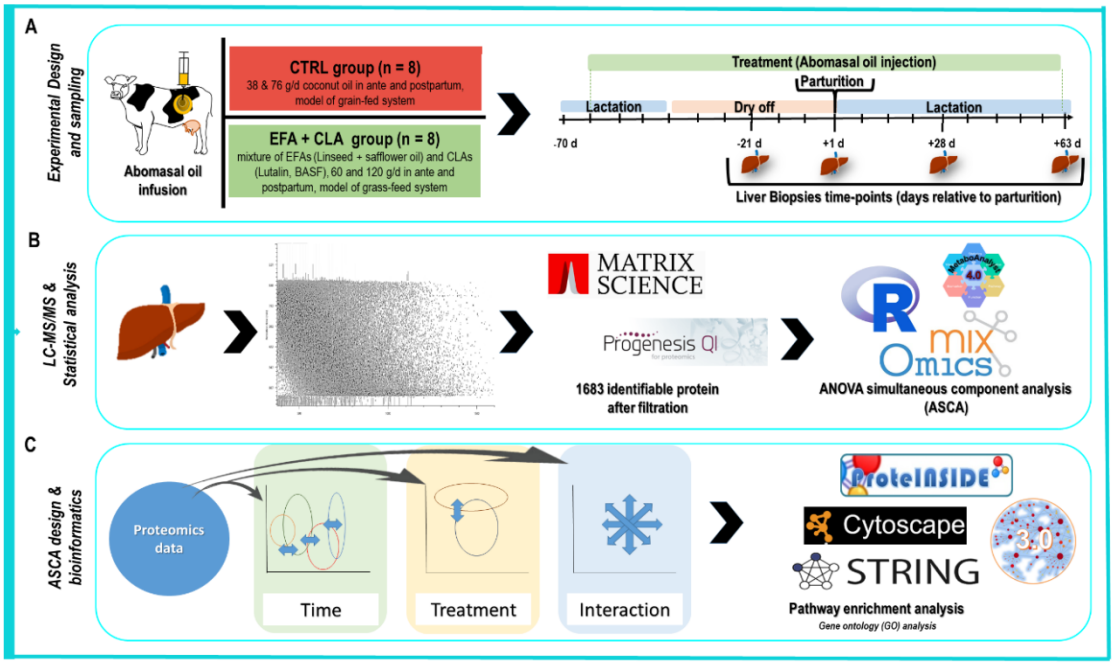


Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow and peptide identification, and (C) statistical analysis and bioinformatics pipeline. (A) Timeline of supplementation (from -63 d ante to +63 d postpartum) and liver biopsy collection (-21 d, +1 d, +28 d, and +63 d relative to parturition). Bold lines indicate liver biopsy sampling timepoints. (B) High-resolution LC-MS/MS analysis, peptide alignment (progenesis), and protein identification (mascot) procedure were performed by Progenesis software coupled with the Mascot search engine, statistical analysis was based on Multivariate Analysis of variance – simultaneous component analysis (ASCA), and (C) ASCA design and bioinformatics analysis.

Results and discussion

Differential proteomic analysis: repeated measurements analysis of variance – simultaneous component analysis

From a total of 1681proteins, 116 proteins during the transition period, 43 proteins between treatments, and 97 proteins in the interaction of them were identified as differentially abundant (Table 1, more details are provided (Veshkini, 2021)). Figure 2 represents the major pattern described by the ASCA model associated with transition time, FA treatment, and their interaction, respectively. Figure 2 A, is a time score plot based on component 1 (52.24% of variation explained) and demonstrated that there is a considerable difference (elbow break) between days 0 and 28. Figure 2 B showed that the groups differed in their principal component (PC) 1 scores (100% of variation explained), with the CTRL and EFA+CLA groups exhibiting

the lowest and highest scores, respectively. Figure 2 C visualizes the major pattern assessed for the interaction effect on PC1 (more than 50% of variation explained) and PC2 (more than 30% of variation explained). Leverage/SPE scatter plots, scree plots, and the permutation tests are provided in Supplementary Figure S1.

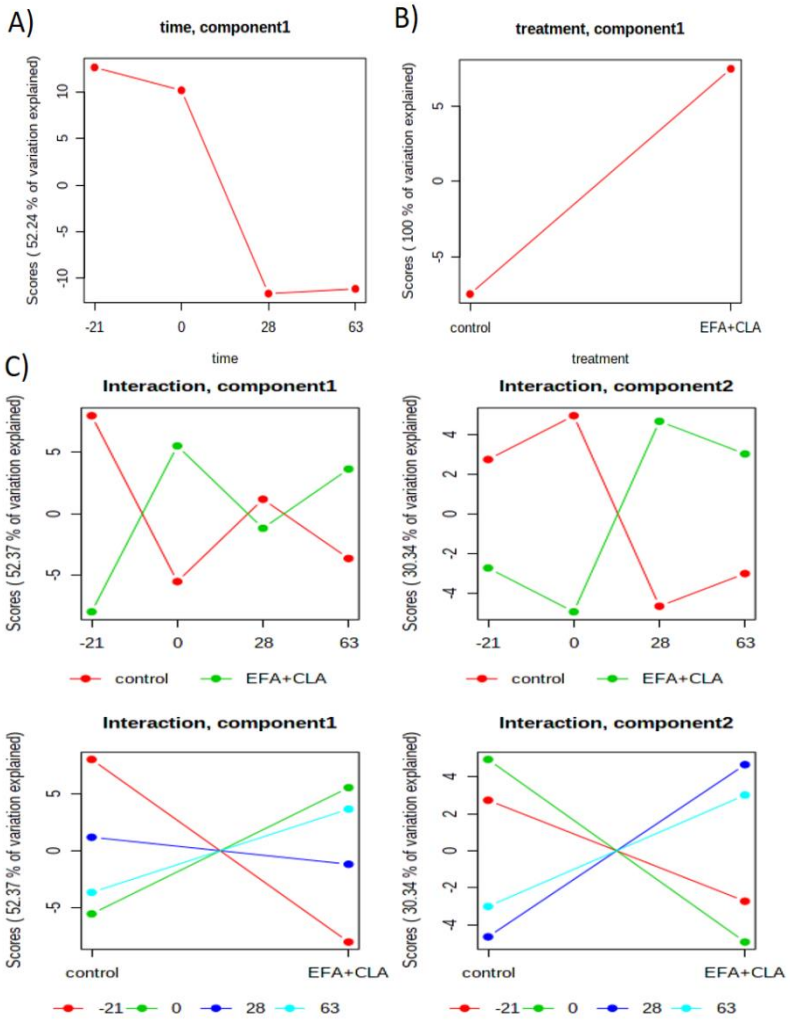


Figure 2) Major patterns associated with transition time (A), FA treatment (B) and their interaction (C) calculated by analysis of variance - simultaneous component analysis (ASCA), in dairy cows supplemented with or without EFA+CLA in 4 time-points (-21, +1, +28, and +63 d relative to parturition. The x-axis indicates the scores and the y axis indicates the variables (different timepoints (a), CTRL and EFA+CLA (b), and interaction of them (ab).

Gene ontology and functional enrichment analyses of differentially abundant proteins during the transition period

The relative abundance of DAP during the transition period is illustrated in a Heatmap (fold changes ranged from -4 to +4) in Figure 3. The protein abundance patterns within timepoints are graphed in the score plot (Figure 2 a), in which the only considerable difference among

them was observed between d +1 and d +28, that was also seen by two separate clusters containing d -21 AP and +1 PP as the first cluster (AP-cluster1) and d +28 and +63 PP as the second one (PP-cluster2). The first cluster (AP-cluster1) was representative of AP; vice versa, PP-cluster2 represented the PP period. Out of the 116 DAP obtained during the transition period, the relative abundance of 93 proteins increased, and 23 proteins decreased in PP-cluster2 compared with AP-cluster1 (Veshkini, 2021).

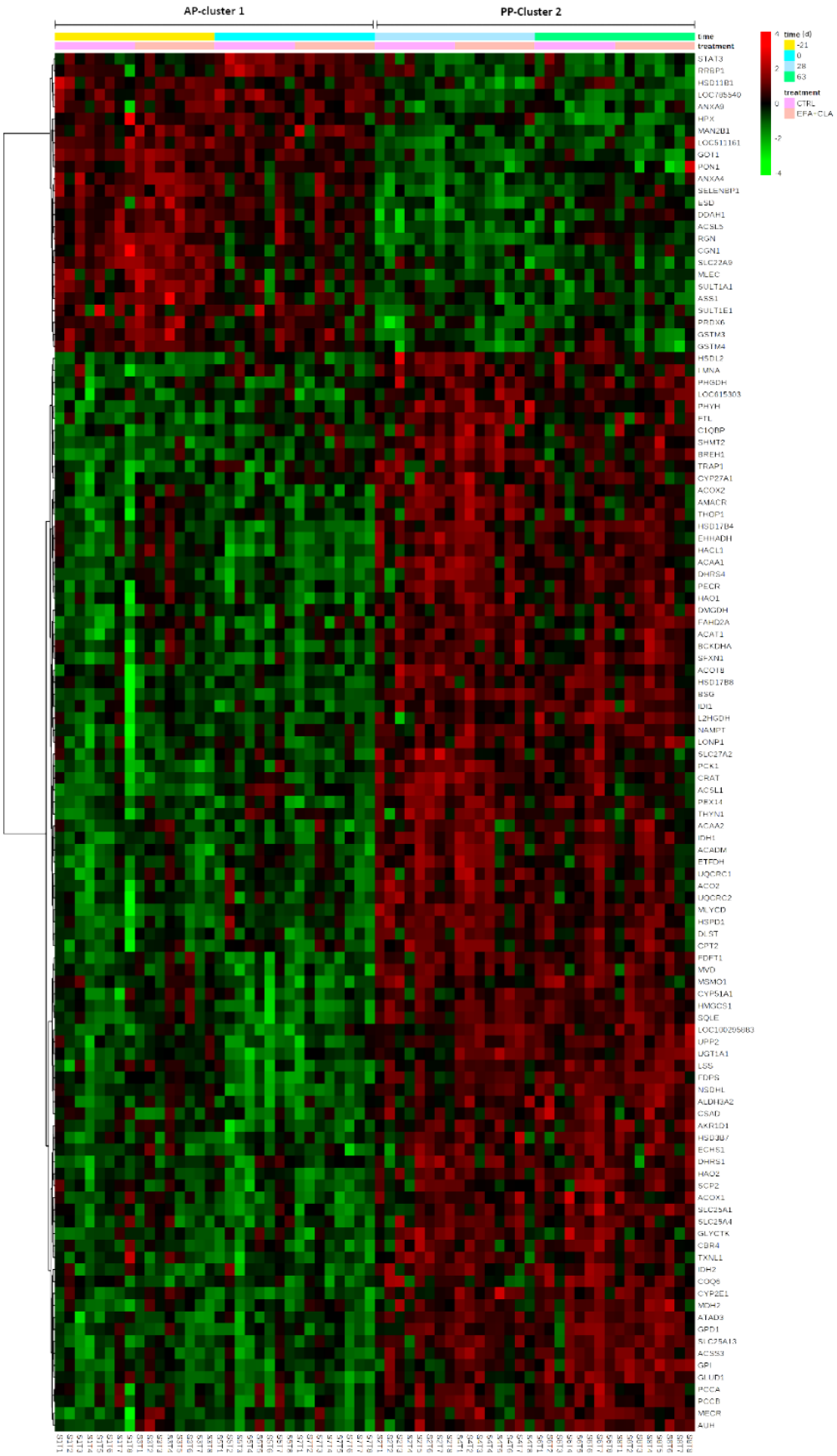


Figure 3) Hierarchical clustering and heatmap representation of differentially abundant proteins during the transition from late gestation to lactation in dairy cows. Rows are respectively sorted by similarity as indicated by the left (proteins) dendrograms. Red and green represent increased and decreased protein abundance, respectively. The colour code for different timepoints and treatments is provided on the right-hand side.

Overabundant proteins (containing phosphoenolpyruvate carboxykinase (GTP) (PCK1), hydroxy acid oxidase 2 (HAO2), 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS1), isocitrate dehydrogenase [NADP] (IDH1), solute carrier family 25 member 13 (SLC25A13), squalene monooxygenase (SQLE), acetyl-CoA acyltransferase 1 (ACAA1), Dihydrolipoamide S-Succinyltransferase (DLST), Acyl-CoA Thioesterase 2(ACOT2), hydroxysteroid (17-beta) dehydrogenase 4 (HSD17B4), isocitrate dehydrogenase [NADP], mitochondrial (IDH2), peroxisomal trans-2-enoyl-CoA reductase (PECR), acyl-CoA synthetase long-chain family member 1 (ACSL1), carnitine O-palmitoyltransferase 2, mitochondrial (CPT2), and cytochrome P450 enzymes (CYP2E1, CYP51A1, and CYP27A1)) were annotated by 98 enriched GO terms in the biological processes (BP) category. They were mainly related to the metabolic processes of energy-related substrates such as carbohydrates, amino acids, lipid and FA, phospholipid, acetyl-CoA, organic cyclic compound, ketone, and carboxylic acid (complete list in (Veshkini, 2021)).
Underbundant proteins including peroxiredoxin-6 (PRDX6), glutathione S-transferase Mu 3 & Mu 4 (GSTM3 & GSTM4), and ASCL5 were annotated by BP GO terms to be related to carbohydrate metabolic process, chemical and ion homeostasis, and glutamine family amino acid catabolic process (complete list provided in (Veshkini, 2021).

Moreover, the functional analysis highlighted the enrichment of 46 KEGG pathways, including peroxisome, FA metabolism, valine, leucine and isoleucine degradation, PPAR signalling pathway, primary bile acid (BA) biosynthesis, steroid biosynthesis, citrate cycle (TCA cycle), biosynthesis of AA, metabolism of xenobiotics by cytochrome P450, pyruvate metabolism, biosynthesis of unsaturated FA, pentose phosphate pathway, synthesis and degradation of ketone bodies, glycolysis/gluconeogenesis, and arachidonic acid metabolism in PP-cluster2 (Figure 4).

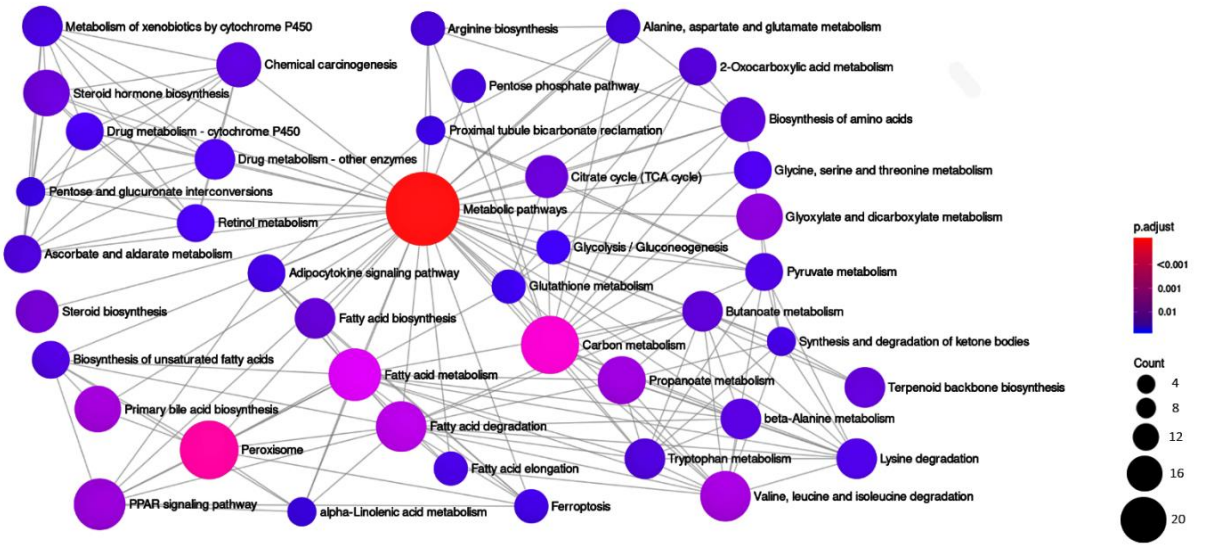


Figure 4) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially abundant proteins (DAP) during the transition from late gestation to lactation in dairy cows. The colour of the nodes represents the –log10 (adjusted P-value); Node size represents the number of DAP contained in the node (smaller indicates lesser DAP, bigger indicates more DAP).

These findings were consistent with previous liver proteome (Xu et al., 2019), and transcriptome (Liang et al., 2017) studies that have reported enrichment of carbohydrates, lipids, and protein metabolism-related pathways in the early and/or peak of lactation compared to the dry period to support milk synthesis. Since none of the cows in any treatment group showed any signs of metabolic disorders, all these massively enriched pathways could be considered as conventional metabolic adaptations to preserve whole-body metabolic homeostasis during the NEB period. Indeed, we have previously reported (Vogel et al., 2020) the elevated plasma concentrations of NEFA and β-hydroxybutyrate (BHB) during the transition from late pregnancy to early lactation, implying that dairy cows from the present study were in a classical physiological NEB state. Thus, within the liver increased gluconeogenesis and ketogenesis are expected to interconvert and metabolize nutrients to support pregnancy and lactation. Consistent with this very general view of liver metabolic adaptations, we have identified proteins involved in ketogenesis, gluconeogenesis, and oxidative capacity through both the TCA cycle and the cytosolic organelles synthesis.

Indeed, once taken up by the liver, NEFA are oxidized either via Acetyl-CoA through the TCA cycle or in ketone bodies from ketogenesis. The over-abundance of ACO2, DLST, IDH1, IDH2, MDH2, and PCK1 related to the TCA cycle, as well as the over-abundance of ACAT1, and HMGCS1 involved in ketogenesis, in the PP period relative to the AP period, strengthened the robustness of the proteome analysis and the ASCA analysis. Indeed most of them were

previously identified by differential proteome during the transition period (Rawson et al., 2012; Moyes et al., 2013; Kuhla and Ingvartsen, 2018; Almughlliq et al., 2019; Xu et al., 2019). Some proteins may be highlighted such as the overabundance of both the ACO2 and IDH mitochondrial enzymes known to induce α-ketoglutarate (AKG) production (from citrate) that serves as an energy source and also as a precursor for glutamine, gluconeogenesis, and synthesis of acute-phase proteins (Tomaszewska et al., 2020). We observed an overabundance of both cytosolic and mitochondrial IDH isozymes (IDH1 and IDH2, respectively) in the PP period, indicating activated IDH2/IDH1 shuttle transferring high energy electrons in the form of NADPH from mitochondria to cytosol (Dai et al., 2018). Moreover, an over-abundance of ACAT1 and HMGCS1 was reported in feed-restricted ketotic cows in the PP period (Loor et al., 2007). Part of the well-known DAP involved in FA oxidation and mevalonate pathway were highlighted in the schematic Figure 5.

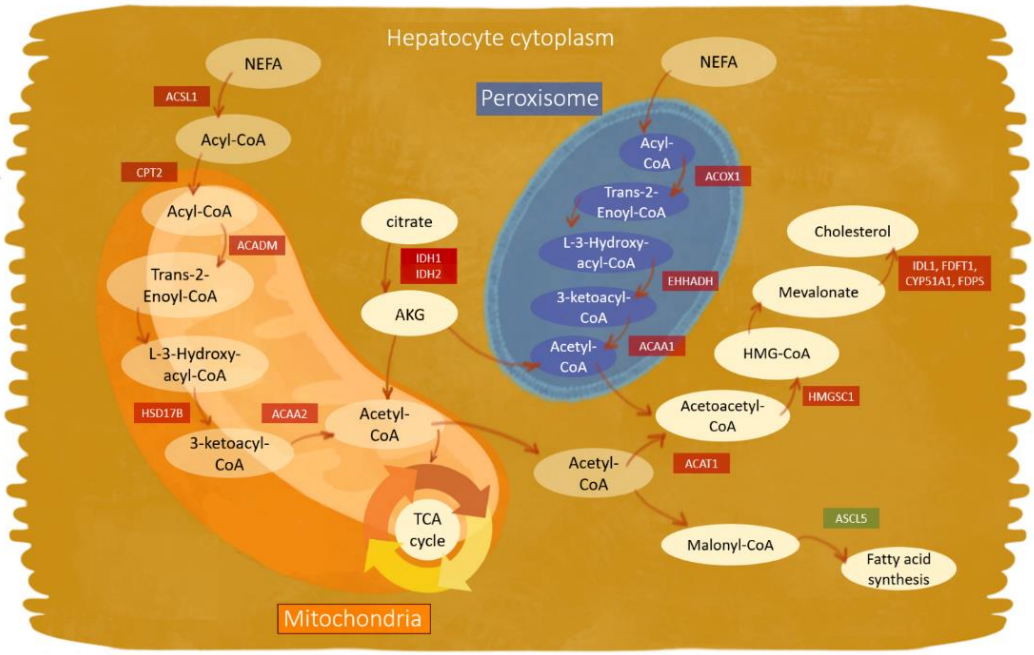


Figure 5) Schematic of fatty acid oxidation in dairy cows' hepatocyte. In the pathway map, only the differentially abundant proteins in the postpartum period are highlighted; red colour indicates upregulation; green designated downregulation.

In dairy cows, propionate as a primary source but also lactate, amino acids (specifically L-alanine), and glycerol can be oxidized indirectly through the TCA cycle to supply carbon for gluconeogenesis. The entry point of these substrates differs and could be through either succinate, oxaloacetate (OAA), or Acetyl-CoA, which is under the control of different isoforms of phosphoenolpyruvate carboxykinase (PEPCK). Here, we observed the PP overabundance of PCK1 (cytosolic form) enzyme, which is a rate-limiting enzyme in gluconeogenesis (Xu et al.,

2020), controlling the entry from amino acids and propionate (Zhang et al., 2015). In line with our results, it has been reported that the expression of PCK1 is elevated with increasing feed intake during early lactation (Greenfield et al., 2000; Agca et al., 2002).

The oxidative capacity of the TCA cycle is dependent on the supply of OAA (carbon carrier) from pyruvate by the action of pyruvate carboxylase (PC) to maintain a 1:1 relationship between OAA and acetyl-CoA (White, 2015). The results revealed an overexpression trend (fold change = 1.65) of the PC enzyme, although its expression was not modeled as differentially abundant. It is critical to balance the synthesis of metabolic intermediates (anaplerosis) and the extraction of metabolic intermediates for breakdown (cataplerosis), especially during the transition period to fuel gluconeogenesis and maintaining carbon homeostasis (White, 2015). Therefore, it can be concluded that the overabundance of both PC and PCK1 probably concur to increase the gluconeogenesis capacity while keeping the balance between anaplerosis and cataplerosis.

Moreover, we observed an enrichment of the peroxisome proliferator-activated receptors (PPAR) pathway, which is known to have a pivotal role in cycling lipid and carbohydrate substrates into glycolytic/gluconeogenic pathways favoring energy production (Wilbanks et al., 2014). Accordingly, an overabundance of Acyl-CoA dehydrogenase (ACADM) which is involved in PPAR signaling and carbon and FA metabolism, combined with the overabundance of long FA transporter (SLC25A1 and SLC25A13) in the PP period, suggest a higher transport activity of FA from the plasma into the hepatocytes, thus supporting a higher level of FA α and β-oxidation for energy supply. In the PPAR pathway, the relative abundance of Enoyl-CoA Hydratase and 3-Hydroxyacyl CoA Dehydrogenase (EHHADH) along with Enoyl-CoA Hydratase, Short Chain 1 (ECHS1) was increased; both proteins have been previously reported to be involved in milk FA metabolism in humans (Banasik et al., 2011) and cow (Shi et al., 2019) studies, not only through the PPAR but also through AMPK (5' AMP-activated protein kinase) signaling pathways. The significant effects of ECHS1 on long-chain unsaturated, medium-chain saturated FA, and milk FA traits in dairy cattle were discussed elsewhere (Shi et al., 2019). The enrichment of the PPAR pathway is also in line with the repeatedly reported role of PPARs as a sensor of NEFA levels (Sanderson et al., 2009; Busato and Bionaz, 2020).

Besides, PPAR are also involved in transcriptional regulatory mechanisms coordinating the abundance and enzyme content of organelles (Fransen et al., 2017). In this regard, we observed the enrichment of pathways related to organelles, in particular, peroxisomes and mitochondria

in PP-cluster2, with more than 20 DAP in the peroxisome, including IDH, Acyl-CoA dehydrogenases (ACADs), Sterol Carrier Protein 2 (SPC2), and ACADM. Both peroxisomes and mitochondria are remarkably dynamic adapting their number and activity depending on the prevailing environmental conditions i.e., excessive NEFA can thus be used directly as substrate and indirectly through PPAR activation (Fransen et al., 2017). Along with mitochondria, peroxisomes play a crucial role in cellular lipid hemostasis, in which the overabundance of SPC2 indicates activation of the peroxisomal cholesterol transport from the cytoplasm and an induced FA β-oxidation (Schroeder et al., 2007).

Moreover, amino acid metabolism, including glycine, serine, isoleucine, threonine, and tryptophan metabolism, was enriched in synchronized with mobilizing skeletal muscle protein during the PP NEB period. The released amino acids were primarily not metabolized in the liver to support mammary glands' milk protein synthesis (Reynolds et al., 2003; Larsen and Kristensen, 2013). Considering the differences between the amino acid profile of muscle and milk (Harper et al., 1984; Mackle et al., 1999), the enrichment of various amino acids metabolism was probably a counter-regulation to maintain the amino acid ratio, precisely because amino acids are only available in limited quantities.

Interestingly, we observed the degradation of the branched-chain amino acids (BCAA, i.e., valine, leucine, and isoleucine) among the most significantly enriched pathways in the PP-cluster2. In this regard, BCAA, in contrast to other amino acids, are less degraded in the liver (first-pass hepatic catabolism) and are preferentially metabolized in extrahepatic tissues (Wessels et al., 2016; Sadri et al., 2017). Activated hepatic degradation of BCAA, in particular during the transition period may indicate that they primarily converted to other amino acids or fed into TCA cycle/ketogenesis pathways. The present results suggest a strong relationship between ketogenesis and BCAAs, accordingly to what was previously reported (Kuhla and Ingvartsen, 2018), in such a way that when citrate synthesis (intensively driven by BCAA degradation but also FA oxidation) exceed the TCA capacity, its surplus is directed to ketogenesis. In this pathway, 11 DAP were involved among which ECHS1, EHHADH, ACAA, and ACADM were discussed previously. Here, the overabundance of the α and β subunits of the propionyl-CoA carboxylase enzyme (PCCA and PCCB) that catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA, revealed an activated gluconeogenesis pathway using propionate as a substrate, and thus feeds the TCA cycle with limiting intermediates.

Proteomic results provided an in-depth overview of metabolic adaptations during the NEB period. To summarize, FA metabolism and degradation, PPAR signaling pathway, peroxisome, and TCA cycle were enriched to enhance lipid and carbohydrate catabolic processes that fuel glycolytic/gluconeogenic pathways favoring energy production rather than storage. Also, the enrichment of pathways related to FA biosynthesis, elongation, and biosynthesis of unsaturated FA, along with α-linolenic acid metabolism, suggest that the identified proteins are involved in providing intermediates/backbones to be used later by the mammary gland for milk fat synthesis. Furthermore, metabolic adaptations were initiated in response to NEB by mobilizing energy substrate to fuel the TCA cycle with OAA, succinate, and α-ketoglutarate, by activating a broad range of pathways related to carbohydrate, lipid, AA, and energy metabolism.

Gene ontology and functional enrichment analyses of differentially abundant proteins between treatment groups

We have previously reported in detail proteins and their associated pathways affected by FA supplementation at several timepoints around parturition (Veshkini et al., 2021). Here, we pooled all timepoints and reported the enriched pathways affected by EFA+CLA treatment (regardless of time). Of the 43 DAP modeled within the treatment, 31 proteins had higher, and 12 proteins had lower abundance in EFA+CLA with a fold change ranging from -3 to 3 (Figure 6).

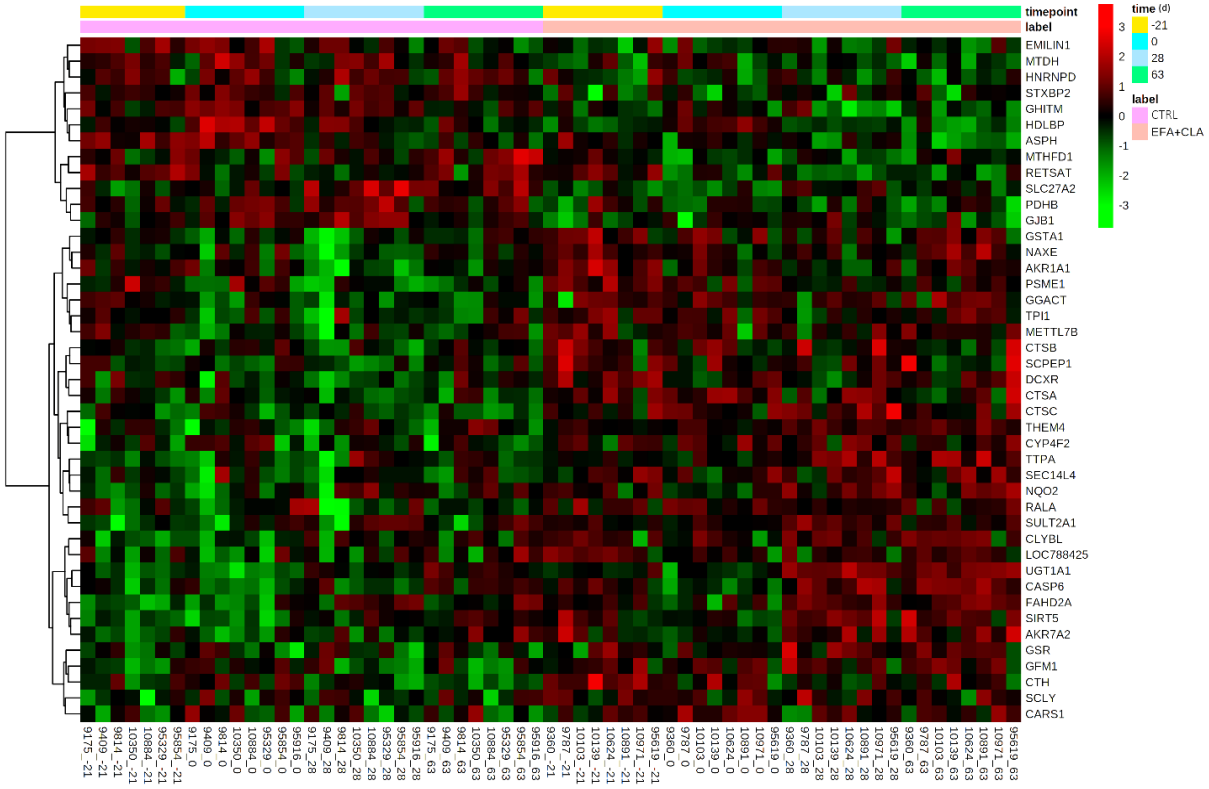


Figure 6) Hierarchical clustering and heatmap presentation of differentially abundant proteins between CTRL and EFA+CLA. Rows are respectively sorted by similarity as indicated by the left (proteins) dendrograms. Red and green represent increased and decreased proteins abundance, respectively. The colour code for different timepoints and treatments is provided on the right-hand side.

Pathway and gene ontology analyses revealed that overabundant proteins were annotated by 28 enriched GO terms within the BP category, including carboxylic acid biosynthetic process (GO:0046394) and metabolic process (GO:0019752), proteolysis (GO:0006508), glucose metabolic process (GO:0006006), cellular metabolic process (GO:0044237), NADP metabolic process (GO:0006739), oxidoreduction coenzyme metabolic process (GO:0006733), coenzyme (GO:0006732) and cofactor (GO:0051186) metabolic process, and carbohydrate catabolic process (GO:0016052). Underabundant proteins did not annotate to any pathways.

Moreover, 11 KEGG pathways were found to be enriched when the 43 DAP were considered: metabolism of xenobiotics by cytochrome P450, pentose and glucuronate interconversions, glycolysis/gluconeogenesis, lysosome, apoptosis, glutathione metabolism, retinol metabolism, chemical carcinogenesis, drug metabolism - cytochrome P450, and drug metabolism - other enzymes (Figure 7). The most significantly enriched KEGG pathway was the metabolism of xenobiotics by cytochrome P450 with four DAP, including an overabundance of glutathione S-transferase A1 (GSTA1), aldo_ket_red domain-containing protein (AKR7A2), sulfotransferase family 2A member 1 (SULT2A1), UDP-glucuronosyltransferase family 1

member A1 (UGT1A1). Cytochrome P450 (CYP) pathways constitute a superfamily of more than 1000 enzymes containing heme, capable of affecting various metabolic and biosynthetic processes by oxidizing different structural compounds, including steroids, prostaglandins, FA, derivatives of retinoic acid, and xenobiotics (Anzenbacher and Anzenbacherova, 2001; Zanger and Schwab, 2013). For instance, the involvement of CYP enzymes in the hepatic biotransformation of cholesterol, its degradation to bile acids (BA), detoxification, and metabolic homeostasis has been the subject of many research studies (Kato et al., 2003; Finn et al., 2009). We have previously reported the involvement of specific CYP enzymes in different timepoints during the transition period that could be time-dependent or related to the fluctuating concentration of FA (NEFA and supplemented FA) serving as specific substrates (Veshkini et al., 2021). Different CYP enzymes are capable of catalyzing the oxidative biotransformation of FA which is known as hepatic ω-oxidation of FA and functions primarily to facilitate their elimination when mitochondrial β-oxidation is saturated. Compared to β-oxidation, ω-oxidation take place in the endoplasmic reticulum and involves the oxidation of the ω-carbon of FA to provide succinyl-CoA (Veshkini et al., 2021).

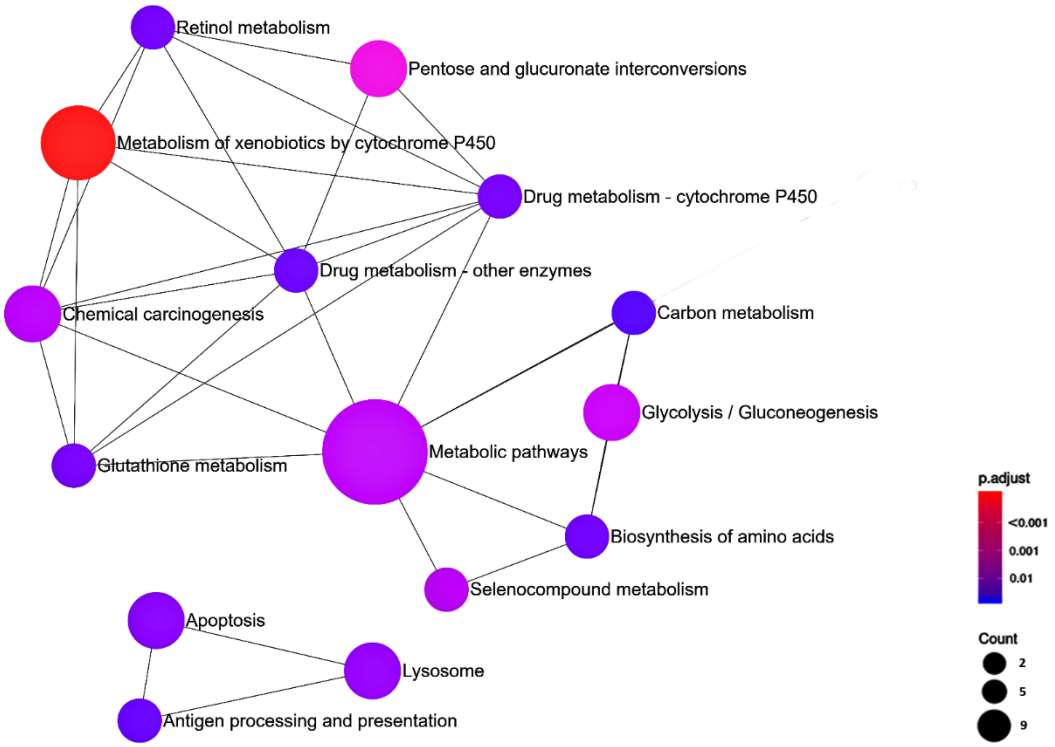


Figure 7) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially abundant proteins (DAP) between CTRL and EFA+CLA. The colour of the dots represents the –log 10 (adjusted P-value); the size of the dots represents the number of DAP in the pathway.

Another mechanism that regulates CYPs expression is through the activation of the PPAR pathway (Shi et al., 2017). It has been shown that PUFA, especially ω-3 FA, compete with NEFA for ligand activation of PPAR (Busato and Bionaz, 2020), suggesting a potential role of these receptors in drug metabolism as well as metabolic homeostasis related to FA metabolism. All these pieces of evidence imply that the cytochrome P450 system may play a key role in regulating hepatic lipid homeostasis, as proposed earlier (Finn et al., 2009).

Another study indicated the involvement of CYP genes in steroidogenesis converting cholesterol to pregnenolone and consequently to dehydroepiandrosterone (Grasfeder et al., 2009). In this study, the steroid hormone biosynthesis pathway was enriched by the overabundance of the LOC100138004 protein. Steroid biosynthesis mainly occurs in the gonads and the adrenal glands, while the liver is considered a site for steroid hormone inactivation (Salleh et al., 2017). Recent observations in dairy cows have shown that providing a gluconeogenic feed (propylene glycol) or treatment with insulin infusion decreased the hepatic expression of CYP enzymes (CYP2C and CYP3A activity) responsible for hepatic progesterone catabolism, which could result in early fetal losses (Lemley et al., 2008). In the current study, neither the concentration of insulin (Vogel et al., 2021) nor the hepatic abundance of CYP2C and CYP3A enzymes were affected by the treatment. Hence, identified CYP enzymes were time-specific; they were not presented at all timepoints to be considered DAPs with the repeated measurement ASCA model. Thus, the ASCA method has identified additional proteins with the CYP pathways that exemplify first the benefit of combining ASCA and PLS-DA analysis, and second the centrality of CYP pathways in responses to EFA+CLA supplementation in dairy cows.

The enrichment of glutathione metabolism indicates a role in the maintenance and regulation of the thiol-redox status against generated ROS during the CYP catalytic cycle. As previously discussed, an elevated rate of peroxisomal and mitochondrial FA oxidation in dairy cows during early lactation is accompanied by greater oxidative production, which may be counteracted by activation of the anti-oxidative machinery system in the liver. Within this pathway, the abundances of two key enzymes, glutamate-cysteine ligase catalytic subunit (GCLC), which is a rate-limiting enzyme in glutathione metabolism, and glutathione reductase (GSR) that converts oxidized GSH to the reduced form were elevated.

Associated with the glutathione and cytochrome metabolism pathway, GSTM 3 and 4 both belonging to the glutathione S-transferase (GST) superfamily, were downregulated. Members

of the GST family are upregulated in response to oxidative stress and are involved in catalyzing the xenobiotic-derived electrophilic metabolites, in steroid hormone biosynthesis, in eicosanoid metabolism and, and in MAPK pathway (for review, see (Hayes et al., 2005)). Moreover, PRDX6, a member of the peroxiredoxin antioxidant enzymes family, is involved in the detoxification process against oxidative stress through glutathione peroxidase. In this regard, Abuelo et al. (Abuelo et al., 2013) reported a gradual increase in oxidative stress status after calving due to fat mobilization. Due to the higher ω-oxidation capacity in EFA+CLA supplemented cows (Veshkini et al., 2021), it seems conceivable to activate GSH synthesis for avoiding oxidative stress. Collectively, the results indicated that EFA+CLA supplementation enriched cytochrome P450 as a core affected pathway.

It is worth mentioning that identifying DAP between CTRL and EFA+CLA group in each timepoint (Veshkini et al., 2021) provided partially different patterns (only a few proteins in common) in comparison to identifying DAP between pooled CTRL and EFA+CLA group (without considering time). This is because we observed a time-specific pattern for DAP, which would not be detectable by the ASCA model. The ASCA would only consider a protein as DAP if it had a constantly lower/higher abundance in all timepoints. Interestingly, metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, drug metabolism - other enzymes, and retinol metabolism were enriched as the main affected pathways by both methods.

Gene ontology and functional enrichment analyses of differentially abundant proteins within the interaction of transition period and fatty acid supplementation

Herein, 97 proteins were found to be affected by the interaction of time and FA supplementation (with a fold change ranging from -6 to +6); proteins were fluctuating between two independent parameters (α*β) and therefore reporting the individual over- or under-abundancy for each protein is not feasible. The relative abundance of proteins modelled in the interaction effect is graphically presented in a Heatmap (Figure 8). The GO enrichment analysis revealed that these proteins were annotated by 65 enriched GO terms within the BP category such as cellular process (GO:0009987), organonitrogen compound metabolic process (GO:1901564), protein metabolic process (GO:0019538), peptide biosynthetic process (GO:0043043), gene expression (GO:0010467), translation (GO:0006412), electron transport chain (GO:0022900), and response to stress (GO:0006950) (Veshkini, 2021).

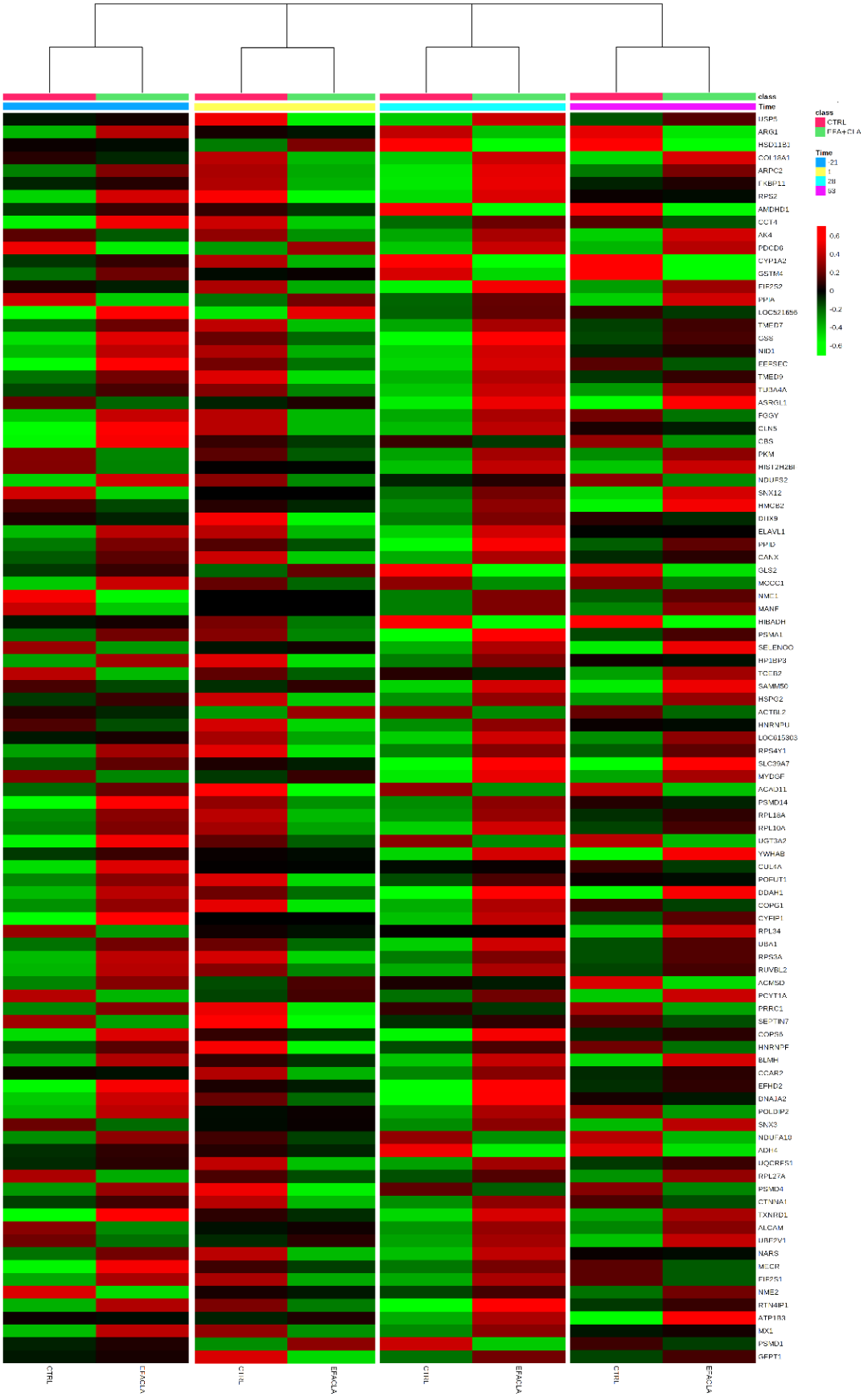


Figure 8) Hierarchical clustering and heatmap representation of differentially abundant proteins by interaction effect. Rows are the average of protein abundaces in each group at each timepoint. Red and green represent increased and decreased protein abundance, respectively. The colour code for different timepoints and treatments is provided on the right-hand side.

Also, the functional analyses of the DAP revealed the enrichment of nine KEGG pathways, including metabolic pathways, metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, drug metabolism - other enzymes, retinol metabolism, steroid hormone biosynthesis, ribosome, chemical carcinogenesis, proteasome (Figure 9) which were mainly the same pathways found enriched for the DAP in the FA-supplemented group (β). Among the KEGG enriched pathways, ribosome (with seven DAP including different ribosomal proteins (RP) S2, S3A, L34, L18A, L10A, L27A, and S4Y1), metabolism of xenobiotics by cytochrome P450 (with five DAP, CYP1A2, UGT2B4, alcohol dehydrogenase 4 (ADH4), hydroxysteroid 11-ßeta dehydrogenase 1 (HSD11B1), and GSTM4), and proteasome (with four hits including different proteasome subunits, PSMD1, PSMD4, PSMA1, and PSMD14) were the top enriched ones. In line with current results, a previous in vitro study on human liver microsomes indicated an inhibitory effect of PUFA containing linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid and, docosahexaenoic acid on CYP1A2 (Yao et al., 2006). In contrast, the inhibition of CYP1A2 was not observed in an in vivo study when linseed oil was infused directly into the abomasum of dairy cows (Piccinato et al., 2010). However, the authors concluded that the infusion might not have achieved sufficient concentrations to inhibit the key enzymes involved in steroid metabolism. The enrichment of ribosome and proteasome pathways is probably due to elevated protein biosynthesis and turnover in the PP-cluster2 and the EFA+CLA group.

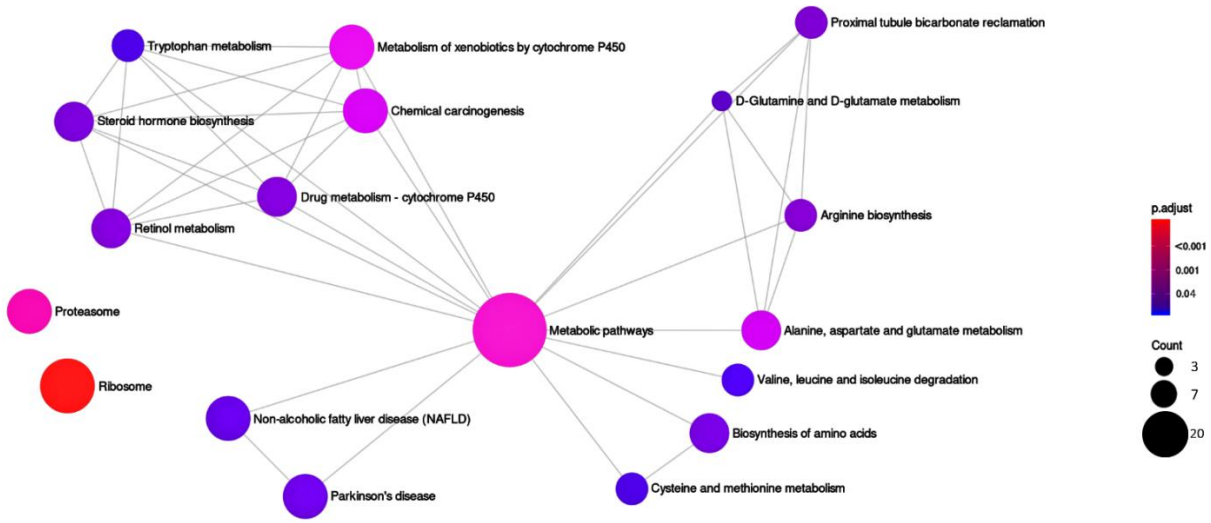


Figure 9) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of proteins identified by the interaction effect. The colour of the dots represents the –log 10 (adjusted P-value); the size of the dots represents the number of differentially abundant proteins in the pathway.

Metabolism of xenobiotics by cytochrome P450 was commonly enriched during the transition to lactation (α), between treatments (β), and interaction of them (αβ), and thus can likely be considered as central mechanisms responsible for maintaining the metabolic homeostasis in response to NEFA mobilization and FA supplementation. Given that CYP are involved in the metabolism of both endogenous and exogenous substrates, it could be speculated that supplemented FA and their intermediate metabolites had xenobiotic-like potential and induced a series of reactions initiated by the ligand activation of PPAR. Consequently, CYP enzymes and their associated pathways such as retinol and glutathione metabolism and steroid hormone biosynthesis were being activated to regulate lipid homeostasis. However, further studies are required to verify this notion.

Comparison of ASCA with PLS-DA method

Choosing a suitable statistical model is always challenging, and it is related to the specific purpose of the study. ASCA design is perfectly suited for time course issues, although sometimes it would be a good complement for classical methods (i.e., PLS-DA) to provide extra information on additive effects that remained uncovered. This is because each method answers a specific question of your study. In this regard, by applying splitting in time PLS-DA, we focused explicitly on the molecular signature of the FA supplementation at each timepoint. Although, repeated measurements ASCA method entirely separated the additive effects of transition time (α), FA treatment (β), and most importantly, their interaction effect (αβ), which is not computable with the other methods (even considering the consecutive PLS-DA(s) on each variable separately). These separations provided us with extra information and a clear view of how FA reacted to or was affected by metabolic adaptations during the transition period.

Conclusion

The present results revealed the molecular signature of metabolic shifts during the transition from gestation to lactation in dairy cows and its interaction with supplemented EFA+CLA using the repeated measurement ASCA model. During the transition from gestation to lactation, DAP enriched metabolic pathways were mainly related to FA metabolism and degradation, amino acids metabolism, biosynthesis and degradation, and carbohydrate and

energy metabolism in favor of energy production. Herein, the NEFA ligand activation of the nuclear PPAR orchestrates lipid metabolism, involving regulation of hepatic mitochondrial and peroxisome metabolism. Supplemented EFA+CLA amplified FA oxidation mechanisms induced by NEFA. The enrichment of cytochrome P450 as an interaction effect was to maintain metabolic homeostasis by oxidation/detoxifying endogenous and exogenous produced xenobiotics. Collectively, it could be concluded that EFA+CLA supplementation in dairy cows having a low level of these two FA, had some marginal beneficial effects on hepatic lipid metabolism and metabolic health.

Acknowledgements

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Supplementary information

Supplementary files were deposited at the INRAe portal as “Gene ontology of hepatic differentially abundant proteins during the transition to lactation, between different fatty acid treatments, and their interaction in Holstein cows”.

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5. Article 3 ((Journal of scientific reports, Volume 12, 05 April 2022, 5648)

Plasma proteomics reveals crosstalk between Lipid Metabolism and Immunity in Dairy Cows receiving Essential Fatty Acids and Conjugated Linoleic Acid

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Abstract

Essential fatty acids (EFA) and conjugated linoleic acids (CLA) are unsaturated fatty acids with immune-modulatory effects, yet their synergistic effect is poorly understood in dairy cows. This study aimed at identifying differentially abundant proteins (DAP) and their associated pathways in dairy cows supplied with a combination of EFA and CLA during the transition from antepartum (AP) to early postpartum (PP). Sixteen Holstein cows were abomasally infused with coconut oil as a control (CTRL) or a mixture of EFA (linseed + safflower oil) and CLA (Lutalin, BASF) (EFA+CLA) from -63 to +63 days relative to parturition. Label-free quantitative proteomics was performed on plasma samples collected at days -21, +1, +28, and +63. During the transition time, DAP, consisting of a cluster of apolipoproteins (APO), including APOE, APOH, and APOB, along with a cluster of immune-related proteins, were related to complement and coagulation cascades, inflammatory response, and cholesterol metabolism. In response to EFA+CLA, specific APO comprising APOC3, APOA1, APOA4, and APOC4 were increased in a time-dependent manner; they were linked to triglyceride-enriched lipoprotein metabolisms and immune function. Altogether, these results provide new insights into metabolic and immune adaptation and crosstalk between them in transition dairy cows divergent in EFA+CLA status.

Keywords

Fatty acid, lipoprotein, cholesterol homeostasis, complement and coagulation cascades, inflammation, postpartum

Introduction

Dairy cows regardless of health status undergo a state of non-pathogenic inflammation during the transition from late pregnancy to early lactation (periparturient period), which consequently activates the immune system[1]. Recent evidence suggested that systemic inflammation may further challenge nutrient homeostasis mechanisms, with consequences that may result in decreased feed intake, increased non-esterified fatty acids (NEFA), hyperketonemia, and disorders/diseases such as hepatic steatosis[1]. Supplementing specific fatty acids (FA) with immunomodulatory properties could be a natural strategy to tolerate immune activation and prevent negative outcomes in dairy cows around parturition[2-4].

Essential FA (EFA) including linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3), along with the stereo-isomers of LA, named as conjugated linoleic acid (CLA), are involved in numerous metabolic pathways such as lipogenesis, inflammation, modulation of immune functions, and gene expression regulation[4,5]. It has recently been reported that the combination of EFA and CLA supplementation during the periparturient period improves dairy cows' energy balance by inducing milk fat depression[6] (MFD) and, as shown by the changes of specific markers, positively affects immune[2] and metabolic homeostasis[7,8]. However, the exact underlying mechanisms by which EFA and CLA impact the metabolism of dairy cows largely remained a "black box" [3].

Plasma is a pool with a high dynamic range of proteins, secreted by all body tissues the most active being the liver, reflecting an organism's metabolic or physiological status[9]. There is limited data on plasma proteins linked to nutritional modifications, particularly during the periparturient period, probably because the large-scale description of plasma protein pool faces methodological challenges[10]. Proteomics approaches may overcome this issue by providing high accuracy for understanding the proteome shifts in different physiological statuses and under different treatments in dairy cows, as recently described[11-13].

Given this background, we aimed to gain insight into the plasma proteome of dairy cows supplied or not with combined EFA and CLA during the periparturient period. Longitudinal label-free quantitative proteomics was performed on plasma samples selected from a previous study according to plasma metabolite results[6]. To the best of our knowledge, this is the first report in dairy cows using longitudinal plasma proteomics to study *in vivo* metabolic and immune adaptation around parturition and its interaction with infused FA.

Results

At 1% peptide false discovery rate (FDR), a total of 241 unique proteins with at least two unique peptides were identified in plasma at each individual time-point (Supplementary S1, Table S1). Of these, 160 belonged to different classes including 41 modifying enzymes (PC00260), 36 protein-binding activity modulators (PC00095), 28 metabolite interconversion enzymes (PC00262), 21 transfer/carrier proteins (PC00219), 9 defense/immunity proteins (PC00090), 9 structural proteins (PC00211), 9 extracellular matrix proteins (PC00102), and 7 intercellular signal molecules (PC00207) (obtained under PANTHER database, http://pantherdb.org/) (Supplementary S1, Table S2).

1 Differential plasma proteome during the antepartum and postpartum period

From day 21 antepartum (AP) to day 1 postpartum (PP), the relative abundance of 14 proteins was increased, and of 49 proteins decreased (Log2 fold change range from -4 to +2, p-value ≤ 0.05) in the plasma regardless of treatment (Figure 1 A, Supplementary S2 Table S3). The KEGG analysis mapped differentially abundant proteins (DAP) to cholesterol metabolism and complement and coagulation cascades pathways. Underabundant proteins were annotated by biological process (BP) gene ontology (GO) terms mainly related to immune regulation and glucose homeostasis, particularly through activation of immune response GO:0002253, acute-phase response GO:0006953, inflammatory response GO:0006954, complement activation GO:0006958, regulation of glucose metabolic process GO:0010906, regulation of tumor necrosis factor production GO:0032680, cellular response to insulin stimulus GO:0032869 (Figure 1 B). Overabundant proteins were annotated by BP GO terms including, response to stress GO:0006950, acute-phase response GO:0006953, cellular oxidant detoxification GO:0098869, and lipid transport GO:0006869 (Figure 1 B). The DAP were assigned to the extracellular region and lipoprotein particles based on the cellular component (CC) GO category (Supplementary S2 Tables S4, S5, S6, S7, S8).

From day +1 to day +28 PP, 29 proteins were over- and 10 proteins were underabundant. Over and under-abundant proteins are displayed in the Volcano plot (Figure 1 C, Supplementary S3 Table S9). The DAP were mapped to the phagosome, NF-kappa B signaling pathway, complement and coagulation cascades, and toll-like receptor signaling pathway using the KEGG pathway analysis (Supplementary S3 Table S10). The GO analysis showed enrichment of identical BP terms by both up- and downregulated proteins, including response to stress GO:0006950, regulation of cytokine production GO:0001817, inflammatory response

GO:0006954, and cellular oxidant detoxification GO:0098869 (Figure 1 D) (Supplementary S3 Tables S11, S12, S13, S14).

Twenty-five proteins, including one over and 24 under-abundant ones, were identified as DAP in d+63 over day +28 (Figure 1 E, Supplementary S4 Table S15). In total, three KEGG pathways, including complement and coagulation cascades, S*taphylococcus aureus* infection, and thyroid hormone synthesis, have annotated the DAP. Insulin-like growth factor-binding protein 2 (IGFBP2) as the only over-abundant protein was not annotated by a GO term, but under-abundant proteins were annotated by GO BP terms related to the regulation of immune response GO:0050776, regulation of response to stimulus GO:0048583, homeostatic process GO:0042592, GO:0010951 negative regulation of endopeptidase activity and regulation of primary metabolic process GO:0080090 (Figure 1 F) (Supplementary S4 Tables S16, S17, S18).

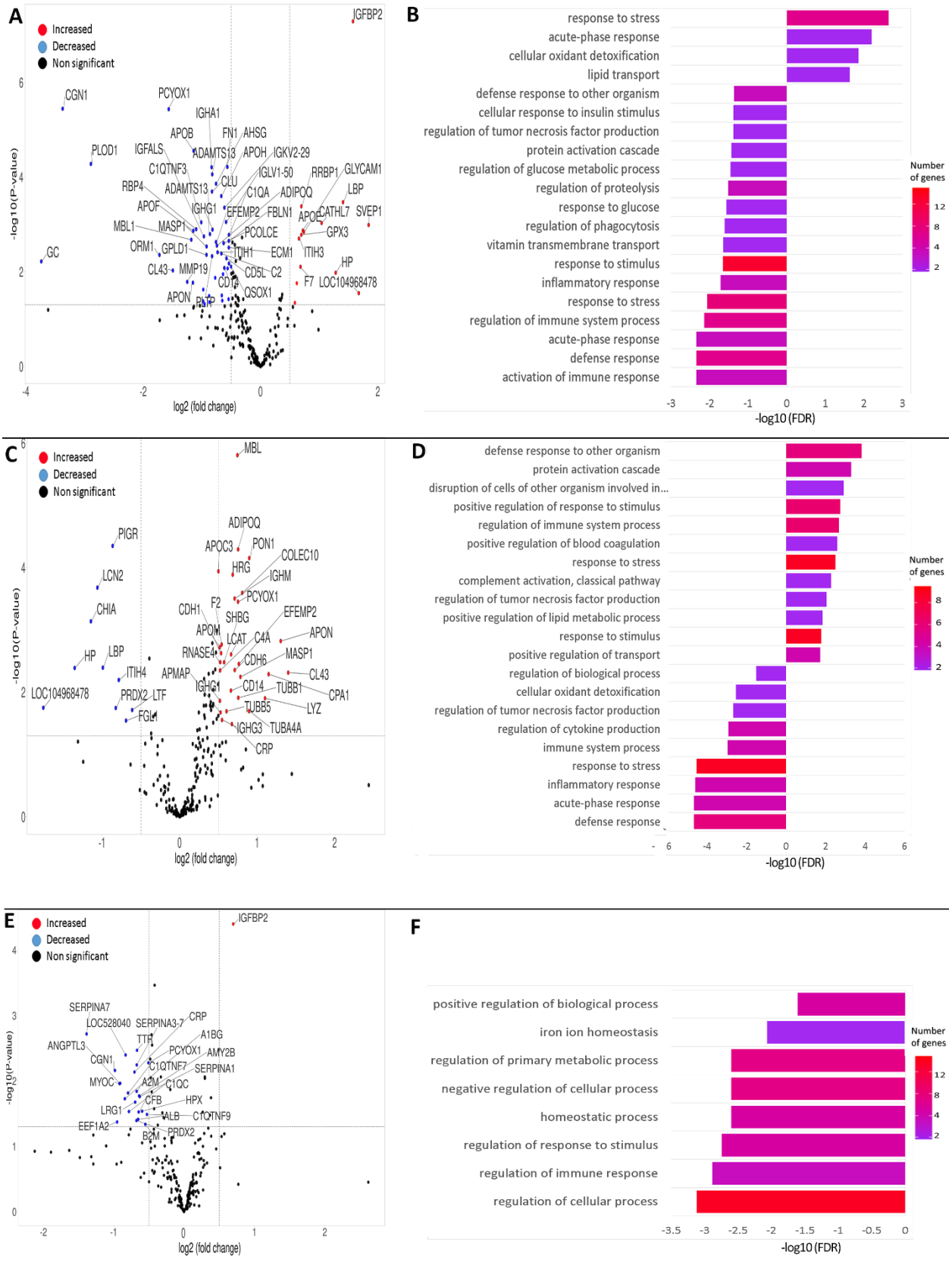


Figure 1) A. Volcano plot representing differentially abundant proteins (DAP) between day -21 and +1 relative to parturition; increased (red dots in top right) and decreased (blue dots in top left) proteins in d+1/d-21 are highlighted ($P < 0.05$ and log2 fold change (FC) > 1.3). B. Biological Process for the DAP Proteins after semantic synthesis by Revigo; Bars indicate proportional to the false discovery rate (FDR) adjusted P-value, the intensity

of color bars is gene count (GC) represents the amount of differentially abundant proteins enriched in the pathway. C. Volcano plot representing DAP between day +1 and +28 postpartum. D. Biological Process for the DAP between day +1 and +28 postpartum. E. Volcano plot representing DAP between day +28 and +63 postpartum. F. Biological Process for the DAP between day +28 and +63 postpartum (For the high quality figure, the reader is referred to the web version of this article).

2- Differential plasma proteome between EFA+CLA and control groups

Overlap between differentially abundant proteins in response to the fatty acid treatment at different timepoints

The Venn diagram in Figure 2 A represents the intersection of proteins identified in all timepoints in response to the EFA+CLA supplementation. The relative abundance of apolipoproteins (APO) C3 (APOC3), APOA1, and APOA4 was greater at all timepoints in response to EFA+CLA supplementation as compared to the CTRL group ("overlapping proteins") (details in Supplementary S5, Table S19). Also, APOC4 and hemoglobin subunit alpha (HBA) were more abundant in the EFA+CLA group in the whole PP period. These APO were mapped to KEGG pathways such as the PPAR signaling pathway, fat digestion and absorption, and cholesterol metabolism (Figure 2 B, Supplementary S6, 7, 8, and, 9, Tables S21, S25, S29, S33). Moreover, APO proteins were annotated by enriched BP GO terms, including triglyceride homeostasis (GO:0070328), cholesterol homeostasis (GO:0042632), lipid transport (GO:0006869), plasma lipoprotein particle assembly (GO:0034377), regulation of lipoprotein lipase activity (GO:0051004), and lipoprotein metabolic process (GO:0042157) (Supplementary S6, 7, 8, and, 9, Tables S22, S26, S30, S34). At the CC level, localization of DAP was extracellular region (GO:0005576), extracellular space (GO:0005615), high-density lipoprotein particle (GO:0034364), chylomicron (GO:0042627), and very-low-density lipoprotein particle (GO:0034361) (Supplementary S6, 7, 8, and, 9, Tables S23, S27, S31, S35). In association with EFA+CLA supplementation, the same KEGG pathways and BP and CC GO terms were enriched in all time points ("overlapping pathways"), therefore to avoid repetition, we did not mention them in each timepoint.

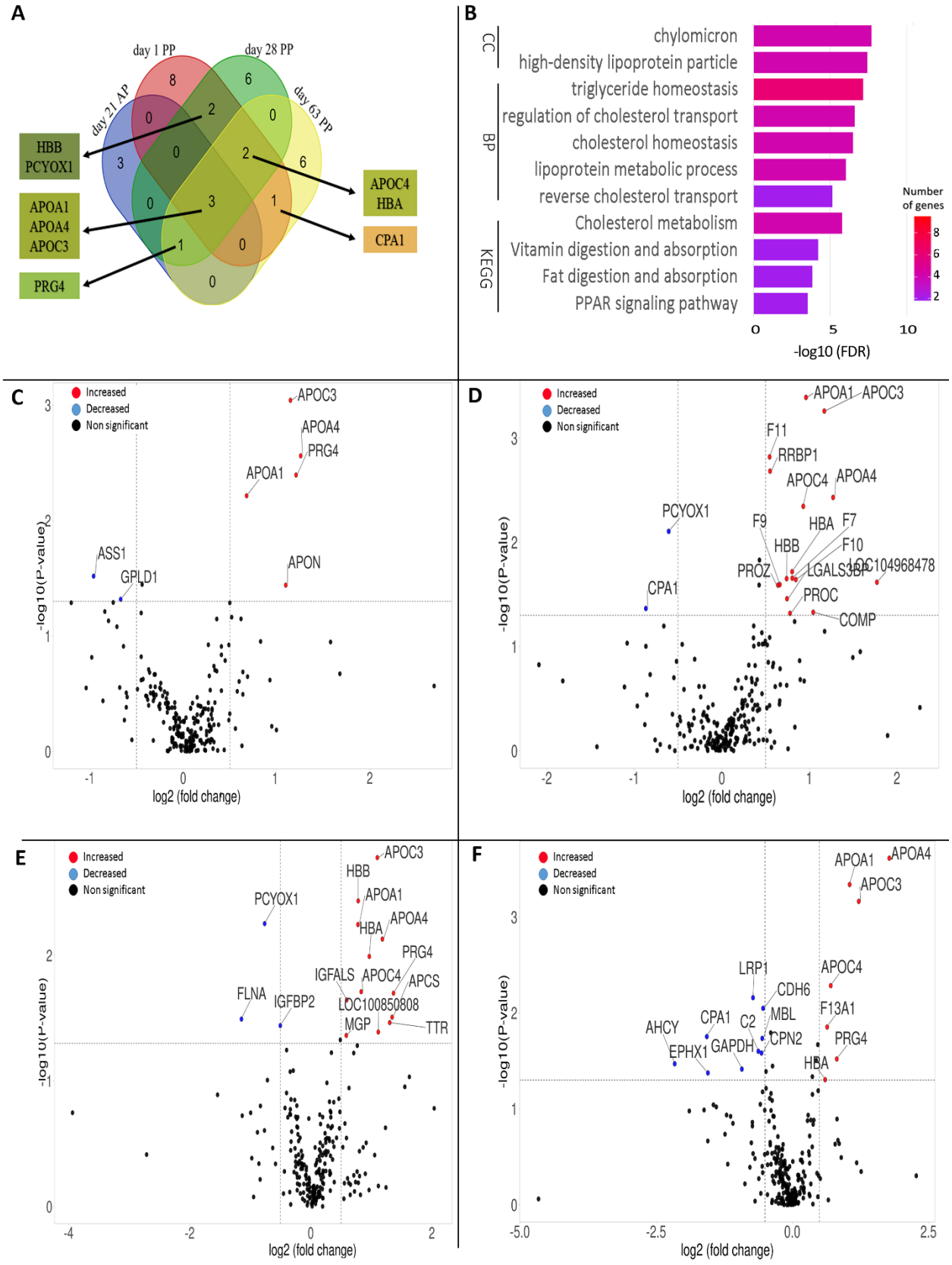


Figure 2) A. Venn diagram representing the overlap between differentially abundant proteins (DAP) in response to EFA+CLA supplementation identified at day -21, +1, +28, and +63 relative to parturition. B. KEGG pathways map[14], biological process (BP), and cellular component (CC) gene ontology terms of DAP after semantic synthesis

by Revigo (overlapping pathways). Bars represents the –log10 (adjusted P-value); the intensity of color bars is gene count (GC) represents the amount of differentially abundant proteins enriched in the pathway. C. Volcano plot representing DAP between Control (CTRL) and essential fatty acid (EFA)+conjugated linoleic acid (CLA) in day 21 antepartum; increased (red dots in the top left) and decreased (blue dots in the top left) proteins in EFA+CLA group are highlighted ($P < 0.05$ and log2 fold change (FC) > 1.5). D. Volcano plot representing DAP between CTRL and EFA+CLA in day 1 postpartum. E. Volcano plot representing DAP between CTRL and EFA+CLA in day 28 postpartum F. Volcano plot representing DAP between CTRL and EFA+CLA in day 63 postpartum (For the high quality figure, the reader is referred to the web version of this article).

Differentially abundant proteins in response to the fatty acid treatment at specific timepoints around parturition

On day 21 AP, seven proteins were differentially abundant between treatments (Volcano plot, Figure 2 C, Supplementary S6 Table S20), in which, beside overlapping proteins, proteoglycan 4 (PRG4), and apoN protein (APON) were more abundant. At the same time, argininosuccinate synthase (ASS1) and phosphatidylinositol-glycan-specific phospholipase D (GPLD1) were less abundant in the EFA+CLA compared to the CTRL group. On day 21 AP, DAP were annotated only by pathways identified for the overlapping proteins (Supplementary S6 Tables S21, S22, S23).

An overview of the proteomic variability between the treatment groups at day 1 PP is given in Figure 2 D (Supplementary S7 Table S24). The relative abundance of 14 proteins was greater and two proteins were lower in the EFA+CLA than in the CTRL group. The overabundant proteins, were annotated by enriched BP GO terms comprised negative regulation of inflammatory response (GO:0050728), and regulation of immune system process (GO:0002682). There were no enriched pathways for the two underabundant proteins in the EFA+CLA group (Supplementary S7 Tables S25, S26, S27).

At day 28 PP, beside overlapping proteins, PRG4, hemoglobin subunit beta (HBB), insulin-like growth factor-binding protein acid-labile subunit (IGFALS), plasma amyloid P-component (APCS), transthyretin (TTR), WAP domain-containing protein (LOC100850808), and matrix Gla protein (MGP) were more abundant in the EFA+CLA group as compared to the CTRL group (Figure 2 E). The PCYOX1 and filamin A (FLNA) were less abundant in EFA+CLA than in CTRL (Figure 2 E, Supplementary S2 Table S28). Herein in response to EFA+CLA supplementation, only negative regulation of the immune system process (GO:0002683) was enriched. The GO analysis was not significant for those proteins that were less abundant in the EFA+CLA group than in the CTRL group (Supplementary S8 Tables S29, S30, S31).

On day 63 PP, we identified 13 DAP, containing overlapping proteins, coagulation factor XIII A chain (F13A1), C3/C5 convertase (C2), and PRG4 with greater abundance, and LDL receptor-related protein 1 (LRP1), carboxypeptidase A1 (CPA1), adenosylhomocysteinase (AHCY), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and epoxide hydrolase (EPHX1) with lower abundance in the EFA+CLA group as compared to the CTRL group (Figure 2 F, Supplementary S9 Table S32). Again at this timepoint, only overlapping pathways have been enriched (Supplementary S9 Tables S33, S34, S35).

Discussion

This study investigated the plasma proteome of dairy cows supplemented with EFA+CLA during the periparturient period. The DAP and their associated pathways are discussed separately during the transition from late pregnancy to early lactation and between treatment groups. Time-affected DAP will be discussed prior to treatment to gain a deeper understanding of dairy cows' metabolic and immune status while confirming some physiological adaptations that increase the reliability of proteomic data. Then FA supplementations will be discussed as they may affect these metabolic adaptations. Protein−protein interaction network analysis depicts that among DAP identified in this study, some belonged to immune system and lipid metabolic process.

From day -21 to +1 of parturition, various immune-related proteins involved in the regulation of pro-inflammatory proteins, such as TNFα, were found to be DAP. This list includes CD14 and adiponectin (ADIPOQ), complement and coagulation cascades such as clusterin (CLU), complement C1q A Chain (C1QA), coagulation factor VII (F7), MASP1, component C2 (C2), CFI, mannose-binding protein C (MBL1), acute-phase response and inflammation comprising haptoglobin (HP), lipopolysaccharide-binding protein (LBP), alpha 2- Heremans-Schmid glycoprotein (AHSG), fibronectin 1 (FN1), and alpha-1-acid glycoprotein 1 (AGP1), and cellular oxidant detoxification protein (GPX3). The involvement of these immune-related proteins is likely due to the activation of the immune system and the development of a systemic inflammation (SI) which is typical in periparturient dairy cows[1], regardless of their health status, to support a new physiological state.

An excessive SI in early PP is generally associated with massive fat mobilization in conjunction with the release of pro-inflammatory cytokines, TNFα, and interleukin IL6[15-17] which in turn alter the secretion of acute-phase proteins (APP). Herein, from day -21 to +1 relative to

parturition and in response to SI, we observed increased abundance of positive APP (HP and LBP) and decreased abundance of negative APP (AHSG and FN1). In addition, AGP1 which is considered a moderate positive APP decreased. Following our results, it is well documented that HP increased from AP to early PP and then gradually decreased[18,19]. In this regard, both HP and LBP are involved in the anti-inflammatory reactions, which are essential to maintain the balanced inflammatory response (for review[1]). Moreover, AHSG, apart from being an APP, is a free fatty acid transporter that enhances cellular lipid uptake and lipogenesis during NEB in dairy cows[20]. Therefore, it seems logical to expect ASHG reduction at the onset of lactation when the lipogenesis pathways were downregulated in favour of energy production. It must be said that the observed AGP1 protein abundances are not in supports of AGP being a positive APP; AGP has been demonstrated to increase during the first and second week of lactation in cows[21] and water buffalos[22], respectively. However, there is no comparable study reporting the AGP1 differences between AP and PP in dairy cows. Remarkably, AGP1 has been reported to act as an anti-inflammatory protein[23], thus being crucial for maintaining immune tolerance. Moreover, it has been reported that both AHSG and AGP1 were reduced before the onset of ketosis in dairy cows[24]. Considering that dairy cows are prone to develop (subclinical) ketosis during the early PP phase, reduced abundance of AHSG and AGP1 in our study is in line with this report.

On the other side, the relative abundance of several complement proteins was reduced at calving. Proteins of the complement system comprise a complex enzymatic cascade that possesses anti-inflammatory functions[25]. The literature on complement proteins in dairy cows plasma, especially during the periparturient period, is scarce. Nevertheless, downregulation of the complement cascade while dairy cows were in SI, implies reduced immune system responsiveness during the early PP period. These results are supported by previous studies that reported immunosuppression in dairy cows before or around calving[26,27].

During transition from AP to PP, five proteins namely phospholipid transfer protein (PLTP), LRP1, and a cluster of APO containing APOE, APOH, and APOB were annotated by the cholesterol metabolism pathway. The relative abundance of all these proteins, except APOE, was downregulated. In accordance, increased ApoE and decreased ApoB100 mRNA abundance have been reported in the liver of dairy cows during the transition from d -35 AP to d 3 PP, whereby APOB was correlated with decreased cholesterol plasma levels[28]. Reduced cholesterol synthesis is also a part of the APP response[1] and is typically observed during early lactation[29].

Hepatic and whole-body cholesterol homeostasis are regulated by the different lipoprotein classes and their APO particles[3]. Concerning the role of the DAP in cholesterol metabolism, PLTP stimulates phospholipid transfer from very-low-density lipoproteins (VLDL) to the high-density lipoprotein (HDL), and its deficiency is associated with decreased plasma HDL, APOA1, and APOB levels[30]. Also, LRP1 deficiency reduced plasma clearance of APOE–containing lipoproteins[31] and thereby accelerated hepatic steatosis[32]. In this regard, ApoE which acts as a receptor-binding ligand is highly associated with both VLDL and HDL in the plasma[31], and its high level leads to the development of lipid-related disorders such as fatty liver in periparturient cows[33]. In addition, decreased ApoB100 is associated with decreased synthesis and secretion of VLDL during the periparturient period. A positive correlation ($r = 0.65$) has been confirmed between ApoB100 and plasma cholesterol in early PP dairy cows[28]. Furthermore, ApoH reduces the intracellular accumulation of cholesterol and blocks the oxidation of LDL[31], which in turn inhibits steatosis development.

In line with our proteomics findings, we have previously reported[6] that the plasma concentration of total cholesterol, LDL-cholesterol, HDL-cholesterol as well as energy balance were at a minimum level on day 1 PP. Decreased APO and lipoproteins suggested reduced cholesterol metabolism (synthesis and reverse transport) and probably initiation of steatosis by the hepatic accumulation of triglycerides (TG) at the onset of lactation.

Interestingly, on day 28 of lactation, some of the immune-related DAP showed the reverse trend compared to d1 PP and tended back to AP levels. In this sense, the APP HP, ITIH4, LBP, and cellular oxidant detoxification protein (PRDX2) were downregulated, and CD14, ADIPOQ, and two proteins related to activation and regulation of the immune system process (HRG, MBL2) were upregulated. Taken together, these observations suggest that the compromised immune system at the beginning of lactation was gradually recovering during the first weeks of lactation. These results support the concept that dairy cows have to adapt to profound metabolic and immune challenges around parturition related to a state of SI. However, they usually recover within few weeks by dampening the inflammatory reaction.

From day 28 to 63 PP, only slight fold changes in protein abundance were observed; the relative abundance of alpha-1-antiproteinase (SERPINA1), alpha-2-macroglobulin (A2M), complement factor B (CFB), protein HP-20 homolog (C1QC) associated with complementing and coagulation cascades were decreased. These results further confirm our previous

conclusion that suboptimal immune and metabolic functions are largely limited to early lactation and then gradually improve.

In response to EFA+CLA supplementation as compared to the CTRL group, APOC3, APOA1, and APOA4 had greater abundances at all timepoints. Genes encoding all these proteins have been shown to share a common enhancer sequence, meaning they operate in synergy[34] probably under the control of transcription factors like PPAR[35]. They are predominantly transcribed, translated, and secreted by hepatocytes in many species including dairy cows[36]. These exchangeable APO have a pivotal role in cholesterol homeostasis as a major component of circulating lipoproteins (chylomicrons, VLDL, LDL, and HDL)[3] and immune cell function[37], also exerting anti- and pro-inflammatory effects[38-40]. Considering the differential expression of APO during the periparturient period and between treatments, as well as their involvement in both lipid metabolism and the immune system, this study suggests APO (as well as their associated molecules lipoproteins) as potential mediators for crosstalk between these two systems.

Besides, fat digestion and absorption and the peroxisome proliferator-activated receptor (PPAR) signaling pathway were among the most important KEGG pathways associated with the identified APO. In that sense, previous studies have demonstrated that PPAR[41] and insulin[42] are inhibitors of ApoC3 gene expression, whereas FA[43] induce ApoC3. It has also been shown that a high-fat diet rich in monounsaturated and omega-3 FA reduced APOC3 in human patients with hypertriglyceridemia and/or hyperlipidemia[44]. In this study, we did not observe any changes in the plasma concentration of insulin by treatment; therefore, it may be proposed that the supplemented FA directly or indirectly increased the abundance of APOC3.

Among APO, APOA4 is associated with the formation and secretion of chylomicrons which are an essential component in the transportation of dietary FA from the intestine to the circulation[3]. More specifically, studies on human hepatocytes and transgenic mice have shown that APOA1[45] and APOA4[46] could reduce hepatic TG accumulation by suppressing endoplasmic reticulum stress and thus restraining the development of steatosis. In this sense, APOA1 is a major component of HDL, and as such involved in bringing cholesterol from peripheral tissues back to the liver (reverse cholesterol transport in dairy cows)[47]. It has been reported that cows with fatty liver and ketosis had lesser serum concentrations of APOA1 which was associated with increased NEFA and decreased cholesterol and phospholipid concentrations[47]. Also, APOC3 is mainly involved in VLDL production and thus it may reduce

intracellular TG accumulation[48,49]. Recently, it has been proposed that the downregulation of plasma APOC3 protein in over-conditioned cows is associated with an impaired assembly of VLDL contributing to hepatic TG accumulation[12].

These results could be a possible explanation for previous observations of specific FA being capable of reducing TG accumulation in cows' liver[4]. Since the ruminants` ability to export TG as a constituent of VLDL from the liver is limited as compared to non-ruminant species[50], these APO may accomplish TG transport from the liver. Following this background, the PP concentrations of NEFA in plasma and of TG in the liver were lower in the EFA+CLA than in the CTRL group[6]. Our results support the common concept that APOC3, APOA1, and APOA4 are essential in the metabolism and transport of supplemented FA to the target organs. In this regard, decreased plasma concentrations of APOB-100, APOA1, and APOC3, along with the induced secretion of HP, were reported to be associated with the development of fatty liver[51]. Our results, therefore, suggest that EFA+CLA supplementation was increasing the abundance of proteins that may avoid fatty liver. Our results are in line with previous results stating that CLA supplementation decreased the liver and plasma TG concentrations during the PP period[6]. Moreover, other studies reported that dietary CLA alleviates non-alcoholic fatty liver disease in humans[52] and rats[53]. On the other hand, there are reports, indicating that CLA supplementation induced fatty liver in mice[54]. This inconsistency can be due to different FA profiles, isomers, and dosage of administrated FA to the animal along with liver physiological differences between species.

Apart from the APOA1–APOC3–APOA4 cluster, a new member of the APO family, APOC4, was found overabundant exclusively in EFA+CLA treated cows when compared to the CTRL cows during the PP period. APOC4 is primarily synthesized in small intestine enterocytes, but its function in hepatocytes is still uncertain[55]. The APOC4 belongs to the APOC family, making another gene cluster together with APOE, APOC1, and APOC2[56]. Both APOC4 and APOE were detected in this study and are reportedly associated mainly with VLDL and TG metabolism in humans[57,58]. As mentioned earlier, the plasma concentration of TG was lower in the EFA+CLA group (data published elsewhere[6]), and these differences were significant on days 14, 28, and 32 PP. There are no reports on APOC4 in cattle, and its roles are yet to be elucidated in dairy cows. Given that APO and probably their associated lipoproteins are increased, it can be concluded that EFA+CLA supplementation elevated the capacity of transferring supplemented FA to the peripheral tissues including the liver, of hepatic TG export, and of reverse cholesterol transport.

At the onset of lactation, coagulation factors 9 and 7, and serum amyloid A (SAA) were more abundant in the EFA+CLA group than in the CTRL group. SAA increases its plasma concentration in cows during the systemic reaction to inflammation[59], although it is worth mentioning that SAA also plays a role in the reverse cholesterol transport as a minor constituent of HDL, and diseases come secondary after a substantial increase in SAA (inflammation) that displaces APOA1 from HDL and form acute-phase HDL (HDL-SAA) (for review[60]). Increased immune-related proteins with FA supplementation coincide with PP immune suppression and may provide another piece of this complex puzzle of the way FA may induce immune-modulatory effects. However, the exact related pathways yet have to be elucidated.

On day 28 PP, the serum amyloid P component (SAP, also known as APCS) was more abundant in the EFA+CLA than in the CTRL group. The SAP is primarily secreted by hepatocytes and macrophages into the circulation and acts as a soluble pattern recognition receptor of the innate immune system[61]. Database searches and functional enrichment analysis demonstrated that SAP associated with HDL might play a role in cholesterol removal from cells[62]. Furthermore, there is mounting evidence that in mice treated with SAP, the plasma paraoxonase1 (PON1) activity was increased[63]. In this regard, a significantly greater paraoxonase activity level was observed in the EFA+CLA group, albeit limited to day 49[2].

At the last timepoint (day 63 PP), concurrent with the shift to a positive EB in the EFA+CLA group, no other pathways were detected or activated apart from common APO pathways (overlapping pathways). This suggests that the beneficial effects of supplemented FA were more relevant during physiological and immunological challenges in the early PP phase.

Conclusion

The plasma protein profiles during the periparturient period provided molecular insight into the systemic inflammatory state, reduced responsiveness of immune response, and impaired cholesterol metabolism at the onset of lactation in dairy cows, which gradually recovered with time PP by reduced their inflammatory response. In addition, this study provides novel knowledge on how EFA+CLA supplementation and the related changes in plasma proteome may act in the crosstalk between lipid metabolism and immune responsiveness in dairy cows. The underlying mechanism might be partly associated with the functions of the APO in regulating hepatic cholesterol and TG metabolism and their emerging role in modulating immune functions in a time-dependent manner. The over-abundance of APOA1–APOC3–APOA4 was explicitly related to EFA+CLA supplementation (regardless of time), although

APOC4 was only elevated during lactation. Collectively, our results suggest a beneficial effect of EFA+CLA supplementation on the prevention of hepatic lipid accumulation during the periparturient period. However, future research should integrate proteomics with other results on specific markers of fatty liver to develop reliable metaphylactic and therapeutic strategies.

Material and Methods

Animals, treatments, and experimental design

All the experimental procedures were carried entirely under animal welfare guidelines and were approved by the ethics of the State Mecklenburg-Western Pomerania, Germany (LALLF M-V/TSD/7221.3-1-038/15). This study was in the frame with a recent comprehensive project described in detail by Vogel et al. (2020)[6]. In brief, 16 Holstein dairy cows in their second lactation from 63 days before to 63 days after parturition were abomasally (10 cm center diameter rumen cannula; Bar Diamond Inc., 106 Parma, ID) infused with one of the two treatments, 1: CTRL, (n = 8; coconut oil, Bio-Kokosöl #665, Kräuterhaus Sanct Bernhard, KG, Bad Ditzenbach, Germany; 76 g/d), this supplement was formulated to compensate for energy intake of EFA treatment and provided no EFA and CLA, 2: EFA+CLA, a combination of linseed oil (DERBY Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany; 78 g/d), safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany; 4 g/d) and Lutalin (CLA, n = 8; cis-9, trans-11, 10 g/d trans- 10, cis-12 CLA, BASF SE, Lampertheim, Germany; 38 g/d). During the dry period, each dose was halved. Oil supplements were provided twice daily at 0700 and 1630 h in equal portions through abomasal infusion lines (Teflon tube [i. d. 6 mm] with two perforated Teflon flanges [o.d. 120 mm], directly into abomasum to avoid ruminant degradation of the fatty acids. The cows were housed in free-stall barns with ad libitum access to a corn silage-based total mixed ration (TMR), formulated according to recommendations provided by the Society for Nutrition Physiology (GfE, 2001[64], 2008[65], 2009[66]) and Deutsche Landwirtschaftliche Gesellschaft (DLG, 2013), for AP and PP [67]. No clinical signs of disease were observed in any of the dairy cows during the experiment.

Ingredients, the chemical composition of the experimental diets, the amount and FA compositions of lipid supplements, FA composition of the experimental diets, and FA composition of the daily infused supplements during lactation are given in Supplementary S10 Tables S36, S37, S38, and S39. The concentrations of NEFA, triglycerides (TG), low-density

lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and total cholesterol (TC) in blood plasma and TG in the liver can be found in Vogel et al., (2020)[6].

Blood Sampling and plasma preparation for proteomics analysis

Blood (about 5 mL) was sampled from each cow on days -21, +1, +28, and +63 relative to calving. Sampling was performed immediately after the morning milking and before feeding by jugular vein puncture using a Vacuette system (Greiner Bio-One In- ternational GmbH, Kremsmünster, Austria) containing K_3EDTA (1.8 g/L) as an anticoagulant. Immediately after collection, the samples were cooled on crushed ice and centrifuged at 1,500 × *g* (4 °C, 20 min). The supernatant was harvested and stored at −80 °C until analysis.

Before protein extraction, plasma samples were thawed slowly in the fridge (2 to 8 °C) and then centrifuged at 10,000 x *g* for 10 min at 4 °C to precipitate cells, debris, and aggregated proteins in the bottom. Enrichment of low-abundance plasma proteins was performed using the ProteoMiner small capacity kit (#163-3006, Bio-Rad Laboratories, Inc., CA, USA), according to the manufacturer's instructions.

Before digestion, the protein concentration was measured by the bicinchoninic acid assay (BCA) kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a reference protein standard. Protein digestion was performed in the S-Trap filter according to the manufacturer's procedure. Briefly, 100 µg of extracted proteins were first mixed with 4% SDS and 20 mM DTT (final concentrations) and boiled for 10 min at 95 °C. After cooling at RT, proteins were alkylated by adding 50 mM iodoacetamide, followed by incubation in the dark for 45 min. The procedure was followed by acidification of samples to a final concentration of 1.2 % phosphoric acid (~ pH 2) for better adhesion to the filter. After that, six volumes of S-Trap binding buffer (90% methanol; 100 mM triethylammonium bicarbonate, TEAB; pH 7.1) were added to the samples. Following a gentle mixing, the protein solution was loaded to a S-Trap filter, centrifuged at 4000 *g* for 10 min at RT, and the flow-through collected and reloaded onto the S-trap filter. This step was repeated two times, and then the filter was washed with 150 µL of binding buffer (two times). Finally, protein digestion was performed by adding 2 µg of sequencing-grade trypsin and 150 µL of digestion buffer (50 mM TEAB) to the filter for overnight at 37 °C.

After the digestion step, peptides were eluted in two steps: in the first step, 40 µL of 50 mM TEAB, 0.2% formic acid in H_2O, and the second step, 50 µL 50% acetonitrile 0.2% formic acid in H_2O were applied. Before injection, peptides were dried in a Speed Vac (Eppendorf

AG, Hamburg, Germany) for 1 h and suspended in 20 µL of equilibration solution (H2O/Trifluoroacetic Acid −99.95/0.05).

Liquid chromatography-mass spectrometry analysis

The plasma peptide mixture was analyzed by label-free LC-MS/MS quantitative proteomics approach using an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific)) coupled to an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) with a Nanospray Flex Ion Source, according to the previously described method by [68]. Briefly, 1 µL of hydrolyzate was first preconcentrated and desalted at a flow rate of 30 µl/mn on a C18 pre-column 5 cm length X 100 µm (Acclaim PepMap 100 C18, 5µm, 100A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water to remove contaminants that could potentially disrupt the efficiency of the mass spectrometry analysis. After 6 min, the concentration column was switched online with a nano debit analytical C18 column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å - SN 10711311) operating at 400 nL/min equilibrated with 96 % solvent A (%99.5 % H2O, 0.5 % formic acid). The peptides were then separated according to their hydrophobicity, thanks to a gradient of 4 to 20% solvent B (99.5 ACN, 0.5 % formic acid) in 60 minutes.

For MS/MS analysis, eluates were electro-sprayed in positive-ion mode at 1.6 kV through a nanoelectrospray ion source heated to 250°C. The Orbitrap Q Exactive HF-X MS was used in HCD top 18 mode (i.e. 1 full scan MS and the 18 major peaks in the full scan were selected for MS/MS). Higher-energy C-trap dissociation (HCD): HCD refers to a CID variation that uses a higher RF voltage to retain fragment ions in the C-trap. Mass spectrometry analysis parameters were as follows: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 60,000 with an injection time of 50 ms on a mass range from 375 to 1600 m/z. Each MS analysis is followed by 18 MS/MS with analysis of MSMS fragments at a resolution of 15,000 with an injection time of 100 ms.

Data processing, statistical analysis, and functional enrichment analysis

Each acquired raw MS/MS spectrum was first aligned to the reference sample (assigned automatically with having the highest of peptide ions coverage) and then processed for peptide ions identification using Progenesis QI software (version 4.2, Nonlinear Dynamics, New Castel upon Tyne, UK), with the default parameter settings (ion charged set to five and Ions ANOVA P-value < 0.05). The identified and the quantified peptide ions were then searched against *Bos*

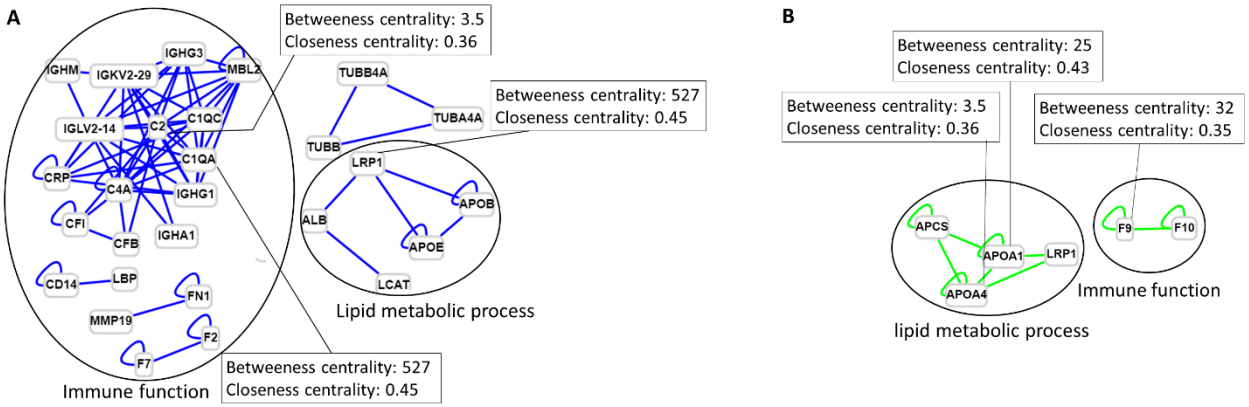
taurus decoy database (Uniprot, download date: 2019/11/07, a total of 37,513 entries) in MASCOT (version 2.5.1) interrogation engine, with the following setting: trypsin as an enzyme digest of a protein, tryptic specificity to cleavage C-terminal after lysine or arginine residues, allowing two missed cleavages, variable modification to carbamidomethylation (C) and oxidation (M), mass tolerance to 10 ppm for precursor ions and 0.02 Da for fragment ions, and FDR<0.01. Accordingly, the corresponding proteins with at least two peptides and two unique validated peptides were identified and quantified based on their intensities.

Before statistical analysis, logarithmic transformation was applied to protein intensities, and missing intensities (with the frequency of less than 50 % of samples) were imputed and replaced by the1/5 of the minimum positive value of each variable in the original dataset. DAP were identified and investigated separately during the time and between treatments. The time associated DAP were identified using paired t-test between each consecutive time-point to cope with carryover effects.

At each timepoint, the most important proteins (VIP) involved in the discrimination of the CTRL and EFA+CLA groups was identified using Partial Least Square Discriminant Analysis (PLS-DA) analysis (mixOmics package in R). The VIP contributed to the projection scores (>1.5) of the first two components (PC1 and PC2) and to cluster separation between treatment groups were ranked. A permutation test (100 random computations) was applied to disprove the over-fitting of the PLS-DA model. However, the significance of permutation test is more relevant for a prediction purpose, which is not the goal of our study. The permutation test was not significant at all timepoints and there was a possibility for false positive detection. Therefore, the second filtration step was applied and only VIP with P-value < 0.05, and log2 (fold change) >1.3 (metaboanalyst R package) were maintained and considered as differentially abundant proteins (DAP) for gene ontology functional enrichment analysis. The P-value was assessed by Student's t-test. Statistical analysis was performed in mixOmics and metaboanalyst R-packages in R statistical software (R version 4.0.0). DAP were visualized according to their expression (by Volcano plot, EnhancedVolcano R package). The GO categorization, including BP, MF, and CC, and KEGG (www.kegg.jp/kegg/kegg1.html)[14] pathway enrichment analysis were conducted using the web-based tool String version 11.0, summarized in REVIGO web-based tool (http://revigo.irb.hr/) and visualized in Cytoscape software (https://cytoscape.org, version 3.8). The *B. taurus* interaction map was set as a background list, and pathways (false discovery rate < 0.05) with at least two protein hits were considered as enriched pathways. Protein protein interaction networks (PPI) were constructed by inputting the time and treatment DAP to proteINSIDE (V 2.0) tool. PPI were searched in *Homo sapiens* to take advantage of

the well-characterised physical interactions between proteins, and PPI agreed by curator review were used to construct PPI networks (Supplementary Figure S1). Within the network, proteins that interact to contribute to cellular or metabolic processes have been highlighted using betweeness or closeness centralities as previously described[69].

Supplementary Figure S1

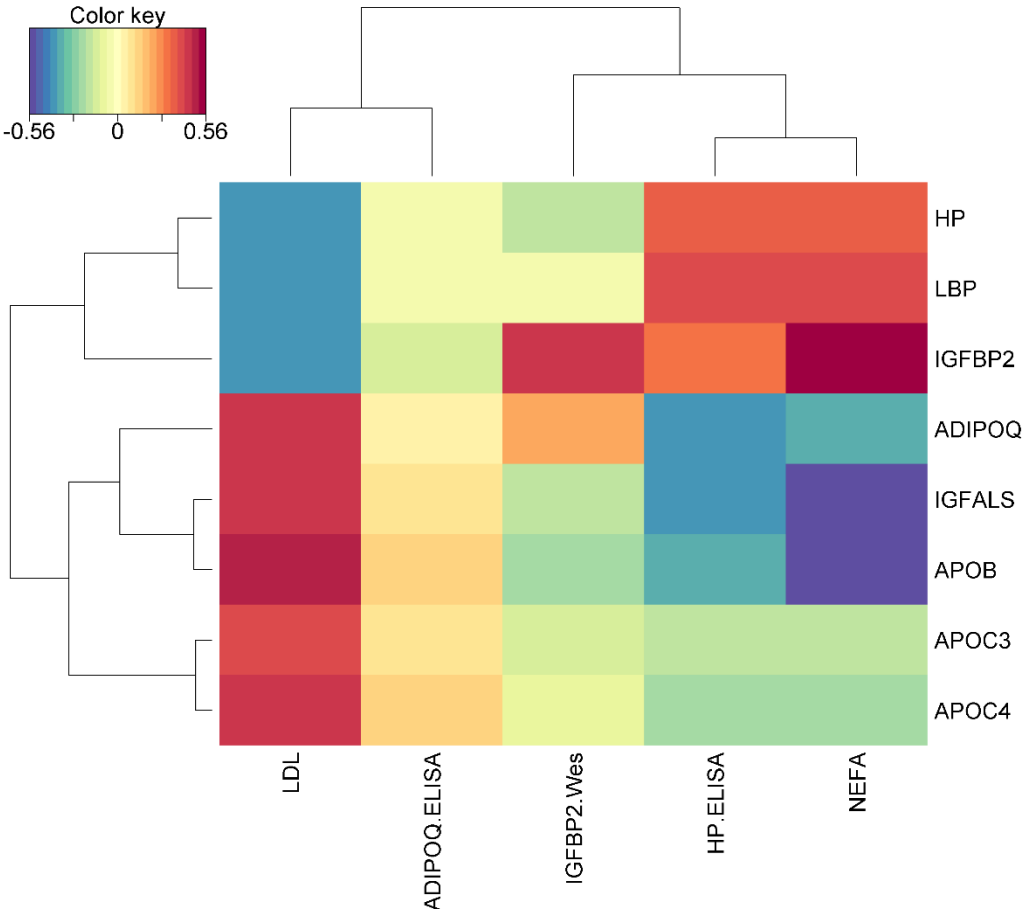


Supplementary Figure S1. Protein−protein interaction network analysis. Networks were constructed by the ProteINSIDE (V 2.0) too. Nodes were DAP identified during the time (A) or between CTRL and EFA+CLA groups (B), and edges were the PPI agreed by curator review in Human species. PPI network were filtered according to betweeness (higher than 0.1, that quantifies how frequently a node is on the shortest path between every pair of nodes for detecting bottlenecks in a network) or closeness (0.1, that quantifies how short are minimal paths from a given node to all others, a large closeness indicates that a node is close to the topological centre of the network) centralities in order to reveal key proteins that play important roles in a network and for a biological pathway.

Proteomics data matched the classical measurements of proteins and metabolites

To validate the proteomics results, we integrated the serum proteome data with plasma metabolites and proteins [6,70] using sparse partial least squares (sPLS) regression model (mixOmics package in R). With X (proteomics data) and Y (classical plasma proteins and metabolites data) as input matrices (logarithmic transformed values), the sPLS model controls many noisy, collinear (correlated) and missing variables in omics datasets, and relates multiple outcome variables across X and Y. Positive and significant correlations were observed between 1- proteomics and ELISA-measured HP; 2- proteomics and Western blot-measured IGFBP2; 3- NEFA with LBP and IGFBP2; 4- LDL with APOB, APOC4, and APOC3 proteins (Supplementary Figure S2). Although, ADIPOQ ELISA results were not correlated (r=0.05, P-value =0.66) with proteomics results. Also, using the bovine AHSG ELISA Kit (Catalog No: DL-aHSG-b, DLdevelop, Wuxi, China), and testing a series of different plasma dilutions, we could not detect AHSG in our plasma samples. This was probably attributable to an inadequate binding specificity of the antibody used in the AHSG ELISA kit. Additionally, despite the accuracies of the mass spectrometry and immunological assays that we classically used to quantify protein abundances, methodological specificities may provide some differences in the abundances assayed.

Supplementary Figure S2



Supplementary Figure S2. Clustered image map of integrated plasma proteins (Y-axis) with metabolites and proteins (ELISA and Western blot, X-axis) using sparse partial least squares (sPLS) model. The correlations between the plasma proteins (proteomics-based) and the metabolite and proteins (ELISA and Western blot-measured) by a color gradient on a two-dimensional colored image. The negatively correlated variables (blue) are represented along the positively correlated variables (red). Dendrograms are added to represent the clusters produced by the hierarchical clustering. Partial Least Squares (PLS) regression is a multivariate methodology which relates two data matrices X and Y, and model multiple outcome variables. Plasma IGF1, HP, and ADIPOQ were measured using a bovine-specific ELISA. Concentrations of plasma IGFBP2 were analyzed via quantitative Western ligand Blot analysis. Plasma metabolites were analyzed using an automatic spectrophotometer (ABX Pentra 400; HORIBA ABX SAS, Montpellier, France) and respective kits: #434 91795 (NEFA; acyl-CoA synthetase – acyl- CoAoidase method) from WAKO Chemicals GmbH (Neuss, Germany); #A11A01640 (TG; lipoproteinlipase – glycerinkinase – glycerin-phosphate-oxidase method), #A11A01638 (LDL-C; direct measurement of cholesterol in LDL by the cholesterinesterase and cholesterinoxiase and LDL cleavage), and #A11A01636 (HDL-C; direct measurement of cholesterol in HDL by accelerator selective detergent method with cholesterinesterase) from HORIBA ABX SAS (Montpellier, France), and #553-126 (TC; cholesterinoxidase method) from mti-diagnostics GmbH (Idstein, Germany). The concentrations of plasma insulin (#RIA-1257) and glucagon (#RIA-1258) were determined via RIA using kits from DRG Instruments GmbH (Marburg, Germany) [2,6,7,69].

Data availability

The data and the related analyses will be made available for the reviewing process upon request.

Conflict of interest

The authors declare no conflict of interest.

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Ethics declaration

All the experimental procedures were carried entirely under animal welfare guidelines (including ARRIVE guidelines) and were approved by the ethics of the State Mecklenburg-Western Pomerania, Germany (LALLF M-V/TSD/7221.3-1-038/15).

Supplementary information

Supplementary files were deposited at the INRAe portal as "Gene ontology and functional enrichment analysis of plasma differentially abundant proteins during the transition to lactation and between different fatty acid treatments in Holstein cows".

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6. General discussion and conclusion

Omics-based studies have added high-quality complementary outcomes and a more holistic view to our traditional understanding of dairy cows physiology, which even led to forming new hypotheses in this regard. The work within this thesis continues the DEFA project initiated at the research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany in 2014 with several publications on the effects of replenishing the supply of EFA and/or CLA after a preceding period of low EFA supply in the transition cows. The overall aim of the present thesis was to get an insight and a comprehensive view into the underlying mechanisms and pathways previously reported in the DEFA project using proteomics analysis.

Liver and plasma samples were subjected to longitudinal proteomics analysis to initially get an insight into the metabolic and immune adaptation during the transition from late gestation to early lactation and also to investigate in depth the molecular mechanisms by which supplemented FA impact health and metabolism. The liver proteomics study (manuscript I & II) was conducted to characterize the specific role of supplemented FA on the hepatic proteome profile and to investigate how FA interact with the metabolic adaptations in dairy cows during the transition period. The plasma proteomics study (manuscript III) was designed to investigate the circulating proteins that may provide clues of different biological processes in tissues and organs, as well as markers of immune and inflammatory responses, affected by FA supplementation. It is important to mention that all omics-based studies were designed without a prior hypothesis, but the results were interpreted based on the previous data collected on the current experimental design. To integrate and summarize the results, the whole metabolic adaptations pathways during the transition to lactation will be first discussed, followed by the specific role of FA in each sample.

I. Metabolic adaptation during the transition to lactation

The dairy cow transition period is associated with considerable physiological changes that affect most organ systems, including liver, mammary glands, adipose, and uterus, also a high incidence of subsequent metabolic and infectious diseases (Wankhade et al., 2017). Within this challenging period, complex metabolic, hormonal, and immunological adaptive reactions occur to support the last stages of fetal growth and the commencement of milk production during early lactation (Wankhade et al., 2017). Most existing literature on the metabolic adaptation during the transition period is based on measuring blood metabolites and hormones, however

recent progress toward proteomics analysis has opened up the next investigation tool in further studies in this topic. Given that the enzymatic activity of proteins is critical in regulating metabolic pathways, integrating proteomics and metabolite data may provide a map of metabolite-protein interactions to understand in depth these complex mechanisms. In this section, we provide an overview of the metabolic and immunological changes during the transition period through the longitudinal characterization of the transition period using proteomics, integration, and bioinformatics analysis of plasma and liver samples.

During the transition period, tissue damage and remodeling in organs such as mammary gland (Atabai et al., 2007), adipose tissue (Kosteli et al., 2010), and placenta (Challis et al., 2009), consequently initiate a mild activation of the immune systemvia molecular patterns of non-pathogenic origin. Therefore, all dairy cows regardless of their health status experience a state of non-pathogenic inflammation and immune activation during the transition period (Horst et al., 2021). Accordingly, despite the traditional dogma suggests immunosuppression in early, a part of the immune system is activated while the other parts are temporarily downregulated to support a new physiological state.

In this regard, our plasma proteomics results indicated the overabundance of positive APP, and underabundance of negative APP, which implies the development of SI and activation of the immune system during the transition from AP to PP. These results are in agreement with previous results reporting a state of systemic inflammation in early lactation dairy cows (reviewed by (Horst et al., 2021)). On the other side, within this timeframe markers of the innate defense system containing a cluster of complement proteins decreased, which could justify the reduced immune system responsiveness during the early PP period. These results are also in line with previous studies reporting immunosuppression in dairy cows during the transition period (Hammon et al., 2006; Sordillo et al., 2009; Esposito et al., 2014). Interestingly, we observed that the affected markers of immune systems were gradually turned back to normal level within a few weeks after parturition in association with the energy status.

As discussed earlier, immune activation in early lactation drains the available glucose which is one of the reasons for the massive fat mobilization in conjunction with excessive NEFA and ketone bodies in the blood (Horst et al., 2021). Following the immune activation, elevated plasma concentrations of NEA and BHB, and the state of NEB in early lactation dairy cows were observed (Vogel et al., 2020). Consequently, several adaptive mechanisms are activated to cope with these physiological changes. The primary reactions are therefore aimed at

enhancing energy production via increasing gluconeogenesis, reducing peripheral insulin sensitivity, and increasing lipolysis (Ceciliani et al., 2018). Our liver proteomics results revealed the enrichment of several pathways directly or indirectly supporting energy-related metabolic processes, including glycolysis/gluconeogenesis, pentose phosphate pathway, TCA cycle, peroxisome biogenesis and proxisomal proteins, FA metabolism, PPAR signaling pathway, primary bile acid (BA) biosynthesis, pyruvate metabolism, biosynthesis of unsaturated FA, and synthesis, and degradation of ketone bodies. The increased capacity of the pathways related to TAG metabolism and FA oxidation was accompanied by rising concentrations of NEFA that reach the liver. Recent evidence (*in vitro)* has propsoed that high NEFA levels (range from 0.6 to 2.4 mM) may faciliate development of ketosis in dairy cows. In this regard, NEFA contribute to reactive oxygen species (ROS) generation which consequently induce transcriptional activation of p53 pathway, transcriptional inhibition of nuclear factor E2-related factor 2 (Nrf2), and release of apoptosis-inducing factor (AIF) in cultured hepatocyte (Li et al., 2020). Heren, we observed an impaired expression of APO, which may negatively affect biogenesis of exchangeable apolipoproteins, thus the export of hepatic TAG as VLDL. In line with our proteomics results, it has been clearly shown that the plasma concentrations of total cholesterol, LDL, and HDL were all at the minimum level right after parturition (Vogel et al., 2020), although VLDL concentration was not measured. It is generally accepted that ruminants are prone to the fatty liver since their liver has essentially a slow capacity of VLDL secretion (Kleppe, Aiello et al. 1988). These results were in accordance with a previous study, in which NGS results revealed downregulation of genes and nuclear receptors associated with cholesterol metabolism and upregulation of FA oxidation pathways in ketosis-induced dairy cows (Loor et al., 2007). Altogether, these situations may lead to the development of (subclinical) ketosis during the early PP, which is known to be closely linked to the occurrence of periparturient diseases.

The integration of large omics datasets with classical blood profiling variables in dairy cow studies may provide complementary insights into the complex pathophysiology of the adaptation to the metabolic needs of lactation. Herein, integration analysis of plasma metabolites and hormones (including NEFA, insulin, BHB, total cholesterol, TAG, LDL, and HDL, as well as growth hormone and IGF-I, and plasma proteome profile (comprising 241 proteins) was performed using sparse partial least squares (sPLS) regression model (using the mixOmics package, R statistical software V 4.0.0) and represented by Pearson's r correlation analysis (corrplot package, R statistical software).

Our findings revealed that IGF-I, NEFA, and LDL were those "classical" variables that were most correlated with plasma proteins, in particular: IGF-I with IGF-binding protein (IGFBP2) (r = -0.71); LDL-C with Apolipoprotein B (APOB) (r = 0.53), IGFBP2 (r = 0.5), and Prenylcysteine Oxidase 1 (PCYOX1) (r = 0.5); NEFA with IGFBP2 (r = 0.59), APOB (r = -0.5), IGFBP Acid Labile Subunit (IGFALS) (r = -0.5), and Alpha 2-Heremans-Schmid Glycoprotein (AHSG, r = -0.47) represented further correlations. Pathway enrichment analysis revealed that ApoB, IGFALS, IGFBP2, and AHSG were interconnected and are involved in the regulation of IGF transport and uptake by IGFBPs. For PCYOX1, bioinformatics analysis in the *Homo sapiens* database indicated an interaction with APOB in LDL metabolism. Interestingly, all the proteins established herein with correlations are associated with glucose metabolism and the somatotropic axis and insulin resistance that were suggested earlier as one of the primary metabolic adaptations.

II. The hepatic proteome as affected by EFA+CLA supplementation

In response to EFA+CLA supplementation, the hepatic relative abundance of proteins related to cytochrome P450 epoxidation/hydroxylation and cholesterol metabolism was affected in a time dependent manner. The cytochrome P450 pathways comprises a wide variety of enzymes such as heme-thiolate monooxygenases that are involved in the oxidation and detoxification of many structurally diverse natural or synthetic chemicals referred to as xenobiotics (Stavropoulou et al., 2018). Xenobiotics, such as drugs and even feed additives, are defined as chemicals that are not naturally produced by the organism and in general harmless but their accumulation may reach toxic levels and lead to cellular and tissue damage (Esteves et al., 2021). Herein, we observed the differential expression of CYP Phase I enzymes, monooxygenases, between the treatment groups. These enzymes catalyze the oxidation and reduction of lipophilic xenobiotics/xenobiotics-like into more polar molecules (reviewed by (Rendic and Guengerich, 2015)). In accordance, increasing evidence suggests the participation of CYP enzymes in PUFA metabolism that can produce a mixture of hydroxy-eicosatetraenes (HETE) depending on the specific CYP enzyme (epoxygenase or hydroxylase activity) as well as the type of PUFA substrate (Sarparast et al., 2020). In this regard, we observed the differential abundant of various CYP enzymes including CYP51A1 (d -21), CYP1A1 & CYP4F2 (d +28), and CYP4V2 (d +63), affected by EFA+CLA treatment in a time dependent manner.

In this regard, SQLE along with 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) are two rate-limiting enzymes in cholesterol synthesis (Scott et al., 2020). Overexpression of SQLE, FDFT1, and CYP51A1 in the AP period eventually yields cholesterol synthesis through the mevalonate pathway. It could be postulated that EFA+CLA supplementation induced hepatic cholesterol synthesis and supported the increased demand for cholesterol during the transition period, although the plasma concentration of TAG and cholesterol remained unchanged between the treatment groups. This is probably due to the fact that high levels of free cholesterol are toxic to cells, therefore cholesterol synthesis is tightly regulated by feedback mechanisms that sense cholesterol and oxysterols levels (Tabas, 2002). The end-products of cholesterol surplus are BA (Bhargava et al., 2020), which could justify the reasons for equivalent plasma cholesterol concentration between treatment groups. Increased cholesterol production probably reflects on the associated pathways such as BA synthesis. At the onset of lactation, proteins related to the activation of BA synthesis and secretion such as BA transporters (SLCO1B3) increased in EFA+CLA group. BA are conserved molecules that not only facilitate VLDL secretion and transport out of hepatocytes, but also act as signal molecules, regulating the regulation of liver lipid metabolism through FXR, LXR, and SREBP-1c pathways (for review see (Lavoie, 2016; Stofan and Guo, 2020). This is clearly shown that BA extraction rate decreased in the liver of fasted cows due to hepatic TAG accumulation and metabolic impairment (Tharwat et al., 2004). Milk cholesterol level was not measured in this study. In spite of no change in milk yield between the treatment groups and a lower milk fat percentage in the EFA+CLA group, the raised cholesterol synthesis are likely explained by other regulatory pathways than milk fat metabolism. Therefore, the results suggest that EFA+CLA supplementation has beneficial effects on hepatic metabolic health and to prevent the development of ketosis .

On day 28 PP, the relative abundance of CYP4F2 was higher and that of CYP1A1 was lower in the EFA+CLA group. The members of the CYP4F and CYP1A family are capable of ω-hydroxylation of PUFA such as ARA and EPA. ω-hydroxylase activity leads to the formation of active eicosanoids, such as HETE, which have different pathological and physiological functions. CYP1A1 is a hydroxylase known to oxidize ARA at a substantial rate (Schwarz et al., 2004). Generally, ARA are the major substrate for ω-hydroxylation, and ARA-derived epoxyeicosatrienoic acids (EET) play a role in the inflammatory system (Tu et al., 2020). Our results suggest that EFA+CLA supplementation shifts the P450-mediated epoxide metabolic profile from ARA-derived EETs (lower CYP1A1) to EPA and DHA-derived 17,18-

EpETE and 19,20- EpDPE, respectively. Opposite to HETE, EpETE have been shown to have potent anti-inflammatory effects (Schunck et al., 2018; Tu et al., 2020).

As discussed earlier, tissue damage and inflammatory stimuli peripartum induce ARA release from membrane phospholipids which are subsequently converted into pro-inflammatory eicosanoids such as PG, leukotrienes (LT), and lipoxins (LX). In contrast, n-3 PUFA regulate tissue regeneration and prevent chronic inflammatory states, by converting to anti-inflammatory bioactive lipid mediators including EPA-derived resolvins (E-series resolvins: RvE1–E3; Fig. 2), DHA-derived resolvins (D-series resolvins: RvD1–D6), protectins (PD1 and PDX) and maresins (MaR1 and MaR2) (reviewed by (Ishihara et al., 2019)). There is no data on CLA-derived lipid mediators, although specific isomers of CLA exert anti-inflammatory properties. In line with the proteomics results, plasma haptoglobin was tended to be lower (AP period) and paraoxonase was higher in the EFA+CLA group compared to the CTRL group at certain timepoints (after 4w) in PP period (Gnott et al., 2020). It could be postulated that the anti-inflammatory effect of n-3 PUFA and CLA is mainly through competing with ARA for conversion to bioactive lipid mediators, although, this claim needs to be further investigated.

Taken together, the proteomics results from liver indicated that EFA+CLA supplementation could be beneficial to prevent hepatic lipid accumulation, i.e., steatosis, but may also inflammation during the transition period.

III. Plasma proteome affected by EFA+CLA supplementation

EFA+CLA supplementation affected the plasma proteome profile of transition dairy cows. The EFA+CLA supplementation induced the relative abundance of APOC3, APOC4, APOA1, and APOA4 during the PP period. We have previously shown and discussed the induction of systemic inflammation in early lactation. It has been demonstrated that inflammatory cytokines such as TNF and IL-1β suppress hepatic APO production, to avoid their anti-inflammatory potential (van Leeuwen et al., 2003; Valladolid-Acebes et al., 2021). This is meaningful additional support to our previous discussion regarding the anti-inflammatory properties of EFA+CLA supplementation at the level of the hepatic proteome during the transition period.

APOA1 plays a pivotal role in the biogenesis and maturation of HDL (Georgila et al., 2019). It stabilizes the ATP-binding cassette transporter 1 (ABCA1) which mediates the efflux of cholesterol across cellular membranes for HDL assembly (in hepatocytes and enterocytes), as well as the reverse cholesterol transport in cells of peripheral tissues (Duong et al., 2008).

Furthermore, APOA1 contributes to innate immunity and exerts anti-apoptotic, anti-oxidative, and anti-inflammatory properties. Therefore, decreased APOAI in the CTRL group could be related to an intensified inflammatory response and may even contribute to a progression to chronic inflammatory states (Georgila et al., 2019). Besides, APOC3 is the most abundant C-APO in HDL and LDL particles in humans (Campos et al., 2001), also known for its actions in the inhibition of lipoprotein lipase (LPL)-mediated lipolysis and the prevention of the hepatic clearance of triglyceride-rich lipoproteins (Valladolid-Acebes et al., 2021). This seems inconsistent with our previously discussed notion that EFA+CLA prevented hepatic TAG overload. However, a recent study proposed that the APOC3/APOE ratio determines the clearance of TAG-rich lipoproteins (Ramms et al., 2019). According to this model, the elevated APOE during the transition period (plasma manuscript) could shift the APOC3 to other metabolic fates.

In the same way, recent findings from genome-wide association studies reported the contribution of the APOE-C1-C4-C2 gene cluster in the regulation of LDL, TG, and HDL-C levels (Willer et al., 2013; Pirim et al., 2019). This data suggests that lower APO in the CTRL group had some disruptive impacts on lipid metabolism pathways which in turn impair HDL-mediated cholesterol efflux and reverse cholesterol transport capacity. These findings not only proved our hepatic proteome results but also provided a novel mechanism by which EFA+CLA supplementation impacts lipid metabolism and inflammation. Although, we observed no differences in the plasma concentration of cholesterol and HDL between groups (Vogel et al., 2020). The reason for these inconsistencies is not clear and requires further investigation in prospective trials.

Altogether, these findings provided novel information and new insights into metabolic and immune adaptation and crosstalk between them in transition dairy cows divergent in EFA+CLA status. Our proteomics results revealed in depth the molecular aspects of immune and metabolic adaptation during the transition from late pregnancy to early lactation. This included a list of proteins and pathways that were not previously considered as being related to metabolic health during the transition period. They may act as key regulators in biological processes inducing lipid metabolism and immune function in dairy cows. Our results suggested a beneficial effect of EFA+CLA supplementation for decreasing steatosis, as well as reducing inflammation during the transition period. However, further research is needed to validate this concept by measuring the specific markers at the level of the metabolome/lipidome.

7. Summary

In dairy cows, remarkable physiological, metabolic, and immunological adaptations occur during the transition from late pregnancy to early lactation (transition period), to support and prioritize milk production. However, high-yielding dairy cows may experience dysregulated metabolic adaptations that result in prolonged negative energy balance (NEB) and metabolic disorders such as ketosis.

Linoleic acid (C18:2 n-6, LA), α-linolenic acid (C18:3 n-3, ALA), and conjugated linoleic acid (naturally occurring isomers of LA) are polyunsaturated fatty acids (PUFA), with proven metabolic and health benefits besides being a source of energy. In particular, cows fed n-3 PUFA reduced production of pro-inflammatory cytokines, while *trans*-10 *cis*-12 CLA improved energy balance by inducing milk fat depression (MFD). When comparared to conventional total mixed feed rations, grass-fed dairy cows have higher levels of essential fatty acids (EFA: LA and ALA) and CLA in the body. Synergistic effects of EFA and CLA supplementation in dairy cows have been studied (in an intensive project called "deficiency in EFA" or DEFA) as a nutritional strategy to repartition energy and to improve immune functions during the transition period, although the underlying mechanisms at the molecular level are yet to be elucidated.

This thesis followed the DEFA experimental design with the aim of investigating 1) the metabolic adaptation during critical timepoints around parturition in both plasma and liver using proteomic analysis, 2) the effect of EFA+CLA supplementation on the liver proteome, 3) the influence of EFA+CLA supplementation on the plasma proteome of transition dairy cows. Late-gestation Holstein cows fed corn silage total mixed rations (low EFA supply) were abomasally infused with either coconut oil (CTRL, n=8, 38 and 76 g/d in gestation and lactation, respectively) or a mixture of EFA (Linseed + safflower oil) and CLA (Lutalin, BASF) (EFA+CLA, n=8, 60 and 120 g/d before and after calving, respectively) from 9 wk ante partum to 9 wk post partum. Extracted proteins from the liver and plasma samples collected at d -21, +1, +28, and +63 relative to calving were subjected to untargeted shotgun proteomics analyses based on nano-liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). For both samples, the raw output of MS/MS spectra was processed with the Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) and aligned using automatic alignment. The list of generated decoys was exported to MASCOT (version 2.5.1) interrogation engine and searched against a *Bos taurus* decoy database (Uniprot, download date: 2019/11/07, a total of 37,513 entries). The corresponding proteins were identified and

quantified based on the intensities of the specific validated peptides. The differentially abundant proteins (DAP) were identified by two methods to first investigate the time effect and second to investigate the effect of FA treatment:

DAP across time were identified in a) the liver using analysis of variance – simultaneous component analysis (ASCA) b) the plasma using paired t-test between each consecutive time-point. DAP between treatment groups were identified at the intersection between multivariate (Partial Least Square Discriminant Analysis (PLS-DA), the most important proteins (VIP) contributing to the projection scores between CTRL and EFA+CLA (>1.5 VIP score) of the first two components (Principal Component 1 and Principal Component 2)) and univariate analyses (P-value < 0.05, and log10 (fold change) >1.3). At each stage, the identified DAP were mined for gene ontology (GO) categorization, including biological process (BP), molecular function (MF), and cellular component (CC), and KEGG enriched pathways using the web-based tools: String (version 11.0) and Cytoscape (version 3.8).

Briefly, DEFA results revealed that EFA+CLA supplementation induced MFD, increased energy balance, decreased postpartum plasma nonesterified fatty acid and triglyceride (TAG) concentrations, and slightly affected markers of hepatic inflammation and (i.e., haptoglobin and paraoxonase), however, dry matter intake, body weight, milk yield, and net energy intake as well as plasma BHB and total cholestrol, were not affected by treatment. In total, 1680 and 241 proteins were identified in the liver and plasma, respectively. During the transition to lactation, 116 proteins were differentially abundant at the hepatic level regardless of treatment effects. Time-affected DAP were annotated by 46 enriched KEGG pathways in the postpartum period, mainly related to the metabolism of carbohydrates, FA, and amino acids, including peroxisome biogenesis and proxisomal proteins, peroxisome proliferator-activated receptors (PPAR) signaling pathway, steroid biosynthesis, citrate cycle (TCA cycle), biosynthesis of amino acids, metabolism of xenobiotics by cytochrome P450, pentose phosphate pathway, synthesis and degradation of ketone bodies, glycolysis / gluconeogenesis. The enrichment of these pathways was in line with the previous results regarding the adaptive metabolic changes in a state of negative energy balance, although novel proteins were identified in those pathways that opened up a better understanding of the underlying mechanisms. In particular, more than 20 DAP were specifically annotated by enriched biological processes related to peroxisomes and mitochondria to maximize the oxidation capacity.

During the transition time, a cluster of apolipoproteins (APO), including APOE, APOH, and APOB, along with a cluster of immune-related proteins, including cluster of differentiation 14

(CD14) and adiponectin (ADIPOQ), complement and coagulation cascades such as clusterin (CLU), complement C1q A Chain (C1QA), coagulation factor VII (F7), MASP1, component C2 (C2), CFI, mannose-binding protein C (MBL1), acute-phase response and inflammation comprising haptoglobin (HP), lipopolysaccharide-binding protein (LBP), alpha 2- Heremans-Schmid glycoprotein (AHSG), fibronectin 1 (FN1), alpha-1-acid glycoprotein 1 (AGP1), and cellular oxidant detoxification protein (GPX3) were differentially abundant in plasma. The time-affected DAP were related to complement and coagulation cascades, inflammatory response, and cholesterol metabolism. The plasma protein profiles during the periparturient period provided molecular insight into the systemic inflammatory state, reduced responsiveness of immune response, and impaired cholesterol metabolism at the onset of lactation in dairy cows, which gradually recovered with time PP by reducing their inflammatory response.

Regarding the FA treatment effect, 29, 12, 27, and 28 were DAP at days -21, +1, +28, and +63, respectively. Between groups, DAP were assigned to the metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, drug metabolism - other enzymes, arachidonic acid metabolism, pyruvate metabolism, steroid hormone biosynthesis, glycolysis/gluconeogenesis, glutathione metabolism, and citrate cycle (TCA cycle) at all time points. Specifically, in d -21, cholesterol metabolism and sterol regulatory element-binding proteins-activated mevalonate pathway, in d +1, the catabolic process and proteolysis and bile acid secretion, and in d +28 and +63 glutamine and glutamate metabolic process were enriched. At each timepoint, cytochrome P450, as a central hub to these annotated pathways, was related to specific CYP enzymes comprising: CYP51A1 (d -21), CYP1A1 & CYP4F2 (d +28), and CYP4V2 (d +63). Supplementation of EFA+CLA in transition cows impacted hepatic lipid metabolism and enriched several common biological pathways at all timepoints that were mainly related to ω-oxidation of FA through the cytochrome p450 pathway. The enrichment of cytochrome P450 can be interpreted as a way to maintain metabolic homeostasis by oxidation/detoxifying endogenously and exogenously produced xenobiotics. In addition, these results suggest that hepatic steatosis during the transition period might be prevented with EFA+CLA supplementation.

In response to EFA+CLA, specific APO comprising APOC3, APOA1, APOA4, and APOC4 were increased in a time-dependent manner; they were linked to TAG-enriched lipoprotein metabolisms and immune function. This study provided novel knowledge on how EFA+CLA supplementation and the related changes in the plasma proteome may act in the crosstalk

between lipid metabolism and immune responsiveness in dairy cows. The underlying mechanism might be partly associated with the functions of the APO in regulating hepatic cholesterol and TAG metabolism and their emerging role in modulating immune functions in a time-dependent manner. The over-abundance of APOA1, APOC3, and APOA4 was explicitly related to EFA+CLA supplementation (regardless of time), although APOC4 was only elevated during lactation.

In summary, our proteomics results provided novel information about metabolic and immune adaptations and their interrelationship with EFA+CLA supplementation in transition dairy cows. Time-affected DAP uncovered the molecular mechanisms underlying the immunological and the metabolic adaptation during the transition from late pregnancy to early lactation, including novel proteins which were not previously considered as being important during the transition period. Moreover, time-dependent effects of EFA+CLA supplementation were observed on the metabolism of cytochrome P450, cholesterol, and TAG via different CYP enzymes and APO, in which the ligand activation of nuclear receptors is presumably responsible. Our results suggested that supplementation with EFA and CLA during the transition period reduced markers of inflammation and attenuated hepatic lipid accumulation and ketosis in early lactation dairy cows. Further studies at metabolite level are needed to characterize in deep the role of EFA and CLA in subclinical ketosis prevention in dairy cows.

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10. List of Publications

Veshkini, A., M. Hammon, H., Sauerwein, H., Tröscher, A., Viala, D., Delosière, M., Ceciliani, F., Déjean, S., Bonnet, M., 2022a. Longitudinal liver proteome profiling in dairy cows during the transition from gestation to lactation: Investigating metabolic adaptations and their interactions with fatty acids supplementation via repeated measurements ANOVA-simultaneous component analysis. J. Proteomics 252, 104435.

Veshkini, A., M. Hammon, H., Vogel, L., Delosière, M., Viala, D., Dèjean, S., Tröscher, A., Ceciliani, F., Sauerwein, H., Bonnet, M., 2022b. Liver proteome profiling in dairy cows during the transition from gestation to lactation: Effects of supplementation with essential fatty acids and conjugated linoleic acids as explored by PLS-DA. J. Proteomics 252, 104436.

Veshkini, A., Bonnet, M., Vogel, L., Tröscher, A., Ceciliani, F., Delavaud, A., Viala, D., Sauerwein, H., & Hammon, H. Plasma proteomics reveals crosstalk between Lipid Metabolism and Immunity in Dairy Cows receiving Essential Fatty Acids and Conjugated Linoleic Acid, 2021 (under review).

Abstracts in conferences

A. Veshkini, M. Bonnet, H. Hammon, L. Vogel, A. Tröscher, M. Delosière, A. Delavaud, D. Viala, H. Sauerwein, F. Ceciliani Machine learning tracking immune-related proteins in serum of periparturient dairy cows, EAAP Annual Meeting 2021, Davos, Switzerland (oral presentation)

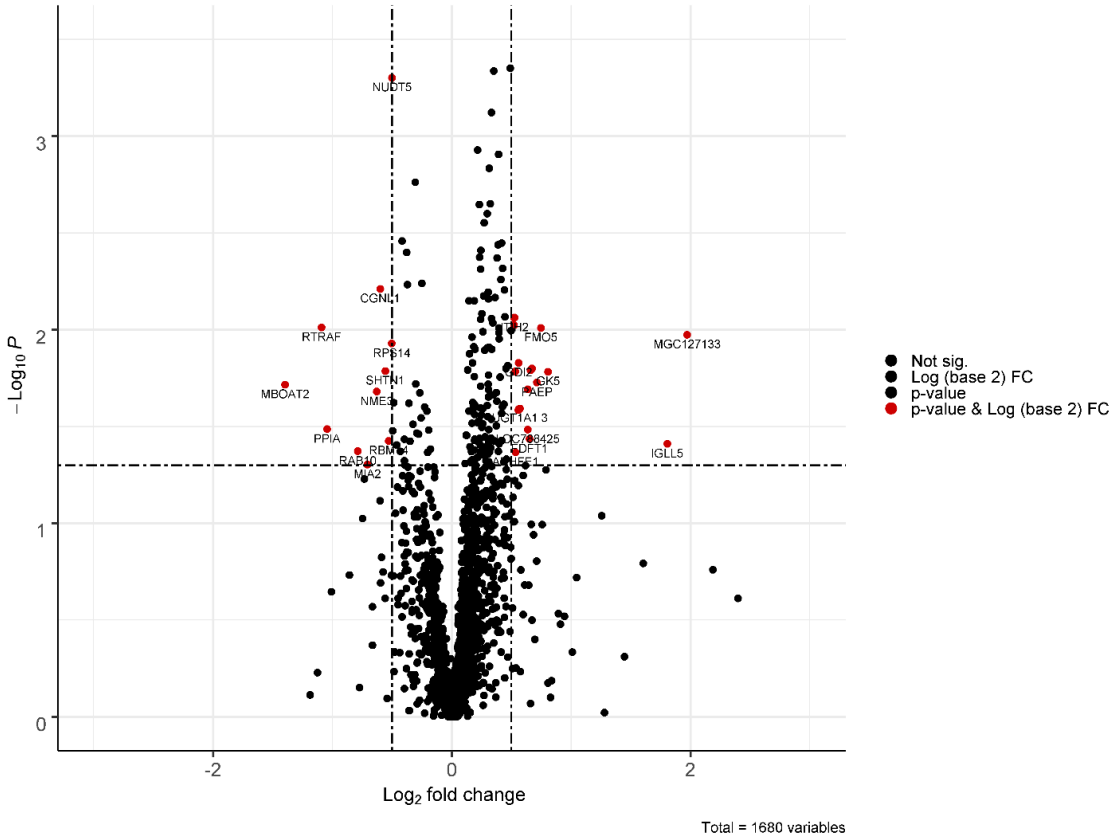
A. Veshkini, S. Déjean, L. Vogel, A. Tröscher, M. Delosière, A. Delavaud, D. Viala, H. Hammon, H. Sauerwein & M. Bonnet. Integrated serum proteome profiles, metabolites, and hormones and their metabolite–protein networks: association with energy metabolism and the somatotropic axis in transition dairy cows, 2021, The 75th GfE (Society for Nutritional Physiology) meeting, poster presentation

A. Veshkini, M. Bonnet, L. Vogel, A. Tröscher, M. Delosière, A. Delavaud, D. Viala, F. Ceciliani, H. Hammon, H. Sauerwein. Differential liver proteomes in periparturient dairy cows supplied or not with essential fatty acids and conjugated linoleic acids, 2020, European Federation of Animal Science, online, Oral presentation

A. Veshkini, H. Hammon, L. Vogel, A. Tröscher, M. Delosière, A. Delavaud, D. Viala, F. Ceciliani, H. Sauerwein & M. Bonnet. Serum proteomics of dairy cows infused with essential fatty acids and conjugated linoleic acids, 2020, European Federation of Animal Science, online, Poster presentation

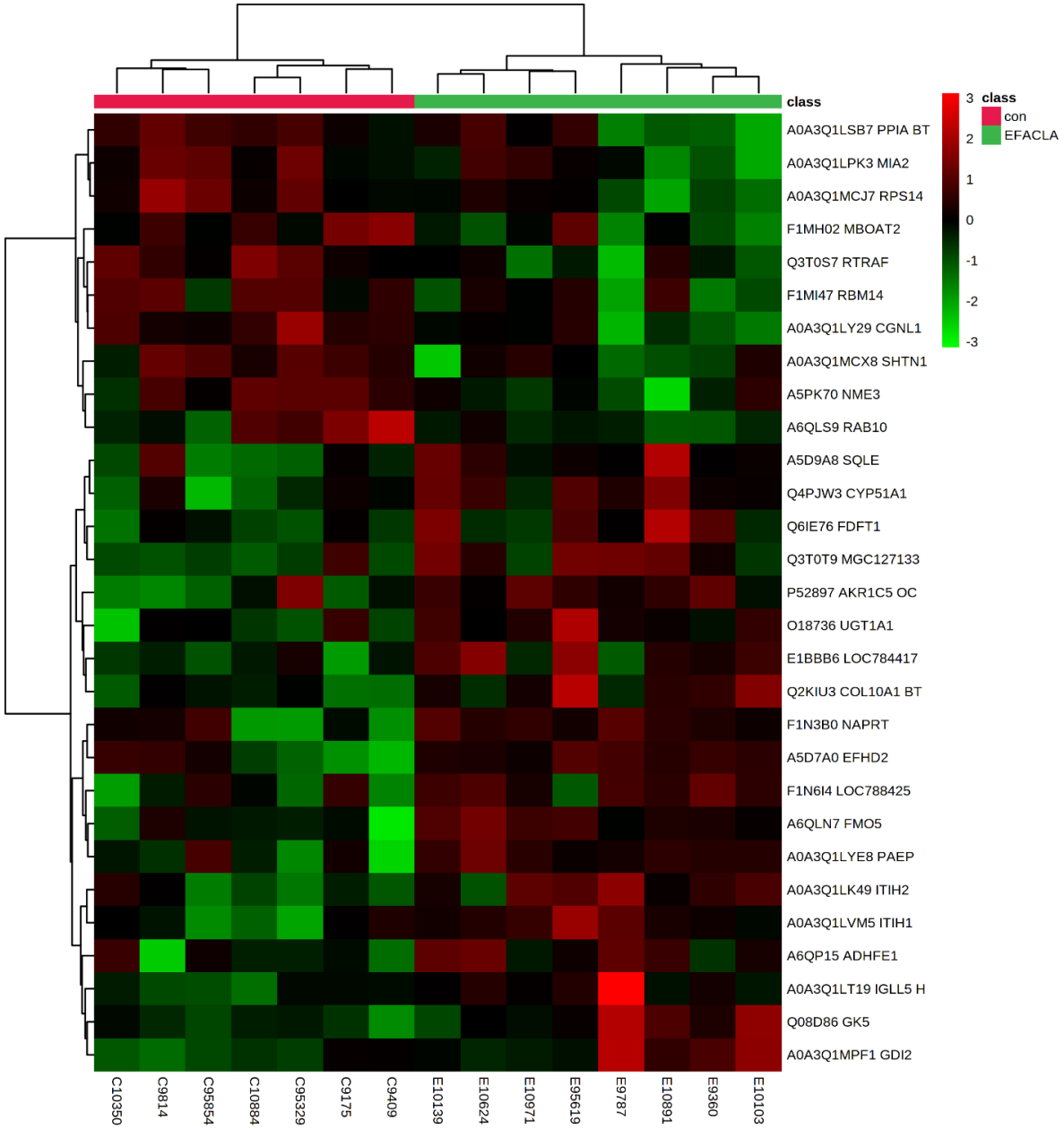
11. Appendix

Appendix 1a.



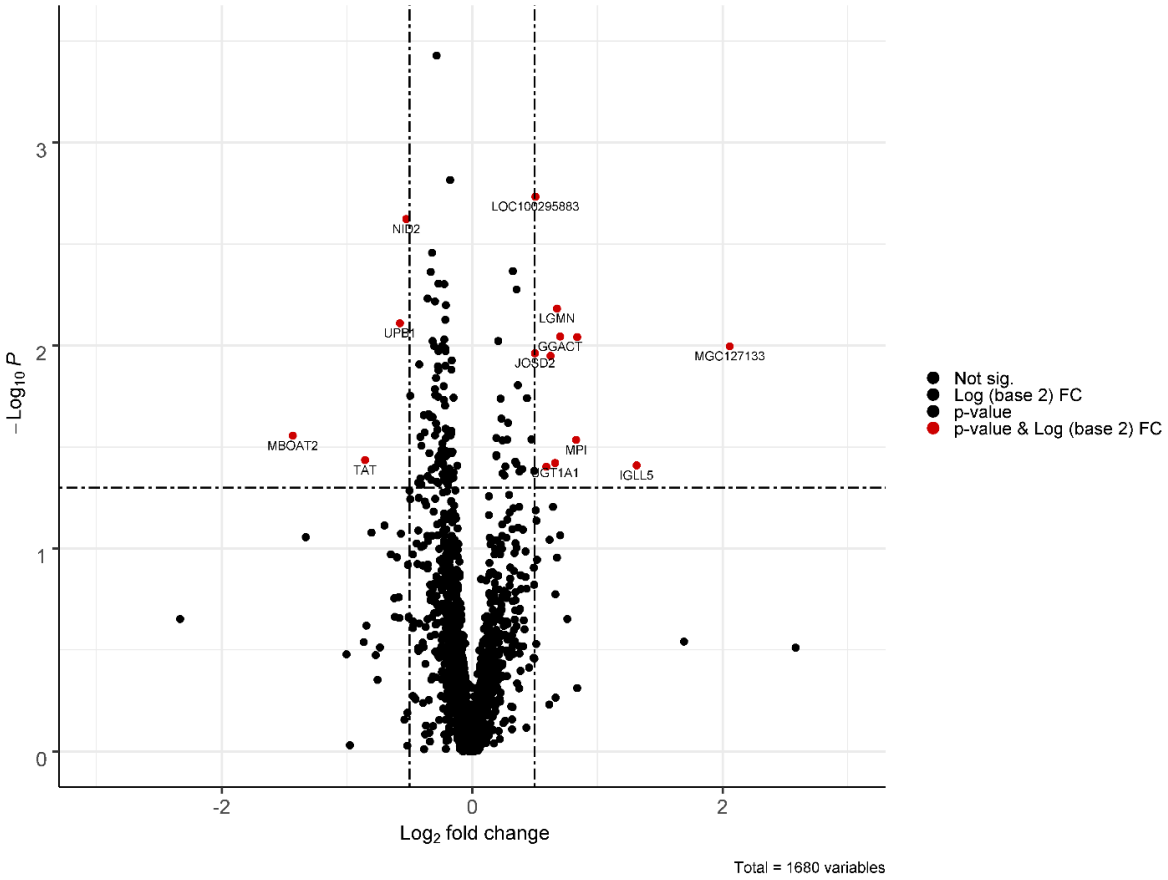
Appendix 1a. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group on day 21 antepartum, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>than 0.58 in a log scale that means a fold change of 1.3).

Appendix 1b.



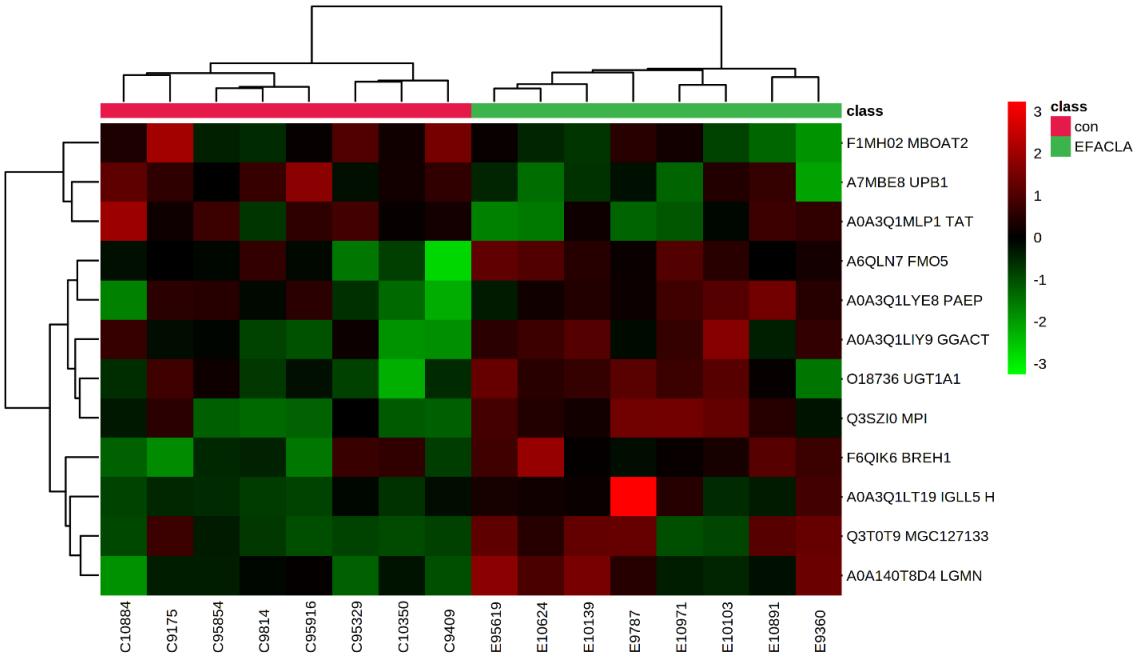
Appendix 1b. Hierarchical clustering heat map analysis of differentially abundant proteins between CTRL and EFA+CLA group on day 21 antepartum; Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively.

Appendix 2a.



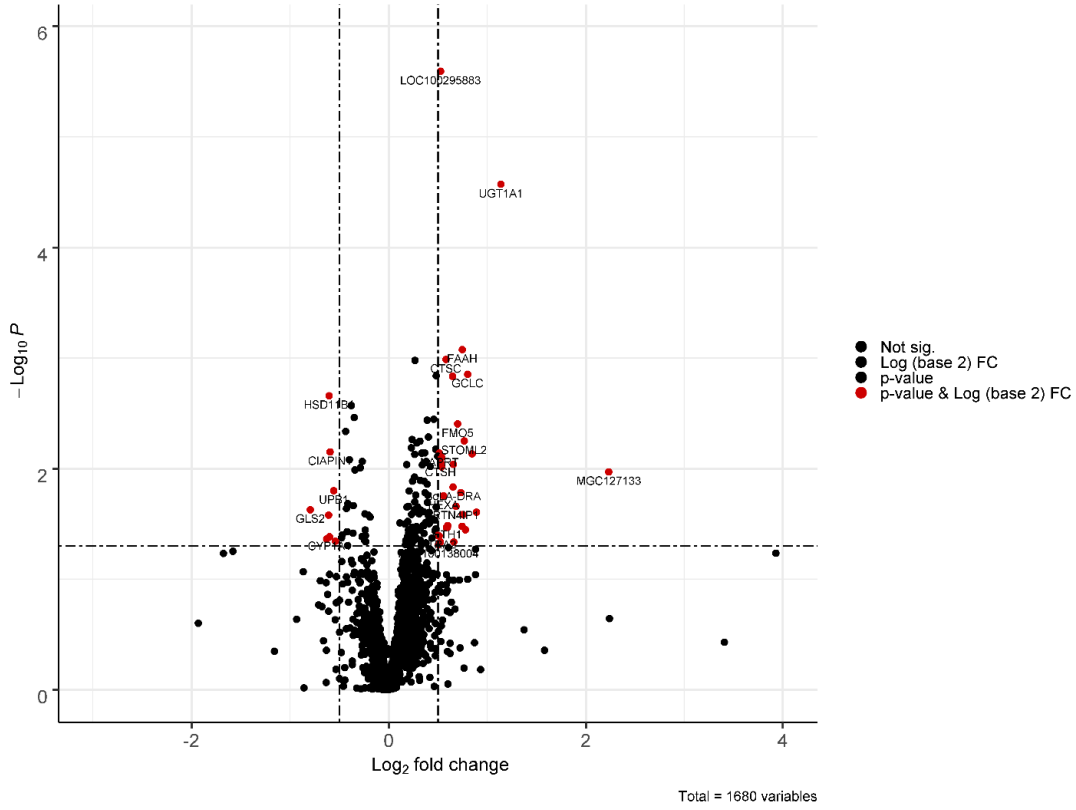
Appendix 2a. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group on day 1 postpartum, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>than 0.58 in a log scale that means a fold change of 1.3).

Appendix 2b.



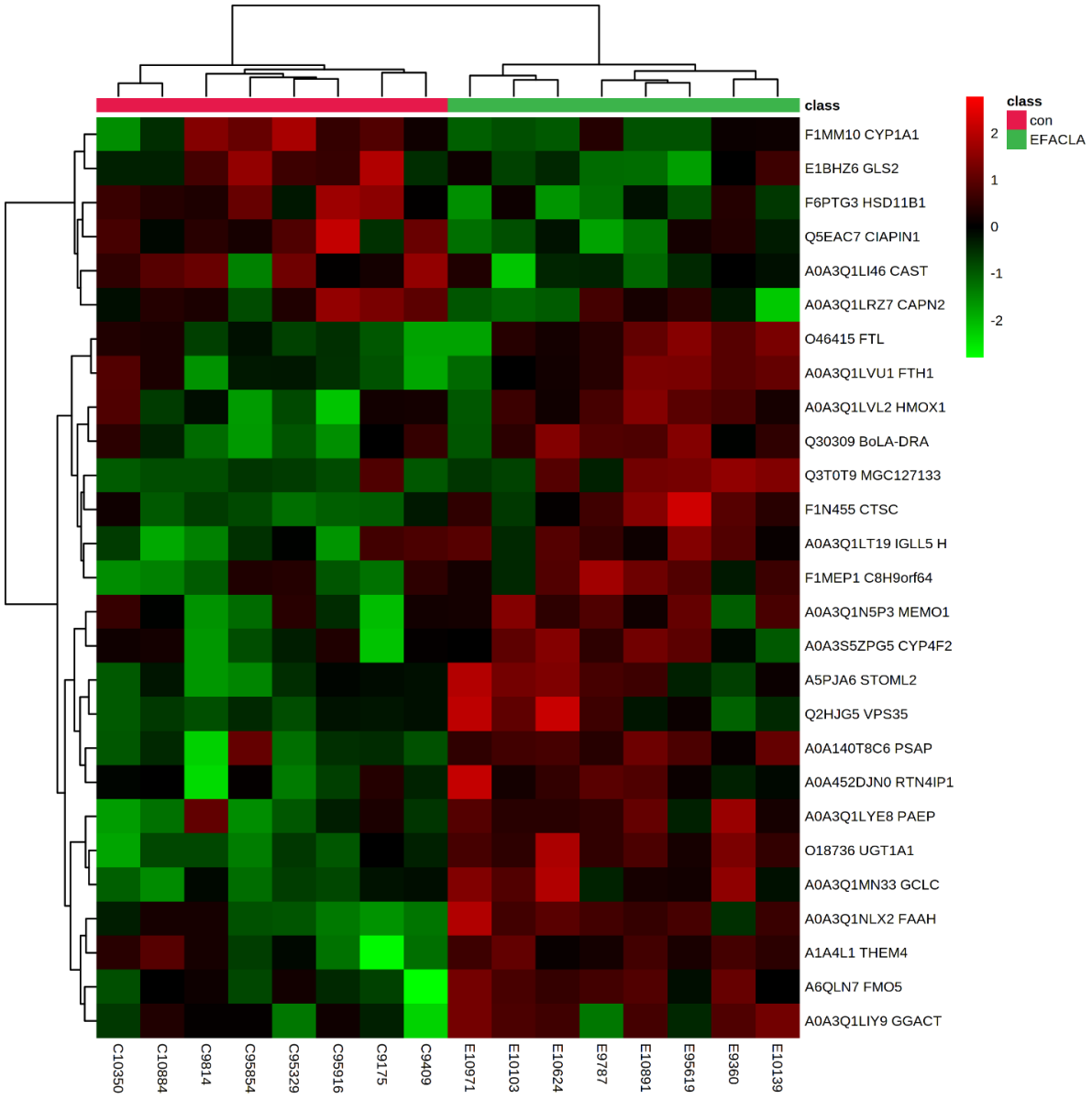
Appendix 2b. Hierarchical clustering heat map analysis of differentially abundant proteins between CTRL and EFA+CLA group on day 1 postpartum; Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively.

Appendix 3a.



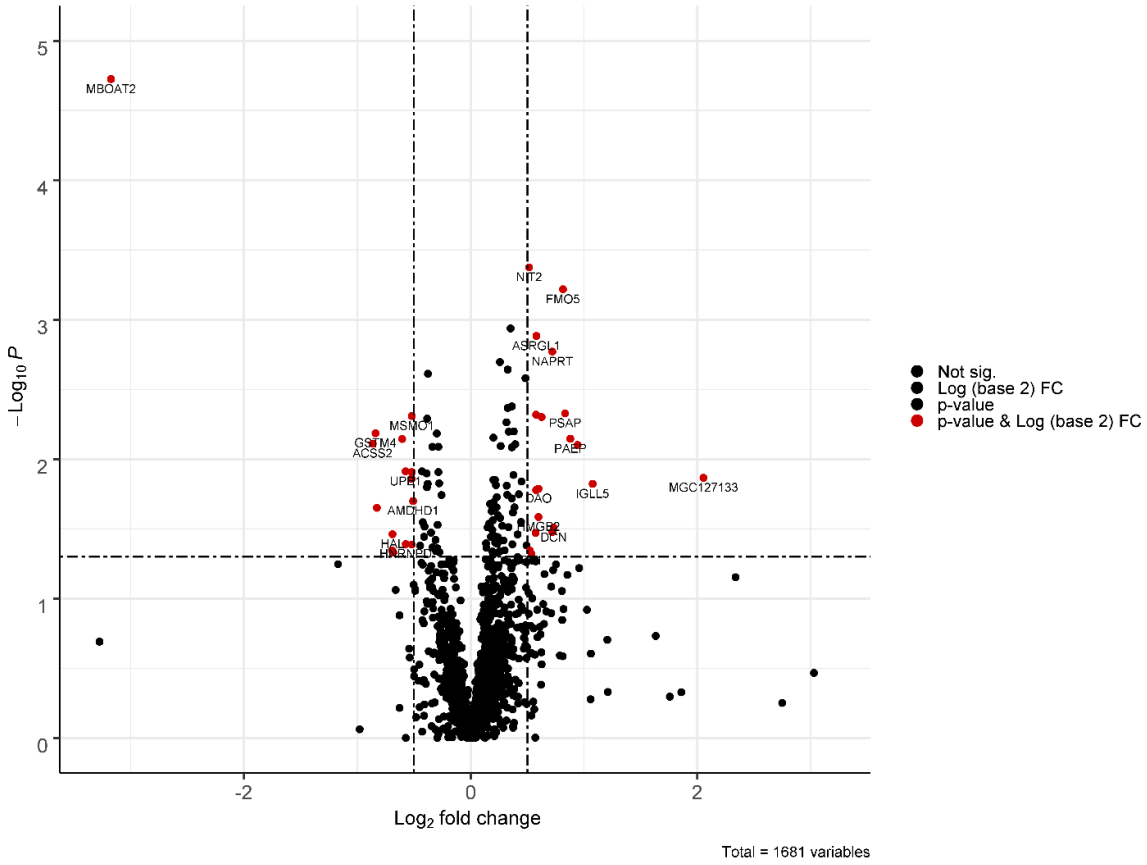
Appendix 3a. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group on day 28 postpartum, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>than 0.58 in a log scale that means a fold change of 1.3).

Appendix 3b.



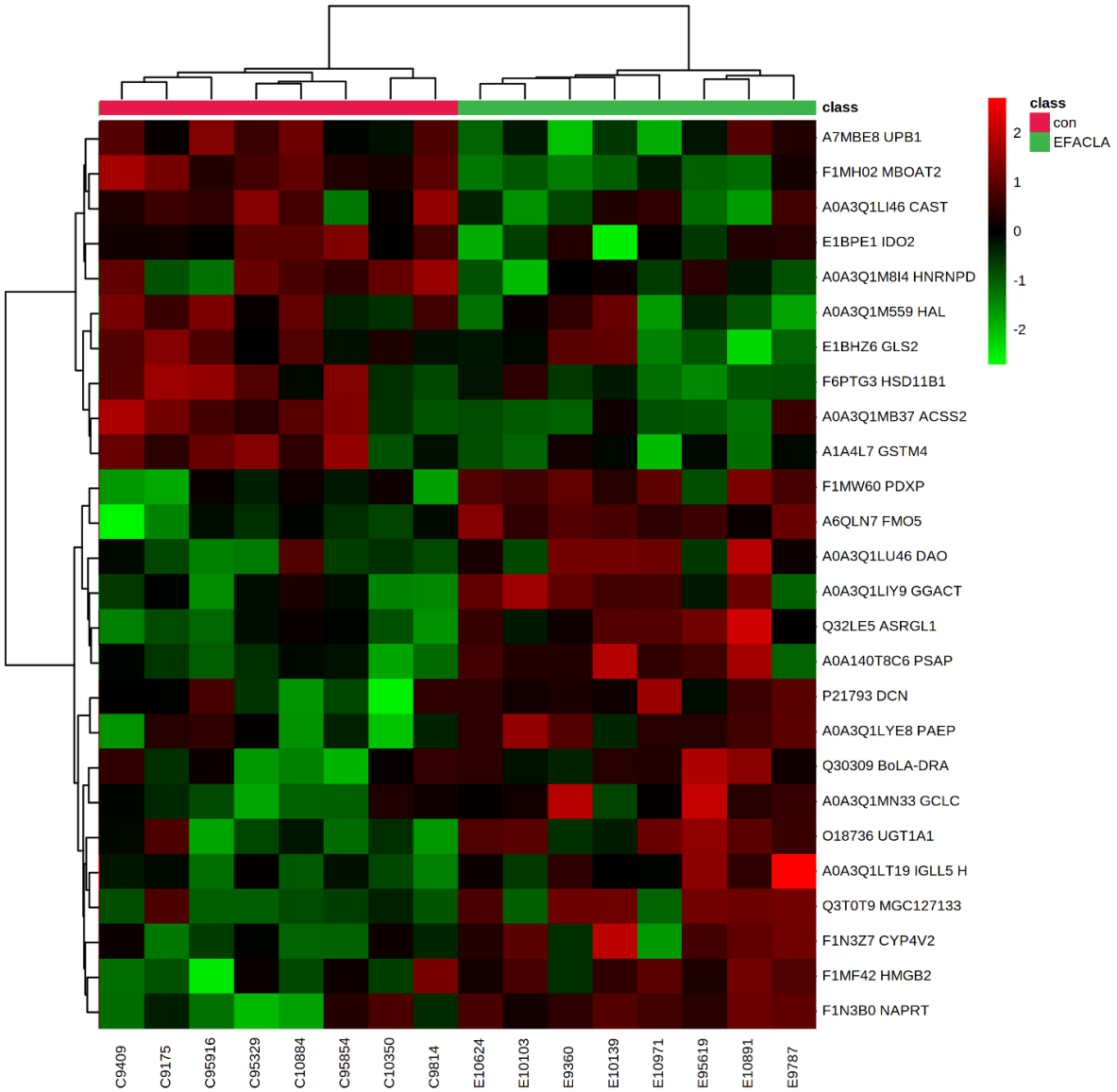
Appendix 3b. Hierarchical clustering heat map analysis of differentially abundant proteins between CTRL and EFA+CLA group on day 28 postpartum; Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively.

Appendix 4a.



Appendix 4a. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group on day 63 postpartum, increased (top right) and decreased (top left) proteins were highlighted in red (P<0.05 and fold change>than 0.58 in a log scale that means a fold change of 1.3).

Appendix 4b.



Appendix 4b. Hierarchical clustering heat map analysis of differentially abundant proteins between CTRL and EFA+CLA group on day 63 postpartum; Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively.